Methods for determining Cytochrome P450 reductase in biological fluids of animals

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Abstract. The study provides a literature review of modern methods for determining Cytochrome P450 reductase. The importance of the enzyme in the body is established. It is shown that Cytochrome P450 reductase determination is used in pharmacology and toxicology. Observed that modern approaches to determining Cytochrome P450 reductase include mass spectrometry, chromatography, electrochemical methods. Spectrometry – allows detection of low concentrations of the substance and study of reaction kinetics. Chromatography (gas, liquid, thin-layer chromatography) is used to separate complex mixtures and determine enzyme activity. Electrochemical methods (voltammetry) help to study the redox transitions of the enzyme. It is confirmed that ELISA is a new era for the Cytochrome P450 reductase determination. Gaps in modern knowledge about Cytochrome P450 reductase are also identified.

1 Introduction

The enzyme CYPOR (cyclosaphene oxidase activity P450) is a key component in the metabolism of various botanical and zoological organisms. Its species specificity manifests itself to varying degrees depending on the species, which is based on the unique properties of the substrates with which it interacts.

In many species, CYPOR is responsible for the detoxification and transformation of exogenous and endogenous compounds such as medications, toxins, and hormones. Particularly in mammals, this enzyme exhibits high levels of activity in relation to oxidative reactions, which allows it to effectively remove health threats.

Research shows that evolutionary changes in CYPOR among species result in a variety of variations that promote adaptation to different ecological niches. For example, in some marine organisms, the enzyme may exhibit improved properties in processing specific salts or organic compounds characteristic of their environment. This diversity highlights the importance of CYPOR as an evolutionary marker and its potential application in biomedical research dedicated to the study of metabolic pathways and the mechanism of action of drugs.

Determination of the enzyme CYPOR (cytochrome P450 reductase) plays a key role in understanding the activity of the cytochrome P450 system, which is responsible for the

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metabolism of many exogenous and endogenous compounds in the body. This system is a complex ensemble of enzymes involved in the oxidation of various biomolecules, and its functionality depends on the correct operation of CYPOR.CYPOR mediates electron transfer from nicotinamide adenine dinucleotide phosphate (NADP) to cytochrome P450s, which is critical for their activation. Improper functioning or deficiency of CYPOR can lead to disruption of the metabolism of medicines and toxins, as well as changes in hormonal activity, which significantly affects the physiological state of the body.

CYPOR activity analysis allows not only to assess the overall metabolic capacity of the cytochrome P450 system but also to predict potential pharmacueticals interactions, which is of critical importance for pharmacology and toxicology. Thus, the study of the CYPOR enzyme represents an important aspect in the field of biochemistry and medical science, providing an in-depth understanding of the functional processes occurring at the molecular level.

The complexity of CYPOR activity determination methods is due to many factors. First, there is a need for high-quality cell systems and samples that ensure the stability of the enzyme. Secondly, the diversity of CYP450 isoenzymes and their interactions with various substrates requires a multistep approach, including both in vitro and in vivo studies. Technologies such as mass spectrometry and chromatographic methods open new horizons in this direction, but challenges remain related to the accuracy and reproducibility of data.

Thus, understanding the role of CYPOR and improving methods for its determination are necessary for progress in pharmacology and toxicology, as well as for the development of more effective and safe medicines. The main goal of the article is to consider the main methods for determining CYPOR in biological fluids of animals.

2 Materials and methods

Studying the methodology is a critical step in the process of any research, since it is at this initial stage that the basis for further steps is formed. The methodology not only determines the approach to data collection and analysis but also provides justification for the choice of certain methods and tools, which in turn affects the reliability and reliability of the results obtained.

Systematic analysis of existing methods and their adaptation to the specifics of the problem under study allows the researcher to avoid mistakes associated with incorrectly chosen directions. During this process, it is necessary to carefully consider not only traditional methods but also modern approaches that can add important perspectives to the research.In addition, studying the methodology at the initial stage helps to establish clear hypotheses and goals that will serve as a compass on the research path. It is important not only to follow specific protocols but also to be flexible, adapting methodological approaches as new facts and circumstances arise.

Thus, a high-quality study of the methodology roots the entire research project, turning it into a structured and targeted event.

This review article uses a variety of materials and methods to provide an in-depth analysis of the selected topic. Initially, a systematic review of the scientific literature was conducted, which included articles published in peer-reviewed journals over the past ten years. The search used PubMed, Scopus, and Web of Science databases using keywords such as “research methods,” “analysis materials,” and “review article.”

Further, standards such as PRISMA and STROBE were used to assess the quality of included studies, thereby eliminating low-quality sources and increasing the reliability of the data obtained. Quantitative and qualitative analysis methods were also used: meta-analysis for quantitative results and content analysis for qualitative studies. This comprehensive methodology allows not only to summarize existing data but also to identify

gaps in current research as well as suggest directions for future research. We are confident that the results of our analysis will be useful for both the scientific community and practitioners in this field.

3 Results and Discussion

First of all, it is crucial to emphasize the role of the CYPOR enzyme in the activation of the cytochrome system.

The catalytic cycle of cytochrome is described as follows:

- The substrate binds close to the heme group, on the side opposite to the axial thiolate. Substrate binding causes a conformational change in the active site, often displacing a water molecule from the distal axial coordination position of the heme iron, changing the heme iron state from low to high spin.
- Binding to the substrate induces electron transfer from NAD(P)H via cytochrome p450 reductase or another related reductase.
- Molecular oxygen binds to the nascent iron heme center at the distal position of axial coordination, initially forming a dioxygenic adduct like oxymyoglobin.
- A second electron is transferred from either cytochrome P450 reductase or cytochrome b5, reducing the Fe-O2 adduct to form a short-lived peroxo state.
- The peroxo group formed in step 4 is rapidly protonated twice, releasing one water molecule and forming highly reactive compounds called P450 compound 1 (or simply compound 1). Compound 1 P450 is an oxo (or ferryl) iron IV with an additional oxidizing equivalent delocalized on top of the porphyrin and thiolate ligands. There is no evidence for an alternative ferric(V)-oxo perferryl.
- Depending on the substrate and enzyme used, P450 enzymes can catalyze any of a wide range of reactions (e.g., hydroxylation). Following release of the product from the active site, the enzyme returns to its original state, with the water molecule returning to occupy the distal coordination position of the iron core.

The enzyme Cytochrome P450 reductase (NADPH-ferrihemoprotein oxidoreductase, NADPH-hemoprotein oxidoreductase, CYPOR) deserves special attention. Microsomal P450-mediated monooxygenase activity, supported by NADPH, requires interaction between the enzyme NADPH-cytochrome P450 reductase and cytochrome P450. This enzyme (CYPOR) and its reference values for different animal species are poorly studied (reference values are not found in the scientific literature). However, from medical practice, it is known that reductase and P450 form a functional 1:1 complex. There is also a 10-20-fold excess of P450 overreductase, which allows us to hypothesize that CYPOR is the limiting factor in the reaction and determines the amount of functionally significant cytochrome.

Thus, activation of cytochrome P450 occurs through the transfer of electrons to it from reduced nicotinamide adenine dinucleotide phosphate (NADPH) during the membrane-binding activity of the enzyme Cytochrome P450 reductase (NADPH-ferrihemoprotein oxidoreductase, NADPH-hemoprotein oxidoreductase, CYPOR), the reference values of which are not found in the scientific literature. But based on the essence of the catalytic reaction of cytochromes, this enzyme is the limiting one in this reaction, which is the basis of our scientific hypothesis. Thus, the study of the potential limiting factor of activation reactions of the cytochrome system, its reference levels, the correlation of these levels with other indicators of the state of the hepatobiliary system, and indicators characterizing the biotransformation of exogenous substances will have scientific novelty.

Modern approaches to CYPOR determination include mass spectrometry, chromatography, and electrochemical methods.

1. Determination of CYPOR by mass spectrometry represents an important step in the field of analytical chemistry, allowing detailed investigation of the composition and structure of complex organic compounds. This technique relies on the ability of a mass spectrometer to separate and identify ions based on their mass and charge. When analyzing CYPOR, which is a key enzyme in the metabolism of many compounds, samples are ionized to create detectable molecular ions. (Figure 1)

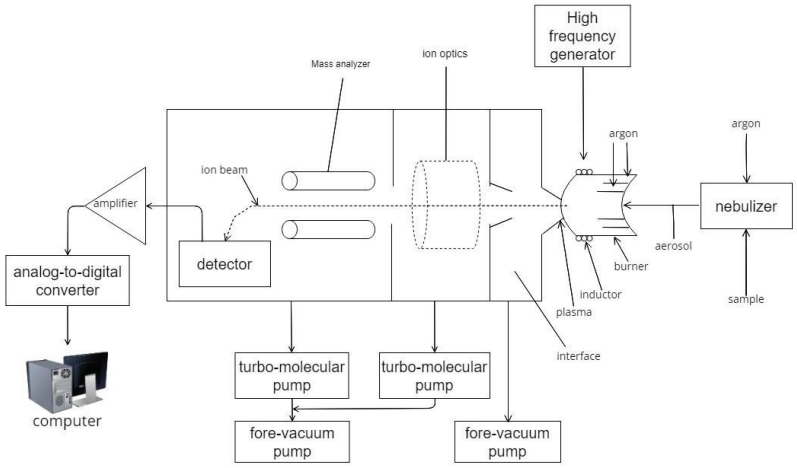


Fig. 1 Mass spectrometry scheme for CYPOR determination.

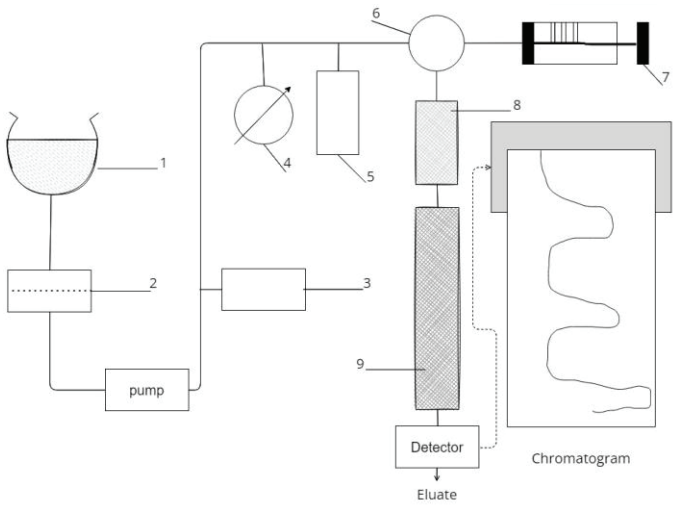
There are several advantages to using mass spectrometry to determine CYPOR. Firstly, the high sensitivity and specificity of the method allow the detection of even minimal concentrations of a substance. Secondly, the possibility of analyzing dynamic processes in real time opens up new horizons for studying the kinetics of enzymatic reactions. In addition, the technique provides the ability to quantitatively analyze, which is critical for understanding biochemical processes.

Thus, the application of mass spectrometry in the determination of CYPOR provides researchers with a powerful tool for in-depth understanding of biological systems and increasing efficiency in the development of new therapeutics.

2. Determination of CYPOR by chromatography represents a key step in the study of medicine metabolism and biochemical processes occurring in the body. The chromatography method allows for the separation of complex mixtures of substances, providing high accuracy and sensitivity of the analysis. This methodology uses several types of chromatography, such as gas chromatography, liquid chromatography, and thin layer chromatography, each with its own advantages and specific applications. (Figure 2)

Determining CYPOR, or cytochrome P450 reductase, requires several preliminary steps, including protein extraction and enzyme isolation. Extraction efficiency directly impacts the determination of CYPOR activity, making the choice of buffer and material processing methods critical.

Analysis of the obtained data is carried out using spectrophotometry or mass spectrometry, which allows more accurate identification of the active forms of the enzyme and assessment of their kinetic parameters. Improvements in chromatographic techniques continue to advance our understanding of the role of CYPOR in pharmacology and toxicology, opening new perspectives for the development of effective therapeutic strategies.



1 - eluent container; 2 - filter; 3 - regulator for equalizing pulsation when feeding eluent; 4 - pressure gauge; 5 - pressure regulating valve; 6 - sample injection tap; 7 - syringe; 8 - safety column; 9 - analytical column

Fig. 2 General scheme of the device for determination of CYPOR by chromatography.

3. The determination of CYPOR (Complex Capacitance Potential Oxide Redox) by electrochemical methods is an important aspect of modern analytical chemistry. The process involves the use of various electrochemical techniques, such as voltammetry, galvanostatics, and impedance spectroscopy, to measure the electrochemical properties and characteristics of compounds. (Figure 3)

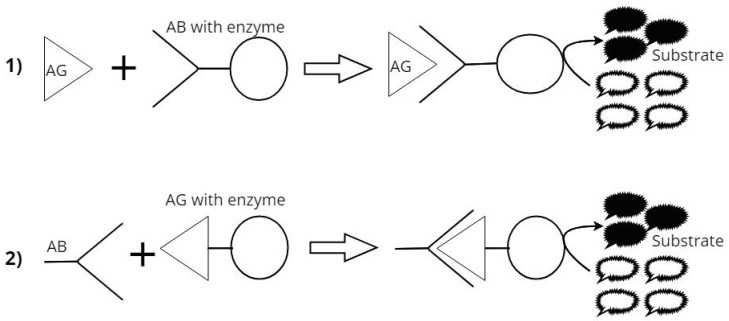


Fig. 3 Basic principle of ELISA for determination of CYPOR. 1 - To detect antigens; 2 - To detect antibodies

Electrochemical methods allow the study of redox transitions, which are key to understanding the reactivity of CYPOR. The most common approach is to use a working electrode modified with special materials, which helps to increase the sensitivity of the method. At the same time, it is important to take into account factors influencing the results, such as temperature, pH of the environment, and concentration of reagents.

However, it should be noted that the development of test systems for the determination of CYPOR by the ELISA method is currently actively developing.

Determination of CYPOR by the ELISA method (enzyme-linked immunosorbent assay) is a highly effective method for the quantitative assessment of specific proteins, in which the pharmacueticals binds to the corresponding antigen. This method is based on the

antigen-antibody principle, allowing one to achieve high sensitivity and specificity in detecting the substance of interest.

The procedure begins with applying the sample to polymer plastic, where binding to primary antibodies specific to the CYPOR being studied occurs. A secondary antibody labeled with an enzyme is then added and binds to the primary antibody, forming a complementary structure. After incubation and washing out the excess, a substrate for the enzyme is added, which, when reacting, causes a color change proportional to the amount of antigen.

By comparing the optical density of the resulting solution with control samples, it is possible to accurately estimate the concentration of CYPOR in the sample. This method is actively used in clinical practice for the diagnosis of various diseases, which emphasizes its importance and effectiveness in modern veterinary medicine.

4 Conclusion

The role of CYPOR in the cytochrome system has not been fully studied; however, researchers are accumulating data on its significance in metabolic processes. CYPOR, or cytochrome P450 reductase, is a key component that ensures the functioning of cytochromes P450, which play an important role in the oxidative metabolism of various compounds, including drugs, toxins, and fatty acids.

The complex mechanics of interaction between CYPOR and cytochrome P450s suggest the presence of multifaceted regulatory pathways that influence the level of activity of these enzymes. It is important to note that CYPOR not only contributes electrons to cytochromes P450s but can also influence their conformation, which in turn changes their catalytic activity.

Recent studies indicate a possible role for CYPOR in the commensal microbiome as well as in responses to stress factors, but the mechanisms underlying these processes require further investigation. To fully understand the function of CYPOR in light of its interactions with other molecules, a comprehensive approach including molecular modeling and experimental biochemistry is required.

The study of methods for determining CYPOR (cytochrome P450 reductase) in biological fluids is becoming increasingly relevant in the light of modern research aimed at understanding the metabolism of medicines and chemical compounds in the body. CYPOR plays a key role in biochemical processes by facilitating the transfer of electrons in the cytochrome P450 system, which in turn affects the synthesis and breakdown of various biologically active molecules.

Methods developed to assess CYPOR allow not only to diagnose metabolic disorders but also to predict responses to pharmacotherapy, which is especially important in the context of individualized medicine. Studying the activity of CYPOR in various biological fluids, such as blood, urine, and saliva, opens new horizons for the development of highly effective biomarkers that can serve as indicators of the health status of animals or their susceptibility to disease.

Acknowledgments

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References

1. A.R. Galiakhmetov, E.A. Kovrigina, C. Xia, J.P. Kim, E.L. Kovrigin, Journal of Biomolecular NMR, **70(1),** 21-31 (2018)
2. P. An. Jian, X. Yu. Ma, B. J. Yi, X. N. Li, Current Developments in Nutrition, **7**, 100202 (2023)
3. B.S. Masters, C.C. Marohnic, Drug Metabolism Reviews, **38(1),** 209-225 (2006)
4. C. Xia, A.L. Shen, P. Duangkaew, R. Kotewong, P. Rongnoparut, J. Feix, J.P. Kim, Biochemistry, **58(19),** 2408-2418 (2019)
5. J. J. P. Kim, A. L. Shen, C. Xia, Handbook of Flavoproteins: Complex Flavoproteins, Dehydrogenases and Physical Methods, **2,** 73-102 (2013)
6. E. V. Proskurnina, M. V. Fedorova, M. M. Sozarukova, A.E. Mitichkin, I.V. Panteleev, E.V. Svetlov, Endocrine, **72(3),** 735-743 (2020)
7. C. C. Marohnic, S. P. Panda, P. Martásek, B. S. Masters, Journal of Biological Chemistry, **281(47)**, 35975-35982 (2006)
8. A. Vonada, L. Wakefield, M. Martinez, C.O. Harding, M. Grompe, A. Tiyaboonchai, Hepatology, **79(5)**, 1088-1097 (2023)
9. M. Kranendonk, C.C. Marohnic, S.P. Panda, M.P. Duarte, J.S. Oliveira, B.S. Masters, J. Rueff, Archives of Biochemistry and Biophysics, **475(2),** 93-99 (2008)
10. L. Jian, Y. Chen, Y. Cao, Q. Zhang, Chinese Science Bulletin (Chinese Version), **66(22),** 2878-2886 (2021)
11. D. Hamdane, C. Xia, S.C. Im, H. Zhang, J.J. Kim, L. Waskell, Journal of Biological Chemistry, **284(17)**, 11374-11384 (2009)