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Summary of the doctoral dissertation:

# Oxidation of selected drugs and natural compounds in reactions catalysed by horseradish peroxidase

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Horseradish peroxidase is an oxidoreductase that contains heme and catalyzes oxidation of various organic compounds using hydrogen peroxide as an acceptor of electrons and protons. In the peroxidative catalytic cycle the enzyme exists in three states: the ground state, compound I, and compound II. Compound I is formed after reaction of the ground state with hydrogen peroxide. Compound II is formed upon reaction of compound I with one molecule of a reducing substrate, and upon reaction with a second molecule of such a substrate, compound II returns to the ground state. Horseradish peroxidase is capable of oxidizing a wide range of compounds, and its isolation is relatively simple. Therefore, it found many practical applications, including enzymatic diagnostic tests used to determine the concentration of many important analytes, such as creatinine, uric acid, glucose, cholesterol, and triglycerides. In these assays, horseradish peroxidase catalyzes the Trinder reaction, which involves the oxidative coupling of 4-aminoantipyrine and phenol or its derivatives. The result of this reaction is the formation of a chromophore with absorption at approximately 500 nm, which enables spectrophotometric determination of the concentration of analytes. Since the introduction of this method into medical diagnostics its interference by many compounds has been detected. However, the mechanisms of these interferences have only been established in a few cases.

The dissertation explains the mechanisms of interference of the Trinder reaction by several groups of compounds. It has been demonstrated that in the case of *p*-diphenols the magnitude of interference depends on the electrochemical properties of compounds (reduction potential) which result from their structure. Homogentisic acid (2,5-dihydroxyphenylacetic acid) had the strongest interfering effect, which resulted from its direct oxidation by horseradish peroxidase and reduction of radical intermediates of the Trinder reaction with phenol as a substrate. For gentisic acid (2,5-dihydroxybenzoic acid), calcium dobesilate, and etamsylate (salts of 2,5-dihydroxybenzenesulfonic acid) only the latter mechanism was significant and the effect on the Trinder reaction was moderate. Additionally, the effect of homogentisic acid and

calcium dobesilate on variants of the Trinder reaction containing sodium 3,5-dichloro-2-hydroxybenzenesulfonate or 3-hydroxy-2,4,6-triiodobenzoic acid, which are worse substrates of horseradish peroxidase than phenol, was checked. The influence of the tested *p*-diphenols was stronger in these systems. The effect of more complex drugs containing a *p*-diphenol group, such as rifampicin, mitoxantrone, and doxorubicin, and a *p*-aminophenol, such as mesalazine, was also tested. Among these compounds, only mesalazine showed moderate interference with the Trinder reaction, and for the remaining compounds, the effect was small. However, in diagnostic tests these interferences should not be significant because the concentrations of these compounds achieved in physiological fluids under standard conditions are too low.

Mechanisms of interference in Trinder-based diagnostic tests by dopamine and dobutamine, a drug used to regulate the work of the heart, were also verified. It was shown that both compounds form aminochrome-type chromophores as a result of their oxidation by horseradish peroxidase. To explain the different oxidation rates of the catechol groups in these two compounds, a dobutamine derivative with a phenolic group blocked by methylation was synthesized. Kinetic analysis and molecular docking of these three compounds to the active site of horseradish peroxidase led to the conclusion that the faster oxidation of the catechol group in dobutamine than in dopamine was caused by two processes: 1) faster oxidation of the phenolic group of dobutamine, which is connected to the amino group by a longer alkyl chain than the catechol group, and the emerging phenoxyl radical mediates the oxidation of the catechol group, 2) hindered access of the dopamine catechol group to the catalytic center caused by its anchoring with an amino group near the entrance to the catalytic center, which is reflected in the much higher $K_m$ value of dopamine than dobutamine.

Inhibition of horseradish peroxidase observed in the test for the determination of uric acid by gallic acid and hispidin, a derivative of caffeic acid, was also verified. Gallic acid, caffeic acid and hispidin have been shown to be rapidly oxidized to unstable products under the conditions of this test. These reactions inhibit the formation of the Trinder reaction chromophore and thus reduce the absorbance values measured in this test. It was found that a similar phenomenon also took place also in the case of selenium analogs of methimazole during oxidation of ABTS by lactoperoxidase. ABTS cation radical was generated by oxidation with sodium persulfate and selenium analogs of methimazole were added to it. The absorbance of the radical generated in this way decreased rapidly in the presence of these compounds. Therefore they only reduce the ABTS cation radical and are not lactoperoxidase inhibitors. By analyzing the course of the

reaction with NMR spectroscopy and high-resolution mass spectroscopy the products of these reactions were identified and the pathway of transformation of the selenium analogs of methimazole was determined.