REVIEW

by Prof. Spaska Stanilova, PhD, DSc

Medical Faculty of Trakia University, Stara Zagora, Bulgaria

on the doctoral thesis for the award of the educational and scientific degree "PhD"

professional field **7.1. Medicine**

doctoral program: ***Medical Biology***

Author: Desislav Grozev Tomov, PhD student on independent preparation

Department: Medical Biology

Topic: Determining Oxidative Stress using Liquid Chromatography with Mass Spectrometric Detection

Supervisor: Prof. Dobrin Svinarov, MD, PhD, DMSc

1. General Introduction to the Procedure and the Doctoral Candidate

The presented set of materials on electronic media is in accordance with Article 70 (1) of Section I. Acquisition of the educational and scientific degree of "DOCTOR" and the scientific degree of "DOCTOR OF SCIENCES" at Medical University - Plovdiv; Regulations of Medical University - Plovdiv dated 28.01.2021.

I have no remarks regarding the submitted documents.

2. Brief Biographical Information about the Doctoral Candidate

The doctoral candidate, Desislav Grozev Tomov, was born on March 12, 1975. In 1999, he graduated in medicine from Medical University - Sofia with a master's degree in medicine. In 2012, he obtained a specialization in Clinical Laboratory at Medical University - Plovdiv and started working in a Clinical Laboratory. From 2018 to 2020, he underwent several specializations related to chromatographic analysis and HPLC; LC-MS method validation. One of these specializations was at Paisii Hilendarski University and the other two were at foreign universities, namely the University of Tartu and the Massachusetts Institute of Technology (MIT) in the USA. Obviously, these specializations define the future development of the doctoral candidate in the field of chromatographic mass spectrometric

analysis. Since 2015, he has been working at the Technological Centre for Emergency Medicine, and from 2019 to the present, he is affiliated with the Department of Bioorganic Chemistry at Medical University - Plovdiv. In this position, he is responsible for conducting chromatographic mass spectrometric analysis and carrying out experimental work related to the doctoral thesis.

3. Relevance of the Topic and Appropriateness of the Set Goals and Objectives

The study is focused on investigating changes in oxidative status through quantitative determination of concentrations of oxidized metabolites in biological fluids using mass spectrometry. The targeted metabolite, 8-isoPGF2-alpha, is quantitatively determined using a validated method involving liquid chromatography followed by mass spectrometry, allowing precise determination of low concentrations for diagnostic purposes. Isoprostanes, including 8-isoPGF2-alpha, are stable molecules and are end products of non-enzymatic oxidation of arachidonic acid esters, present in the composition of phospholipids in cell membranes, with their concentration correlating with that of reactive oxygen species (ROS). As a product of lipid peroxidation, found in all biological fluids including plasma, urine, cerebrospinal fluid, and saliva, it can be used to assess oxidative stress in the body.

Oxidative stress is a state where the balance between processes of free radical synthesis and neutralization is disrupted, which is a prerequisite for cellular structure damage, impaired function, and the occurrence of pathological changes at cellular and organ levels. Over the last decade, numerous experimental data have accumulated linking oxidative stress to the onset and progression of various diseases, including oncological and neurodegenerative conditions. Studying oxidative stress and exploring new approaches to enhancing endogenous antioxidant defence systems and/or suppressing free radical-induced damage in the body, including the use of various natural extracts and substances with established antioxidant functions, represent a novel and relevant area in biomedical and clinical research. The diverse mechanisms and roles that oxidative stress plays in various processes within the body necessitate its measurement and evaluation using appropriate methods.

In conclusion, considering the scientific data on the topic and the presented literature review, the research conducted by the doctoral candidate is highly relevant both in theoretical direction and for specific medical application in clinical diagnostics.

4. Understanding of the Problem

The literature review is well-structured and organized in accordance with the topic, demonstrating that the doctoral candidate is familiar with the achievements of global and local research in the field, as well as the contemporary investigative and diagnostic methods associated with the research problem. A total of 158 literature sources have been cited. Detailed data are presented regarding endogenous and exogenous factors and mechanisms of free radical generation in cellular compartments, their chemical and biochemical transformations, as well as the stability of various ROS and RNS. The role of free radicals as part of physiological processes in the body and their involvement in the regulation of arterial pressure, cytokine secretion and function, growth factors, hormones, and neuromodulation are highlighted. Molecular mechanisms of changes in biological macromolecules (lipids, proteins, nucleic acids, and carbohydrates) under the influence of various types of ROS are thoroughly examined, particularly when regulatory mechanisms are ineffective and their concentration is elevated due to increased synthesis and/or inefficient antioxidant processes. The subsequent structural and functional impairments, leading to cellular, tissue, and organ disruptions mediating pathological changes in the body, are well described. Various methods for assessing oxidative stress are creatively described and analysed, quantitatively determining different end metabolites resulting from the effect of ROS, along with the possibilities for their analysis, depending on their half-life period. Reactive oxygen and nitrogen species include both relatively stable molecules like hydrogen peroxide and alkyl hydroperoxide, as well as highly reactive hydroxyl, superoxide, and alkoxyl radicals with short half-lives and unsuitability for analysis. Special attention is paid to the quantitative analysis of isoprostanes in a biological matrix using liquid chromatography with mass spectrometric detection. Various approaches to ionizing the analysed substance (analyte) are described in detail, as well as the currently used mass analysers (quadrupole, magnetic sector, TOF, QIT, orbitrap, and tandem mass spectrometers), along with their advantages and disadvantages based on the nature of the sample in which the analyte will be quantitatively determined.

This well-written literature review demonstrates that the doctoral candidate comprehensively understands the state of the problem and creatively interprets it. Furthermore, it provides a solid foundation for planning and conducting the doctoral candidate's own research within the outlined theme.

The aim of the dissertation work is precisely and clearly formulated and scientifically justified, aiming to develop and validate methods for analysing 8-isoPGF2-alpha in various biological matrices using liquid chromatography with tandem mass spectrometry and to apply these methods in diagnostics and clinical studies. The set objectives, four in total, emerge from the dissertation's goal and are fully realized in the experimental section.

5. Research Methodology

The primary research methodology involves high-performance liquid chromatography followed by mass spectrometric detection. Mass spectrometric analysis is widely used for detecting atomic and molecular ions based on their mass-to-charge ratio (m/z). During detection, the sample can be directly analysed, but to enhance specificity and sensitivity, it often undergoes initial separation through gas or liquid chromatography. Following separation, the sample is ionized and directed to the mass detector. Currently, one of the most commonly used tandem mass analysers is the triple quadrupole, which consists of three sequentially arranged quadrupoles. The first and third quadrupoles act as mass filters, while the second functions as a collision cell. This detection method has been applied in the present dissertation work. Component separation from the original sample is carried out using an analytical column with a core-shell packing material. The described method has been employed to quantitatively determine 8-isoPGF2-alpha in blood plasma and saliva, following suitable sample processing involving protein precipitation and subsequent analyte isolation.

The chosen research methodology enables the achievement of the set goal and the generation of appropriate answers to the tasks addressed in the dissertation work.

The presented analytical method in the Materials and Methods section represents an original development by the doctoral candidate, encompassing a modification of a previously established method for sample preparation involving liquid-liquid extraction with phase separation, chromatographic separation using a core-shell analytical column, and mass spectrometric detection.

6. Characteristics and Evaluation of the Dissertation Work

In the Results and Discussion section, the obtained experimental data are extensively described, following statistical analysis. This allows for comparisons and appropriate

conclusions to be drawn in line with the set goals and tasks of the doctoral candidate. The volume of presented graphics and tables (26 tables and 31 figures) is suitable for illustrating the results of experimental activities.

The first chapter outlines the experiments related to the development and verification of the liquid chromatographic-mass spectrometric method for quantitative determination of 8-isoPGF2-alpha in blood plasma. Analysing substances with low concentrations like 8-isoPGF2-alpha in a complex matrix such as blood plasma is a significant challenge and requires proper sample preparation. The doctoral candidate compares four extraction procedures for sample preparation to identify the method with the highest yield of the analysed compound. Chromatographic separation is optimized by comparing several analytical columns using injections of a standard solution of 8-isoPGF2-alpha at defined concentrations to determine the best signal-to-noise ratio (S/N) for the peak of the target analyte and the internal standard. Optimization of the mass detector involves selecting appropriate conditions for analyte ionization by adjusting the vaporizer temperature, spray voltage, gas flow pressures (sheath gas, auxiliary gas, ion sweep gas), and capillary temperature. The complete characterization of the validated method is presented, including the lower limit of quantitation for 8-isoPGF2-alpha (5 ng/L), low-level (91.3%) and high-level (96.0%) recoveries, precision and reproducibility, selectivity, and stability.

The second chapter describes a similar procedure applied to validate the liquid chromatographic-mass spectrometric method for the analysis of 8-isoPGF2-alpha in saliva.

In the third chapter, the results of quantitative determination of 8-isoPGF2-alpha using the validated method in the plasma of 21 healthy volunteers and 95 patients with Hashimoto's autoimmune thyroiditis are presented. The study aims to evaluate oxidative stress. Higher average values of 8-isoPGF2-alpha in the Hashimoto's group compared to the control group (8.8 ± 7.8 vs. 5.9 ± 3.4, $p=0.043$) confirm previously published results highlighting the significance of 8-isoPGF2-alpha as a biomarker for free radical damage in this condition. However, the obtained results do not show significant differences based on thyroid hormone levels and disease stage, likely due to the small sample size.

In the final fourth chapter, the results of quantitative determination of 8-isoPGF2-alpha using the validated method in stimulated saliva of dental patients before and dynamically at 2 and 7 hours after the placement of a metal-ceramic restoration are presented and discussed. The analysis indicates significantly lower levels of 8-isoPGF2-alpha in stimulated saliva, which

tend to rise at the second hour and the seventh day after restoration placement. The comparison of 8-isoPGF2-alpha levels in stimulated and unstimulated saliva of 35 dental patients reveals significantly higher levels in unstimulated saliva before treatment ($p=0.001$). A statistically significant difference is also observed at the second hour after restoration placement ($p=0.008$), with higher levels in unstimulated saliva. By the seventh day, no significant differences in 8-isoPGF2-alpha concentrations are detected between the two types of saliva ($p=0.491$). The analysis shows a strong positive correlation between 8-isoPGF2-alpha levels and the investigated metal ions in unstimulated saliva before treatment. Notably, this represents the first literature data on changes in oxidative status in the oral cavity.

7. Contributions and Significance of the Development for Science and Practice

Isoprostanes are stable end products of lipid peroxidation of arachidonic acid and isomers of enzymatically generated compounds such as prostaglandins and leukotrienes. The F2-isoprostane family comprises 64 different isomers, with the most widely studied being 8-isoprostaglandin F2-alpha (8-isoPGF2alpha). The gold standard for measuring 8-isoPGF2alpha is gas chromatography-mass spectrometry (GC/MS), but the solid-phase extraction methods required for sample preparation are labour-intensive and often lead to contamination, artifact generation, and measurement of a mixture of four isomers rather than solely 8-isoPGF2alpha. Quantitative measurement of 8-isoPGF2alpha can also be achieved using ELISA, but this method usually yields higher results than GC-MS, possibly due to cross-reactivity of polyclonal antibodies with other isoprostane metabolites. Liquid chromatography coupled with mass spectrometry, as used in the developed procedure described in this dissertation, allows for more specific measurement of 8-isoPGF2-alpha. This approach includes appropriate sample preparation, liquid chromatographic column fractionation, and quadrupole mass spectrometric detection, enabling higher specificity and sensitivity in quantitative determination of 8-isoPGF2-alpha. Furthermore, this methodology is appropriately adapted for the determination of 8-isoPGF2-alpha in blood plasma and saliva, with diagnostic and quantitative detection applications as a primary marker for oxidative damage in various diseases.

In summary, the doctoral candidate's conclusion that the method for analysing 8-isoPGF2-alpha in blood plasma combines original sample preparation with phase separation, optimal chromatographic and mass spectrometric parameters, boasts a broad concentration range,

excellent analytical reliability, and fulfils all requirements for research and clinical applications. This conclusion also outlines the primary scientific and applied contribution of the dissertation in implementing a new method to demonstrate increased reactive oxygen species (ROS) production in the context of pathological processes.

The research conducted using this method allows for another original scientific conclusion: a positive correlation between increased ROS production in the oral cavity and local inflammatory processes, as well as an assessment of the effect of conducted treatment. Notably, a positive connection is established between local oxidative damage and the application of CoCr alloys in the oral cavity.

In conclusion, the presented dissertation holds both current and contributory value. It contributes to the development of an original methodology for the quantitative determination of 8-isoPGF2-alpha through high-speed liquid chromatography with mass detection and provides an assessment of increased ROS production in patients. Thus, the developed methodology holds high potential for implementation in diagnostic practice.

8. Evaluation of Publications Related to the Dissertation Work

In relation to the dissertation work, three referenced publications have been presented, two of which are in a journal with an Impact Factor (IF) of 2.157 and Q3 according to SJR, while one is in a Q2 journal, surpassing the requirements of MU-Plovdiv as formulated in the Regulations for obtaining the academic degree of "Doctor." All three publications are connected to the dissertation's topic and reflect the main results obtained during the scientific-experimental research process. In the publication concerning the development of the methodology, the doctoral candidate is listed as the first author, while in the other two, they are listed as the second author, illustrating their contribution and personal involvement in the conducted research. Two of the publications have already been cited, with three citations recorded according to Scopus data. Additionally, the doctoral candidate has presented their results in 5 scientific forums, three of which were held abroad.

9. Personal Involvement of the Doctoral Candidate

From the presented materials - the creative autobiography, dissertation, publications, and associated supporting evidence - it is evident that the doctoral candidate has actively

participated in the conducted research, the acquisition and analysis of results, as well as the formulation of conclusions. Additionally, the doctoral candidate has participated in two scientific projects at MU-Plovdiv.

10. Abstract

The provided abstract by the doctoral candidate adequately reflects the essential aspects of the dissertation work. The abstract contains the main part of the results and their discussion, along with all obligatory information about the candidate, the examination committee, and the upcoming public defence.

11. Critical Remarks and Recommendations

Determining isoprostanes, especially 8-isoPGF2alpha, in biological samples is often used to assess oxidative damage caused by free radicals. This is achieved through three main approaches: gas chromatography with mass detection, liquid chromatography with mass detection, and immunological methods (ELISA and RIA). Each of these approaches has its own advantages and drawbacks. It would be beneficial if the doctoral candidate had conducted a comparative analysis of the samples using not only the newly developed method but also another method, such as ELISA, which is more accessible. This would enable a comparison of the results and the identification of any differences. Of course, this suggestion can also be considered as a recommendation for future research by the doctoral candidate.

CONCLUSION

The doctoral thesis ***contains scientific and scientific-applied results that represent an original contribution to the field of science and meet all*** the requirements of the Law for the Development of the Academic Staff in the Republic of Bulgaria (LDASRB), the Regulation for the Implementation of LDASRB, and the corresponding Regulation of Medical University - Plovdiv. The presented materials and dissertation results **fully** comply with the specific requirements of Medical University - Plovdiv. The doctoral thesis demonstrates that the doctoral candidate Desislav Grozev Tomov **possesses** in-depth theoretical knowledge and

professional skills in the scientific field of Medical Biology, and **shows** qualities and abilities for independent scientific research.

Based on the above, I am confident in giving my ***positive evaluation*** for the conducted research, as presented in the reviewed doctoral thesis, abstract, achieved results, and contributions. I ***recommend to the esteemed academic jury to award the educational and scientific degree of 'Doctor'*** to Desislav Grozev Tomov in the doctoral program of Medical Biology.

23.08.2023

Reviewer:

Prof. Spaska Stanilova, PhD, DSc.