Talanta 84 (2011) 710–716

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

A peroxidase-based method for the determination of dopamine, adrenaline, and α-methyldopa in the presence of thyroid hormones in pharmaceutical forms

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ARTICLE INFO

Article history:
Received 3 December 2010
Received in revised form 18 January 2011
Accepted 30 January 2011
Available online 4 February 2011

Keywords:
Horseradish and soybean peroxidases
Catecholamines
Thyroid hormones
Pharmaceutical analysis

ABSTRACT

The analytical properties of two commercial plant peroxidases isolated from horseradish roots and soybean hulls in the catalysis of the transformation of some catecholamines were demonstrated in the absence and presence of thyroid hormones (L-thyroxine and 3,3′,5′-iodothyronine). For the first time the reactions of dopamine, adrenaline, and α-methyldopa oxidation with H_2O_2 catalysed by horseradish peroxidase with the addition of L-thyroxine as the amplification agent were studied and proposed as the indicator reactions for the simple and rapid enzymatic determination of the indicated catecholamines in their concentration ranges 0.5–300, 4–300, and 100–400 μM, respectively. The catalytic activity of the enzyme (characterized by the reaction rate) was controlled spectrophotometrically. The optimum conditions for the indicator reactions were thoroughly characterized. The mechanism of the stimulatory effect of L-thyroxine on the oxidation of the catecholamines was discussed. The developed enzymatic procedures were successfully applied for the determination of dopamine, adrenaline, and α-methyldopa in some pharmaceutical forms.

1. Introduction

Dopamine (DA), adrenaline (AD), and α-methyldopa (MD) belong either to natural products of adrenal glands in human beings or to synthetic biologically active compounds of neurotransmitters class – catecholamines. Insufficient concentrations of DA and AD in body fluids cause such serious diseases as anorexia, schizophrenia, Parkinson's disease etc. Nowadays, catecholamine-based pharmaceuticals are widely used in medicine, therefore, it is extremely important to estimate the quality of these pharmaceuticals in pharmaceutical industry and intra-pharmacy control.

According to the European Pharmacopoeia, catecholamines are determined by their potentiometric titration by $HClO_4$ in formic or glacial acetic acid media [1]. There are many various techniques proposed in the scientific literature for the determination of catecholamines in pharmaceuticals including voltammetric [2–7], spectrophotometric [8–10], luminescent [11–13], chromatographic [14–18], or kinetic [19] procedures. Unfortunately, the existing procedures have some disadvantages such as expensive equipment [17–21], laborious [2–4,6,11,12] and time-consuming [8–10,13] photometric reactions, and difficulties in implementation [5,19]. In addition, nowadays the electrochemical biosensors based on bean sprout peroxidase [20], peroxidases from zucchini [21], polyphenol oxidase from palm tree [22] were developed for the determination of DA in pharmaceutical formulations with the detection limits 10, 26, and 50 μM, respectively. Only one biosensor for the determination of AD with its detection limit 8 nM was reported [23], which employed polyphenol oxidase from banana. It is necessary to emphasize that in all mentioned cases the researchers have applied the crude extracts of the enzymes prepared *in vitro* instead of their commercial preparations. Such approach limits the application of the described biosensors for routine pharmaceutical analysis.

Depending on a pharmaceutical form, concentrations of catecholamines in drugs vary in the rather wide range (1 μM–1 mM). Therefore, it is preferable to develop and use the analytical procedures which provide the determination of catecholamines in different pharmaceutical formulations. A promising approach to the determination of catecholamines, and DA, AD, and MD especially, is the application of well-known, thoroughly studied, highly active, stable, and commercially available peroxidases isolated from horseradish roots (HRP) and soybean hulls (SBP) [24]. In the quality control of catecholamine-based drugs it is expedient and convenient to couple the enzymatic determination of catecholamines with the simple and available spectrophotometric method for monitoring the rate of their biotransformation. The indicated enzymes are known to catalyse the oxidation of the considered substances by H_2O_2 [25], although the produced chromophores have low absorptivity, and oxidation occurs slowly [26]. It should be mentioned that only few procedures for DA, AD, and

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doi:10.1016/j.talanta.2011.01.074

MD determination using native plant peroxidases and spectrophotometry to control the reaction rate could be found in the literature [27]. They are based on the interference of catecholamines with the formation of quinoid dyes produced by coupling of *p*-chlorophenol and 4-aminoantipyrine catalysed by HRP. These procedures are simple and sensitive (c_{min} – 2 and 1 μM for DA and AD, respectively), but they are not rapid and require additional reagents beside the enzyme.

Thyroid hormones are known to stimulate the production of catecholamines in human body *in vivo* [28]. In 1950–1960 while studying the relationship between oxygen consumption of animals and an increase in metabolic rates, Klebanoff [29] reported that L-thyroxine (T_4) and some related compounds stimulated AD oxidation by H_2O_2 catalysed by HRP *in vitro*. The reaction rates were measured in the absence and presence of thyroid hormones as the absorbance change at 300 nm. The maximum stimulatory effect on AD oxidation rate was observed in the case of T_4 and 3,3′,5′-iodothyronine (T_3) at 25 °C in 5 min after mixing the components of the reaction. Despite the obvious analytical outlooks of this indicator system, a procedure for AD determination was not developed. The amplification of other enzymatic reactions by "second" substrates of biocatalysts was also described by other authors (enzymologists) [30]. However, nobody has used this effect yet to develop the procedures for the determination of catecholamines utilizing plant peroxidases.

We studied the effect of thyroid hormones on the oxidation not only of AD, but also of DA and MD, and not only in the presence of HRP but also of SBP. As a result of the present research, novel, simple, and rapid enzymatic procedures for DA, AD, and MD determination in pharmaceuticals were proposed. These procedures are based on the thyroid amplification of catecholamines oxidation by H_2O_2 catalysed by commercial preparations of HRP and SBP using spectrophotometric control of the rates of the indicator reactions.

2. Experimental

2.1. Reagents and materials

2.1.1. Enzymes

HRP and SBP (EC 1.11.1.7) were purchased from Sigma (USA). The solutions of the enzymes were prepared by dissolving the precisely weighted portions of their solid preparations in 0.1 M phosphate buffer solutions (PBS), pH 7.0. The concentrations of HRP and SBP stock solutions were determined by the Soret band absorbance ($\varepsilon_{403} = 1.02 \times 10^5\ M^{-1}\ cm^{-1}$) [31]. Solid preparations and solutions of the enzymes were stored in a refrigerator at +4 °C.

2.1.2. Substrates

Working solutions (5 mM) of DA, AD and MD were prepared by dissolving precisely weighted portions of the solid preparations of dopamine hydrochloride, adrenaline hydrotartrate, and methyldopa sesquihydrate (Sigma, USA) in water. Due to their light instability, these solutions had to be kept in dark vessels and prepared fresh daily. Stock standard solutions (5 mM) of T_4 and T_3 were prepared by dissolving precisely weighted portions of their solid samples (Sigma, USA) in 0.1 M KOH. The standard solution of H_2O_2 (9.6 M) was purchased from Merck (Germany). Working solutions of H_2O_2 were prepared daily by successive dilution of the standard solution with water.

2.1.3. Other reagents

Tris(hydroxymethyl)aminomethane (Tris) (Serva, Germany), K_2HPO_4, KH_2PO_4 (Merck, Germany), imidazol, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Sigma, USA) were used to prepare appropriate buffer solutions according to the described recommendations [32]. Lidocaine hydrochloride, sucrose, lactose, fructose, NaCl, L-cysteine, and $Na_2S_2O_5$ were purchased from Sigma (USA).

All reagents were of analytical grade or higher and used without further purification.

Doubly distilled water with resistibility over 18 MΩ cm^{-1} purified with a Simplicity Proto system (Millipore, France) was used for preparing all aqueous solutions.

2.2. Apparatus

Kinetic measurements were performed using a Shimadzu UV mini-1240A spectrophotometer (Japan, the accuracy ±0.0003 absorbance units) using a microcuvette with l = 1 cm. The pH of aqueous solutions was measured with an accuracy ±0.005 using a potentiometer in a pH-meter mode (Econics-Expert-001, Russia). Reaction mixtures were thermostated using a Model Thermit thermostat (Russia, ±1 °C precision). Micro-dosage devices produced by Biohit (Denmark) and Eppendorf (Finland) were used for sampling.

The accuracy of the results of DA and AD determination in real samples obtained by the enzymatic method was confirmed using HPLC-MS as an alternative one. Chromatographic conditions were as follows: Agilent-1100 chromatograph, Zorbax Eclipse XDB-C18, 150 mm × 4.6 mm column, granulation – 5 μm, eluent: 20 mM ammonium-acetate buffer solution, pH 7.5; detector: MS-SL – quadrupole.

2.3. Procedures

2.3.1. General procedure for the determination of catecholamines

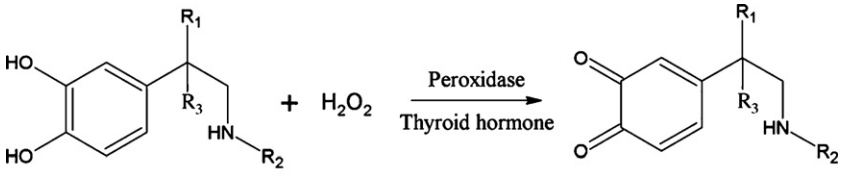
The following components were successively placed into a plastic calibrated test-tube with a stopper: a certain buffer solution with the required pH value, solutions of the enzyme, catecholamine, and thyroid hormone. Finally, a portion of H_2O_2 solution was introduced. The total volume of the reaction mixture was 300 μl. At the moment when H_2O_2 was added and the solutions were mixed, the kinetic curves were registered for 2 min with 1 s interval. Blank experiments were carried out in the absence of thyroid hormone, because the reaction in the absence of the analyte – the enzyme substrate – did not proceed. All the experiments were performed at ambient temperature.

2.3.2. Pretreatment of pharmaceuticals

DA samples were quantitatively transferred from the ampoules into a plastic test-tube. Then, a 1 ml portion of each sample was placed into a glass test-tube and diluted up to 20.00 ml by water with pH 5.5–6.0. AD samples were concentrated by Oasis® MCX cartridge (Waters, USA), then quantitatively outwashed from the cartridge by a 5% NH_3 aqueous solution and 10-fold diluted. All the procedures took not more than 15 min. In the case of MD determination one tablet of the pharmaceutical preparation containing 250 mg of MD was weighed and ground into a fine powder. The obtained powder was quantitatively placed into a 50 ml volumetric flask using 20 ml of water; the content of the flask was shaken thoroughly for dissolving the active component of the drug, MD. Then water insoluble components of the pharmaceutical preparation (magnesium stearate, talc, starch, ethylcellulose, and stearic acid) were separated by a filtration using a filter paper (blue tape). The obtained solution of MD was diluted to 40.0 ml by water and mixed well. The procedures of analysis were carried out immediately in order to avoid the oxidation of catecholamines in the air.

2.3.3. Procedure of DA (AD, MD) determination in the model solutions

Firstly, a 490 (550, 560) μl portion of 0.1 M PBS, pH 5.0 (8.6 or 7.0), a 40 (10, 5) μl portion of 9 μM HRP solution, a 10 μl



R_1 = -H (DA), -OH (AD), -CH_3 (MD)

R_2 = -H (DA), -CH_3 (AD), -H (MD)

R_3 = -H (DA), -H (AD), -COOH (MD)

Fig. 1. The scheme of DA, AD, and MD oxidation catalysed by plant peroxidases.

portion of the standard solution of each catecholamine (DA, AD, or MD) with the concentrations 1.5, 3.0, 4.5, 6.0, 9.0, 12.0 mM (25, 50, 75, 100, 150, 200 μM in the reaction mixture), a 20 (10, 5) μl portion of 1 mM T_4 solution were successively placed into a plastic test-tube with a stopper. Finally, a 40 (20, 20) μl portion of 3 mM H_2O_2 solution was introduced. At the moment when H_2O_2 was added and the reaction solution was mixed, a timer was turned on and the absorbance at analytical wavelengths 465 (480, 481) nm was measured at 1 s intervals for 2 min. The calibration curves were plotted on the coordinates: the rate of the indicator reaction, μM min^{-1} versus the DA (AD, MD) concentration, μM. The initial rate of the indicator reaction (V_0, μM min^{-1}) was calculated as $V_0 = \Delta c/\Delta t = \Delta A/\Delta t \times 1/l\varepsilon = \tan\alpha/l\varepsilon$, where Δc was an increment of the indicator substance concentration at the reaction time (Δt), A was the absorbance, l was the cell length, ε was the molar absorptivity of dopamine-*o*-quinone (2.45×10^3 M^{-1} cm^{-1}), adrenochrome (4.02×10^3 M^{-1} cm^{-1}), or α-methyldopa-*o*-quinone (3.10×10^3 M^{-1} cm^{-1}); tan α was the slope of the absorbance (A) versus time (t, s) plot. The rates of DA (AD, MD) oxidation in the absence of the enzyme were 2 (6, 3) μM min^{-1}. These values did not exceed 5–7% of the rates in the presence of the enzyme (for instance, HRP) which were 45 (80, 50) μM min^{-1}.

2.3.4. Procedure of DA (AD, MD) determination in pharmaceuticals

The enzymatic determination of DA (AD, MD) in real samples was performed using the standard addition method according to the following procedure: a 480 (540) μl portion of a 0.1 M PBS, pH 5.0 (8.6, 7.0), a 40 (10, 5) μl portion of 9 μM HRP solution, a 12 μl portion of the analysed sample (obtained as described in Section 2.3.2), a 20 (10, 5) μl portion of 1 mM T_4 solution were placed successively into a plastic test-tube with a stopper. Finally, a 40 (20, 20) μl portion of 3 mM H_2O_2 solution was introduced. Further experiments were accomplished as described above.

Analogous experiments were carried out in the presence of a sample and three standard additions: 25, 50, 75 μM of DA (AD) or 100, 150, 200 μM of MD standard solutions.

3. Results and discussion

3.1. Choice of the indicator reactions

The reactions of enzymatic oxidation of DA, AD, and MD by H_2O_2 catalysed by HRP or SBP in the presence of thyroid hormone (T_4 or T_3) were chosen as the indicator ones (Fig. 1).

The oxidation of catecholamines leads to the formation of the coloured quinone-containing products of DA, AD, and MD oxidation absorbing maximally at 440–485, 470–500, and 460–490 nm, respectively. In our experiments, the analytical wavelengths for studying DA, AD, and MD oxidation were 465, 480, and 481 nm, respectively. The absorption spectra of the products of AD, DA, and MD oxidation carried out under the conditions reported earlier for AD oxidation by Klebanoff [29] (PBS, pH 7.0, concentrations: HRP – 0.8 μM, AD – 160 μM, T_4 – 42 μM, H_2O_2 – 0.33 mM) and the kinetic curves of these indicator reactions are presented in Fig. 2a and b, respectively.

The data in Fig. 2a show two absorbance maxima in all the spectra. The first maximum (403 nm) corresponds to the absorbance of the HRP–H_2O_2 complex; another one – in the range 460–500 nm, refers to the absorbance of the oxidation products of catecholamines. It should be emphasized that the addition of hormones, e.g. T_4 (Fig. 2b), into the indicator systems results in a 5.2, 5.0, and a 10-fold increase in the rates of DA, AD, and MD enzymatic oxidation, respectively.

In order to choose the preferable source of a biocatalyst, which is one of the major components of the indicator reaction, the kinetic parameters of these reactions proceeding according to the Michaelis–Menten mechanism were calculated. In the case of DA oxidation the values of V_{max} and k_{cat} ($k_{cat} = V_{max}/[\text{enzyme}]$) [33]

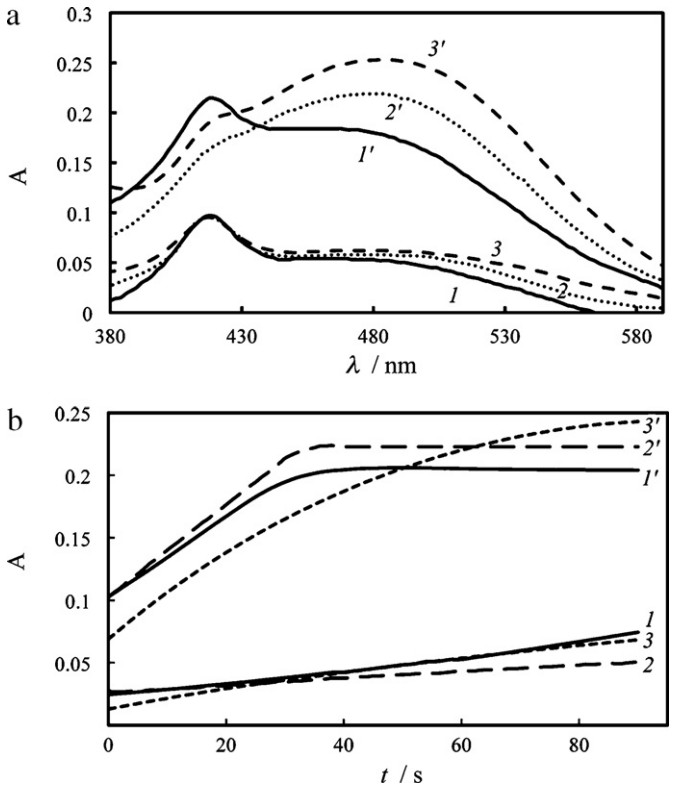


Fig. 2. Absorbance spectra of the products after 1 min of the reaction start (a) and kinetic curves (b) of DA (1, 1′), MD (2, 2′), and AD (3, 3′) oxidation in the absence (1–3) and presence (1–3′) of T_4, respectively (0.1 M PBS, pH 7.0; concentrations: HRP – 600 nM, DA (AD, MD) – 100 μM, T_4 – 40 μM, H_2O_2 –0.4 mM; blank solution – 0.1 M PBS, pH 7.0; wavelengths for Fig. 4b – 465 (DA), 481 (MD), and 480 (AD) nm).

Table 1
The dependence of the rate of catecholamine oxidation on the nature of the added hormone (0.1 M PBS, pH 7.0; concentrations: catecholamine – 100 μM, HRP – 600 nM; hormone – 40 μM; H_2O_2 – 0.4 mM; $P = 0.95$, $n = 4$).

Catecholamine	DA		AD		MD
Hormone	T_4	T_3	T_4	T_3	T_4
ΔV[a] ($\mu M\ min^{-1}$)	55 ± 5	20 ± 5	115 ± 10	35 ± 5	80 ± 10

[a] $\Delta V = V - V_0$, where V and V_0[b] – the rates of the indicator reactions in the presence and absence of hormone, respectively.
[b] V_0 for DA, AD, and MD were 6, 17, and 10 $\mu M\ min^{-1}$, respectively.

were $(70 \pm 5)\ \mu M\ min^{-1}$ and $(180 \pm 20)\ min^{-1}$ in the presence of HRP, and $(45 \pm 10)\ \mu M\ min^{-1}$ and $(125 \pm 10)\ min^{-1}$ in the presence of SBP, respectively ($P = 0.95$, $n = 4$). The values of the maximum rate and the catalytic constant were higher in the case of HRP as compared to those of SBP; therefore, the application of HRP as a biocatalyst appeared to be preferable. The similar situation was observed while determining the kinetic parameters of the enzymatic oxidation of AD and MD by H_2O_2. The affinity between the enzyme and the substrate (characterized by k_{cat}) in all cases seemed to be stronger in the presence of HRP. Thus, HRP was chosen as an optimum biocatalyst for further studies.

As the highest rates of AD oxidation by H_2O_2 catalysed by HRP had been observed previously in the presence of T_4 and T_3 [29], these hormones were used in our experiments. The data of Table 1 show that the rate of the indicator reactions in the presence of T_4 is significantly higher than that in the presence of T_3. In the case of MD oxidation, the experiment with T_3 was not performed, as its effect on the reactions of DA and AD oxidation was minor. Therefore, it seemed to be preferable to utilize the stimulatory effect of T_4 in the considered enzymatic indicator reactions for the development of the procedures for the determination of catecholamines.

After the indicator systems based on the revealed stimulatory effect of T_4 on HRP-catalysed oxidation of catecholamines had been chosen, the optimum conditions of these reactions were determined.

3.2. Choice of the nature and pH of the buffer solution

The proper selection of the nature and composition of a buffer solution allows controlling the properties of the enzyme (catalytic activity, stability, and substrate specificity) and the analyte [34]. Since the buffer solution usually plays an important role in enzymatic catalysis, the effect of PBS, Imidazol–HCl, HEPES–KOH, and Tris–HCl buffers on the catalytic activity of HRP was studied in order to choose the appropriate buffer solution for the development of sensitive procedures for DA, AD, and MD determination. The results of these experiments are presented in Fig. 3a.

It has been found that the rates of the reactions of DA, AD, and MD oxidation are maximal when PBS is used (Fig. 3a). Their values in other buffer solutions were about 50% of those in PBS (with just one exception in the case of MD oxidation in imidazol buffer solution: its rate was 85% of that in PBS). Thus, PBS is the most favorable for the effect of T_4 on the both catecholamines oxidation, because unlike the organic buffer solutions [35,36], the PBS molecules cannot capture free radicals produced in the process of T_4 transformation, which participate in DA, AD, or MD oxidation.

The data obtained while studying the dependence of the rate of the indicator reactions on pH of the selected buffer solution are demonstrated in Fig. 3b. Fig. 3b shows, that the optimal pH ranges are 5.2–5.6 and 7.2–8.0 for DA, 8.0–8.8 for AD, and 6.7–7.5 for MD oxidation. So, DA is oxidized better if it is completely (pH < 5.8) or for more than 90% protonated (pH < 8.0) in the indicator system ($pK_{a,1} = 9.05$) [37]. The rate of AD oxidation is the highest when AD is half-dehydrogenated ($pK_{a,1} = 8.02$; $pK_{a,2} = 9.46$) [38]. However, the reaction rate of DA oxidation was found not to depend on DA concentration in the indicator system at pH 7.2–8.0. MD is completely oxidized when –COOH group of MD molecules is dehydrogenated ($pK_{a,1} = 2.3$; $pK_{a,2} = 8.7$) [39]. Thus, the values of pH 5.4, 8.6, and 7.0 were chosen as the optimum values for further investigations.

It was found that the effect of a buffer concentration on the rate of the indicator reactions was insignificant in the range of 0.05–0.4 M (the reaction rate changed up to not more than 2% of its value in 0.1 M PBS); therefore, 0.1 M PBS was used in all further experiments.

3.3. Choice of the concentrations of HRP, T_4, and H_2O_2

The influence of the concentrations of HRP, T_4, and H_2O_2 on the rate of the indicator reactions is illustrated by Fig. 4a–c, respectively.

The concentrations of the components were chosen so that the reaction rate was high and it could be conveniently registered by spectrophotometry. The resultant optimum conditions for the determination of the catecholamines are summarized in Table 2.

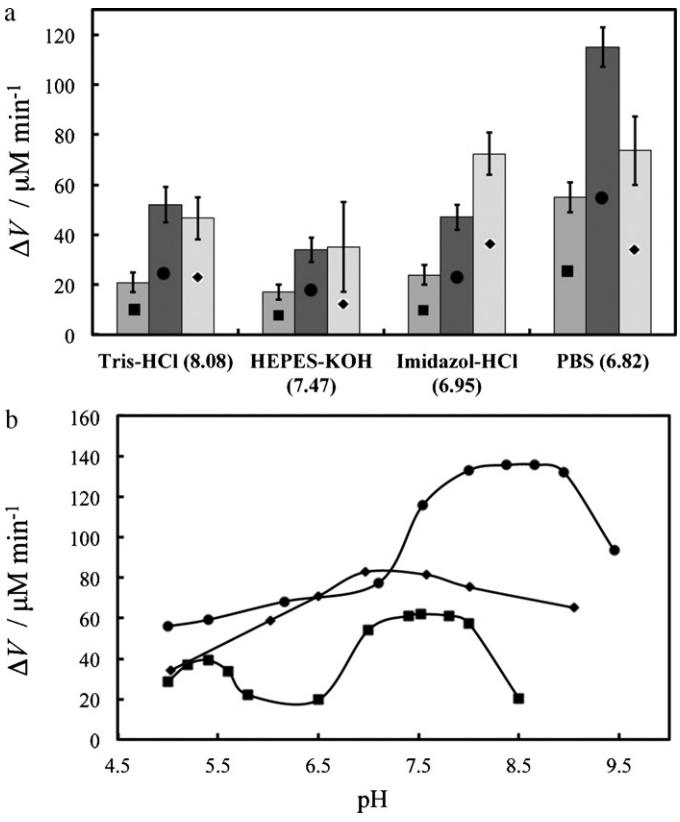


Fig. 3. The dependence of the rates of DA (■), AD (●), and MD (♦) oxidation on the nature (a) and pH (b) of buffer solution (in the brackets – the value of pK_a of a buffer-forming compound; pH 7.0 (a); concentrations: DA (AD, MD) – 100 μM, HRP – 600 nM; T_4 – 40 μM; H_2O_2 – 0.4 mM; $P = 0.95$, $n = 3$ (a)).

Table 2
The optimal conditions for carrying out the indicator reactions of DA, AD, and MD oxidation (0.1 M PBS).

Catecholamine	λ_{max} (nm)	pH	Concentration		
			HRP (nM)	T_4 (μM)	H_2O_2 (μM)
DA	465	5.4	650	30	250
AD	480	8.6	150	15	100
MD	481	7.0	50	8	100

3.4. Mechanism of the enzymatic oxidation of the catecholamines in the presence of T_4

It is known that the enzymatic oxidation of one substrate may be accelerated as a result of the addition of easier oxidized substrate into the indicator system. This effect is associated with "the substrate–substrate activation" and is applied for the determination of slowly oxidized substrates of HRP [30]. However, the mechanism of the substrate–substrate activation assumes that both substrates, taking part in the indicator reaction, are oxidized. First, it seemed to be reasonable to explain the effect of T_4 on the oxidation of the catecholamines according to the mentioned mechanism. But our experiments have revealed that the molecules of T_4 are not oxidized under the optimum conditions used for carrying out the enzymatic reactions. No products of T_4 enzymatic oxidation are observed in the absorption spectra of the products of the indicator reactions in 1 min after mixing their components. Thus, the mechanism of the studied indicator processes seemed to differ from the substrate–substrate activation. The scheme of the proposed mechanism which is realized in the presence of T_4 in the peroxidase-catecholamine system is shown in Fig. 5. Takayama and Nakano [35] observed the similar mechanism while studying the reaction of reduced nicotinamide adenine dinucleotide (NADH) oxidation catalysed by HRP in the presence of T_4.

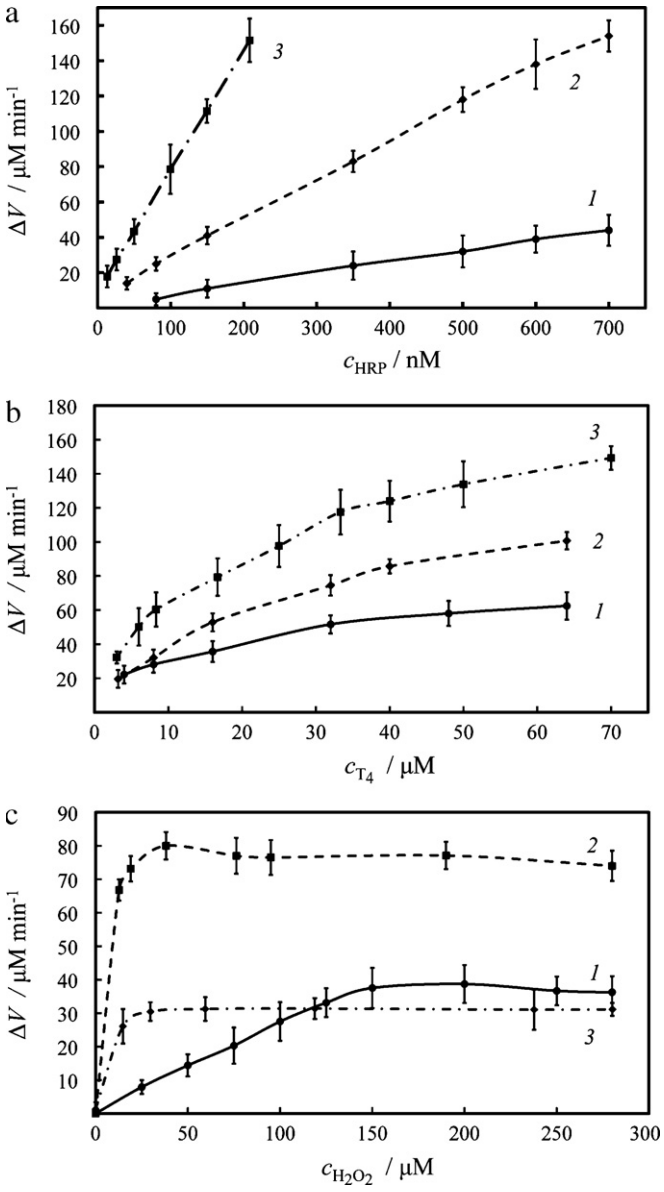


Fig. 4. The dependence of the rates of DA (1), AD (2), and MD (3) oxidation on concentrations of HRP (a), T_4 (b), and H_2O_2 (c) (0.1 M PBS, pH 5.4 (1), 7.0 (2), and 8.6 (3), concentrations: (a) catecholamine – 100 μM, HRP – from 15 to 700 nM, T_4 – 40 μM, H_2O_2 – 0.4 mM; (b) HRP – 650 (1), 150 (2), 50 (3) nM, catecholamine – 100 μM, T_4 – from 2 to 70 μM, H_2O_2 – 0.4 mM; (c) HRP – 650 (1), 150 (2), 50 (3) nM, catecholamine – 100 μM, T_4 – 30 (1), 15 (2), 8 (3) μM, H_2O_2 – from 20 to 280 μM; $P = 0.95$, $n = 3$).

Our affirmation was confirmed by HPLC-MS analysis of various compositions of the components of the indicator reactions. The results are presented in Supplementary Data. Mass spectra of the initial substances and the products of their enzymatic oxidation proved that T_4 molecules were not oxidized. The products of the enzymatic oxidation of the catecholamines were found to be identical in the presence and absence of T_4. Thus, the mechanism presented in Fig. 5 more likely characterizes the transformation processes in the indicator reactions. Here, T_4 acts as stimulatory agent due to the formation and accumulation of free radicals required for the further oxidation of the catecholamines.

3.5. Enzymatic procedures for the determination of catecholamines

The enzymatic procedures for the determination of DA and AD in the presence of T_4 were developed under the revealed optimum conditions (Table 2). The experiments were carried out according to the procedure described in Section 2.3.3. The analytical parameters of the proposed procedures are summarized and compared with those of the existing spectrophotometric procedures in Table 3.

Table 3 shows that the lower limits of DA and AD determination in the presence of T_4 are 10 and 5-fold (respectively) lower, than those in its absence. However, the procedure for MD determination is not so sensitive though its sensitivity is compared with that for some spectrophotometric procedures (Table 3). In addition, its reproducibility is poor even at high concentrations of MD (RSD – 21% at 100 μM, 24% at 400 μM). Nevertheless, this procedure may be used for the determination of MD in some pharmaceutical preparations.

It should be noted that the proposed enzymatic procedures for the determination of DA and AD are superior to the existing spectrophotometric procedures in terms of sensitivity in 2 and 5 times (Table 3), respectively. The important advantages of the proposed procedures are their simplicity and rapidity (the analysis time does not exceed 15 min); the equipment for analysis is also simple.

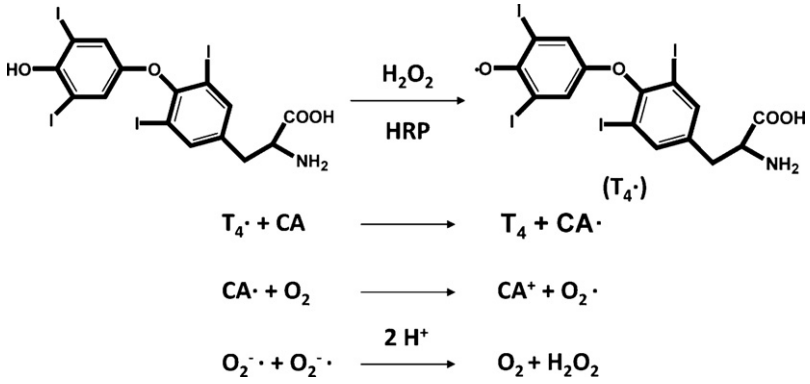


Fig. 5. The mechanism of the oxidation of catecholamines catalysed by HRP in the presence of T_4 [35].

Table 3
Analytical characteristics for DA, AD, and MD determination using the reaction of their oxidation by H_2O_2 catalysed by HRP and T_4 and their comparing with existing enzymatic and spectrophotometric procedures.

Reagents	Analyte	Applicable concentration range (μM)	λ_{max} (nm)	Remark	Ref.
Enzymatic					
HRP and T_4	DA	0.5–300[a] 5–300[b]	465	Total time of analysis is less than 15 min	Our work
	AD	4–300[a] 20–250[b]	480		
	MD	100–400[a]	481		
p-Chlorophenol and 4-aminoantipyrine	DA	5–50	505	Required a long period of time	[27]
	AD	10–45	505		
Poliphenol oxidase	DA	200–6000	470	I^-, SO_3^{2-} interfere seriously	[40]
	MD	200–6000	480		
Spectrophotometric					
$NaIO_4$ and 4-aminobenzoic acid	DA	1.3–113	458	Time-consumption photometric reaction	[9]
	MD	2.4–95	460		
Diazotised sulphanilamide with molybdate ions	DA	2.7–38.2	500	Waiting to develop colour	[41]
	AD	2.4–33			
	MD	2.4–33			
Sulphanillic acid	DA	4–100	475	Required a methanol solution, laborious reaction	[42]
	AD	5–150	475		
	MD	4–80	507		
K_2CrO_4 and sulphanillic acid	DA	13–72	495	Products are unstable	[43]
	MD	7–190	490		
NBS and isoniazide	DA	50–200	490	Time-consumption photometric reaction	[8]
	MD	10–350	480		[44]
Barbituric acid	MD	50–1200	593	Heating at 100 °C for 20 min	[45]
4-Aminoantipyrine and $[Cu(NH_3)_4]^{2+}$ ions	DA	310–2700	505	Laborious photometric reaction	[46]

[a] In the presence of T_4 calibration equations: $y = (0.9 \pm 0.1)\,x - (1.5 \pm 0.4)$ for DA (RSD, 8%), $y = (1.4 \pm 0.2)\,x + (1.4 \pm 0.4)$ for AD (RSD, 10%), and $y = (0.04 \pm 0.01)\,x + (7 \pm 1)$ for MD (RSD, 25%), where, $y = V_0$ (μM min^{-1}); $x = c_{DA}$, c_{AD}, or c_{MD} (μM).
[b] In the absence of T_4.

3.6. Analysis of pharmaceuticals

The developed procedures were applied for the determination of DA and AD in some pharmaceutical formulations. Preliminarily, the influence of other widespread components, which can occur as additional constituents in the pharmaceuticals containing DA and AD, on the rate of the indicator reactions was examined. It should be noted that a single pharmaceutical formulation could not contain more than one catecholamine. The results are shown in Table 4. The tolerance was defined as the concentration ratio of foreign species that produced an error exceeding 5% in the value of the reaction rate. In the case of MD determination in tablets all other existing components of the pharmaceutical preparation were not soluble in water and were separated by filtration.

The additional components were found not to cause fluctuations of the analytical signal at the level of their concentrations in pharmaceuticals. Thus, the proposed procedures for the determination of DA and AD are free of interference of compounds at the level of their concentration in pharmaceutical formulations. The pretreatment and analysis of pharmaceuticals were performed according to the procedures described in Sections 2.3.2 and 2.3.4. The results of

Table 4
Effects of the supplementary components of the pharmaceutical preparations on the determination of 10 μM DA and AD in the presence of T_4 using the proposed enzymatic procedures.

Analyte	Tolerance ratio: supplementary component/catecholamine	Supplementary component
DA	1×10^2	NaCl
	10	Sucrose, fructose, lactose
	2	$Na_2S_2O_5$, L-cysteine
AD	1×10^3	NaCl, lidocaine
	10	Sucrose, fructose, lactose
	2	$Na_2S_2O_5$

Table 5
The results of the determination of catecholamines in pharmaceuticals by the enzymatic and HPLC-MS methods ($P = 0.95$, $n = 5$).

Pharmaceutical (producer)	Found		Producer's data
	Enzymatic	HPLC-MS	
"Dopamine – Ferein"		g	
(Ferein, Russia)	4.6 ± 0.2	5.2 ± 0.5	5
"Xylocaine 2% mit adrenaline 1:200,000"		$\mu g\,ml^{-1}$	
(AstraZeneca GmbH, Germany)	5.1 ± 0.3	4.8 ± 0.4	5
"Dopegyt"		μg	
(Egis, Hungary)	239 ± 13	245 ± 16	250

the determination of DA, AD, and MD by the developed peroxidase-based procedures are given in Table 5.

Table 5 shows that the results of the determination of DA, AD, and MD in pharmaceuticals obtained by the enzymatic procedures are in a good agreement with those obtained by HPLC-MS and Producer's data.

4. Conclusions

The source of plant peroxidase isolation and the buffer solution nature were shown to play important roles in the oxidation of the studied catecholamines. For the first time, the stimulatory effect of L-thyroxine was applied for the enzymatic determination of dopamine, adrenaline and α-methyldopa. The results demonstrate that the application of such original for biochemical methods approach as a stimulatory effect of hormones expands the possibilities of enzymatic analysis, especially in those cases when analytes (enzyme substrates) are slowly oxidized and their oxidation products are characterized by low absorptivity.

Such advantages of the developed enzymatic procedures as their simplicity, rapidity, and high sensitivity are attractive for their application in analysis of different pharmaceutical forms (injections, tablets) containing an active component of a drug (some catecholamine) over its concentration range of 1 μM–1 mM. The sensitivity of the proposed enzymatic procedures is higher or comparable to the sensitivity of the determination of catecholamines employing electrochemical biosensors (with just one exception [23] where laboratory-isolated enzyme preparation was used).

Acknowledgements

This work was done with the financial support of Ministry of Education and Science of Russian Federation (contract No. P868 from 2010, 25 May) and Russian Foundation for Basic Research (grant No. 09-03-00823-a).

The authors thank Prof. Dr. A.V. Pirogov and Dr. E.B. Pashkova (Analytical Chemistry Division of Moscow State University) for their help in the determination of the catecholamines in pharmaceuticals by HPLC-MS.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.01.074.

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