Analysis of Some Phenothiazine Drugs and Their S-Oxides using Enzymatic Method of Cholinesterase Inhibition

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In this work, a highly sensitive spectrophotometric method was developed for the determination of cholinesterase (AChE) inhibitors - phenothiazine derivatives (PhTs). The assay involves spectrophotometric measurement of a mixture of buffer, solutions of acetylcholine (Ach), a sample containing AChE, H_2O_2, and PhTs. The rate of the enzymatic hydrolysis of Ach reaction was determined by the tangent method of the linear part of the kinetic curve in the A (358 nm) – t-coordinate. Results from the determination of PhTs and S-oxide PhTs with the AChE regulated spectrophotometric system showed the limit of quantitation of 5 ng/mL (IE_{20}) and a linear dynamic range from 5 to 30 ng/mL for Chlorpromazine, Promethazine and from 0.5 to 10 ng/mL for Chlorpromazine S-oxide, from 1 to 10 ng/mL for Promethazine S-oxide, 12 to 40 ng/mL for Thioridazine 2,5-disulfoxide respectively. RSD for concentrations of PhTs as low as $1.5 \cdot 10^{-8}$ mol/L did not exceed 6.7%, while their corresponding sulfoxides at $1.50 \cdot 10^{-9}$ didn't exceed +6.5%. $\delta < +3.8\%$.
Findings in this work demonstrate that this method may be used for the determination of phenothiazine based drugs, and sensitive tests for rapid PhT monitoring without the addition of other exogenous catalysts.

Keywords: analytical method, assay, phenothiazine derivatives, S-oxides, cholinesterase, pharmaceutical analysis, spectrophotometric determination

Phenothiazines are an important group of neuroleptics used in the treatment of moderate and severe mental and emotional conditions. They are also known for their antiemetic, anaesthetic, analgesic, sedative and antihistaminic effects. Although the exact mechanism of phenothiazine neuroleptics is currently unknown, scientists believe they may work by blocking the action of dopamine in the brain. Phenothiazine neuroleptics are used when patients do not respond to other neuroleptics [1].

Two phenothiazine derivatives (PhTs)and three metabolites have been studied in this work, namely: Chlorpromazine (CPZ) "3-(2-chloro-phenothiazin-10-yl)-N,N-dimethylpropan-1-amine hydrochloride", Promethazine (PTZ) "N,N-α-trimethyl-10H-phenothiazine-10-ethanamine; hydrochloride", Chlorpromazine 5-sulfoxide base (CPZ S-oxide) "3-(2-chloro-5-oxophenothiazin-10-yl)-N,N-dimethylpropan-1-amine", Promethazine 5-sulfoxide base (PMZ S-oxide) "N,N-dimethyl-1-(5-oxophenothiazin-10-yl)propan-2-amine", Thioridazine disulfoxide (THZ 2S, 5S-dioxide) "10-[2-(1-methylpiperidin-2-yl)ethyl]-2-methylsulfinylphenothiazine 5-oxide; 10-[2-[(2RS)-1-Methylpiperidin-2-yl]ethyl]-2-(methylsulfinyl)-10H-phenothiazine 5-Oxide" (Mesoridazine 5-Sulfoxide). Their chemical structures are shown in Figure 1.

Chlorpromazine hydrochloride is the most important compound in the large group of phenothiazine derivatives. Chlorpromazine and many other phenothiazine derivatives, are very lipophilic molecules that readily bind with membranes and proteins. Approximately 95-98 % of the drug is bound in the plasma; 85 % of the drug is bound to the plasma protein albumin. Highest concentrations of the drug can be found in the brain, lung, and other tissues that receive a high supply of blood.

Phenothiazines undergo a series of metabolic transformations in the organism. The corresponding sulfoxide derivatives (S-oxide PhTs) are the major human urinary metabolites. Like other phenothiazines, it is easily oxidized in an acidic medium by the action of various oxidizing agents, which leads to the formation of highly colored oxidation products. In aqueous solution in the presence of oxygen, a colorless chlorpromazine sulfoxide is formed as the final product.

Both sulfur atoms present in thioridazine, are susceptible to S-oxidation. Cytochrome P450IID6 (CYP2D6) is involved in the formation of thioridazine 2-sulfoxide (2-SO) from thioridazine and probably in the formation of thioridazine 5-sulfoxide (5-SO), but not in the formation of thioridazine 2-sulfone (2-SO2) from thioridazine 2-SO [2].

Due to the clinical importance, it is pertinent to establish a simple and sensitive method for the determination of PhTs and their main metabolites in biological fluids.

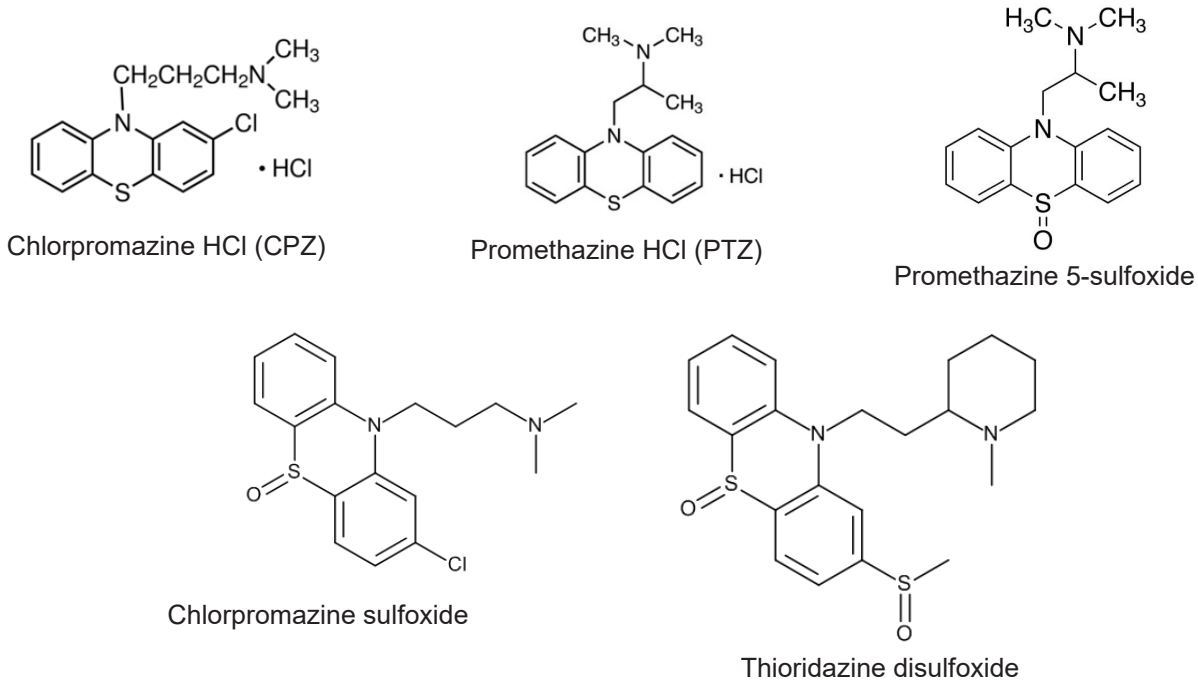

Figure 1: Chemical structures of phenothiazine derivatives and three major metabolites.

Chlorpromazine, Promethazine and Thioridazine, like other phenothiazines, are very aromatic and have intense UV absorption and fluorescent characteristics, which have been successfully used in spectrophotometric and spectrofluorimetric methods of analysis [3-15], as well as in the sensitive and specific determination of a drug by HPLC using UV and fluorescent detectors [16-19].

Thioridazine can be spectrophotometrically determined in urine after extraction. Some of the non-conjugated metabolites of chlorpromazine and thioridazine can be detected and determined by spectrofluorometric method. The drug is extracted with heptane, reextracted into acetic acid and then oxidized with hydrogen peroxide to the corresponding fluorophores. The measurement is carried out against a blank of plasma; for thioridazine λ_{ex}: 355 nm, λ_{em}: 430 nm, and analysis at 370-480 nm. This procedure distinguishes between the common phenothiazines but not between a drug and its metabolites in all cases. A similar spectrofluorimetric method for quantification of thioridazine and mesoridazine in plasma, and urine after treatment with 0.1 mol L^{-1} H_2SO_4 and 0.1% $KMnO_4$; λ_{ex}: 355 nm and λ_{em}: 440 nm has been described [20, 21].

Some other analytical methods have been explored by scientists for same purpose [22-34]. Although phenothiazine pharmaceuticals are the most prescribed drugs worldwide, in recent years [35-37], their final state in the environment has received little attention compared to micropollutants of other pharmaceuticals.

Numerous products were found after irradiation of thioridazine dissolved in pure water. Two main were identified as 5-sulfoxide and 2-sulfoxide [38].

Chromatographic methods, especially HPLC, are methods of choice when conducting research on degradation of pharmaceuticals. Gas chromatography is used rarely because most of the drugs possess limited volatility – in such cases derivatization (transformation into more volatile substance) may be necessary [39].

Phenothiazine derivatives such as chlorpromazine and thioridazine have been reported to inhibit cholinesterases [40, 41]. Generally, most of these compounds are selective inhibitors of butyrylcholinesterase [42-47].

Biochemical methods for the determination of cholinesterase inhibitors also deserve special attention. Methods have been developed and the possibility of quantitative determination of some phenothioazine derivatives by their inhibitory activity in the biochemical reaction of hydrolysis of the enzyme cholinesterase has been demonstrated [48, 49].

Among the above-reported methods, some quantify compounds of interest with the aid of sophisticated instruments like LC-MS, GC-MS, NMR etc. However, the cost of these analyses for routine check is very high. While some vary in the degree of stability-sensitivity, others are expensive and time consuming. Therefore, it is important to develop a new simple and sensitive method for determination of PTZ and their S-oxides.

Our recent work focused on the study of anticholinesterase activity of some phenothiazine derivatives using a developed biochemical spectrophotometric method [50].

The aim of the present study was to develop

a new simple kinetic spectrophotometric method suitable for the determination of phenothiazine derivatives and their major metabolites based on their anticholinesterase activity.

Materials and methods

Acetylcholine Chloride (Pharm Grade) - 0.02 g per amp/5 mL, produced by "VECTOR" – State Science Centre of virology and biotechnology in Russian Federation" (Russia).

Sodium Phosphate dibasic, $Na_2HPO_4 \cdot 12H_2O$ (puriss.), CAS -7558-79-4, produced by «ReaChem», Kharkiv, Ukraine.

Dry protein drug of cholinesterase from horse serum - 80 mg / fL (VI class), 27 AU/mg, produced by SMU "Biomed", Russia.

Remark: The catalytic activity of 1 unit (U) has such amount of the given enzyme preparation which coverts 1 μmole of the given substrate in 1 min at the given reaction conditions.

"Stabilized Hydrogen Peroxide 30-40 %", (puriss.), (LLC "Inter - Synthes", Boryslav, Ukraine); The content of hydrogen peroxide was determined by the State Pharmacopoeia of Ukraine (SPU) according to the monograph "High-test hydrogen peroxide solution 27.5-31.0%. High purity double distilled water.

Chlorpromazine hydrochloride ≥ 98 % (TLC); CAS Number: 69-09-0 – Sigma-Aldrich. $C_{17}H_{19}ClN_2S \cdot HCl$ (CPZ), 2-Chloro-10-(3-dimethylaminopropyl) phenothiazine hydrochloride;

Promethazine hydrochloride 98 %; CAS Number: 58-33-3 – Sigma-Aldrich. $C_{17}H_{20}N_2S \cdot HCl$ (PMZ), 10-[2-(Dimethylamino)propyl]phenothiazine hydrochloride;

Thioridazine hydrochloride ≥ 99 %; CAS Number:130-61-0 – Sigma-Aldrich. $C_{21}H_{26}N_2S_2 \cdot HCl$ (THZ), 10-[2-(1-Methyl-2-piperidyl)ethyl]-2-(methylthio)-10H-phenothiazine hydrochloride.

The purity of S-oxides of Chlorpromazine and Promethazine was determined by High performance liquid chromatography method (HPLC).

Diperoxyhexanedioic acid was obtained by the acylation reaction of hydrogen peroxide with the appropriate carboxylic acids in the presence of sulfuric acid according to known method (see reference [51]). Diperoxyadipic acid, $HO_3C(CH_2)_4CO_3H$, 98%, Mp, 114.5 °C (dec.), AO,% (Theoretical), 17.5 (17.9), molecular weight (theoretical), g / mol 177 ± 2 (178.2);

Chlorpromazine S-oxide base, promethazine S-oxide base and thioridazine disulfoxide were synthesized by an analogous scheme as was obtained of diperoxyhexanedioic acid.

More detailed information on promethazine S-oxide, chlorpromazine S-oxide and thioridazine 2,5-disulfoxide can be found in "Blazheyevskiy, M.Ye; et al" [50].

Preparation of solutions: $5.0 \cdot 10^{-3}$ M of phenothiazine derivatives stock solutions was prepared daily by dissolving accurately weighed samples phenothiazine derivatives in 0.01 mol L^{-1} solution HCl (thioridazine - 96 % ethanol) and diluting the solution to 100 mL with double distillated water in a 100-mL volumetric flasks. Suitable aliquots of the stock solutions of of phenothiazine derivatives were transferred into separate glass-stopper tubes.

Working standard solutions (WSS) of phenothiazine derivatives were prepared from the stock solutions by appropriate dilution with double distillate water or 0.01 M hydrochloric acid. All solutions were stored at room temperature in a cool dark place.

Preparation of 0.2 mol L^{-1} Phosphate buffer solution (pH 8.35) 35.75 g Crystallized disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$) was dissolved in 500 mL flask using double-distilled water. 19 mL of 0.1 M solution of Hydrochloric acid solution was added. pH of the final solution was controlled potentiometrically.

Preparation of 10% (wt) Hydrogen peroxide solution: The solution was prepared by the appropriate high-test hydrogen peroxide dilution with double-distilled water. The content of hydrogen peroxide in the working 10% solution was determined by permanganatometric method.

Preparation of 1% (wt) p-Phenetidine hydrochloride solution: p-Phenetidine hydrochloride (*p*-Ph) was extracted from the base by Hydrogen Chloride precipitation in the chloroform solution. 1.00 g of *p*-phenetidine hydrochloride was dissolved in 80 mL of double-distilled water in 100 mL volumetric flask and then brought to the mark.

Preparation of Acetylcholine chloride solution (ACh): The ampoule's content of 0.02 g pharmacopoeia drug Acetylcholine Chloride was dissolved in 20 mL of double-distilled water.

Preparation of Cholinesterase *solution (ChE):* The ampoule's content of 80 mg was dissolved in 20 mL of double-distilled water.

General recommended procedure:

The first part: 10.0 mL portion of 0.2 M phosphate buffer solution (pH = 8.35) was transferred into 20 mL graduated test tube with ground plug, 1.0 mL of 1 mg/mL acetylcholine solution was added and then 1.6 mL of 10 % hydrogen peroxide solution was added and the stopwatch was switched on. After that the solution was shake thoroughly and was thermostated for 10 min. Then 0.5 mL of 1% *p*-Ph solution was added and brought to the mark 16 ml with double distilled water in a 20 mL volumetric flask. The stopwatch was switched on and every minute each solution was scanned spectrophotometrically (λ=358 nm) for 15 min and 1.0 cm cuvette were used. The rate of reaction was determined as a slope of the kinetic curve A *vs* time [(ACh + H_2O_2) + *p*-Ph] ($tg\alpha_0$, min^{-1}).

The second part: 10.0 mL portion of 0.2 M phosphate buffer solution (pH = 8.35) was transferred into 20 mL graduated test tube with ground plug and 1.0 mL of 1 mg/mL acetylcholine solution was added. Next, 0.5 ml portion of cholinesterase was added, and then

1.6 mL of 10 % hydrogen peroxide solution was added while stirring. The mixture was shaken thoroughly and kept for 10 min in a thermostat. Then 0.5 mL of 1 % *p*-Ph solution was added and brought to the mark 16 ml with double distilled water. The stopwatch was switched on and the solution was scanned spectrophotometrically (λ=358 nm) every minute for 15 mins in a 1.0 cm cuvette. Buffered solution with double distilled water was used as reference solution. The rate of reaction was determined as a slope of the kinetic curve A *vs* time: $(ChE + ACh)_t + H_2O_2 + p$-Ph, ($min^{-1}$) switched on a stopwatch, and thermostated for 10 min, [(ChE + ACh) + H_2O_2 + *p*-Ph] tgα (min^{-1}).

The third part (method for quantitative determination of inhibitors): 10.0 mL portion of 0.2 M of phosphate buffer solution (pH = 8.35) was transferred into 20 mL graduated test tube with ground plug. The accurate volumes (from 0.40 to 3.20 ml) of test solution of Inhibitor (WSS *solution*) were added into a standard flask. 0.5 mL of cholinesterase was added while stirring, the stopwatch was switched on. Every solution was shaken thoroughly and thermostated for 10 min. 1.0 mL of 1 mg/mL acetylcholine solution was quickly added, shaken and thermostated for 10 min. 1.6 mL of 10 % Hydrogen peroxide solution was then added, kept for 10 min in thermostat. 0.5 mL of 1% *p*-Ph solution was added and brought to the mark 16 ml with double distilled water. Using the stopwatch the solution was scanned spectrophotometrically (λ=358 nm) every minute for 15 minutes in a 1.0 cm cuvette. Buffer solution with double distilled water as reference solution was used. The rate of the reaction was determined as a slope of the kinetic curve A *vs* time) [(ChE + Inh) + Ach] +H_2O_2 + *p*-Ph], $tg\alpha_i$ (min^{-1}).

The inhibiting efficiency (IE, %) of the enzymatic hydrolysis of acetylcholine in the presence of PhT derivatives was determined by the following equation:

$$IE\ (\%) = ((tg\alpha_i - tg\alpha)/(tg\alpha_0 - tg\alpha)) \cdot 100,$$

where $tg\alpha_i$ (min^{-1}) is the tangent of the angle tangent to the linear part of the kinetic curve in the coordinates A *vs* t (a slope of the kinetic curve) for the reaction [(ChE + Inh) + ACh] +H_2O_2 + *p*-Ph] in the presence of AChE and an inhibitor;

$tg\alpha_0$ is the tangent of the angle to the linear part of the kinetic curve in the coordinates A *vs* t (a slope of the kinetic curve) for the reaction [(ACh + H_2O_2) + p-Ph] in the absence of AChE and inhibitor;

tgα is the tangent of angle of the linear part of the kinetic curve in the coordinates A vs t (a slope of the kinetic curve) for the reaction [(ChE + ACh) + H_2O_2 + *p*-Ph] in the presence of AChE and the absence of an inhibitor.

Measurements of absorbance of solutions were performed in a 1 cm cuvette on an Evolution 60S UV-Visible Thermo-Scienticfic Spectrophotometer (USA) against double-distilled water (compensation solution).

Measurements were performed at +37 °C, the temperature of the reaction mixture was maintained by its thermostating in water, pH of the solutions was monitored potentiometrically using a glass electrode.

The 200 MHz proton magnetic resonance spectra of S-oxides of phenothiazine derivatives (dissolved in DMSO-d6) were recorded on a Varian XL-200 Spectrometer.

Measurements were performed at +37 °C, the temperature of the reaction mixture was maintained by its thermostating in water, pH of the solutions was monitored potentiometrically using a glass electrode.

Results and discussion

Detection is based on the fact that ACh catalyzes the oxidation of the substrate 4-Ethoxyaniline (p-Phenetidine) (p-Ph) by H_2O_2 to form a yellow-brown product (4,4'-Azoxyphenetole) with an absorption peak at 358 nm, but that oxidation is suppressed if ACh previously is hydrolyzed by AChE to form choline and acetic acid. In the presence of inhibitor, the activity of AChE is inhibited, thereby inducing the recovery of the yellow-brown coloration. Based on these findings, a highly sensitive method is herein developed for the determination of AChE and its inhibitors. The assay only requires mixing of buffer, solutions of ACh, a sample containing AChE, H_2O_2 and p-Ph and spectrophotometric measurement. The rate of the enzymatic hydrolysis of Ach reaction was determined by the tangent method of the linear part of the kinetic curve in the A (358 nm) – t coordinate.

This developed analytical system for the determination of AChE inhibitors using coupled reactions of ACh perhydrolysis and peroxyacid oxidation of p-Ph as an indicator substance is summarized in Figure 2.

As an example, the kinetic curves of the indicator reaction of p-Ph oxidation in the system (Ach- (AchE + Inh) -H_2O_2-p-Ph) depending on the concentration of chlorpromazine is presented in Figure 3. Characteristics of methods for the determination of some phenothiazine derivatives and their S-oxides influencing anticholinesterase activity are presented in table 1.

The obtained values of IE_{20} inhibition efficiency for sulfoxides of the corresponding phenothiazine derivatives were an order of magnitude lower than those of the corresponding phenothiazine derivatives. Due to the AChE-catalyzed hydrolysis of acetylcholine, the peroxidase-like activity was altered, which was used for the highly sensitive determination of AChE activity. Furthermore, due to inhibition by some phenothiazine drugs (PhTs) and their S-oxides (S-oxide PhTs) on AChE, PhTs and S-oxide PhTs were also determined with the AChE regulated spectrophotometric system yielding LOQ of 5 ng mL^{-1} (IE_{20}) and a linear dynamic range from 5 to 30 ng mL^{-1} for Chlorpromazine, Promethazine and from 0.5 to 10 ng mL^{-1} for Chlorpromazine S-oxide, from 1 to 10 ng mL^{-1} for Promethazine S-oxide, 12 to 40 ng mL^{-1} for Thioridazine 2,5-disulfoxide respectively (see Table 1).

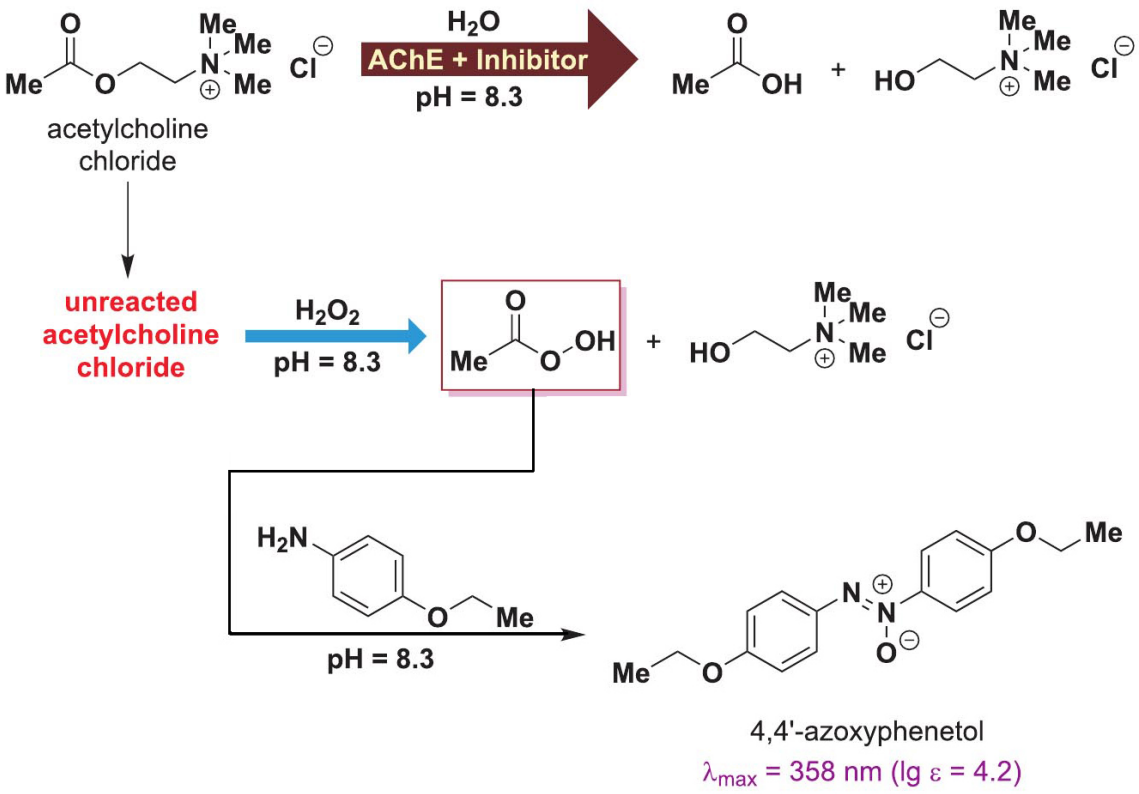

Figure 2. Analytical system for the determination of AChE inhibitors using coupled reactions of ACh perhydrolysis and peroxyacid oxidation of *p*-Ph as an indicator substance.

Table 1. Characteristics of methods for determining some phenothiazine derivatives and their S-oxides by anticholinesterase activity.

Test substance	Linear dynamic range of concentrations/ ng mL^{-1}	Linear regression equations (tg α, min^{-1} vs c, mol L^{-1})	LOQ (IE_{20})/ ng/mL
Chlorpromazine hydrochloride	5 to 30	tg α = (1.77±0.64)·10^{-2} log *c*+ (15.3±4.9)·10^{-2} (r=0.995)	5
Promethazine hydrochloride	5 to 30	tg α=(2.18±0.73)·10^{-2} log *c*+ (17.9±5.4)·10^{-2} (r=0.999)	5
Chlorpromazine S-oxide	0.5 to 10	tg α = (1;60±0.79)·10^{-2} log *c*+ (15.5±6.5)·10^{-2} (r=0.987)	0.5
Promethazine S-oxide	1 to 10	tg α = (1.57±0;52)·10^{-2} log *c*+ (14.3±4.2)·10^{-2} (r=0.995)	1
Thioridazine 2,5-disulfoxide	12 to 40	tg α =(1;46±0;60)·10^{-2} log *c*+ (12.1±4.3)·10^{-2} (r=0.991)	12

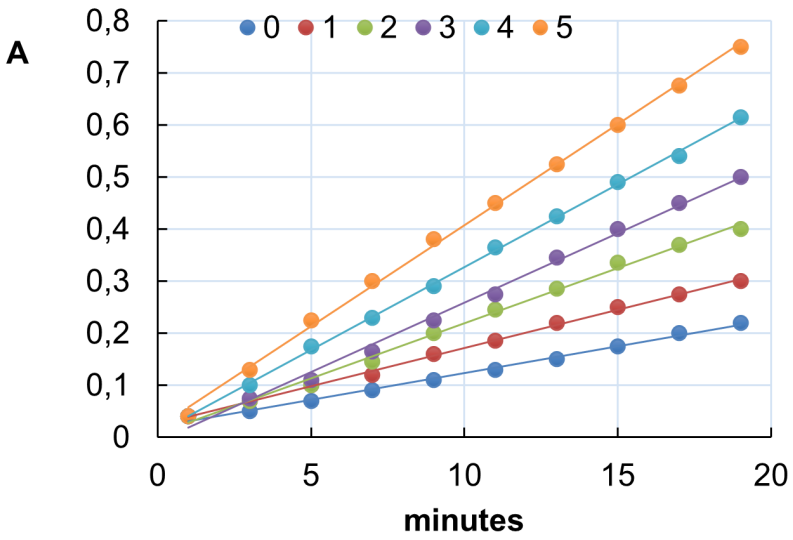

Figure 3. Kinetic curves of the *p*-Ph oxidation indicator reaction in the system. (Ach-(AchE + Inh)-H_2O_2-p-Ph) depending on the concentration of Inhibitor(Chlorpromazine S-oxide), ng mL^{-1}: 0 – blank reagent (Ach-(AchE)-H_2O_2-p-Ph) (c(Inh)=0); 1 – 0.53; 2 – 1.00; 3 – 1.90; 4 – 5.3; 5 - (Ach-H_2O_2-p-Ph) (control ACh).

Statistical processing of results of the analysis of cholinesterase inhibitors of phenothiazine derivatives are presented in Table 2. As seen, percentage systematic error (δ) is less than $t_\alpha \times RSD/n^{1/2}$, which indicates the correctness (accuracy) of the results. RSD for concentrations of CPZ and PMZ $1.5 \cdot 10^{-8}$ mol L^{-1} does not exceed 6.7%, and for their corresponding sulfoxides for concentrations $1.50 \cdot 10^{-9}$ and $3.10 \cdot 10^{-9}$ mol L^{-1} – +6.5%. For Thioridazine 2,5-disulfoxide at a concentration of $3.00 \cdot 10^{-8}$ mol L^{-1} RSD is 6.45%.

Table 2. Statistical output of results from the analysis of phenothiazine derivatives cholinesterase inhibitors.

Metrological characteristics	Cholinesterase inhibitors				
	Chlorpromazine hydrochloride	Promethazine hydrochloride	Chlorpromazine S-oxide	Promethazine S-oxide	Thioridazine 2,5-isulfoxide
μ[1], mol L^{-1}	1.50×10^{-8}	1.50×10^{-8}	1.50×10^{-9}	3.10×10^{-9}	3.00×10^{-8}
$\bar{x}$, mol L^{-1}	1.56×10^{-8}	1.55×10^{-8}	1.53×10^{-9}	3.20×10^{-9}	3.10×10^{-8}
±S	1.00×10^{-9}	0.90×10^{-9}	1.00×10^{-10}	1.80×10^{-10}	2.00×10^{-9}
$\pm\Delta\bar{x}$	1.20×10^{-9}	1.11×10^{-9}	1.24×10^{-10}	2.25×10^{-10}	1.00×10^{-10}
RSD	6.7	6.0	6.5	5.6	6.45
δ[2]	+3.8	+3.3	+2.0	+3.2	+3.3

Note: [1]μ - true value (taken for analysis); [2]$\delta = (\bar{x}-\mu)100\%/\mu$.

Conclusion

A new sensitive and selective spectrophotometric method for the quantitative determination of acetylcholinesterase inhibitors (phenothiazine derivatives) has been developed. Results suggest that this method is applicable in the determination of phenothiazine antipsychotic drugs in model solutions. The sensor platform can also be implemented on test sensors for rapid PhT monitoring. Thus, this extremely simple spectrophotometric method, without the addition of other exogenous catalysts, has great potential for the determination of phenothiazine neuroleptics in situ and can be further used for sensitive applications in areas related to environmental protection and food safety, as well as for chemical-toxicological analysis of biological fluids.

Conflict of Interest

No conflict of interest.

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