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ІНСТИТУТ ОРГАНІЧНОЇ ХІМІЇ НАН УКРАЇНИ
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У журналі розглянуто проблеми синтезу й аналізу органічних та елементоорганічних сполук, аналогів природних сполук і лікарських субстанцій, наведено результати фізико-хімічних досліджень у вищезазначених напрямах. Також з погляду (біо)органічної, фармацевтичної, аналітичної та фізичної хімії проаналізовано питання з різних аспектів рослинництва, ґрунтознавства й дослідження навколишнього середовища.

Для працівників науково-дослідних установ, вищих навчальних закладів та фахівців хімічного, фармацевтичного, біологічного, медичного і сільськогосподарського профілів.

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New phosphorus-containing polycycles with a spiroamine group

Abstract

Aim. To synthesize hexahydrospiro[cyclopropane-1,10'-pyrido[1,2-*c*]quinazoline] and 2-λ^5-benzo[*f*][1,4,2]diazaphosphepine derivatives – new N-P containing heterocyclic compounds with the 6-azaspiro[2.5]octane fragment.

Results and discussion. A new analog of the powerful electrophilic reagent – "Alder dimer" – was obtained from the interaction of triflic anhydride and spiro(4-cyclopropane) piperidinyl formamide, and further used to synthesize new *N*'-P^V- and P^{III}-substituted *N*'-phenyl, *N*''-hexahydro(azaspiro)octylformamidinium salts – precursors of acyclic N-phosphorylated diamino carbenes with a spiroamine group. It has been shown that acyclic N-phosphorylated diaminocarbenes are transient species affording various products. The structure of the final product is primarily determined by nature of the phosphorus-bearing substituent, namely a phosphoryl or phosphino-group. *N*-P^V-substituted carbene undergoes a 1,2-phosphorus shift with the formation of (selenophosphoryl)formamidine in a high yield. For *N*-P^{III}-substituted carbene a compatible 1,3-*H* shift also occurs with the formation of an intermediate azomethine ylide converted into a new heterocyclic system – hexahydrospirocyclopropane -1,10'-pyrido[1,2-*c*]quinazoline. Under the action of acid an unexpected further expansion of the 6-member ring occurs with the formation of a diazepine derivative.

Experimental part. The reaction of Alder reagent with N-P^V-seleno phosphoryl arylamides afforded N-phosphorus substituted formamidinium salts, which are easily reduced to P^{III} analogues. In addition to the corresponding formamidines, the new *N*-phosphorylated spiroamine-containing polycyclic system was isolated from the reaction mixture formed by the deprotonation of such salts with a strong non-nucleophilic base.

Conclusions. The Alder reagent approach allows synthesizing precursors of acyclic formamidine carbenes with the spiroamine group. Such carbenes are unstable. By converting these compounds *N*-P^{III}-substituted tetrahydropyrimidine and diazaphosphepine derivatives with the 6-azaspiro[2.5]octane fragment have been obtained for the first time.

***Keywords*:** transient acyclic carbenes; 1,2-phosphorus shift; 6-azaspiro[2.5]octane; diazaphosphepine; N-P bond

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Нові фосфоровмісні поліциклічні сполуки зі спіроаміногрупою

Анотація

Мета. Синтезувати похідні гексагідроспіро[циклопропан-1,10'-піридо[1,2-*c*]хіназоліну] та 2-λ^5-бензо[*f*][1,4,2]діазафосфепіну – нові гетероциклічні N-P-вмісні сполуки із фрагментом 6-азаспіро[2.5]октану.

Результати та їх обговорення. У результаті взаємодії ангідриду трифлатної кислоти з формамідом (спіроциклопропан)піперидину отримано потужний електрофільний реагент – новий аналог «димера Альдера», за допомогою якого синтезовано нові *N*'-P^V- та P^{III}-заміщені солі *N*'-феніл, *N*''-азаспірооктил формамідинію – прекурсори ациклічних *N*-фосфорильованих діамінокарбенів зі спіроаміногрупою. Виявлено, що останні не є стабільними сполуками і зазнають *in situ* перетворень з утворенням різних продуктів, будова яких визначається насамперед типом фосфоровмісного замісника: фосфорильна або фосфіно-групи. *N*-P^V-заміщений карбен зазнає 1,2-фосфорного зсуву з утворенням селенофосфорилформамідину з високим виходом. У *N*-P^{III}-заміщених карбенах відбувається також рівнобіжний 1,3-Н зсув з утворенням проміжного азометин іліду, який циклізується в нову гетероциклічну систему – гексагідроспіро[циклопропан-1,10'-піридо[1,2-*c*]-хіназолін]. Неочікуване подальше розширення 6-членного остова з утворенням похідної діазепіну відбувається під дією кислоти.

Експериментальна частина. Оригінальною реакцією реактиву Альдера з *N*-селенофосфорил ариламідами одержано *N*-фосфорил заміщені формамідинові солі, які легко відновлюються до P^{III}-аналогів. Окрім відповідних формамідинів,

нову N-фосфорильовану спіроаміновмісну поліциклічну систему було виділено з реакційної суміші, що утворюється за депротонування таких солей сильною ненуклеофільною основою.

Висновки. Використання підходу з реактивом Альдера дозволяє синтезувати прекурсори ациклічних формамідинових карбенів зі спіроаміногрупою. Такі карбени не є стійкими. Перетворенням цих сполук уперше отримано *N*-P^{III}-заміщений тетрагідропіримідин та похідні 1,2,4-діазадигідрофосфепіну з фрагментом 6-азаспіро[2.5]октану.

Ключові слова: транзитні ациклічні карбени; 1,2-фосфорний зсув; 6-азаспіро[2.5]октан; діазафосфепін; N-P-зв'язок

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■ Introduction

Since the early 1990s, there was the booster progress in *N*-heterocyclic carbenes chemistry owing to their excellent properties as ligands for transition metals [1, 2]. But today they rate far beyond privileged homogeneous catalysis transformations [3]. Their inherent structural and electronic features make them highly useful in different eminent areas of research, such as organometallic materials [4, 5], metallopharmaceuticals [6, 7] and as organocatalysts [8]. Alongside, carbenes still appear as reactive intermediate species in organic transformations, which could lead to rarely accessible heterocyclic compounds [9].

In our previous works, we presented a new type of acyclic diaminocarbenes: *N*-P^{III}- and P^{V}-substituted diaminocarbenes **A** (X = lone pair or Se) (Figure) [10–12]. It was found that diaminocarbenes **A** (X = lone pair) bearing bulky alkyl groups (R = *t*Bu or Ad) and N(*i*Pr)$_2$ were the most stable (Figure). Both increase and decrease in the size of the dialkylamino group N(R^2)$_2$ made them unstable leading to various transformations. Nevertheless, these P^{III} carbenes were characterized spectroscopically unlike their P^{V} congeners that gave C-phosphorylated formamidines **B** (X = Se) [10, 11]. The carbenes **A** (X = lone pair) featuring N(R^2)$_2$ groups (piperidino, pyrrolidino, azetidino and dimethylamino) were found to be unstable eventually producing a mixture of products. The major product in the mixture appeared to be *N*-P^{III}-substituted tetrahydroquinazolines [12]. Compared to N-heterocyclic carbenes, acyclic diaminocarbenes (ADCs) are known to lack a geometrical rigidity that drives for better σ-donor properties and facilitates in some cases the creation of a chiral environment. However, flexibility of these systems makes them less stable, and the σ-donicity strongly depends on their conformation [13, 14] with both controlled by steric encumbrance of the *N*,*N*'-substituents attached to the C^2 carbon centre. In addition to dimerization, ADCs are disposed to undergo different routes of decomposition. *β*-Fragmentation of stable [*bis*(diisopropyl)amino]carbene was reported by *Alder* [15]. The above-mentioned 1,2-migrations are described for both cyclic azole-based analogs and imidazolylidenes that have other heteroatom centered groups (B, Si, N) attached at the adjacent nitrogens [16–18]. Analogous transformation by C-H bond insertion is less known [19]. An example of cyclization into a novel bicyclic compound by an acid-promoted 1,3-isomerization of a stable aminocarbene into a transient azomethine ylide was reported by *Bertrand* [20]. Moreover, among the intramolecular cyclizations, which are rare ways to larger phosphorus heterocycles, ring-enlargement reactions with the insertion of phosphorous are not common. Among a few examples there is the

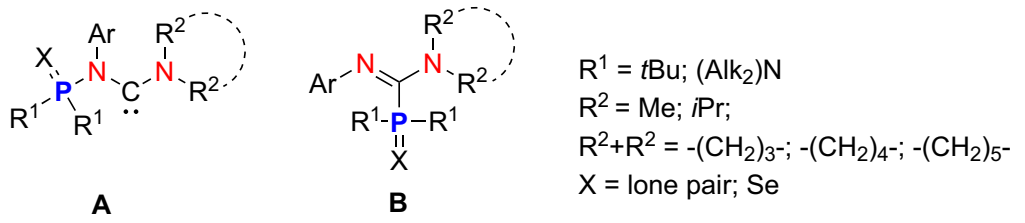

Figure. N-phosphorylated diaminocarbenes and C-phosphorylated formamidines

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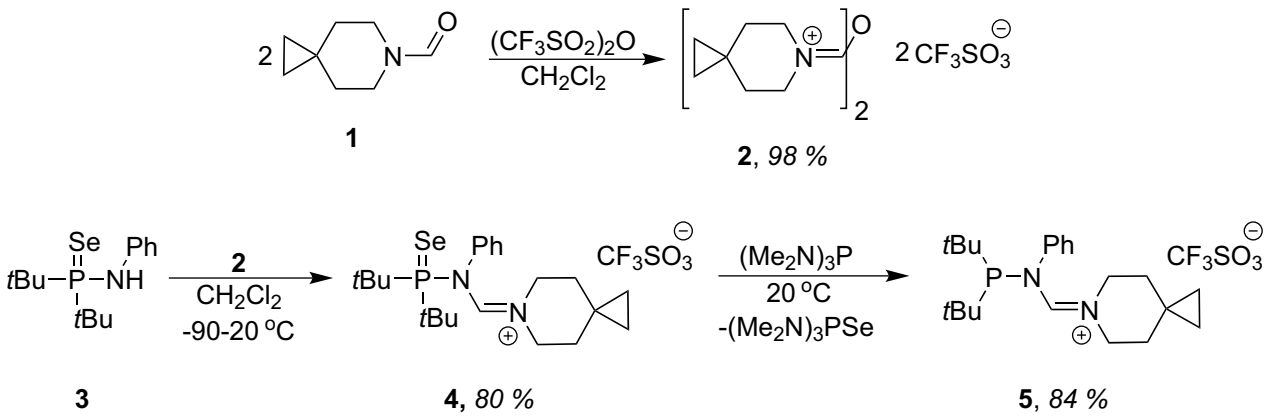


Scheme 1. The synthesis of P^{III} formamidinium salt

insertion of a phosphorus atom into the C-O linkage of oxazolidine that is a key step in the synthesis of α-amino phosphonic acids [21]. When benzylated 1-O-acetyl-D-hexopyranoses reacted with triethyl phosphite and trimethylsilyl trifluoromethanesulfonate as a catalyst the similar phosphorus insertion opened a way to seven-membered nucleoside phostones [22].

We were interested in using the method described above to obtain derivatives of higher complexity amines actually used as a structural fragment in the synthesis of therapeutic agents. With the growth of pandemic agents' activity in the world, the generation of new models of compounds with the biological activity does not lose its relevance. This work focuses on derivatives containing the residue of spirocyclopropyl piperidine – 6-azaspiro[2.5]octane. Namely, spiroamino compounds of this type are associated with the synthesis of the immune checkpoint inhibitor, chemokine receptor antagonists [23, 24]. Astra Zeneca synthesized such derivatives as a potent histamine H3 receptor antagonist [25]. It is believed that the antibacterial activity of DV-7751 correlate with the (*S*)-amino-6-azaspiro[3.4]octane moiety [26].

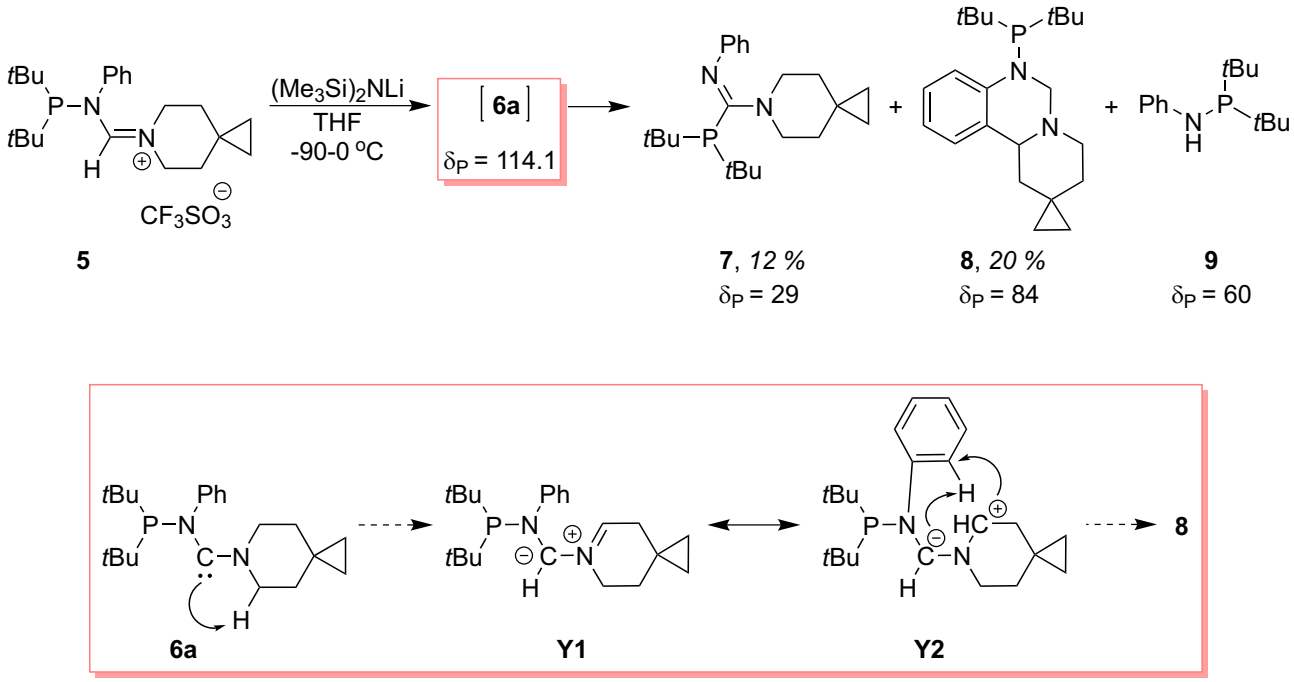
■ Results and discussion

Compound **2** is an analog of the Alder reagent bearing the residue of spirocyclopropyl piperidine. It can be quite easily synthesized from the available starting substances by the interaction of formamide **1** with triflic acid anhydride (Scheme 1) [27]. Mixing the reagents at the temperature range of -90–-70°C in dichloromethane gave a pure compound **2** that gradually precipitated as a colorless crystalline solid. It is well stored in a solid state at +4°C; if necessary, it can be crystallized from acetonitrile.

Previously, we showed that *N*-Aryl phosphinoselenoic amides **3** reacted readily with Alder dimers in a molar ratio of 1:1 giving hydrolytically stable P^{V}-*N*-substituted salts like **4** with a yield of more than 90% [12, 28]. Similarly, formamidinium triflate **4** was prepared in dichloromethane in a good yield (Scheme 1). The selenophosphoryl group of salt **4** was readily reduced when treated with hexamethylphosphorus triamide to give the corresponding *N*-P^{III}-substituted formamidinium salt **5** (Scheme 2). In ^{1}H NMR spectra both compounds had a weak signal at δ_{H} 8–9 ppm, which was distinctive for the N-CH=N– fragment. Equivalent signals as the most downfield doublet for **4** and singlet for P^{III}- **5** were present in their ^{13}C NMR spectra (δ_{C} 155–157 ppm). A distinctive quartet with a typical constant 320 Hz was observed for the CF_3-group.

As noted above, *N*-P^{V}- and P^{III}-substituted formamidinates like **4** and **5** meet the requirements as precursors of carbenes. However, it was found that regardless of the nature of the substituents at the adjacent *N*-atoms after deprotonation of *N*-P^{V}-substituted derivatives like **4**, the reaction mixture contained *C*-selenophosphoryl-formamidines when reaching the room temperature [10, 12]. In some cases, carbene **C** (P^{V}-) (Figure) was recorded using ^{31}P NMR at low temperatures.

As we were interested in the formation of phosphorus heterocycles, we studied directly deprotonation of P^{III}-derivatives. Like in the case of other formamidinium carbenes with cyclic secondary amino groups the formation of **6a** by addition of lithium hexamethyldisilazide to salt **5** was confirmed by ^{31}P NMR (δ_{P} = 114 ppm, -10°C) [12]. The final reaction mixture contained tetrahydroquinazolines **8**, as well as phosphanes **7** and tBu$_2$PNHPh (**9**). The compounds **8** and **7** were isolated, and their structure was confirmed by



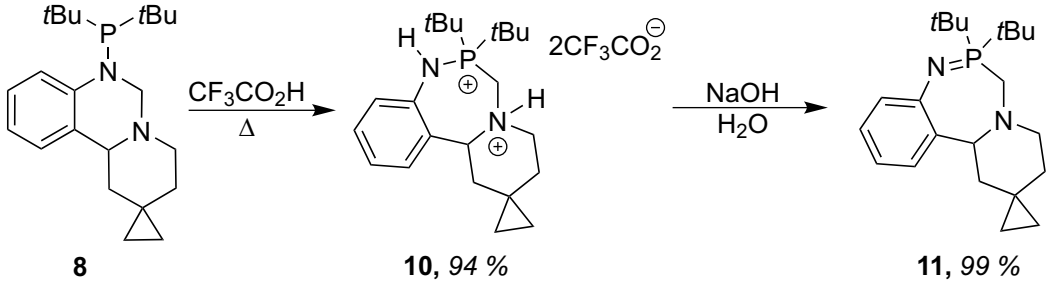
Scheme 2. Deprotonation of formamidinium salt **5**

NMR spectroscopy and mass-spectrometry. Amide **9** was identified by comparing the ^{31}P NMR signal with the tabular value. As seen in Table, the stability of carbene **6** as well as the content of the target cyclic product **8** is quite low compared to the previous results with azetidine (**6b**), pyrrolidine (**6c**) and piperidine (**6d**) [12]. Taking into account the mechanism of this cyclization earlier calculated according to DFT [12], aza-ylide **Y1** formed as an intermediate can be unstable in case of large secondary amines, such as 6-azaspiro[2.5]octane. As a result, the decomposition of carbene to phosphinous amide under the reaction conditions proceeds to a greater extent.

It is well known that amidophosphinous derivatives $(R^1)_2$P-N$(R^2)_2$ are readily hydrolyzed when treating with aqueous acids to give the corresponding phosphinic acids [29]. Similarly to the derivatives of quinazolines containing a pyrrolidine or piperidine fragment, the action of trifluoroacetic acid on tricyclic compound **8** led to the formation of phosphonium compounds **10**, but not to the hydrolysis of the P-N bond [30]. Subsequent addition of an alkali to a solution of such

Table. Properties of carbenes **6**

Compound No.	*t*Bu$_2$PNHPh Yield, %	Yield of **7**, %	Yield of **8**, %	$t_{1/2}$ of carbene **6**
6a	50	22	21	3 days (-4 °C)
6b	25	-	54	4 days (-4 °C)
6c	18	12	60	54 min (20 °C)
6d	30	23	46	75 min (20 °C)



Scheme 3. The ring enlargement reaction of compound **8**

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a salt also smoothly gave phosphazene **11** with a cycle size of one atom larger (Scheme 3). In favor of changes in the structure of these substances spectral data are indicative. Thus, in ^{31}P NMR spectra the signals of compounds **8**–**10**–**11** shifted naturally in a range of δ_P 85–69–42 ppm, in the area of a strong field corresponding to P^V compounds. The most downfield doublet observed in the ^{1}H NMR spectrum of salt **10** apparently corresponded to the ammonium proton and disappeared after the treatment of salt with a base. In ^{13}C NMR spectra of seven-membered cycles **10** and **11**, the signal of the methylene carbon atom of –N-$\underline{CH_2}$-N– group had a greater coupling constant and was shifted by almost 30 ppm to a strong field, reflecting a change in the coordination number of the phosphorus atom and the formation of the C-P bond. The appearance of additional conjugation of double bonds in the diazaphosphepine cycle of **11** was noticeably displayed by the change of chemical shifts of the benzene ring carbon atoms.

Conclusions

New *N*-P^{III}-substituted tetrahydropyrimidine and diazaphosphepine derivatives with the 6-azaspiro[2.5]octane fragment have been obtained as a result of inherent transformations of acyclic formamidinium carbenes.

Experimental part

All procedures with air- and moisture-sensitive compounds were performed under dry argon in flame-dried glassware. Solvents were purified and dried by standard methods. Melting points were determined with an electrothermal capillary melting point apparatus. ^{1}H NMR spectra were recorded with a Bruker Avance DRX 500 (500.13 MHz) or a Varian VXR-300 (299.94 MHz) spectrometer. ^{13}C NMR spectra were recorded with a Bruker Avance DRX 500 (125.75 MHz) spectrometer. ^{31}P NMR spectra were recorded with a Varian VXR-300 (121.4 MHz) spectrometer. Chemical shifts (δ) were given in ppm downfield relative to internal tetramethylsilane (TMS) for ^{1}H and ^{13}C and external 85% H_3PO_4 for ^{31}P. Elemental analyses were performed at the analytical laboratory of the Institute of Organic Chemistry, National Academy of Sciences of Ukraine. Mass spectra were recorded on an Agilent 1200 LCMSD SL instrument (atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI)) or Agilent 7820A gas chromatograph system (electron impact ionization (EI), ionization energy −70 eV).

The synthesis of 6-azaspiro[2.5]octane-6-carbaldehyde (1)

To the solution of 6-azaspiro[2.5]octane (8.9 g, 0.08 mol) in *o*-xylene (30 mL), formic acid (4.0 g, 0.087 mol) was added. The reaction mixture was heated at 170°C for 2 h and another 1 h at 190°C with the distillation of *o*-xylene by the help of a Dean-Stark nozzle. The residue was kept at the reduced pressure (12 mmHg) at 95°C with a condensate detachment, then re-distilled twice in high vacuo (b. p. 60°C/0.05 mmHg) to give aldehyde **1**. Anhydrous compound **1** was obtained by distillation under P_2O_5.

A white solid. Yield – 10.1 g (92%). M. p. 26–28°C (hexane). ^{1}H NMR (500 MHz, $CDCl_3$), δ, ppm: 0.35 (4H, s, CH_2); 1.31–1.38 (4H, m, CH_2); 3.34 (2H, t, J = 5.5 Hz, CH_2); 3.52 (2H, t, J = 5.5 Hz, CH_2); 8.02 (1H, s, CH). ^{13}C NMR (125.7 MHz, $CDCl_3$), δ, ppm: 11.4 (s, CH_2); 17.9 (s, *C*); 34.2 (s, CH_2); 35.8 (s, CH_2); 39.7 (s, CH_2); 45.9 (s, CH_2); 160.59 (s, CH). GC-MS (EI), m/z, peak area: 139, 99.6%.

The synthesis of the Alder dimer 6,6'-(oxybis(methaneyl-6-ylidene))bis(6λ^4-azaspiro[2.5]octane) trifluoromethanesulfonate (2)

To a cooled to -90°C solution of trifluoromethanesulfonic anhydride (3.4 g, 12 mmol) in dichloromethane (30 mL) the solution of formamide **1** (3.5 g, 25 mmol) in dichloromethane (30 mL) was added. The reaction mixture was stirred until the temperature increased to 16°C, and then for 1 h. The precipitate was filtered under argon, washed with dichloromethane (3×20 mL) and dried to give a pure target compound. The analytical sample was obtained by crystallization from acetonitrile.

A white powder. Yield – 6.52 g (98%). M. p. 191–193°C (decomp.). Anal. Calcd. for $C_{18}H_{26}F_6N_2O_7S_2$, %: C 38.57; H 4.68; N 5.00. Found, %: C 38.93; H 4.75; N 5.07. ^{1}H NMR (500 MHz, CD_3CN), δ, ppm: 0.58 (4H, s, 2×CH_2); 1.77 (2H, br. s, CH_2); 1.82 (2H, br. s, CH_2); 4.14 (4H, br. s, 2×CH_2); 9.31 (1H, s, CH). ^{13}C NMR (125.74 MHz, $CDCl_3$), δ, ppm: 11.16 (s, CH_2); 16.07 (s, C); 32.94 (s, CH_2); 33.58 (s, CH_2); 49.72 (s, CH_2); 54.52 (s, CH_2); 120.8 (q, J_{CF} = 320 Hz, CF_3); 158.32 (br. s, CH).

The synthesis of 6-(((di-*tert*-butylphosphoroselenoyl)(phenyl)amino)methylene)-6-azaspiro[2.5]octan-6-ium trifluoromethanesulfonate (4)

To the suspension of Alder dimer **2** (6.5 g, 11.5 mmol) cooled to -90°C in dichloromethane (30 mL) the solution of *P*,*P*-di-*tert*-butyl-

N-phenylphosphinoselenoic amide **3** (3.8 g, 12.0 mmol) in dichloromethane (20 mL) was added. After reaching the room temperature (16°C) the reaction mixture was stirred for 20 min. The solvent was evaporated at reduced pressure. Diethyl ether (50 mL) was added to the oil-like residue, then it was shaken to its solidification. The crystalline product formed was filtered under argon using a reverse rinse filter, washed with diethyl ether (4×20 mL), dried and then dissolved in THF (20 mL). The solid product formed at 2°C was separated by filtration under argon, washed with THF (2×15 mL) to give compound **4**. The mother liquor was evaporated in vacuo, the solid residue was washed with water (4×5 mL) to the neutral pH, the insoluble precipitate was dried in vacuo to give an additional amount of compound **4**.

A white powder. Yield – 5.4 g (80%). M. p. 177–178°C. Anal. Calcd. for $C_{23}H_{36}F_3N_2O_3PSSe$, %: C 47.02; H 6.18; N 4.77. Found, %: C 46.92; H 6.02; N 4.99. ^{1}H NMR (500 MHz, $CDCl_3$), δ, ppm: 0.32 (2H, s, CH_2); 0.41 (2H, s, CH_2); 1.15 (2H, s, CH_2); 1.58 (18H, d, J_{HP} = 17.5 Hz, 6×CH_3); 1.68 (2H, s, CH_2); 2.92 (2H, s, CH_2); 4.01 (2H, s, CH_2); 7.4–7.6 (5H, m, Ph); 8.75 (1H, s, CH). ^{13}C NMR (125.74 MHz, $CDCl_3$), δ, ppm: 11.7 (s, CH_2); 16.5 (s, CH_2); 29.2 (s, CH_3); 34.2 (s, CH_2); 35.8 (s, CH_2); 45.0 (d, J_{CP} = 28 Hz, $\underline{C}(CH_3)_3$); 49.1 (s, CH_2); 59.3 (s, CH_2); 120.9 (q, J_{CF} = 320 Hz, CF_3); 129.8 (s, CH); 130.0 (s, CH); 130.1 (s, CH); 136.6 (s, *C*); 155.4 (d, J_{CP} = 9 Hz, CH). ^{31}P NMR (202.4 MHz, $CDCl_3$), δ, ppm: 141.4 (J_{PSe} = 832 Hz). LC-MS (ESI), *m/z*: 439 $[M–CF_3SO_3+1]^+$; 149 $[CF_3CO_3]^-$; 316 $[M-124]^-$.

The synthesis of 6-(((di-*tert*-butylphosphanyl)(phenyl)amino)methylene)-6-azaspiro[2.5]octan-6-ium trifluoromethanesulfonate (5)

To the suspension of compound **4** (4.0 g, 6.8 mmol) in dichloromethane (15 mL) at 20°C, hexamethylphosphorous triamide (1.2 g, 7.4 mmol) was added dropwise with stirring. In 30 min, the solvent was evaporated to dryness at reduced pressure. A dry diethyl ether (30 mL) was added to the residue. The flask was shaken until a crystalline solid was formed. The solid was collected by filtration under argon, washed with diethyl ether (4×20 mL), and dried to constant weight in vacuo (0.05 Torr).

A white powder. Yield – 2.9 g (84%). M. p. 93–94°C. Anal. Calcd. for $C_{23}H_{36}F_3N_2O_3PS$, %: N 5.51; P 6.09. Found, %: N 5.17; P 5.93. ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 0.31 (2H, s, CH_2); 0.40 (2H, s, CH_2); 1.21 (2H, s, CH_2); 1.34 (18H, d, J_{HP} = 13.2 Hz, 6×CH_3); 1.65 (2H, t, J_{HH} = 5.4 Hz, CH_2); 2.98 (2H, br. t, J_{HH} = 5.6 Hz, CH_2); 4.0 (2H, br. s, CH_2); 7.3-7.5 (5H, m, Ph); 8.04 (1H, br. s, CH). ^{13}C NMR (125.7 MHz, $CDCl_3$), δ, ppm: 11.6 (s, CH_2); 16.4 (s, CH_2); 29.24 (d, J_{CP} = 18 Hz, *C*); 33.96 (s, CH_2); 35.1 (s, CH_2); 36.9 (d, J_{CP} = 35 Hz, *C*); 48.0 (s, CH_2); 56.7 (s, CH_2); 120.9 (q, J = 320 Hz, CF_3); 126.0 (s, CH); 128.6 (s, CH); 130.5 (s, CH); 144.2 (s, *C*); 156.8 (s, CH). ^{31}P NMR (202.4 MHz, $CDCl_3$), δ, ppm: 131.5.

The procedure for the synthesis of compounds 7 and 8.

To the solution of salt **5** (2.9 g, 5.8 mmol) in THF (20 mL) at -90°C the solution of lithium hexamethyldisilazide (930 mg, 5.6 mmol) in THF (20 mL) was added dropwise over 5 min. After reaching -60°C, the reaction mixture was stirred for another 10 min, then kept stirring at 0°C for 10 days (or at -4°C for 13 days). The solvent was evaporated in vacuo, the solid residue was extracted with pentane (50 mL), the precipitate was filtered off under argon using the reverse rinse filter, washed with pentane (3×20 mL). The filtrate was evaporated in vacuo, the residue was purified by fractionation at reduced pressure. Amidophosphonite **9** (1.27 g; b. p. 60–110°C/0.05 mmHg) was obtained in the first fraction. The second fraction (b. p. 150–160°C/0.05 mmHg) contained mixture of products **7** and **8** (930 mg). This mixture was recrystallized from pentane (2.5 mL), the precipitate formed at -18°C was collected to give compound **8** (400 mg, 20%; m. p. 96–97°C). The mother liquor was concentrated at reduced pressure to 1/4 of volume, in 24 h at -18°C the precipitated compound **7** (280 mg, 12%; m. p. 110–112°C) was collected.

[(6-Aza-spiro[2.5]oct-6-yl)-(di-tert-butyl-phosphanyl)-methylene]-phenyl-amine (**7**)

Yellowish crystals. Yield – 0.28 g (12%). M. p. 110–112°C (hexane). Anal. Calcd. for $C_{22}H_{35}N_2P$, %: C 73.71; N 7.81. Found, %: C 73.66; N 7.74. ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 0.25 (4H, s, 2×CH_2); 1.26 (4H, br. s, 2×CH_2); 1.33 (18H, d, J_{PH} = 12 Hz, 6×CH_3); 3.30 (4H, br. s, 2×CH_2); 6.72 (2H, d, J_{HH} = 8 Hz, Ph); 6.88 (1H, t, J_{HH} = 8 Hz, Ph); 7.25 (1H, t, J_{HH} = 8 Hz, Ph); 7.26 (1H, t, J_{HH} = 8 Hz, Ph). ^{13}C NMR (125.7 MHz, $CDCl_3$), δ, ppm: 11.3 (s, CH_2); 17.5 (s, CH_2); 30.14 (d, J_{CP} = 14 Hz, $\underline{C}(CH_3)_3$); 33.15 (d, J_{CP} = 20 Hz, *C*); 35.0 (s, CH_2); 49.25 (d, J_{CP} = 16 Hz, C); 120.0 (s, CH, Ph); 120.6 (s, CH, Ph); 128.7 (s, CH, Ph); 163.15 (d, J_{CP} = 10 Hz, C-P). ^{31}P NMR (81 MHz, $CDCl_3$), δ, ppm: 29.5. LC-MS (ESI), *m/z*, peak area: 359.2 $[M+H]^+$, 94%.

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5'-(Di-tert-butylphosphino)-5',6',8',9',11',11a'-hexahydrospiro[cyclopropane-1,10'-pyrido[1,2-c]quinazoline] (**8**)

A white solid. Yield – 0.2 g (20%). M. p. 96–97°C. Anal. Calcd. for $C_{22}H_{35}N_2P$, %: C 73.71; H 9.84; N 7.81. Found, %: C 73.70; H 9.78; N 7.75. ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 0.36-0.41 (3H, m, CH_2); 0.86 (1H, d, J_{HH} = 12.6 Hz, CH_2); 1.30 (9H, d, J_{HP} = 12.3 Hz, 3×CH_3); 1.31 (9H, d, J_{HP} = 12.6 Hz, 3×CH_3); 1.92 (1H, t, J_{HH} = 12 Hz, CH_2); 2.16 (1H, t, J_{HH} = 12 Hz, CH_2); 2.34 (1H, t, J_{HH} = 11 Hz, CH_2); 2.87 (1H, d, J_{HH} = 10 Hz, CH_2); 3.53 (1H, d, J_{HH} = 10.5 Hz, CH_2); 3.89 (1H, d, J_{HH} = 10.5 Hz, CH_2); 4.36 (1H, d, J_{HH} = 10.5 Hz, CH_2); 6.75 (1H, t, J_{HH} = 7.5 Hz, Ph); 6.94 (1H, d, J_{HH} = 7.5 Hz, Ph); 7.07 (1H, t, J_{HH} = 7.5 Hz, Ph); 7.66 (1H, t, J_{HH} = 7.0 Hz, Ph). ^{13}C NMR (125.7 MHz, $CDCl_3$), δ, ppm: 11.2 (s, CH_2); 12.9 (s, CH_2); 18.3 (s, *C*); 29.5 (d, J_{CP} = 16 Hz, *C*); 30.3 (d, J_{CP} = 18 Hz, *C*); 34.85 (s, CH_2); 35.7 (d, J_{CP} = 24 Hz, *C*); 37.04 (d, J_{CP} = 30 Hz, *C*); 41.8 (s, CH_2); 51.8 (s, CH_2); 62.3 (s, CH_2); 70.1 (d, J_{CP} = 10 Hz, *C*); 118.6 (s, CH); 119.2 (d, J_{CP} = 30 Hz, CH); 125.2 (s, CH); 126.2 (s, CH); 126.77 (d, J_{CP} = 4 Hz, *C*); 147.7 (d, J_{CP} = 23 Hz, *C*). ^{31}P NMR (81 MHz, $CDCl_3$), δ, ppm: 84.9. LC-MS (ESI), *m/z*, peak area: 359.2 $[M+H]^+$, 97%.

The synthesis of 6,6-di-*tert*-butyl-5,6,7,8,9,10,12,12a-octahydrospiro[benzo[*f*]pyrido[1,2-*d*][1,4,2]diazaphosphepine-11,1'-cyclopropane]-6,8-diium *bis*(2,2,2-trifluoro acetate) (**10**)

The mixture of **8** (200 mg, 0.56 mmol) and trifluoroacetic acid (2 g, 4.4 mmol, at least 2.5 equivalents) was refluxed at 95–100°C for 1 h. The mixture was concentrated at reduced pressure, the residue was dissolved in Et_2O (5 mL) and kept at -18°C until the residue solidifies, the solid product was collected, washed with Et_2O (2×2 mL) and dried in vacuo.

A white solid. Yield – 0.300 g (94%). M. p. 126–127°C. ^{1}H NMR (500 MHz, $CDCl_3$), δ, ppm: 0.32 + 0.35 (4H, 2×br. s, CH_2); 0.75 (1H, d, J = 13.5 Hz, CH_2); 0.86 (1H, d, J = 13 Hz, CH_2); 1.20 (9H, d, J_{HP} = 14.5 Hz, 3×CH_3); 1.62 (9H, d, J = 15 Hz, 3×CH_3); 2.06-2.11 (2H, m, CH_2); 2.896 (1H, t, J = 11 Hz, CH_2); 3.05 (1H, d, J = 11 Hz, CH_2); 3.64–3.76 (3H, m, CH + CH_2); 7.00 + 7.046 (2H, d + t, J = 7.5 Hz + J = 7.5 Hz, ArH); 7.12 (1H, t, J = 7.5 Hz, ArH); 7.39 (1H, d, J = 8.0 Hz, ArH); 7.72 (1H, d, J = 8.0 Hz, NH). ^{13}C NMR (125.7 MHz, $CDCl_3$), δ, ppm: 10.8 (CH_2); 12.5 (CH_2); 18.0 (*C*); 26.97 (CH_3); 27.9 (CH_3); 35.0 (CH_2); 35.8 (d, $^1J_{CP}$ = 46.5 Hz, *C*); 38.5 (d, $^1J_{CP}$ = 31 Hz, *C*); 45.0 (d, $^1J_{CP}$ = 69 Hz, CH_2); 46.9 (CH_2); 57.2 (d, $^3J_{CP}$ = 11 Hz, CH_2); 71.3 (CH); 116.0 (q, $^1J_{CF}$ = 290.5 Hz); 125.5 (CH); 126.6 (d, $^4J_{CP}$= 6.3 Hz, CH); 128.5 (CH); 130.8 (CH); 132.9 (*C*); 136.9 (d, $^2J_{CP}$= 5 Hz, *C*); 160.4 (d, $^1J_{CF}$ = 3.8 Hz). ^{31}P NMR (80.9 MHz, $CDCl_3$), δ, ppm: 69.5. LC-MS (ESI), *m/z*: 359.2 $[M–CF_3CO_2+1]^+$; 113 $[CF_3CO_2]^-$.

The synthesis of 6,6-di-*tert*-butyl-9,10,12,12a-tetrahydro-7*H*-6λ^5-spiro[benzo[*f*]pyrido[1,2-*d*] [1,4,2]diazaphosphepine-11,1'-cyclopropane] (**11**)

To the solution of salt **10** (280 mg, 0.9 mmol) in H_2O (3 mL) the solution of NaOH (500 mg) in H_2O (1 mL) was added. The mixture was heated at 80°C with stirring for 1 h. Then after cooling benzene (2×4 mL) was added, the organic phase was collected, concentrated under vacuum, dried and distilled (180°C/0.05 mmHg) to give **11** as a mushy solid recrystallized from pentane (2.5 mL).

A beige solid. Yield – 0.150 g (99%). M. p. 102–103°C (pentane). Anal. Calcd. for $C_{22}H_{35}N_2P$, %: C 73.71; H 9.84; N 7.81. Found, %: C 73.67; H 9.97; N 7.75. ^{1}H NMR (500 MHz, $CDCl_3$), δ, ppm: 0.29-0.33 (4H, m, CH_2); 0.76 + 0.825 (2H, d + d, J = 13.5 Hz, J = 15 Hz, CH_2); 1.11 (9H, d, J_{PH} = 12.5 Hz, 3×CH_3); 1.44 (9H, d, J_{PH} = 13.5 Hz, 3×CH_3); 2.06–2.11 (1H, m, CH_2); 2.24 (1H, t, J = 9.5 Hz, CH_2); 2.76 (1H, t, J = 11.0 Hz, CH_2); 2.92 (1H, d, J = 12.0 Hz, CH_2); 3.11 (1H, dd, J_1 = 15.0 Hz, J_2 = 6.5 Hz, CH); 3.44 (1H, d, J = 13.0 Hz, CH_2); 3.61 (1H, d, J = 10.5 Hz, CH_2); 6.58-6.60 (1H, m, ArH); 6.83 (1H, d, J = 8.0 Hz, ArH); 6.92 (2H, br. s, ArH). ^{13}C NMR (125.7 MHz, $CDCl_3$), δ, ppm: 10.9 (CH_2); 12.7 (CH_2); 18.2 (*C*); 27.5 (CH_3); 28.2 (CH_3); 35.2 (d, $^1J_{CP}$= 70 Hz, *C*), 35.4 (CH_2), 38.56 (d, $^1J_{CP}$= 47 Hz, *C*); 46.3 (CH_2); 46.56 (d, $^1J_{CP}$= 50 Hz, CH_2); 57.7 (d, $^2J_{CP}$ = 11 Hz, CH_2); 71.0 (CH); 117.4 (d, $^4J_{CP}$ = 2.5 Hz, CH); 126.9 (CH); 128.55 (d, $^3J_{CP}$ = 15 Hz, CH); 129.4 (d, $^4J_{CP}$ = 2.5 Hz, CH); 133.4 (d, $^3J_{CP}$ = 5 Hz, *C*); 153.5 (d, $^2J_{CP}$ = 5 Hz, *C*). ^{31}P NMR (80.9 MHz, $CDCl_3$), δ, ppm: -41.7. LC-MS (ESI), *m/z*: 360.1 $[M+2]^+$.

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The synthesis and the anticonvulsant activity screening of new 5-substituted 2-imino-4-thiazolidinone derivatives

Abstract

Aim. To synthesize 5-ene-4-thiazolidinones containing heterocyclic rings in the molecule as potential anticonvulsants, and screen their anticonvulsant activity on a model of pentylenetetrazole (PTZ) seizures.

Results and discussion. A straightforward and convenient synthesis of novel 5-ene-derivatives of thiazol/oxazole-bearing 4-thiazolidinones as possible anticonvulsant agents was performed. Compounds were characterized using methods of spectral analysis (^{1}H NMR and LC-MS). 5-Chloro-3-methyl-1-phenyl-1*H*-pyrazole-4-carbaldehyde underwent the aminolysis on a chlorine atom by a molecule of monoethanolamine (MEA) in the Knoevenagel reaction with thiazole/oxazole-bearing 4-thiazolidinones. The preliminary screening of the anticonvulsant activity was performed for the compounds synthesized on the model of PTZ-induced seizures, and active derivatives were identified.

Experimental part. Commercially available 2-aminothiazole and 5-methylisoxazol-3-amine were used as starting compounds for the synthesis of 2-chloro-*N*-(hetaryl)acetamides. The latter were transformed into thiazole/oxazole-bearing 4-thiazolidinones by the treatment with ammonium isothiocyanate. Modification at C5 position of the heterocycles synthesized was performed by the Knoevenagel reaction with aromatic/heteroaromatic aldehydes and MEA as a catalyst (either equimolar or 0.1 mol% amount) in the ethanol medium. The structure of novel derivatives was confirmed by ^{1}H NMR and LC-MS spectra. The anticonvulsant activity of all derivatives synthesized was studied *in vivo* on the model of PTZ-induced seizures. Latency of the seizures, the number of clonic-tonic seizures in one mouse, the percent of animals with clonic and tonic seizures, the duration of the seizure period, and the lifetime of the animals before death were evaluated and calculated.

Conclusions. The results obtained are promising for further design of potential anticonvulsants among oxazole-bearing 4-thiazolidones with the possible mechanism of the anticonvulsant action through the GABA-ergic impact and inhibition of the prostaglandin and leukotriene synthesis.

Keywords: 4-thiazolidinones; Dimroth reaction; Knoevenagel reaction; antiepileptic drugs; epilepsy; anticonvulsant activity

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Синтез та скринінг протисудомної активності нових 5-заміщених 2-іміно-4-тіазолідинонів

Анотація

Мета. Синтезувати 5-ен-4-тіазолідинони з гетарильними фрагментами в молекулі як потенційні протисудомні засоби та провести скринінг їхньої протисудомної активності на моделі пентилентетразолових судом.

Результати та їх обговорення. Проведено простий і зручний синтез нових 5-ен-похідних тіазол/оксазоловмісних 4-тіазолідинонів як потенційних протисудомних засобів. Структуру нових сполук досліджено та підтверджено методами

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спектрального аналізу (^{1}H ЯМР та LC-MS). 5-Хлор-3-метил-1-феніл-1*H*-піразол-4-карбальдегід зазнає амінолізу за атомом хлору дією моноетаноламіну (МЕА) в реакції Кньовенагеля з тіазол/оксазолвмісними 4-тіазолідинонами. Проведено первинний скринінг протисудомної активності синтезованих сполук на моделі пентилентетразол-індукованих судом та ідентифіковано активні сполуки.

Експериментальна частина. Комерційно доступні 2-амінотіазол і 5-метилізоксазол-3-амін було використано як вихідні сполуки для одержання 2-хлор-*N*-(гетарил)ацетамідів. Останні обробленням амоній ізотіоціанатом було перетворено на тіазол/оксазолвмісні 4-тіазолідинони. Модифікацію за положенням С5 сполук проводили за реакцією Кньовенагеля з ароматичними/гетероароматичними альдегідами в присутності МЕА як каталізатора (в еквімолярній або 0,1 моль% кількості) в середовищі етанолу. Структуру нових синтезованих речовин підтверджено даними ^{1}H ЯМР-спектроскопії та LC-MS. Протисудомну активність синтезованих похідних вивчали *in vivo* на моделі PTZ-індукованих судом. Оцінювали та розраховували латентність судом; кількість клоніко-тонічних судом в однієї миші; відсоток тварин із клонічними та тонічними судомами; тривалість судомного періоду та тривалість життя тварин.

Висновки. Отримані результати є перспективними для наступного дизайну потенційних протисудомних засобів серед оксазолвмісних похідних 4-тіазолідинонів з можливим механізмом протисудомної дії через ГАМК-ергічний вплив та пригнічення синтезу простагландинів і лейкотриєнів.

Ключові слова: 4-тіазолідинони; реакція Дімрота; реакція Кньовенагеля; протиепілептичні засоби; епілепсія, протисудомна дія

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■ Introduction

The antiepileptic drug design is an important area in current medicinal chemistry [1]. Despite the significant progress in the treatment of seizures and the discovery/introduction of innovative antiepileptic drugs, there are still plenty of problems associated with adverse effects, as well as drug resistance taking place under long-term application of approved drugs [2–4]. In this regard, the search for new potent anticonvulsant agents is a logical and actual approach to achieve a positive effect on the frequency and severity of spontaneous epileptic seizures, negative cognitive and psychosomatic consequences in patients [2–4].

Nitrogen-containing heterocyclic rings are important structural elements in many known anticonvulsant drugs [5–8]. In our previous studies 4-thiazolidinones containing 2-aminothiazole moiety are reported as promising anticonvulsant agents; they are structural analogs of a potential dual cyclooxygenase-2 and 5-lipoxygenase (COX-2/5-LOX) inhibitor darbufelon (Figure 1) [9–11]. Data on the anticonvulsant activity of classical non-steroidal anti-inflammatory drugs [12–17], the polypharmacological futures of 4-thiazolidinones and our experimental findings are sound arguments for further design of novel hetarylsubstituted 2-imino/amino-4-thiazolidinones as potential anticonvulsants.

■ Results and discussion

Synthesis of the target compounds

Commercially available 2-aminothiazole (**1**) and 5-methylisoxazol-3-amine (**2**) were used as starting compounds and were converted to 4-thiazolidinones **3** and **4**, respectively (Scheme 1) [9, 18]. Initially, the appropriate 2-chloro-*N*-(hetaryl)acetamides **1.1** and **1.2** were obtained by the reaction of amines **1**, **2** with chloroacetyl chloride in the presence of triethylamine in the dry dioxane and further transformed to derivatives **3**, **4** by the interaction with ammonium isothiocyanate.

It was reported that the process mentioned above (Scheme 1) did not stop at the stage of the nucleophilic substitution and underwent a spontaneous heterocyclization to the 4-thiazolidinone cycle formation as the intramolecular Dimroth type rearrangement of substituents in positions 2 and 3 took place [18]. It should be noted that compounds **3** and **4** synthesized are characterized by the existence in the imino-form in the solution and the crystalline state as previously proven using spectral and X-ray diffraction methods [9, 18]. The structure modification of 2-(aryl/hetaryl)-imino-4-thiazolidones at C5 position leads to a significant impact on the appearance, as well potency of the anticonvulsant activity [9–11]. Derivatives **3** and **4** synthesized are active methylene compounds.

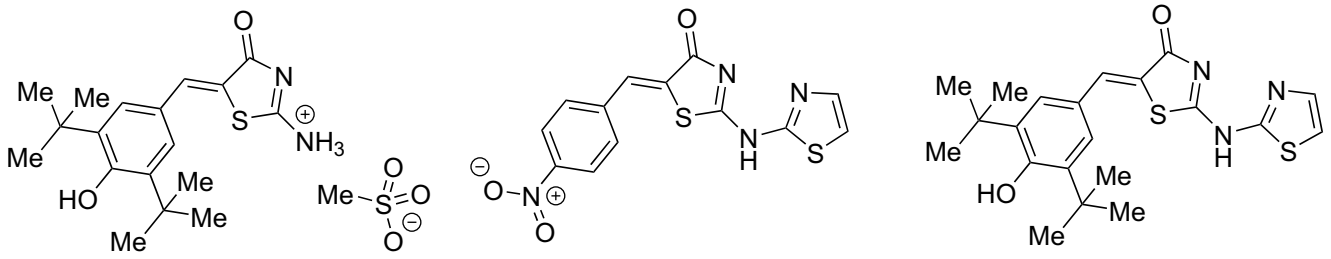


Figure 1. The structures of promising anticonvulsant agents previously found

Hence, the Knoevenagel condensation was used for further modification of the structure at C5 atom. A set of aromatic/heteroaromatic aldehydes was used, and the reaction was carried out in the soft conditions using monoethanolamine (MEA) as a catalyst (the equimolar amount in case of **3.1**, and 0.1 mol% in case of other derivatives) in the ethanol medium (Scheme 2).

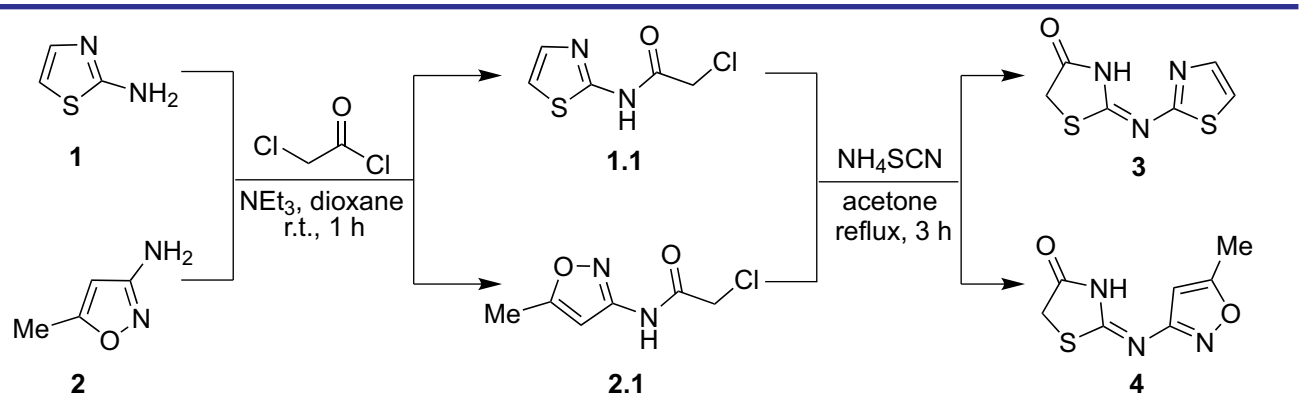
The structure of novel derivatives **3.1**–**3.3**, **4**, **4.1**–**4.3** synthesized was characterized by ^{1}H NMR, and LC-MS spectra. The aminolysis of the chlorine atom by the molecule of MEA took place during the synthesis of compound **3.1**. Two broad singlets at 3.53 and 3.59 ppm, corresponding to four methylene-groups protons, as well as broad singlet at 9.71 ppm corresponding to the aminoethanole residue of the NH-group proton were observed in the ^{1}H NMR spectrum of **3.1**. The molecular ion peak observed at m/z value of 427.0 $[M+H]^+$ in a positive ionization mode in the mass spectrum confirmed the formation of compound **3.1.** Signals of the endocyclic N-H, as well as OH-group protons for **3.1** were absent in the relevant ^{1}H NMR spectrum due to deuteroexchange. The signal of the methylidene group protons appeared at δ ~7.6–7.9 ppm and clearly indicated the *Z*-configuration of ylidene derivatives **3.1**–**3.3**, **4.1**–**4.3** synthesized [19].

It should be noted that in the ^{1}H NMR spectrum (solvent DMSO-d_6) of compound **4.2** the prototropic amino-imine tautomerism was observed (Scheme 3). The signal of the amino-form of the NH-proton of compound **4.2** was observed at 11.95 ppm, and there was the signal of that one for the imino-form at 12.35 ppm. Additional studies using variety of conditions for NMR experiments (the change of the solvent, temperature, etc.) were not performed as the feature mentioned was well-known and widely described for such type of heterocycles [20]. The molar ratio of tautomers based on the integral intensity curve was 1:6 (amine and imine forms, respectively).

The anticonvulsant activity screening

The anticonvulsant activity of derivatives **3.1**–**3.3**, **4**, **4.1**–**4.3** was studied *in vivo* on the model of PTZ-induced seizures. The results of the experiment are presented in Table and Figure 2.

Substances **3.1**, **3.2, 4.1** and **4.2** were indifferent to PTZ-induced seizures. The compounds did not have a significant effect on all parameters of the convulsive syndrome model, including the integrated indicator – mortality. The reduction in the duration of the convulsive period under the effect of compound **3.1** is not a manifestation of anticonvulsant properties in the context of other characteristics of the convulsive syndrome since it correlates with a decrease in the time from the appearance of the first paroxysms to death with a constant severity of attacks (during this



Scheme 1. The synthesis of 2-(thiazol-2-ylimino)thiazolidin-4-one (**3**) and 2-((5-methylisoxazol-3-yl)imino)thiazolidin-4-one (**4**)

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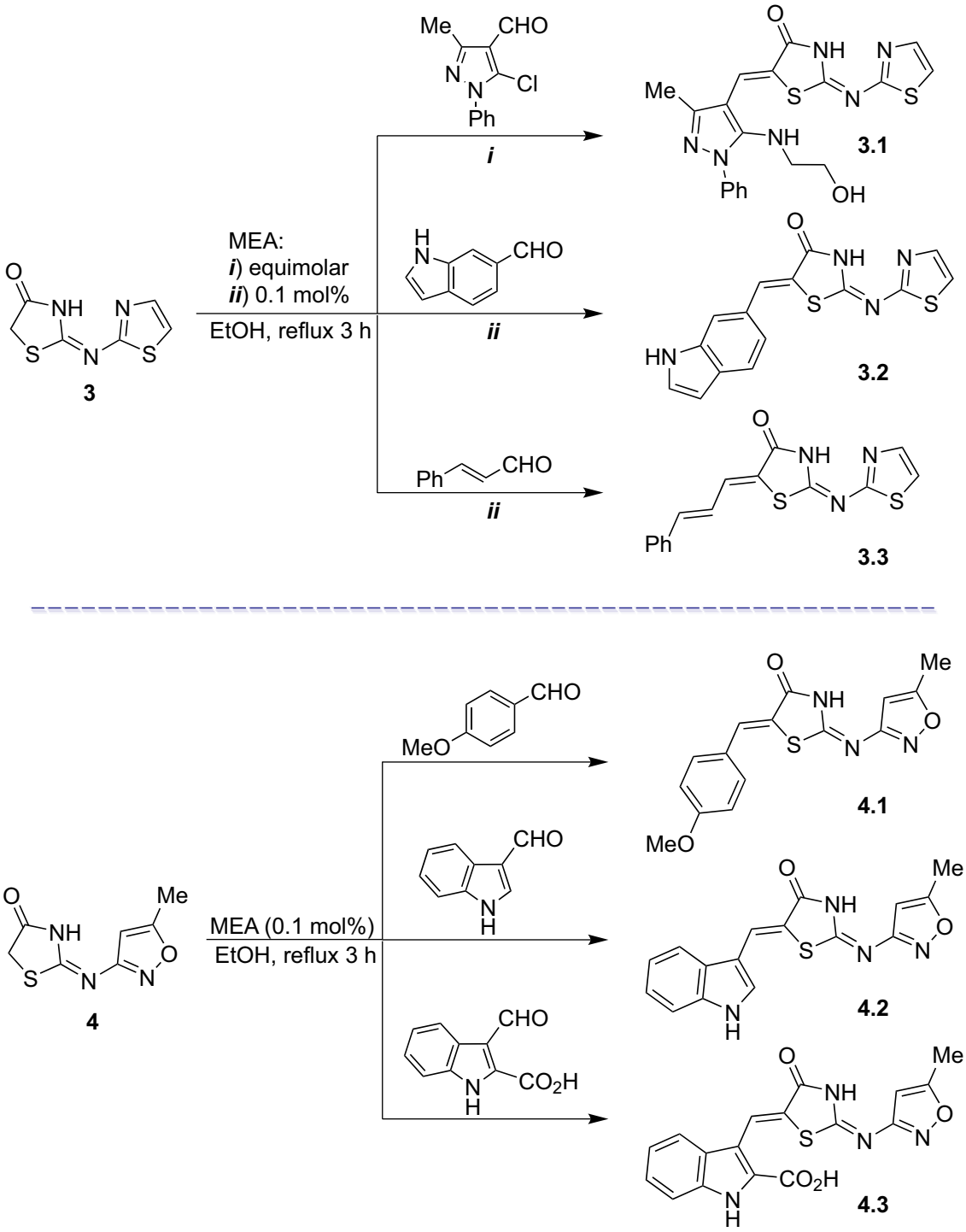
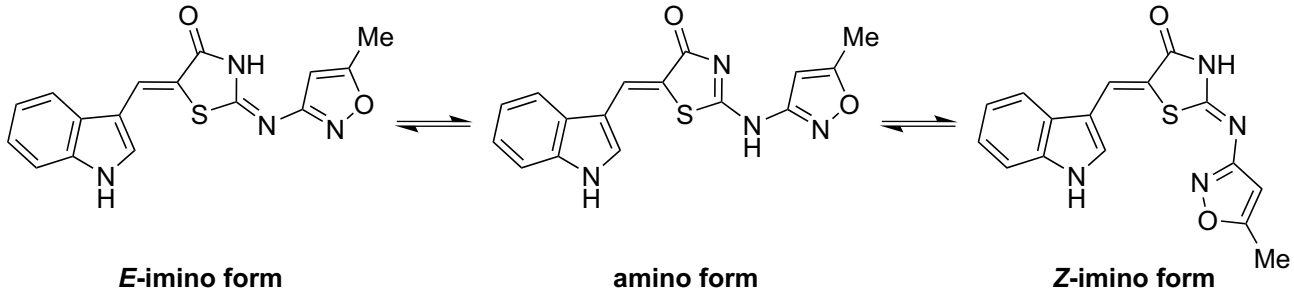


Scheme 2. The synthesis of 5-ylidene derivatives (**3.1-3.3**, **4.1-4.3**) of 2-(thiazol-2-ylimino)thiazolidin-4-one (**3**) and 2-((5-methylisoxazol-3-yl)imino)thiazolidin-4-one (**4**)

shorter time the animals have time to die from a slightly smaller number of attacks), but mortality is not reduced.

Derivative **3.3** possesses pro-convulsant properties. Under the effect of this compound, the number of seizures per one animal increased by 5.5%, in contrast to the control group; all animals, without exception, recorded a lethal tonic extension – 6 points and 100% mortality.

Compounds **4** and **4.3** showed the anticonvulsant activity, which could be determined as moderate. Under the action of **4** the number of animals with tonic convulsions decreased by 21.67% ($p < 0.05$), correlating with the animal's survival as mortality decreased by 23.33% ($p < 0.05$). Compound **4.3** significantly reduced the number of seizures in animals by 29.5% ($p < 0.05$), even reduced the percentage of clonic seizures in animals



***E*-imino form** **amino form** ***Z*-imino form**

Scheme 3. Possible tautomeric forms for compound **4.2**

by 8.33% ($p < 0.05$). Administration of the compound statistically significantly ($p < 0.05$) reduced the life expectancy of animals to death by 1.9 folds, and the duration of the convulsive period by 62.16%. The integral indicator – mortality significantly decreased compared to the control group by 23.33%.

The screening results obtained are promising for further design of potential anticonvulsants among oxazole-bearing 4-thiazolidones. It is important to note that the possible mechanism of the anticonvulsant action of the derivatives synthesized could indicate/combine GABA-ergic effects and inhibition of the prostaglandin and leukotriene synthesis [9, 10]. The new derivatives **3.1**–**3.3**, **4**, **4.1**–**4.3** synthesized and studied in this work are found less active than early found hits [9, 10], as well as darbufelon, however the data presented are extremely useful for the rational design of potential anticonvulsants among heterylsubstituted 4-thiazolidones.

■ Conclusions

In the present paper, a straightforward and convenient synthesis of novel 5-ene-derivatives of thiazol/oxazole-bearing 4-thiazolidinones as possible anticonvulsant agents has been described. Compounds have been characterized using methods of spectral analysis. The preliminary screening of the anticonvulsant activity has been performed for the compounds synthesized on the PTZ-induced seizures, and active derivatives have been identified. The results obtained are promising for further design of potential anticonvulsants among oxazole-bearing 4-thiazolidones with the possible mechanism of the anticonvulsant action through the GABA-ergic impact and inhibition of the prostaglandin and leukotriene synthesis.

■ Experimental part

Synthetic part

Melting points were measured in open capillary tubes on a BÜCHI B-545 melting point apparatus (BÜCHI Labortechnik AG, Flawil, Switzerland) and were uncorrected. The elemental analyses (C, H, N) were performed using a Perkin-Elmer 2400 CHN analyzer (PerkinElmer, Waltham, MA, USA) and were within ±0.4% of the theoretical values. ^{1}H NMR spectra (DMSO-d_6) were recorded on a Varian Unity Plus 400 (400 MHz) spectrometer (Varian Inc., Paulo Alto, CA, USA). All spectra were recorded at room temperature unless otherwise indicated and were referenced internally to the solvent reference frequencies.

Table. The anticonvulsant effect of the test compounds **3.1-3.3**, **4**, **4.1**–**4.3** in the PTZ model. Each value represents the mean ± S.E.M

Group	No.[a]	Dose, mg kg^{-1}	Number of clonic-tonic seizures per mouse	% of mice with seizures		Severity of seizures, points (0–6)	Mortality, %
				Clonic	Tonic		
Control	30	90	2.37 ± 0.26	100	96.67	5.77 ± 0.13	90
Sodium valproate	13	300	0.62 ± 0.46**	23.08**	15.38**	1.15 ± 0.64**	15.38**
3.1	6	100	1.33 ± 0.21$^{\#\#}$	100$^{\#\#}$	83.33$^{\#\#}$	5.50 ± 0.50$^{\#\#}$	83.33$^{\#\#}$
3.2	6	100	1.83 ± 0.48$^{\#\#}$	100$^{\#\#}$	100$^{\#\#}$	5.83 ± 0.17$^{\#\#}$	83.33$^{\#\#}$
3.3	6	100	2.50 ± 0.56$^{\#\#}$	100$^{\#\#}$	100$^{\#\#}$	6.00 ± 0.00$^{\#\#}$	100$^{\#\#}$
4	12	100	1.83 ± 0.42$^{\#\#}$	100$^{\#\#}$	75*$^{\#\#}$	5.08 ± 0.40$^{\#\#}$	66.67*$^{\#\#}$
4.1	6	100	3.00 ± 0.58$^{\#\#}$	100$^{\#\#}$	83.33$^{\#\#}$	5.50 ± 0.5$^{\#\#}$	83.33$^{\#\#}$
4.2	6	100	2.17 ± 0.31$^{\#\#}$	100$^{\#\#}$	83.33$^{\#\#}$	5.50 ± 0.50$^{\#\#}$	83.33$^{\#\#}$
4.3	12	100	1.67 ± 0.53*$^{\#\#}$	91.67*$^{\#\#}$	83.33$^{\#\#}$	5.00 ± 0.52$^{\#\#}$	66.67*$^{\#\#}$

Note: [a] the number of animals in each group; * $p \le 0.05$, ** $p \le 0.01$ compared to the control; $^{\#}$ $p \le 0.05$, $^{\#\#}$ $p \le 0.01$ compared to sodium valproate

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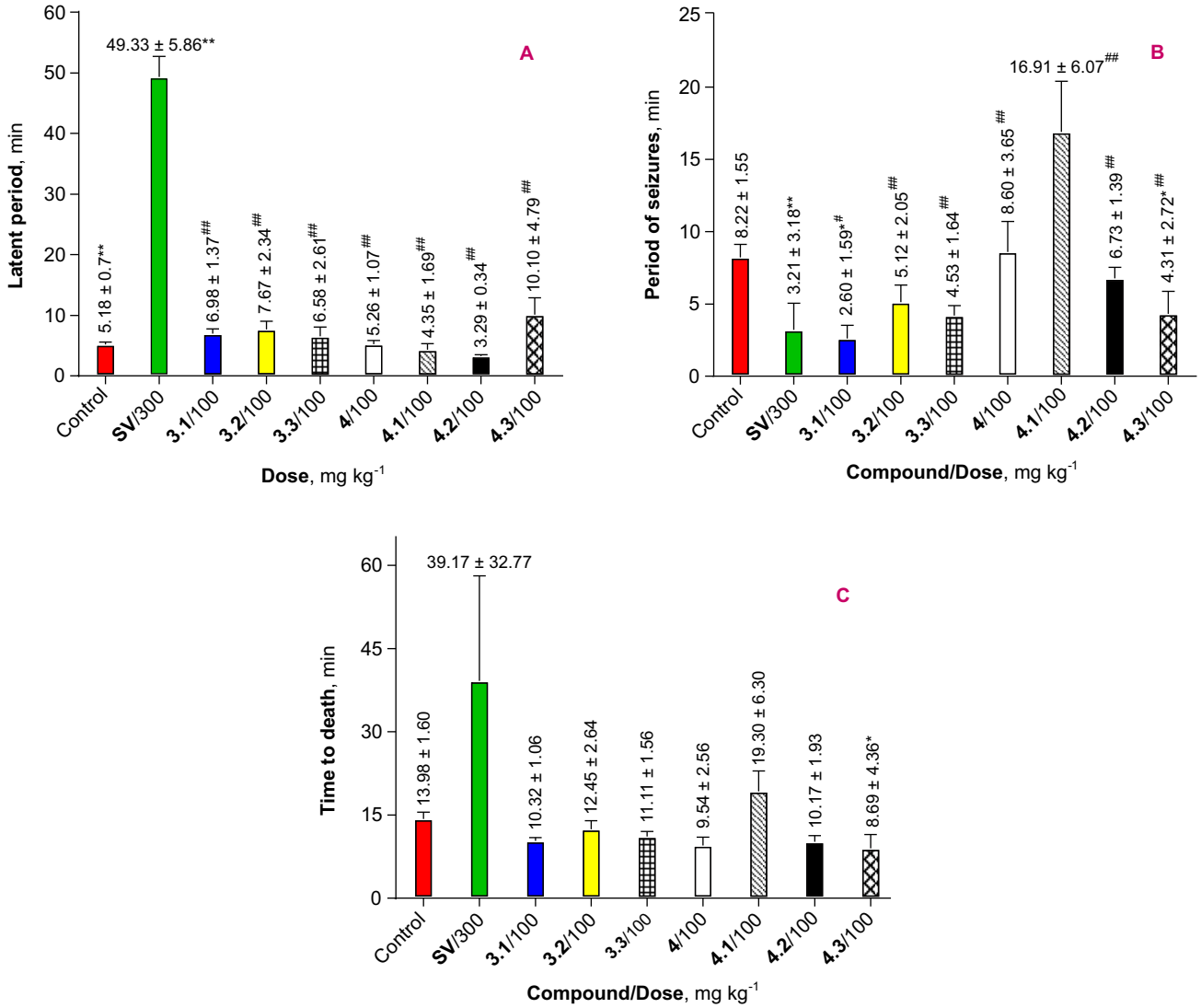

Figure 2. The anticonvulsant activity of the reference drug and derivatives **3.1**–**3.3**, **4**, **4.1**–**4.3** in the PTZ test: (**A**) latency to the first seizure episode; (**B**) duration (period) of seizures; (**C**) time to death. Each value represents the mean ± S.E.M. SV – sodium valproate; * $p < 0.05$, ** $p < 0.01$ compared to the control; # $p < 0.05$, ## $p < 0.01$ compared to sodium valproate

Chemical shifts (δ) were expressed in ppm, and coupling constants (J) were reported in Hz. LC-MS spectra were obtained on a Finnigan MAT INCOS-50 device (Thermo Finnigan LLC, San Jose, CA, USA). The reaction mixture was monitored by thin layer chromatography (TLC) using commercial glass-backed TLC plates (Merck Kieselgel 60 F_{254}). Solvents and reagents were commercially available and used without further purification. The protocol for the synthesis of compounds **3** (as well as its properties) was described in [9, 18]; compound **4** was synthesized similarly.

2-((5-Methylisoxazol-3-yl)imino)thiazolidin-4-one (**4**)

Yellow powder. Yield – 0.450 g (68%). M. p. – 230–232°C (EtOH). Anal. Calcd. for $C_7H_7N_3O_2S$, %: C 42.63; H 3.58; N 21.31. Found, %: C 42.50; H 3.51; N 21.40. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 2.36 (3H, s, CH_3); 4.04 (2H, s, CH_2); 6.09 (1H, s, isox.); 12.00 (1H, s, NH). LC-MS (ESI), m/z, peak area: 198.0 $[M+H]^+$, 100.0%.

The general procedure for the synthesis of 5-ylidene derivatives of compounds 3.1–3.3 and 4.1–4.3

To a stirred solution of compound **3** or **4** (10 mmol) in EtOH (10 mL) the appropriate aldehyde (11 mmol) and MEA (in the case of 5-chloro-3-methyl-1-phenyl-1*H*-pyrazole-4-carbaldehyde MEA is used in the equimolar amount, in all other cases – in 0.1 mol% amount) were added, and the mixture was then refluxed for 4 h. The resulting solids of compounds **3.1**–**3.3** and **4.1**–**4.3** were collected by filtration, washed with ethanol (5–10 mL), and crystallized from the appropriate solvent.

5-((5-((2-Hydroxyethyl)amino)-3-methyl-1-phenyl-1H-pyrazol-4-yl)methylene)-2-(thiazol-2-ylimino)thiazolidin-4-one (**3.1**)

A yellow powder. Yield – 0.310 g (69%). M. p. – 182–184°C (EtOH). Anal. Calcd. for $C_{17}H_{12}N_4O_4S$, %: C 53.51; H 4.25; N 19.70. Found, %: C 53.80; H 4.50; N 19.90. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 2.55 (3H, s, CH_3); 3.53 (2H, br.s, CH_2); 3.59 (2H, br. s, CH_2); 7.12 (1H, br. s, thiaz.); 7.33 (1H, t, J = 7.3 Hz, phenyl); 7.43 (1H, br. s, thiaz.); 7.60 (2H, t, J = 7.4 Hz, phenyl); 7.75 (2H, d, J = 8.0 Hz, phenyl); 7.90 (1H, s, =CH); 9.71 (1H, br. s, NH); 1H (NH, thiazolidine) and 1H (OH) are in exchange. LC-MS (ESI), m/z, peak area: 427.0 $[M+H]^+$, 100.0%.

5-((1H-Indol-6-yl)methylene)-2-(thiazol-2-ylimino)thiazolidin-4-one (**3.2**)

A brown powder. Yield – 0.450 g (71%). M. p. – 287–289°C (DMF). Anal. Calcd. for $C_{15}H_{10}N_4OS_2$, %: C 55.20; H 3.09; N 17.17. Found, %: C 55.40; H 3.30; N 17.40. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 6.51 (1H, s, thiaz.); 7.28 (1H, d, J = 8.2 Hz, indol.); 7.46 (1H, s, indol.); 7.55 (1H, s, thiaz.); 7.69 (2H, m, indol.); 7.74 (1H, s, indol.); 7.83 (1H, s, =CH); 11.55 (1H, s, NH, indol.); 12.55 (1H, s, NH, thiazolidin.). LC-MS (ESI), m/z, peak area: 327.0 $[M+H]^+$, 100.0%.

5-(3-Phenylallylidene)-2-(thiazol-2-ylimino) thiazolidin-4-one (**3.3**)

A yellow-brown powder. Yield – 0.380 g (72%). M. p. – 232–234°C (AcOH). Anal. Calcd. for $C_{15}H_{11}N_3OS_2$, %: C 57.49; H 3.54; N 13.41. Found, %: C 57.80; H 3.60; N 13.40. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 7.12 (1H, d, J = 15.0 Hz, =CH); 7.27 (1H, d, J = 15.0 Hz, =CH); 7.36-7.43 (5H, m, phenyl); 7.47 (1H, s, thiaz.); 7.65 (1H, s, thiaz.); 7.69 (1H, s, =CH); 12.50 (1H, s, NH). LC-MS (ESI), m/z, peak area: 314.0 $[M+H]^+$, 100.0%.

5-(4-Methoxybenzylidene)-2-((5-methylisoxazol-3-yl)imino)thiazolidin-4-one (**4.1**)

A yellow powder. Yield – 0.460 g (91%). M. p. – 254–256°C (dioxane). Anal. Calcd. for $C_{15}H_{13}N_3O_3S$, %: C 57.13; H 4.16; N 13.33. Found, %: C 57.50; H 4.30; N 13.40. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 2.40 (3H, s, CH_3); 3.82 (3H, s, OCH_3); 6.21 (1H, s, isox.); 7.12 (2H, d, J = 8.5 Hz, phenyl); 7.57 (2H, d, J = 8.5 Hz, phenyl); 7.66 (1H, s, =CH); 12.55 (1H, s, NH). LC-MS (ESI), m/z, peak area: 316.0 $[M+H]^+$, 100.0%.

5-((1H-Indol-3-yl)methylene)-2-((5-methylisoxazol-3-yl)imino)thiazolidin-4-one (**4.2**)

A brown-yellow powder. Yield – 0.290 g (56%). M. p. – 236–238°C (AcOH). Anal. Calcd. for $C_{16}H_{12}N_4O_2S$, %: C 59.25; H 3.73; N 17.27. Found, %: C 59.40; H 3.50; N 17.40. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 2.41 (3H, s, CH_3); 6.22 (1H, s, isox.); 7.19 (1H, t, J = 7.7 Hz, indol.); 7.24 (1H, t, J = 7.7 Hz, indol.); 7.52+7.60 (1H, d+d, J = 7.7 Hz, indol.); 7.74 (1H, s, indol.); 7.82+7.87 (1H, d+d, J = 7.7 Hz, indol.); 7.97 (1H, s, =CH); 11.86+12.05 (1H, s+s, NH); 11.95+12.35 (1H, s+s, NH). LC-MS (ESI), m/z, peak area: 325.0 $[M+H]^+$, 100.0%.

3-((2-((5-Methylisoxazol-3-yl)imino)-4-oxothiazolidin-5-ylidene)methyl)-1H-indole-2-carboxylic acid (**4.3**)

A white-yellow powder. Yield – 0.290 g (43%). M. p. – 251–253°C (dioxane). Anal. Calcd. for $C_{17}H_{12}N_4O_4S$, %: C 55.43; H 3.28; N 15.21. Found, %: C 55.60; H 3.30; N 15.40. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 3.69 (3H, s, CH_3); 5.07 (1H, s, isox.); 7.29–7.32 (3H, m, indol.); 7.54 (1H, d, J = 7.0 Hz, indol.); 7.99 (1H, s, =CH); 9.10 (1H, s, NH); 12.79 (1H, s, NH); 1H (COOH) is in exchange. LC-MS (ESI), m/z, peak area: 369.0 $[M+H]^+$, 100.0%.

Pharmacology

The study of the anticonvulsant activity of the derivatives **3.1**–**3.3**, **4**, **4.1**–**4.3** synthesized was performed on 97 randomly bred mice of both sexes weighing 18–23 g; they were kept under standard conditions in the vivarium of the Central Research Laboratory of the Educational and Scientific Institute of Applied Pharmacy of the National University of Pharmacy, Kharkiv, Ukraine.

A basic screening model of PTZ seizures was used in accordance with the guidelines in the experimental study [21]. During the experiment, the rules and principles adopted by the Helsinki Declaration on Humane Animal Welfare (2000), the Directive of the Council of the European Union on the protection of animals used for scientific purposes (2010) were observed. All animal procedures were approved by the Local Ethical Committee in the National University of Pharmacy, Kharkiv, Ukraine (Approval No: 3/2019).

Animals were randomized into the following groups: group 1 – control pathology (Control, untreated PTZ-seizures), group 2 – animals with model seizures, treated with the reference drug sodium valproate, groups 3–9 – animals with seizures, treated with derivatives **3.1**–**3.3**, **4**, **4.1**–**4.3** synthesized.

Animals were administered the test compounds once in the form of a thin suspension stabilized with polysorbate-80 (Tween-80), in the dose of 100 mg kg^{-1} intragastrically 30 min before the seizure induction. The dose was chosen on the data of 4-thiazolidinone derivatives effectiveness in the study [9–11].

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As a reference drug sodium valproate (Depakine, Sanofi-Aventis, France) was used in the dose of 300 mg kg^{-1} intragastrically as an oral syrup [21]. In the control group, animals were administered purified water intragastrically in the appropriate volume, 30 min before the PTZ seizure induction.

The mechanism of PTZ seizures is associated with inhibition of the GABA-receptor complex [21]. Animals were administered PTZ (Corazol, Sigma, USA) as an aqueous solution in the dose of 90 mg kg^{-1} subcutaneously 30 min after compounds application.

Each animal was placed in a separate cylindrical plastic container (5 L) and continuously monitored for 60 min. Latency of the seizures; the number of clonic-tonic seizures in one mouse; % of animals with clonic and tonic seizures; the duration of the seizure period (from the first to the last attack); and the lifetime of the animals before death (in mice with a lethal outcome) were calculated. The severity of seizures was evaluated according to the scale ranging from 1 to 6: 1 – trembling; 2 – circus movement; 3 – clonic seizures; 4 – clonic-tonic seizures with a lateral position; 5 – tonic extension; 6 – tonic extension leading to the animal's death. When seizures were not observed within 1 h, it was considered that the latent period was 60 min. The protection of animals from the development of clonic and tonic seizures and lethality were treated as the most significant indicators of the anticonvulsant activity of the compounds [21].

The statistical analysis was performed using a Statistica 10.0 software by the methods of variation statistics. The average values and standard errors were calculated. The significance of the differences between groups was estimated according to the Student's criterion (t) in the case of normal distribution, and according to the nonparametric Mann-Whitney criterion (U) in the case of the absence of normal distribution. The results determined in an alternative form (presence/absence of a certain feature) were evaluated using the Fisher's criterion (φ).

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Application of the enzymatic method for the quantitative determination of dequalinium chloride in lozenges

Abstract

Aim. To develop a new kinetic spectrophotometric enzymatic method suitable for the quantitative determination of dequalinium chloride in lozenges.

Materials and methods. An enzymatic kinetic spectrophotometric method for the quantitative determination of dequalinium chloride in lozenges has been proposed. It is based on the ability of dequalinium chloride to inhibit the enzymatic hydrolysis reaction of acetylcholine by cholinesterase in the presence of the acetylcholine (ACh) excess and H_2O_2. The degree of inhibition was determined by the kinetic method using two conjugated reactions: ACh perhydrolysis (interaction with an excess of hydrogen peroxide) followed by oxidation with the peroxyacid formed. Peracetic acid formed *in situ* by the reaction between unreacted ACh and H_2O_2 interacts with *p*-phenetidine forming a product, which absorbs at λ_{max} = 358 nm, in the phosphate buffer solution with pH 8.3 at room temperature.

Results and discussion. The linear dependence of the calibration graph for the quantitative determination of dequalinium chloride was in the concentration range of 0.2–0.8 μg mL^{-1} (r = 0.999). LOD and LOQ were 0.01×10^{-6} and 0.03×10^{-6} mol L^{-1}, respectively. For the quantitative determination of dequalinium chloride in lozenges, RSD ≤ 2.65 % (accuracy, δ = -1.10...+1.78 %).

Conclusions. A new enzymatic kinetic spectrophotometric method has been developed, and its applicability to the quantitative determination of dequalinium chloride in lozenges has been shown. It does not require a complicated treatment of the analyte and a tedious extraction procedure. The method proposed is sensitive enough to determine a small amount of the active pharmaceutical ingredient. These advantages encourage the application of the method proposed in routine quality control of the drugs studied in analytical laboratories.

Keywords: dequalinium chloride; cholinesterase; acetylcholine; quantification

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Застосування ензимного методу для кількісного визначення декваліній хлориду в пігулках для розсмоктування

Анотація

Мета. Розробити новий ензимний кінетико-спектрофотометричний метод, придатний для кількісного визначення декваліній хлориду в пігулках для розсмоктування.

Матеріали та методи. Було запропоновано ензимний кінетико-спектрофотометричний метод кількісного визначення декваліній хлориду в пігулках для розсмоктування. Метод заснований на здатності декваліній хлориду інгібувати реакцію ензимного гідролізу ацетилхоліну холінестеразою з наступним визначенням ступеня інгібування кінетичним методом з використанням двох спряжених реакцій: пергідролізу ацетилхоліну (реакція з надлишком гідроген пероксиду) та наступного пероксикислотного окиснення. Пероцтова кислота, утворена *in situ* в результаті реакції між H_2O_2 та непрореагованим ACh, реагує з *n*-фенетидином за кімнатної температури у фосфатному буферному розчині з pH 8,3 з утворенням продукту з максимумом світлопоглинання λ_{max} = 358 нм.

Результати та їх обговорення. Лінійна залежність градуювального графіка для кількісного визначення декваліній хлориду зберігається в інтервалі концентрацій 0,2–0,8 мкг $мл^{-1}$ (r = 0,999). LOD та LOQ були $0{,}01 \times 10^{-6}$ та $0{,}03 \times 10^{-6}$ моль $л^{-1}$ відповідно. За кількісного визначення декваліній хлориду в пігулках для розсмоктування RSD ≤ 2.65 % (точність, δ = -1.10...+1.78 %).

Висновки. Розроблено новий ензимний кінетико-спектрофотометричний метод, який легко можна застосувати до визначення декваліній хлориду в пігулках для розсмоктування. Він не вимагає складного обробляння аналіту та виснажливої

процедури екстрагування. Запропонований метод є достатньо чутливим, щоб дозволити визначати малу кількість активного фармацевтичного інгредієнта. Ці переваги спонукають до застосування запропонованого методу в рутинному контролі якості досліджуваних препаратів у аналітичних лабораторіях.

Ключові слова: декваліній хлорид; холінестераза; ацетилхолін; кількісне визначення

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■ Introduction

For more than 60 years dequalinium chloride (DQ) has been used as an anti-infective drug, mainly to treat local infections. Nowadays, it is a common ingredient of sore-throat lozenges. As a lipophilic *bis*-quaternary ammonium salt (Figure 1) the drug exhibits membrane effects and selectively targets mitochondria to deplete DNA and block energy production in cells. Beyond its mitochondriotropic property, DQ can interfere with the correct functioning of diverse proteins. A dozen of DQ protein targets have been identified; their implication for the antibacterial, antiviral, antifungal, antiparasitic, and anticancer properties of the drug has been discussed [1]. The clinical efficacy and potential attractiveness of this substance have been confirmed by numerous publications [2–5]. Today, the Ukrainian pharmaceutical industry produces a number of local antiseptics with DQ, which are used to treat diseases of the upper respiratory tract. Thus, the question of choosing the method of analysis for lozenges with DQ is relevant.

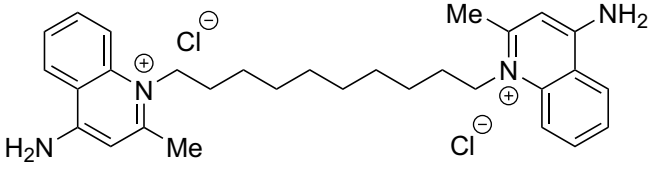
To quantify DQ, the European Pharmacopoeia proposes the method of non-aqueous titration. Acetic anhydride and formic acid are used as solvents, 0.1 M perchloric acid solution is applied as a titrant, and the end point of the titration is recorded potentiometrically [6]. The British Pharmacopoeia also recommends non-aqueous titration to quantify DQ with the perchloric acid solution in a mixture of dioxane/glacial acetic acid with addition of mercury(II) acetate, and crystalline violet is used as an indicator [7]. Additionally, the method of high-performance liquid chromatography is also widely used in the analysis of DQ [8–11]. Several works proposed the method of spectrophotometry for the quantitative determination of DQ [12–14]. All the methods mentioned above have certain advantages; however, they require specific equipment and trained staff.

Thereby, the aim of the current study was to develop a simple, fast, sensitive and affordable method for the quantitative analysis of DQ, particularly in lozenges for treating throat diseases. Previously, we developed a simple, fast, sensitive kinetic-enzymatic spectrophotometric method for the determination of another quaternary ammonium salt – benzalkonium chloride in various dosage forms [15].

■ Materials and methods

Reagents and equipment

Dequalinium chloride (pp. 1410001632, OLON, Italy) with the active substance content of 94.8% was used in the work.



INN: Dequalinium dichloride
Chemical name: 1,1'-(decane-1,10-diyl)bis(4-amino-2-methylquinolin-1-ium)
Molecular weight: 527.6

Figure 1. The structure of dequalinium chloride

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p-Phenetidine, 98% (Sigma-Aldrich); *p*-Phenetidine hydrochloride was prepared by dissolution of *p*-phenetidine in chloroform followed by precipitation of the salt by gaseous HCl.

Acetylcholine chloride, 0.2 g of the substance in ampoules, was of pharmaceutical grade (the State Science Center of Virology and Biotechnology "Vector", Russia).

Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$), puriss. p.a. ("ReaChem", Kharkiv, Ukraine), and a dry cholinesterase from horse serum (SMU "Biomed", Russia), 80 mg (VI class, activity 22 AU mg^{-1}), were used in the study. The catalytic activity of 1 activity unit (AU) is manifested by such an amount of this enzyme preparation that converts 1 μmol of the substrate in 1 min under given reaction conditions.

Stabilized hydrogen peroxide, 30–40% solution, puriss. p.a. (LLC Inter-Synthes, Boryslav, Ukraine) was used; the precise content of hydrogen peroxide was determined by permanganatometry according to the State Pharmacopoeia of Ukraine [16].

High purity double-distilled water was used during our experiments.

"Amilar IC" – orange-flavored lozenges. Active ingredients (in 1 tablet) are: dequalinium chloride, 0.25 mg, dibucaine hydrochloride, 0.03 mg, manufactured by "InterChem SLC" (Ukraine), batch number s.02501118.

"Dekvadol" – white tablets. Active ingredients (in 1 tablet) are: dequalinium chloride, 0.25 mg, dibucaine hydrochloride, 0.03 mg, manufactured by JSC "Kyiv Vitamin Plant" (Ukraine), batch number VM 491219.

"Lizak" – grey to brown tablets. Active ingredients (in 1 tablet) are: dequalinium chloride, 0.25 mg, lysozyme hydrochloride, 10 mg, manufactured by JSC "Farmak", Ukraine, batch number s. 150920.

The absorbance measurements were performed on a colorimeter (CFC-2) (Zagorsky optical and mechanical plant, Russia) using quartz cells with a width of 1 cm.

The pH measurements were performed with a combined glass electrode (SP20B) together with an EAL-1M3.1 reference standard silver chloride electrode.

Preparation of 0.2 M phosphate buffer solution (pH 8.3)

Disodium hydrogen phosphate dodecahydrate (35.75 g) was dissolved in 500 mL of double-distilled water, and a 0.1 M solution of hydrochloric acid (19 mL) was then added. The pH of the final solution was controlled by potentiometry.

Preparation of 10% hydrogen peroxide solution

The solution was prepared by dilution of hydrogen peroxide (30–40%) with the required amount of double distilled water. The content of hydrogen peroxide in 10% working solution was determined by permanganatometry.

Preparation of 1% *p*-phenetidine hydrochloride solution

1.00 g of *p*-phenetidine hydrochloride was dissolved in 80 mL of double-distilled water in a 100 mL volumetric flask and diluted to the volume with the same solvent.

Preparation of cholinesterase (ChE) solution

In a flask containing a dry powder of cholinesterase (80 mg) double-distilled water (10 mL) was added, and the flask was moved to a thermostat for 10 min at a temperature of 37–40°C.

Preparation of acetylcholine chloride (ACh) solution

The ampoule content (0.2 g of ACh) was dissolved in 200 mL of double-distilled water. For this purpose, an ampoule was opened, 4.0 mL of water was pipetted and added to the ampoule and then shaken until ACh was completely dissolved. Then the ACh solution was transferred into a 200 mL volumetric flask and diluted to the volume with double-distilled water [17].

Preparation of the stock solution

0.05277 g (accurate weight) of the DQ substance (the active substance content $w = 94.8\%$) was dissolved in double-distilled water in a 100.0 mL volumetric flask, and the solution was diluted to the volume with the same solvent (with the concentration of 0.5 mg mL^{-1}) at 20°C. The content of the flask was mixed thoroughly.

10.00 mL of the initial solution was pipetted into a 100.0 mL volumetric flask, and the solution was diluted to the volume with double-distilled water at 20°C. The content of the flask was mixed thoroughly (with the concentration of 50 μg mL^{-1}).

The general procedure for the quantitative determination

The first part: 10.0 mL of 0.2 M phosphate buffer solution (pH = 8.3) was transferred into a 20 mL graduated test tube with a ground stopper, 1% ACh solution (1.0 mL) and 10% hydrogen peroxide solution (2.0 mL) were consecutively added, and the stopwatch was started. After that, the solution was shaken thoroughly and thermostated for 10 min at a temperature of 37°C. Then 1% *p*-phenetidine hydrochloride solution (1.0 mL) was added to the test tube, and

the solution was diluted to the volume with double-distilled water. The stopwatch was started again, and the solution was scanned photometrically every minute over the period of 15 min on a photoelectric colorimeter, the color filter No.2 (λ_{max} = 358 nm) and a 1.0 cm cuvette were used. The solution containing only 0.2 M phosphate buffer (10.0 mL, pH = 8.3) was used as a reference solution. The relative rate of the reaction [(ACh + H_2O_2) + *p*-Ph] ($tg\alpha_{V_{max}}$, min^{-1}) was determined as a slope of the the optical density (*A*) *vs* time (*t*, min) kinetic curve.

The second part: 0.2 M phosphate buffer solution (10.0 mL, pH = 8.3), 1% ACh solution (1.0 mL) and the ChE solution (2.0 mL) were transferred into a 20 mL graduated test tube with a ground stopper. After that, the solution was shaken thoroughly and thermostated for 10 min at a temperature of 37°C. Then 10% hydrogen peroxide solution (2.0 mL) was introduced into the test tube while stirring. The mixture was shaken thoroughly and kept for 10 min in a thermostat at the same temperature. Then 1% *p*-phenetidine hydrochloride solution (1.0 mL) was added, and the solution was diluted to the volume with double-distilled water. The stopwatch was started, and every minute the solution was scanned photometrically over the period of 15 min on a photoelectric colorimeter; the color filter No. 2 (λ_{max} = 358 nm) and a 1.0 cm cuvette were used. The solution containing only 0.2 M phosphate buffer (10.0 mL, pH = 8.3) was used as a reference solution. The relative rate of the reaction [(ChE + ACh) + H_2O_2 + *p*-Ph] ($tg\alpha_{min}$, min^{-1}) was determined as a slope of the optical (*A*) *vs* time (*t*, min) kinetic curve.

The third part: 0.2 M phosphate buffer solution (10.0 mL, pH = 8.3) was transferred into a 20 mL graduated test tube with a ground stopper. The accurate volumes of the working standard solutions of DQ or test solutions of the drugs (see below) were added into the test tube. The ChE solution (2.0 mL) was added while stirring, a stopwatch was started, and every solution was shaken thoroughly and thermostated at 37°C for 10 min. Then 1% acetylcholine solution (1.0 mL) was quickly added to the test tube, the stopwatch was started, and the mixture was shaken thoroughly and thermostated for 10 min again. After that, 10% hydrogen peroxide solution (2.0 mL) was added, the mixture was kept in the thermostat for 10 min; further 1% *p*-phenetidine hydrochloride solution (1.0 mL) was added, and the solution was diluted to the volume with double-distilled water. The stopwatch was started, and the solution was scanned photometrically on a photoelectric colorimeter every minute over a period of 15 min; the color filter No. 2 (λ_{max} = 358 nm) and a 1.0 cm cuvette were used. The solution containing 0.2 M phosphate buffer (10.0 mL, pH = 8.3) was used as a reference solution. The relative rate of the reaction [[(ChE + DQ) + ACh] +H_2O_2 + *p*-Ph] ($tg\alpha_{c_i}$, min^{-1}) was determined as a slope of the optical density (*A*) *vs* time (*t*, min) kinetic curve.

The calibration graph procedure

Working standard solutions 1-7 used for calibration

4.00, 6.00, 8.00, 10.00, 12.00, 14.00, 16.00 mL of the stock solution were pipetted into a series of 50.0 mL volumetric flasks, and the solutions were diluted to the volume with double-distilled water at 20°C (final concentrations were 4.0; 6.0; 8.0; 10.0; 12.0; 14.0; 16.0 μg mL^{-1}, respectively).

The performance of the method proposed was verified on the sample concentrations of 0.2; 0.3; 0.4; 0.5; 0.6; 0.7; 0.8 μg mL^{-1} according to the *General procedure*.

The inhibition degree of the enzymatic hydrolysis of acetylcholine (*U*, %), in the presence of DQ was calculated by the following formula:

$$U(\%) = \frac{tg\alpha_{c_i} - tg\alpha_{min}}{tg\alpha_{V_{max}} - tg\alpha_{min}} \times 100\ \%$$

where $tg\alpha_{V_{max}}$ (min^{-1}) – is the relative rate of the *p*-phenetidine oxidation reaction with peroxyacetic acid formed in the reaction of ACh perhydrolysis (in the absence of ChE and DQ);
$tg\alpha_{min}$ (min^{-1}) – is the relative rate of the *p*-phenetidine oxidation reaction with peroxyacetic acid formed in the reaction of perhydrolysis of unreacted ACh (in the presence of ChE and in the absence of DQ);
$tg\alpha_{c_i}$ (min^{-1}) – is the relative rate of the *p*-phenetidine oxidation reaction with peroxyacetic acid formed in the reaction of perhydrolysis of unreacted ACh (in the presence of ChE and DQ with the concentration c_i).

■ Results and discussion

Parameters that can affect the performance of the method proposed were studied to reach the optimum working conditions and reagent concentrations [18].

The measurement velocity of changing the absorption *vs* time ($\Delta A/\Delta t$, min^{-1}) made it possible

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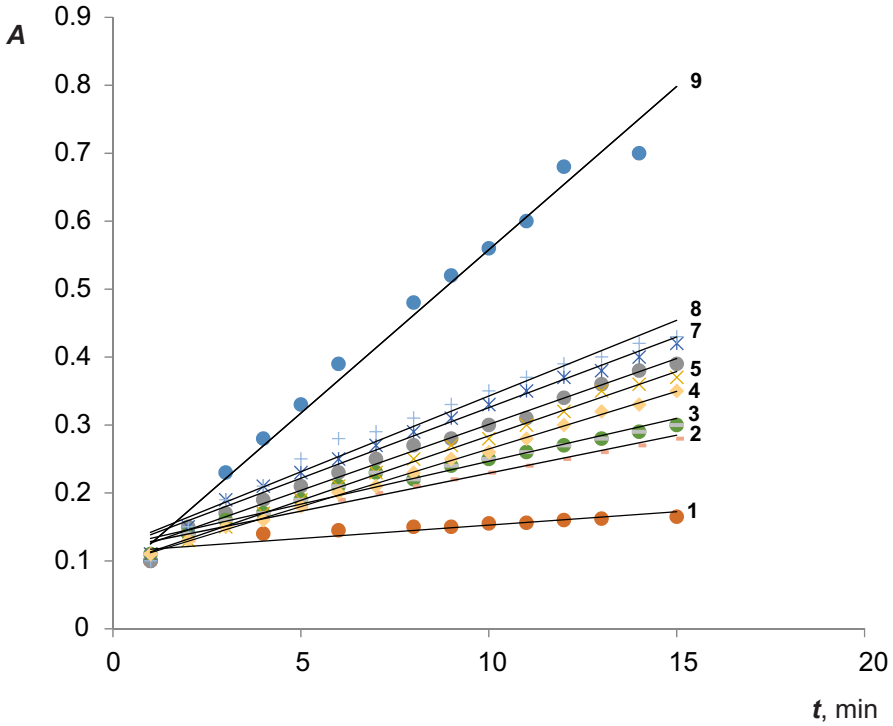

Figure 2. Kinetic curves of *p*-phenetidine oxidation by hydrogen peroxide in the presence of the system: 1 – ACh + ChE, 2–8 –ACh + (ChE + DQ), 9 – ACh; *w* (ACh) = 1%; ChE = 22 AU; *c*(DQ), μg mL^{-1}: 2 – 0.2, 3 – 0.3, 4 – 0.4, 5 – 0.5, 6 – 0.6, 7 – 0.7, 8 – 0.8

Table 1. Metrological characteristics of the linear dependence of the inhibition degree of the enzymatic hydrolysis of acetylcholine (*U*, %) on the concentration of the dequalinium chloride solution

Linearity parameters	$U = b_1X + a_1$	$Y = b_2X + a_2$
b	97.1×10^6	1.45
S_b	0.9×10^6	0.01
a	-14.1	-0.42
S_a	±0.5	1.50
R	0.99997	0.9997
RSD_0	0.49	1.47%
LOD	0.01×10^{-6} mol L^{-1}	–
LOQ	0.03×10^{-6} mol L^{-1}	–

to quantify DQ in the substance. Figure 2 shows the kinetic curves of the reaction of *p*-phenetidine oxidation by hydrogen peroxide in the presence of different concentrations of DQ. They have a linear character at the initial stage; it allows us to use the slope (tgα) of kinetic lines as the value of an analytic signal that corresponds to a certain content DQ in the sample.

The range of application of this procedure was 0.2–0.8 mg mL^{-1}. The calibration graph (Figure 3) was constructed in the coordinates of the concentration (mol L^{-1}) *vs* the inhibition degree (%).

According to the data obtained on the inhibition degree of ChE by various concentrations of DQ, the calibration dependence was constructed in absolute and normalized coordinates (normalization was performed according to the nominal

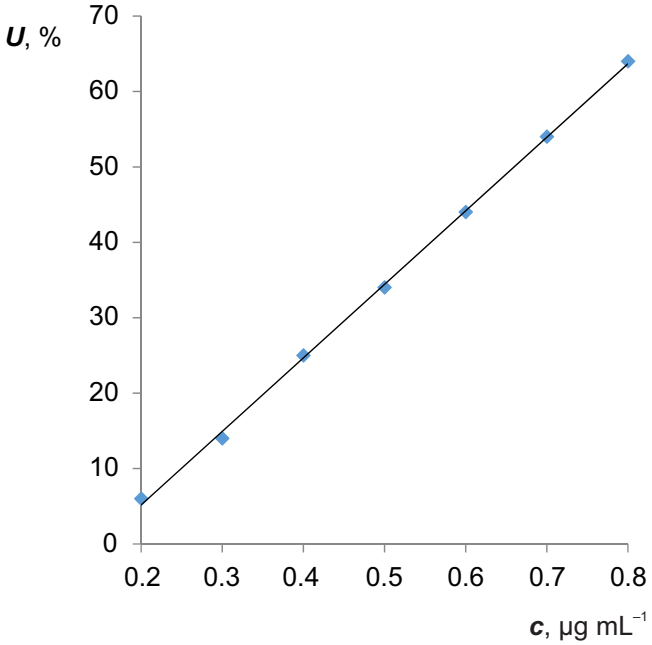

Figure 3. The calibration graph of the dependence of the inhibition degree of the enzymatic hydrolysis of acetylcholine (*U*, %) on the concentration of the dequalinium chloride solution

content in the drug): $U = b_1X + a_1$ and $Y = b_2X + a_2$, respectively (Tables 1 and 2). The least squares method was used to calculate the metrological characteristics of the calibration dependences obtained (angular coefficient b and its standard deviation S_b, free term a, and its standard deviation S_a), as well as correlation coefficient (R) and residual standard deviation RSD_0 (Table 1).

Table 2. The results of the analysis of the working standard solutions and their statistical processing

The actual concentration of DQ in the solutions (C_{crit} = 0.5 mg mL^{-1})		Degree of inhibition ChE U_i, %	Found (%) to $U_{reference}$ Y_i, % ($U_{reference}$ = 33.5%)	The calculated concentration of DQ in the solutions X_i^{calc}, %	RR_i, % = $\frac{X_i^{calc}}{X_i} \times 100$ %
C_i, mg mL^{-1}	X_i, %				
0.2	40	5.02	14.99	39.31	98.26
0.3	60	15.32	45.73	60.51	100.85
0.4	80	24.15	72.09	78.69	98.36
0.5	100	33.95	101.34	98.87	98.87
0.6	120	45.21	134.96	122.05	101.71
0.7	140	52.65	157.16	137.37	98.12
0.8	160	62.54	186.69	157.73	98.58
				$\overline{RR}$, %	99.25
				δ, % = |100 – $\overline{RR}$|	0.75
				δ, % ≤ 0.32 · $max\Delta_x$ = 0.96%	satisfied
				RSD_{RR}, %	1.43
				Δ_{RR}, % = RSD_{RR} · t(95%, n – 1)	2.76
				Δ_{RR}, % ≤ $max\Delta_x$ = 3.05%	satisfied

Table 3. The results of the quantitative determination of DQ in lozenges by the method proposed

Drug	Manufacturer, pharmaceutical factory	The content of DQ (mg)	DQ Found, ($\overline{X}$ ± ΔX), mg[a]	RSD, %	Accuracy (δ, %)[b]
Amilar IC	Ukraine, ALC "Interhem" s. 02501118	0.2422	0.246 ± 0.0081	2.65	+1.56
Dekvadol	Ukraine, PC "Kyiv Vitamin Plant", VM 491219	0.2513	0.2518 ± 0.0030	1.17	-1.10
Lizak	Ukraine, PC "Farmak" s. 150920	0.258	0.2534 ± 0.0058	1.86	+1.78

Notes: [a] Mean of 5 measurements (P = 0.95); [b] $\delta = (\overline{X} - \mu) \times 100\% \times \mu^{-1}$; μ is the actual content of DQ according to the Certificate

From Table 1 one can conclude that the parameters of the calibration linear dependence constructed in normalized coordinates are characterized by satisfactory linearity for the given range of concentrations.

The recommended procedure for the analysis of "Amilar IC", "Dekvadol" and "Lizak" drugs

Twenty (20) tablets of each "Amilar IC", "Dekvadol" and "Lizak" drugs were crushed to homogeneity, and 2.5097 g (accurate weight) of this powder was dissolved in double-distilled water in a 100 mL volumetric flask. The solution was filtered, the filter was washed, and diluted to the volume with double-distilled water. The analysis was performed according to the *General procedure*.

The quantitative content (x) of dequalinium chloride ($C_{30}H_{40}Cl_2N_2$) in one tablet (lozenge) was calculated by the following equation:

$$x\ (g) = \frac{m_{st} \times w\% \times U_{sample} \times 100 \times 20 \times \overline{m}_{tabl}}{m_s \times U_{st} \times 100\ \%}$$

where m_{st} – is the mass of the standard sample of the DQ substance, 0.05277 g;

w – is the active substance content in the working standard solutions of DQ, %;

U_{sample} – is the cholinesterase inhibition degree by the working sample, %;

U_{st} – is the cholinesterase inhibition degree by the standard sample, %;

$\overline{m}_{tabl}$ – is the average mass of the drug working samples, g;

100, 10, 20 – is dilution coefficients.

The degree of inhibition (U_{sample} and U_{st} in %) was calculated according to the procedure described in the literature [19].

The data obtained and presented in Table 3 indicate the possibility to use the method proposed for the quantitative determination of DQ in lozenges.

■ Conclusions

A new enzymatic kinetic spectrophotometric method has been developed, and the possibility of the quantitative determination of dequalinium chloride in lozenges has been shown. The linearity range for the quantification of dequalinium chloride by the enzymatic method was 0.2–0.8 μg mL^{-1}; RSD ≤ 2.65% (accuracy, δ = –1.10…+1.78%); LOQ = 0.03×10^{-6} mol L^{-1}.

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Determination of the extraction frequency of green tea leaves by the antioxidant method

Abstract

Aim. To determine the optimal extraction frequency of green tea leaves with 60 % ethanol by the antioxidant method.

Materials and methods. Chun Myn green tea leaves were the object of the study, the raw material was collected in Anhui province (China) from March to April. Dry green tea leaves were standardized according to the European Pharmacopeia 9.0. Spectrophotometry was used to quantify biologically active substances. The antioxidant activity was determined by the potentiometric method. Potentiometric measurements were performed on a HANNA 2550 pH meter (Germany) with a combined platinum EZDO 50 PO electrode (Taiwan). A UV-1000 spectrophotometer (China) was used to measure the optical density.

Results and discussion. The total content of phenolic compounds was 9.60 ± 0.17, 1.30 ± 0.03 and 0.12 ± 0.002 %, catechins – 9.20 ± 0.18, 1.20 ± 0.02 and 0.07 ± 0.002 %, flavonoids – 0.27 ± 0.005, 0.04 ± 0.001, 0.005 ± 0.001, hydroxycinnamic acids – 0.49 ± 0.01, 0.07 ± 0.002 and 0.007 ± 0.001 %, dry residue – 10.75 ± 0.11, 1.59 ± 0.02 and 0.15 ± 0.002 %, the antioxidant activity was 474.08 ± 9.48, 67.70 ± 1.35 and 7.01 ± 0.14 mmol-equiv $m_{dry\ res}^{-1}$ for the first, second and third extraction, respectively. According to the results obtained, the optimal number of extractions of the raw material with 60% ethanol was found to be two.

Conclusions. The dynamic of extractions of biologically active substances of green tea leaves has been studied by triple extraction of the raw material to find the optimal extraction frequency; for the first time, a method for determining the extraction frequency based on the antioxidant activity of the extracts has been developed and proposed. It has been found that the optimal extraction rate is 2 times. The results obtained will be used in the further production of herbal medicines, dietary supplements, and cosmetic products with a green tea extract.

Keywords: green tea; leaves; analysis; extract; extraction; antioxidant activity

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Визначення кратності екстракції листя зеленого чаю антиоксидантним методом

Анотація

Мета. Визначити оптимальну кратність екстракції листя зеленого чаю 60 % етанолом антиоксидантним методом.

Матеріали та методи. Об'єктом дослідження було листя зеленого чаю сорту Чун Мін, зібране в провінції Анхуй (Китай) з березня до квітня. Сухе листя було стандартизовано відповідно до Європейської фармакопеї 9.0. Для кількісного визначення біологічно активних речовин використовували спектрофотометрію. Для визначення антиоксидантної активності використовували потенціометричний метод. Потенціометричні вимірювання виконували на pH-метрі HANNA 2550 (ФРН) із комбінованим платиновим електродом EZDO 50 PO (Тайвань). Для вимірювання оптичної густини використовували спектрофотометр UV-1000 (Китай).

Результати та їх обговорення. Сумарний вміст фенольних сполук становив 8,60 ± 0,17, 1,30 ± 0,03 і 0,11 ± 0,002 %, катехінів – 9,20 ± 0,18, 1,20 ± 0,02 і 0,07 ± 0,002 %, флавоноїдів – 0,27, 0,04 і 0,005 %, гідроксикоричних кислот – 0,49 ± 0,01, 0,07 ± 0,002 і 0,007 ± 0,001 %, сухий залишок – 10,75 ± 0,11, 1,59 ± 0,02 і 0,15 ± 0,002 %, антиоксидантна активність становила 474,08 ± 9.48, 67,70 ± 1.35 і 7,01 ± 0.14 ммоль-екв. $m_{сух.зал.}^{-1}$ для першої, другої і третьої екстракції відповідно. Одержані результати свідчать, що двократна екстракція досліджуваної сировини 60 % етанолом є оптимальною.

Висновки. Для визначення оптимальної кратності екстракцій було проведено дослідження динаміки екстракцій біологічних активних речовин листя зеленого чаю шляхом трикратної екстракції сировини, а також уперше розроблено

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й запропоновано метод визначення кратності екстракції, заснований на визначенні антиоксидантної активності екстрактів**.** Виявлено, що двократна екстракція є оптимальна. Отримані результати будуть використані для створення фітопрепаратів, дієтичних добавок та косметологічної продукції з екстрактом зеленого чаю.

***Ключові слова*:** зелений чай; листя; аналіз; екстракт; екстракція; антиоксидантна активність

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■ Introduction

Oxidative stress is a part of many diseases, such as diabetes mellitus, cancer, atherosclerosis, Alzheimer's disease, and chronic obstructive pulmonary disease [1]. Literature data evidence represents that oxidative stress is caused by the imbalance between the production and degradation of reactive oxygen species (ROS). Examples of ROS include free radicals, such as the superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), and nitric oxide radical ($NO^{\bullet}$) [2].

The defense system of the human body involves endogenous enzymes inactivating ROS. The members of this group are superoxide dismutase, catalase, and glutathione peroxidase [3]. However, there are cases when the endogenous defense system is exhausted by chronic diseases, stress, and not a good diet. As a result, it requires exogenous antioxidants, such as phenolic compounds, carotenoids, vitamin E, and amino acids. Among them, the leading position in the top of antioxidants is taken by catechins – a subgroup of phenolic compounds [4].

Green tea leaves are the major source of catechins. The content of catechins there varies from 25 to 35% [5]. Moreover, green tea leaves contain other subgroups of phenolic compounds. Thus, flavanols (1–2.5%), flavanones (1.5–3%), phenolic acids (2–5%); caffeine (1.5–2.5%), amino acids (1–5.5%), organic acids (1–1.8%) have been isolated from green tea leaves [6].

Owing to the chemical composition of green tea leaves, the extract from this raw material can be used in developing and creating herbal medicines, dietary supplements, and cosmetic products, which further can be used by patients with cardiovascular diseases, diabetes mellitus, cancer, polycystic ovary syndrome, infectious diseases, etc. Today, the optimal extraction frequency is determined by measuring the content of biologically active substances, as well as the dry residue in the extracts obtained. However, no one has previously described the determination of the extraction frequency by measuring the antioxidant activity of the extracts. Our recent study [3] has revealed that 60% ethanol is the optimal solvent for obtaining an extract from green tea leaves. Therefore, the current work aimed to study the dynamics of extraction of biologically active substances from green tea leaves with 60% ethanol to determine the optimal extraction frequency by the method of measuring the antioxidant activity.

■ Materials and methods

Chun Myn green tea leaves were the object of the study, the raw material was collected in Anhui province (China) from March to April. Dry green tea leaves were standardized according to the European Pharmacopeia 9.0 [7]. All solvents and other chemicals used in the study were of analytical grade.

Potentiometric measurements were performed on a HANNA 2550 pH meter (Germany) with a combined platinum EZDO 50 PO electrode (Taiwan). The quantitative analysis of biologically active compounds was performed on a UV-1000 spectrophotometer (China) with matched 1 cm quartz cells.

The weighing was done using an AN100 digital analytical balance (AXIS, Poland) with d = 0.0001 g.

Extraction procedure

Five (5.0) g of the grinded leaves was mixed with 100 mL of 60% ethanol. The extraction was carried out within 1 hour on a water bath with a condenser, then it was repeated two times with a new portion of the solvent. After that, the extracts

obtained were filtrated and concentrated to 10 mL using a rotary evaporator.

The dry residue of the extracts was determined by the gravimetric method according to the State Pharmacopeia of Ukraine (SPhU) [8].

Preparation of the standard solutions

To plot the calibration curve of the dependence of absorbance on the amount of gallic acid, the stock solution (250 mg mL^{-1}) was prepared by dissolving 50.0 mg (accurate weight) of gallic acid in 96% ethanol, and the solution was diluted to 200.0 mL with the same solvent. The stock solution was diluted with the solvent to prepare the model solutions 1–5 with concentrations of 1.0; 2.0; 3.0; 4.0; 5.0 μg mL^{-1}, respectively.

To plot the calibration curve of the dependence of absorbance on the amount of (–)-epigallocatechin gallate, the stock solution (10 mg mL^{-1}) was prepared by dissolving 250.0 mg of (–)-epigallocatechin gallate in 96% ethanol, and the solution was diluted to 25.0 mL with the same solvent. The stock solution was diluted with the solvent to prepare the model solutions 1–5 with the concentrations of 100; 150; 200; 300; 400 μg mL^{-1}, respectively.

The standard solution of rutin was prepared by weighing 10 mg (accurate weight) of rutin, transferred in a 25 mL volumetric flask, dissolved in 70% ethanol while heating on a water bath, and diluted to the volume with the same solvent. 1.0 mL of the solution prepared was transferred into a 25 mL volumetric flask and diluted to the volume with the same solvent.

The total content of phenolic compounds was measured by the Folin-Ciocalteu assay, the optical density was measured at 760 nm [9]. The calibration curve was plotted with interval concentrations of 1.0–5.0 μg mL^{-1}, the calibration equation $Y = 0.1055X + 0.1745$ ($R^2 = 0.9951$). The total content of phenolic compounds with reference to gallic acid was calculated according to the following equation:

$$X(\%) = \frac{C_x \times K_{dil} \times 100}{V}$$

where C_x – is the concentration of gallic acid according to the calibration curve, $C \times 10^{-6}$, g mL^{-1}; V – is the volume of the extract, mL; K_{dil} – is the dilution coefficient.

The vanillin reagent assay was applied to determine the total amount of catechins [5], and the absorbance was measured at 505 nm. The calibration curve was plotted within interval concentrations of $(100–400) \times 10^{-6}$ g mL^{-1}, the calibration equation $Y = 0.0025X – 0.0851$ ($R^2 = 0.9951$). The total catechins content in the extracts with reference to (–)-epigallocatechin gallate was calculated according to the following equation:

$$X(\%) = \frac{C_x \times K_{dil} \times 100}{V}$$

where C_x – is the concentration of (–)-epigallocatechin gallate according to the calibration curve, $C \times 10^{-6}$ g mL^{-1}; V – is the volume of the extract, mL; K_{dil} – is the dilution coefficient.

The total flavonoids were determined using the assay of the complex formation with 2% $AlCl_3$, the absorbance was measured at 417 nm [10].

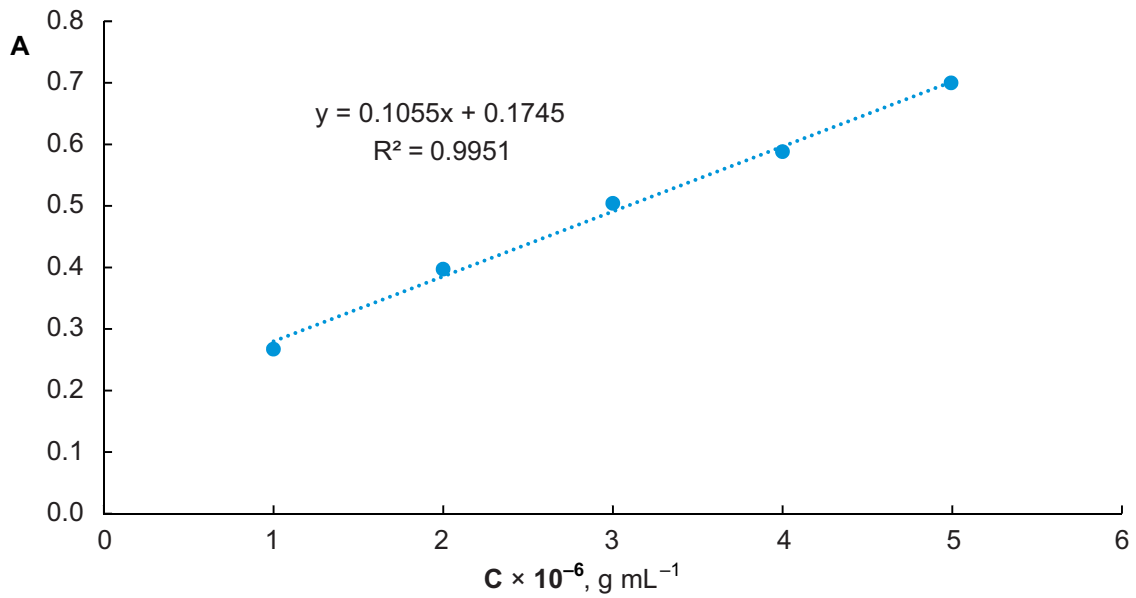

Figure 1. The calibration curve of the absorbance against the concentration of gallic acid

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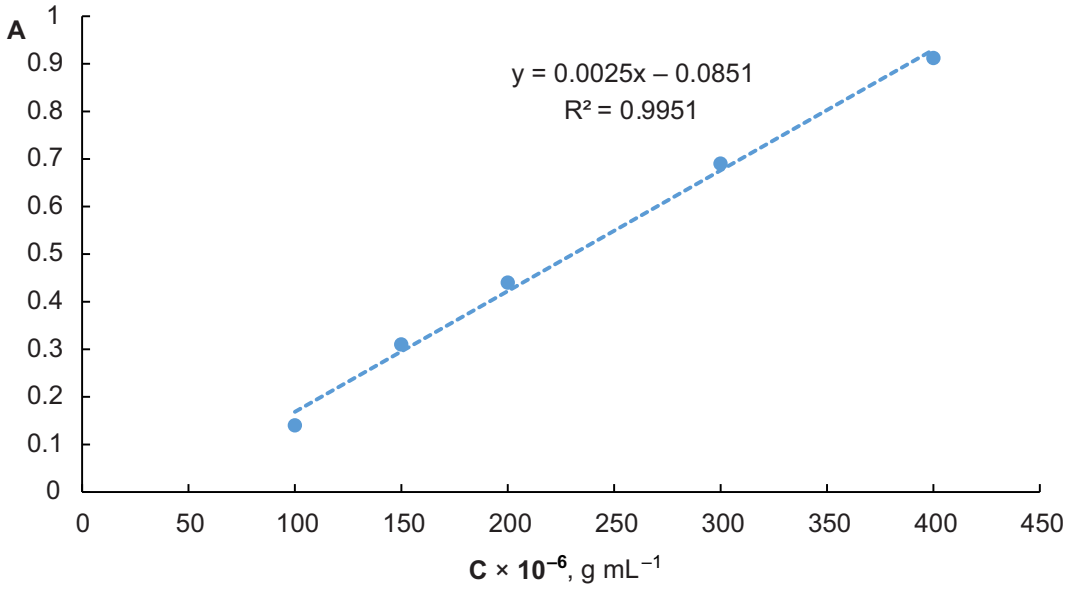

Figure 2. The calibration curve of the absorbance against the concentration of (–)-epigallocatechin gallate

The total content of flavonoids in the extracts with reference to rutin was calculated according to the following equation:

$$X(\%) = \frac{A \times K_{dil} \times 100}{A_{st} \times V}$$

where A – is the absorbance of the solution analyzed; A_{st} – is the absorbance of the standard solution of rutin; V – is the volume of the extract, mL; K_{dil} – is the dilution coefficient.

The total content of hydroxycinnamic acids was measured by the assay of complex formation with $NaNO_2$-Na_2MoO_4, and the absorbance was measured at 505 nm [11]. The total content of hydroxycinnamic acids in extracts with reference to chlorogenic acid was calculated according to the following equation:

$$X(\%) = \frac{A \times K_{dil}}{188 \times V}$$

where A – is the absorbance of the solution analyzed; 188 – is the specific absorption coefficient of chlorogenic acid; V – is the volume of the extract, mL; K_{dil} – is the dilution coefficient.

The antioxidant activity of the extracts was measured by the potentiometric method [12] calculated according to the following equation, and expressed as mmol-equiv $m_{dry\ res.}^{-1}$:

$$AOA = \frac{C_{ox} - \alpha \times C_{red}}{1 + \alpha} \times K_{dil} \times 10^3 \times \frac{m_1}{m_2}$$

where $\alpha = C_{ox}/C_{red} \times 10^{(\Delta E - E\text{ethanol})nF/2.3RT}$; C_{ox} – is the concentration of $K_3[Fe(CN)_6]$, mol L^{-1}; C_{red} – is the concentration of $K_4[Fe(CN)_6]$, mol L^{-1}; $E_{ethanol} - 0.0546 \times C_{\%} - 0.0091$; $C_{\%}$ – is the concentration of ethanol; ΔE – is the change of the potential; $F = 96485.33$ C mol^{-1} – is the Faraday constant; n = 1 – is the number of electrons in the electrode reaction; $R = 8.314$ J mol^{-1} K^{-1} – is the universal gas constant; T – 298 K; K_{dil} – is the dilution coefficient; m_1 – is the mass of a dry residue; m_2 – is the mass of a dry residue in 1.0 mL of the extract.

For all experiments, five samples were analyzed, and all assays were performed 5 times. The results were expressed as mean values with confidence intervals. MS EXCEL 7.0 and STATISTIKA 6.0 were used to perform the statistical analysis.

■ Results and discussion

The total content of phenolic compounds was determined by the Folin-Ciocalteu method. According to the results obtained given in Table, the total content of phenolic compounds was 9.60±0.17% in the first extraction, whereas in the second extract the amount of phenolic compounds decreased by 86%, and in the case of the third extraction by 99%. The summary content of phenolic compounds was equal to 11.02% in the total extract.

The amount of catechins was measured using the vanillin reagent assay. Table represents that in the first extraction the content of catechins was 9.20±0.18%, in the second extraction – 1.20±0.02%, and in the third one – 0.07±0.002%. The total catechins were equal to 10.47% in the total extract. Hence, the contribution of the first

Table. The total content of phenolic compounds, catechins, flavonoids, and hydroxycinnamic acids in green tea extracts

	1st extraction	2nd extraction	3rd extraction	Sum
Dry residue, %	10.75 ± 0.11	1.59 ± 0.02	0.15 ± 0.005	12.49
Total phenolic compounds, %	9.60 ± 0.17	1.30 ± 0.03	0.12 ± 0.002	11.02
Total catechins, %	9.20 ± 0.18	1.20 ± 0.02	0.07 ± 0.002	10.47
Total flavonoids, %	0.27 ± 0.005	0.04 ± 0.001	0.005 ± 0.001	0.32
Total hydroxycinnamic acids, %	0.49 ± 0.01	0.07 ± 0.002	0.007 ± 0.001	0.56
Antioxidant activity, mmol-equiv $m_{dry\ res.}^{-1}$	474.08 ± 9.48	67.70 ± 1.35	7.01 ± 0.14	548.79

extraction to the overall extract was 87.87%, the second one – 11.46%, and the third one – 0.67%.

The total amount of flavonoids in extracts was relatively low compared to other biologically active substances. In the first extract their content was 0.27±0.005%, in the second one – 0.04±0.001%, and the third one – only 0.005±0.001%.

Table also shows that the total content of hydroxycinnamic acids was 0.56% in the total extract. The first extraction provided 0.49±0.01%, the second one – 0.07±0.002%, and the last one – 0.007±0.001% to the total extract. Among all phenolic compounds, hydroxycinnamic acids took second place after catechins.

The dry residue of the extracts analyzed was determined by the gravimetric method of analysis. Table shows that the dry residue in the first extraction was 10.75±0.11%, in the second one – 1.59±0.02%, and the third one – 0.15±0.005%. The total dry residue of three extractions was 12.49%.

Today the main method of finding the extraction frequency from various types of the raw material is based on determining the dry residue and the amount of biologically active substances in extracts. According to the data obtained (Table, Figure 3), the optimal extraction frequency equals two. The third extraction with a new portion of the solvent does not significantly increase the amount of biologically active substances in the total extract.

However, in our view, this way of determining the extraction frequency is not rather suitable due to several disadvantages of the method. Among others, one should note that the gravimetric method and the measuring amount of all

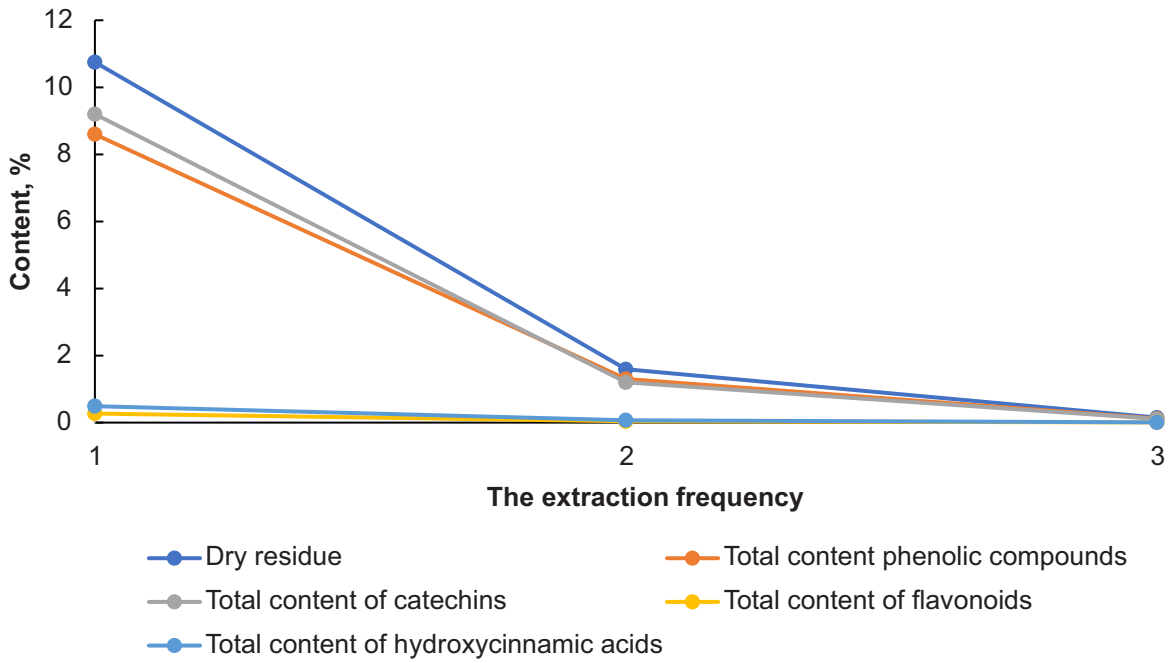

Figure 3. Curves of the dry residue, total phenolic compounds, catechins, flavonoids, hydroxycinnamic acids versus the extraction frequency

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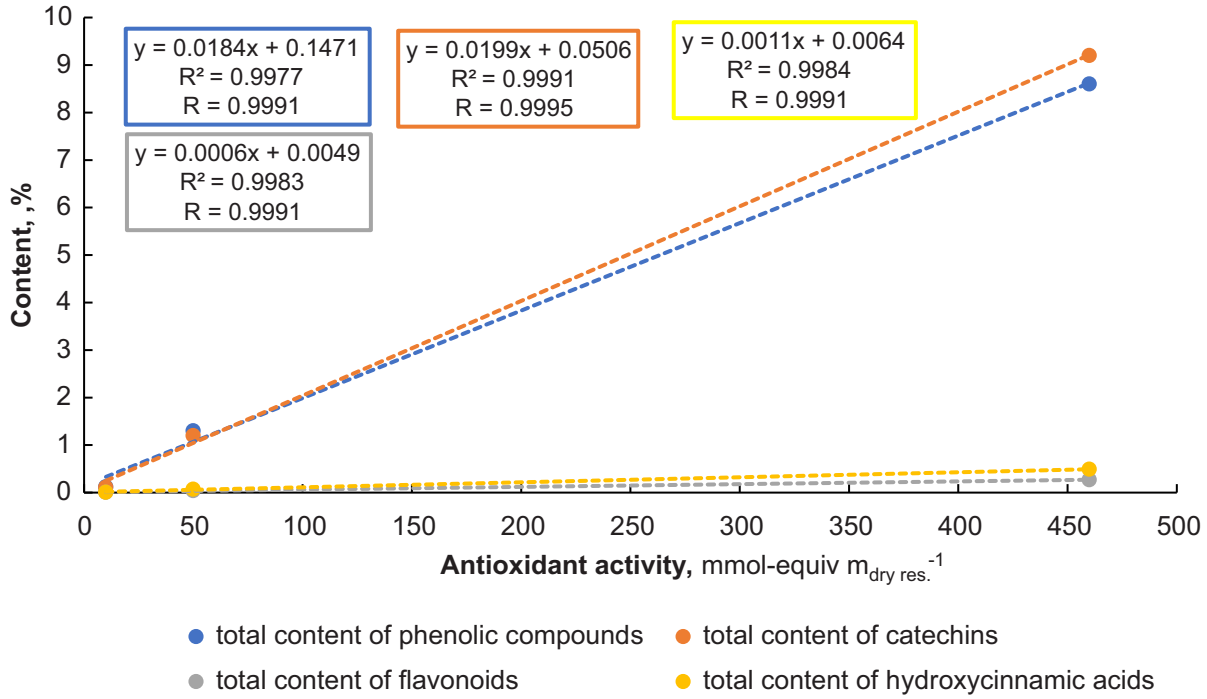

Figure 4. The correlation between the antioxidant activity and the content of total phenolic compounds, catechins, flavonoids, hydroxycinnamic acids in extracts of green tea leaves

biologically active substances in extracts are rather time-consuming processes.

Therefore, herein we suggest determining the optimal extraction frequency using the antioxidant activity of the extracts obtained and the total antioxidant activity of the raw material. There are some reasons for that. Firstly, the potentiometric method of the antioxidant activity determination is simple and not time-consuming. Secondly, the accuracy of the method is not inferior to that one of gravimetry. Moreover, it does not need expensive reagents and equipment for analysis. In addition, in many scientific studies the antioxidant activity correlates with the anti-inflammatory effect, this gives an idea of other possible effects of the extracts under research [14].

In our previous study [15] it was found that the total antioxidant activity of green tea leaves equaled 660.75 mmol-equiv $m_{dry\ res.}^{-1}$. In our opinion, the most optimal value for the acceptance criterion of the antioxidant activity is 80% of the total antioxidant activity of the raw material, which is due to the fact that, depending on the type of green tea, the antioxidant activity of its extracts may vary. Thus, in our case, the acceptance criterion equals 528 mmol-equiv $m_{dry\ res.}^{-1}$. Table represents that the antioxidant activity of the 1st extract was 474.08 mmol-equiv $m_{dry\ res.}^{-1}$, the 2nd extract – 67.70 mmol-equiv $m_{dry\ res.}^{-1}$ and the 3rd extract – 7.01 mmol-equiv $m_{dry\ res.}^{-1}$. The antioxidant activity of the total extract was equal to 548.79 mmol-equiv $m_{dry\ res.}^{-1}$ as shown in Table. The 1st extraction contributes 86% to the total antioxidant activity, the 2nd extraction – 12.73%, and the 3rd one only 1.27%. According to the results, the total antioxidant activity is equal to 542 mmol-equiv $m_{dry\ res.}^{-1}$ after two extractions of green tea leaves, and it meets the requirement of the acceptance criteria as it is not less than 528 mmol-equiv $m_{dry\ res.}^{-1}$. Therefore, the 3rd extraction was redundant and only led to overspending of the solvent, energy, and labor resources, and decreasing the labor productivity.

To compare the methods of determining the extraction frequency, the correlation between antioxidant activity and the content of biologically active substances in green tea extracts was assessed. For this purpose, the linear regression analysis and Pearson's coefficient (R) were used. As a result, it was found that there was a very high correlation between the antioxidant activity and the amount of phenolic compounds (R = 0.9991), catechins (R = 0.9995), flavonoids (R = 0.9991), and hydroxycinnamic acids (R = 0.9991). Thus, there is no difference in the results and the method of the antioxidant activity can be applied to determine the optimal extraction frequency from green tea leaves. The results obtained are shown in Figure 4.

■ Conclusions

The dynamic of extractions of biologically active substances of green tea leaves has been studied

by triple extraction of the raw material to find the optimal extraction frequency; for the first time, a method for determining the extraction frequency based on the antioxidant activity of the extracts has been developed and proposed. Thus, it has been found that the optimal extraction rate is 2 times. The results obtained will be used in the further production of herbal medicines, dietary supplements, and cosmetic products with a green tea extract.

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A comparative study of the effect of phenothiazine derivatives and their S-oxides on cholinesterase investigated by a new kinetic spectrophotometric method

Abstract

Aim. To develop a new kinetic spectrophotometric method for determining acetylcholinesterase (AChE) inhibitors – phenothiazine antipsychotic drugs (PhT) and their sulfoxide metabolites (S-oxides) without adding an exogenous catalyst to obtain a chromogenic agent.

Materials and methods. The bases of S-oxides of promethazine (PMZ), chlorpromazine (CPM) and thioridazine (THZ) were obtained by oxidizing the corresponding PhT hydrochlorides with diperoxyadipic acid. The structure of the S-oxides of the corresponding PhT synthesized was proven by melting points, spectral characteristics (^{1}H NMR and IR methods) and oscillopolarography results. ^{1}H NMR spectra were recorded on a Varian XL-200 spectrometer. IR spectra were recorded within the range of 4000-400 cm^{-1} on a SPECORD M-80 spectrometer (Zeiss, Jena, Germany). To register polarograms, a "PO 03 CLA" oscillopolarograph with a three-electrode cell was used. The purity of S-oxides was determined by the high-performance liquid chromatography method on a Zorbax SB, C-18 (250×4.6) mm column. Measurements of absorbance of solutions were performed in a 1 cm cuvette on an Evolution 60S UV-Visible Thermo-Scientific Spectrophotometer (USA) (λ = 358 nm).

Results and discussion. Acetylcholine (ACh) was found to mimic the activity of peroxidase; based on it, a spectrophotometric system containing ACh–H_2O_2–*p*-phenetidine for a sensitive and selective assessment of the AChE activity and determination of its inhibitors was developed. According to the plots of inhibition efficiency *vs* inhibitors concentration, the inhibiting ability of chlorpromazine, promethazine and thioridazine and their S-oxides was determined. The IC_{50} values of CPM, PMZ and THZ and their metabolites in relation to the AChE activity were estimated as 11 ng mL^{-1} (CPM) and 1.8 ng mL^{-1} (CPM S-oxide), 17 ng mL^{-1} (PMZ) and 2.5 ng mL^{-1} (PMZ S-oxide) and 27 ng mL^{-1} (THZ 2S,5S-dioxide). The results obtained indicate that S-oxides of the corresponding PhT are selective and potent inhibitors of AChE. The values of the inhibition efficiency obtained for S-oxides of PhT derivatives were an order of magnitude lower than those of the corresponding PhT derivatives.

Conclusions. The spectrophotometric method proposed without the addition of other exogenous catalysts holds promise for the on-site determination of PhT antipsychotics and can be additionally used for sensory applications in areas related to environmental protection and food safety, as well as in the chemical-toxicological analysis.

Keywords: acetylcholine; acetylcholinesterase; phenothiazine derivatives; S-oxides; photometric methods of analysis

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Порівняльне вивчення впливу похідних фенотіазину та їх S-оксидів на холінестеразу за допомогою нового кінетичного спектрофотометричного методу

Анотація

Мета. Розробити новий кінетико-спектрофотометричний метод визначення інгібіторів ацетилхолінестерази (AChE), фенотіазинових антипсихотичних засобів (PhT) та їх сульфоксидних метаболітів (S-оксидів) без додавання екзогенного каталізатора для отримання хромогенного агента.

Матеріали та методи. Основи S-оксидів прометазину, хлорпромазину та тіоридазину синтезували шляхом окиснення відповідних гідрохлоридів PhT дипероксиадипіновою кислотою. Будову синтезованих S-оксидів відповідних PhT доводили за температурами плавлення, спектральними характеристиками (^{1}H ЯМР- та ІЧ-методи) та результатами осцилополярографії. Спектри ^{1}H ЯМР записували на спектрометрі Varian XL-200. ІЧ-спектри реєстрували в діапазоні

4000-400 см$^{-1}$ на спектрометрі SPECORD M-80 (Zeiss, Jena, Німеччина). Для реєстрації полярограм використовували осцилополярограф ПО, модель 03 ЦЛА з триелектродним елементом. Чистоту S-оксидів визначали методом ВЕРХ на колонці Zorbax SB, C-18 (250 × 4,6) мм. Вимірювання поглинання випробуваних розчинів S-оксидів відповідних PhT проводили за довжини хвилі λ = 358 нм у кюветі завтовшки 1 см на спектрофотометрі Evolution 60S UV-Visible Thermo-Scienticfic (США).

Результати та їх обговорення. З'ясовано, що ацетилхолін (ACh) імітує активність пероксидази, на основі чого було розроблено спектрофотометричну систему, що містить ACh-H_2O_2-*п*-фенетидин для чутливого селективного оцінювання активності AChE та визначення інгібіторів ферменту. Відповідно до графіків залежності інгібувальної здатності від концентрації інгібіторів визначено інгібувальну здатність хлорпромазину, прометазину, тіоридазину та їх S-оксидів. IC_{50} хлорпромазину, прометазину, тіоридазину та їх метаболітів щодо активності AChE оцінено так: 11 нг мл$^{-1}$ – хлорпромазин, 1,8 нг мл$^{-1}$ – хлорпромазин S-оксид, 17 нг мл$^{-1}$ – прометазин, 2,5 нг мл$^{-1}$ – прометазин S-оксид та 27 нг мл$^{-1}$ – тіоридазин 2S,5S-діоксиду. Одержані результати свідчать про те, що S-оксиди відповідних PhT є селективними та потужними інгібіторами AChE. Отримані значення ефективності інгібування для S-оксидів похідних PhT були на порядок нижчими, ніж у відповідних похідних PhT.

Висновки. Розроблена спектрофотометрична методика без додавання інших екзогенних каталізаторів має перспективи для визначення PhT нейролептиків на місці і може бути додатково використана для сенсорних застосувань у сферах, пов'язаних із захистом навколишнього середовища та безпекою харчових продуктів, а також у хіміко-токсикологічному аналізі.

***Ключові слова*:** ацетилхолін; ацетилхолінестераза; похідні фенотіазину; S-оксиди; фотометричні методи аналізу

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■ Introduction

Acetylcholinesterase (AChE) is an important enzyme in the central and peripheral nervous system [1]. Its primary biological function is to catalyze the breakdown of acetylcholine (ACh) and some other choline esters that function as neurotransmitters [2]. Nowadays, it is accepted that Alzheimer's disease (AD), common dementia for older people worldwide, is related to a low level of ACh in the hippocampus and cortex [3]. AChE inhibitors can penetrate the blood-brain barrier and have been suggested to increase the level of ACh to treat AD [4]. However, ACh excess results in neuromuscular paralysis or fatal consequences [5].

Various analytical techniques, such as Ellman's method [6], electrochemical methods [7–10], the liquid crystals-based method [11] and chemiluminescent and fluorescent methods [12–19], have been developed for the AChE activity and screening of its inhibitors.

However, some of these methods suffer from drawbacks of the time-consuming sample pretreatment, sophisticated instrument manipulation or high costs. Moreover, it is reported that Ellman's method may lead to a false-positive effect [20].

Hence, developing a simple, highly sensitive and selective method for probing the AChE activity and screening for its potential inhibitors is highly needed.

Photometric methods have attracted significant attention because of their simplicity and low-cost advantages. Moreover, they can circumvent the relative complexity inherent in other detection methods by relying on unaided visual readouts instead of complicated instruments, which is especially useful for on-site detection in real-time [21, 22].

Earlier, we proposed a new kinetic spectrophotometric method for determining the AChE activity. It consists of spectrophotometric measurement of the rate of the ACh enzymatic hydrolysis through two conjugated reactions – perhydrolysis of the non-hydrolyzed ACh residue with a hydrogen peroxide excess and oxidation of the indicator substance *p*-phenetidine (*p*-Ph) by peroxyacetic acid formed to 4,4'-azoxyphenetol (λ_{max} = 358 nm, lgε 4.2) [23]. The rate of the enzymatic hydrolysis of ACh was measured by the tangent method using the linear part of the

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kinetic curve in the light absorbance (*A*, 358 nm) *vs* time (*t*, min) coordinates. The linear inversely proportional dependence of the conditional reaction rate on the enzyme concentration was observed. The same principle has been used to quantify compounds capable of inhibiting AChE enzyme as a change in the activity of AChE causes a change in the amount of unreacted ACh in the enzymatic hydrolysis reaction. The latter is quantified by the kinetic-spectrophotometric method as mentioned above (Scheme) [24].

■ Materials and methods

Acetylcholine chloride (pharmaceutical grade), 0.02 g per amp/5 mL, produced by "VECTOR", State Science Centre of Virology and Biotechnology, Russia.

Disodium hydrogen phosphate dodecahydrate, $Na_2HPO_4 \cdot 12H_2O$ (puriss. p.a.), produced by "ReaChem", Kharkiv, Ukraine.

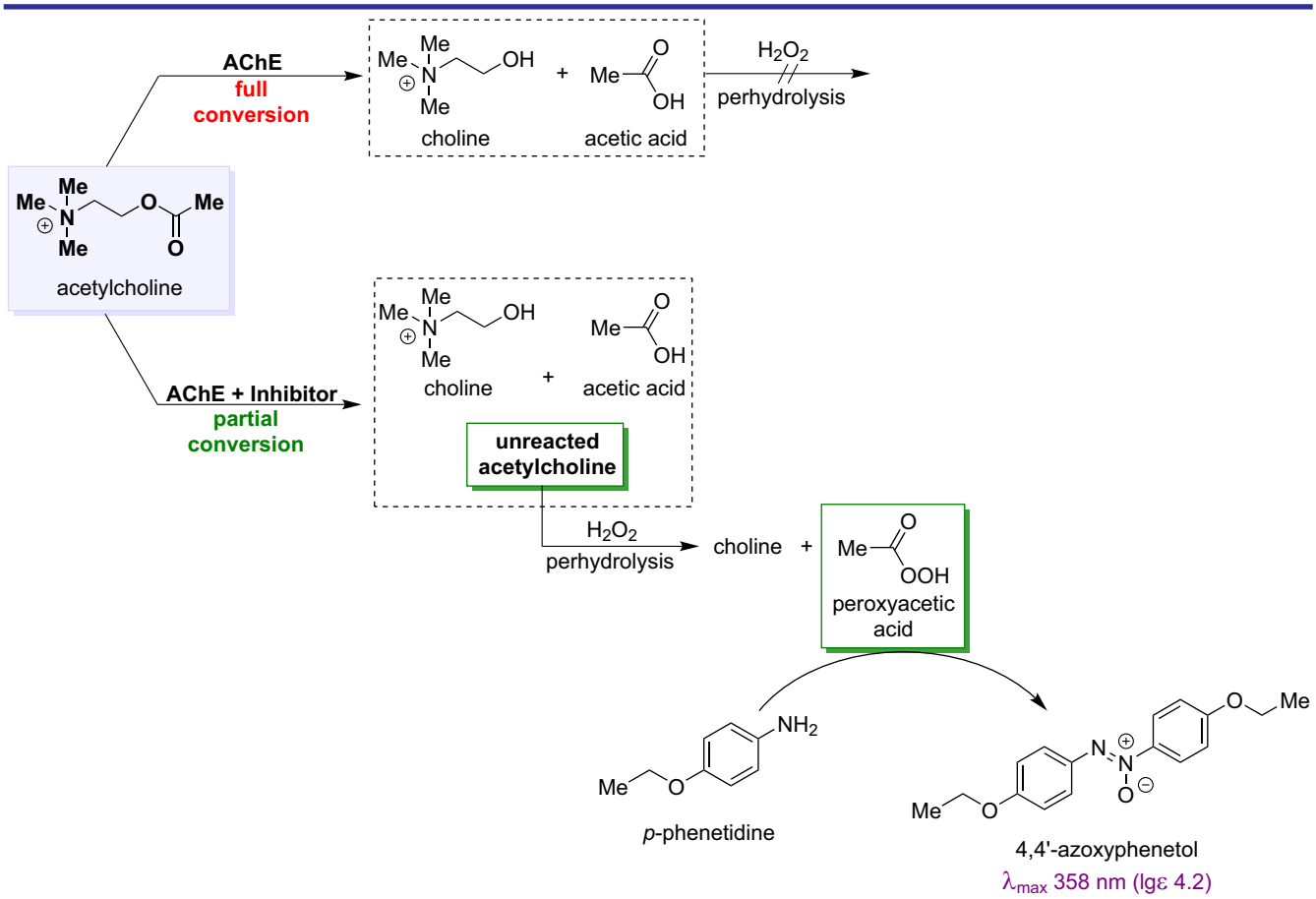
A dry acetylcholinesterase enzyme from horse serum – 80 mg per vial (VI class) with the known specific activity of 27 AU mg^{-1} (according to the certificate) produced by SMU "Biomed" (Russia). The catalytic activity of 1 activity unit (AU) is manifested by such an amount of this enzyme preparation that converts 1 μmole of this substrate in 1 min under given reaction conditions.

A stabilized hydrogen peroxide 30–40% solution, puriss. p.a. (LLC Inter-Synthes, Boryslav, Ukraine). The precise content of hydrogen peroxide was determined according to the State Pharmacopoeia of Ukraine [25].

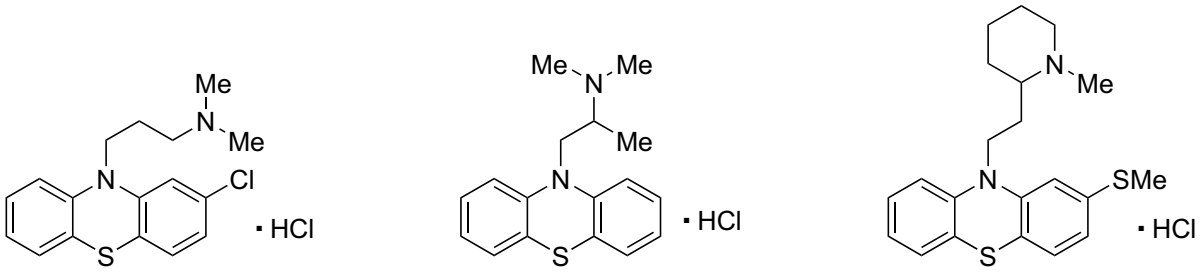
Chlorpromazine hydrochloride ≥98% (TLC); CAS 69-09-0, Sigma-Aldrich. $C_{17}H_{19}ClN_2S \cdot HCl$ (CPZ), 2-chloro-10-(3-dimethylaminopropyl)phenothiazine hydrochloride.

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

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



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



chlorpromazine hydrochloride | promethazine hydrochloride | thioridazine hydrochloride

Figure 1. The structures of the phenothiazines tested

High purity double distilled water was used in all experiments.

Chlorpromazine hydrochloride, promethazine hydrochloride, thioridazine hydrochloride and thioridazine 2S,5S-disulfoxide were obtained from commercial sources and used without further purification.

The structures of the phenothiazine antipsychotic drugs and the corresponding S-oxides are given in Figure 1.

^{1}H NMR spectra were recorded on a Varian XL-200 Spectrometer (200 MHz) using DMSO-d_6 as a solvent and TMS as an internal standard.

IR spectra were recorded within the range of 4000–400 cm^{-1} on a SPECORD M-80 spectrometer (Zeiss, Jena, Germany) in KBr pellets (200 mg of KBr and 2 mg of the test compounds).

Voltammograms were recorded on a PO 03 CLA oscilllographic polarograph (Rostov-on-Don Research Institute, Russia) in a three electrode thermostated cell at 20°C; the indicator microelectrode was a dropping mercury one, the reference electrode was a saturated calomel electrode, and the auxiliary electrode was a platinum electrode. The potentials of peak maxima were measured with a V7-21 digital voltmeter with the precision of ±1 mV. Triangular-shaped polarizing voltage was applied to the cell electrodes at a scanning rate of = 0.5 V s^{-1}. The potential was varied in the range from –0.2 to –1.4 V. Dissolved oxygen was removed from solutions by blowing purified argon over 20 min. The polarography conditions were selected in each case.

The pH measurements were performed with a combined glass electrode (SP20B) together with an EAL-1M3.1 reference standard silver chloride electrode.

The measurements of absorbance (*A*) of solutions were performed in a 1.0 cm cuvette on an Evolution 60S UV-Visible Thermo-Scientific Spectrophotometer (USA) against the buffer solution with double distilled water (compensation solution).

The preparation procedure for the promethazine S-oxide base

0.64 g (0.002 mol) of PMZ was dissolved in 15 mL of distilled water; 0.44 g (0.0025 mol) of diperoxyadipic acid was added with stirring and left at room temperature for 15 min. The mixture was poured with 2 mL of 50% sodium hydroxide solution, and the resulting precipitate was extracted with diethyl ether (3×10 mL). The combined organic phases were washed with water cooled to 10°C (3×20 mL), dried over anhydrous sodium sulfate, and the solvent was evaporated at room temperature. Acetone was added to the residue, and the mixture was cooled. In 2 days, white crystals precipitated; then they were filtered and dried at room temperature. The yield of PMZ S-oxide was close to the quantitative one.

Diperoxyadipic acid was obtained by the interaction between hydrogen peroxide and adipic acid in the presence of sulfuric acid according to the known method [24]. Diperoxyadipic acid, $HO_3C(CH_2)_4CO_3H$, 98%, m. p. 114.5°C (dec.), active oxygen content, % (theor.), 17.5 (17.9).

Promethazine S-oxide base. M. p. 118-119°C. ^{1}H NMR (200 MHz, DMSO-d_6), δ, ppm: 0.75 (3H, d, CH_3); 2.42 (6H, s, $2NCH_3$); 2.81–2.95 (1H, m, CH); 4.18–4.60 (2H, t, CH_2); 7.38–7.92 (8H, m; ArH). IR (KBr), ν, cm^{-1}: 1028 (S=O).

CPZ S-oxide base was synthesized by the similar procedure as for PMZ S-oxide base.

Chlorpromazine S-oxide base. M. p. 111-112°C. ^{1}H NMR (200 MHz, DMSO-d_6), δ, ppm: 1.82 (2H, q, CH_2); 2.15 (6H, s, $2\times NCH_3$); 2.35 (2H, t, $CH_3NC\underline{H}_2$); 4.38 (2H, t, NCH_2); 7.22–8.11 (7H, m, ArH).

Analysis of oxidation products of phenothiazine derivatives by HPLC

In addition, the purity of S-oxides of CPZ and PMZ was determined by the high-performance liquid chromatography method on a Zorbax SB, C-18 (250×4.6) mm column. The mobile phase was the solution of camphorsulfonic acid (2.9 g L^{-1}), which pH was adjusted to 5.4 with 5 mol L^{-1}

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sodium hydroxide solution. The flow rate was 1 mL min^{-1}. The volume of the injected sample was 20 μL. Detection was performed by UV-spectrophotometry at 262 nm. The sulfoxides of PhT derivatives studied were dissolved in the mobile phase. The concentration of S-oxides, mg mL^{-1}, was: CPZ – 0.402, PMZ – 0.406. The percentage of the active substance calculated by the normalization method was: for CPZ S-oxide – 94.66%, S-oxide PMZ – 97.87% with the purity of the prominent peaks of 99.85%, 99.84%, respectively.

Oscillopolarograms (0.1 mol L^{-1} KCl, pH 5.5), E_p^k (V): CPM S-oxide (1.8×10^{-5} mol L^{-1}) –0.785, –1.058 (E_p^A –0.935); PMZ S-oxide (1.8×10^{-5} mol L^{-1}) –1.140, –1.385 (E_p^A –1,13); THZ 2S,5S-disulfoxide (1.8×10^{-5} mol L^{-1}) –1.10, –1.295; (0.1 mol L^{-1} KCl, 0.01 M HCl), E_p^k (V): CPM S-oxide (1.8×10^{-5} mol L^{-1}) –0.865, –1.084 (E_p^A –0.938); PMZ S-oxide (1.8×10^{-5} mol L^{-1}) –0.937; THZ 2S,5S-disulfoxide (1.8×10^{-5} mol L^{-1}) –0.841, –0.975.

Preparation of the solutions

Stock solutions of PhT derivatives

Daily 5.0×10^{-3} mol L^{-1} of PhT derivatives stock solutions were prepared by dissolving accurately weighed samples of PhT derivatives in 0.01 mol L^{-1} HCl solution (THZ in 96 % ethanol) and diluting the solution to 100 mL with double distilled water in volumetric flasks. The corresponding aliquots of the stock solutions of PhT derivatives were transferred into separate glass-stopper tubes.

Working standard solutions of PhT derivatives

Working standard solutions (WSS) of PhT derivatives were prepared from the stock solutions by the corresponding 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)

Disodium hydrogen phosphate dodecahydrate (35.75 g) was dissolved in a 500 mL flask using double-distilled water. Then 19 mL of 0.1 mol L^{-1} solution of the hydrochloric acid solution was added. The pH of the final solution was controlled by potentiometry.

Preparation of 10% hydrogen peroxide solution

The solution was prepared by the corresponding high-test hydrogen peroxide dilution with double-distilled water. The hydrogen peroxide content in 10% working solution was determined by the permanganatometric method.

Preparation of 1% p-phenetidine hydrochloride solution

p-Phenetidine hydrochloride (*p*-Ph) was prepared by dissolution of *p*-phenetidine in chloroform followed by precipitation of the salt by gaseous HCl. 1.00 g of *p*-phenetidine hydrochloride was dissolved in 80 mL of double-distilled water in a 100 mL volumetric flask and diluted to the volume with the same solvent.

Preparation of acetylcholine chloride solution

The ampoule content (0.02 g of ACh) was dissolved in 20 mL of double-distilled water. The ampoule was opened, 4.0 mL of water was pipetted and added to the ampoule, then shaken until acetylcholine was completely dissolved. Then the ACh solution was transferred into a 20 mL volumetric flask and diluted to the volume with double-distilled water.

Preparation of acetylcholinesterase solution

The ampoule content of 80 mg of AChE was dissolved in 20 mL of double-distilled water. The ampoule was opened, 4.0 mL of water was pipetted, and shaken until AChE was completely dissolved. Then the AChE solution was transferred into a 20 mL volumetric flask, and diluted to the volume with double-distilled water.

The procedure generally recommended

The first part: 10.0 mL of 0.2 M phosphate buffer solution (pH = 8.35) was transferred into a 20 mL graduated test tube with a ground stopper, and 1.0 mL of 1 mg mL^{-1} ACh solution was added. Then 1.6 mL of 10% hydrogen peroxide solution was added, and the stopwatch was started. After that the solution was shaken thoroughly and thermostated for 10 min. Then 0.5 mL of 1% *p*-Ph solution was added, and the solution was diluted to the volume with double distilled water. The stopwatch was switched on again, and the solution was scanned photometrically every minute over the period of 15 min (λ = 358 nm). The solution containing only 0.2 M phosphate buffer (10.0 mL, pH = 8.3) was used as a reference solution. The rate of the reaction [(ACh + H_2O_2) + *p*-Ph] was determined as a slope of the kinetic curve *A vs t*, ($tg\alpha_0$, min^{-1}).

The second part: 10.0 mL of 0.2 M phosphate buffer solution (pH = 8.35) was transferred into a 20 mL graduated test tube with a ground stopper, and 1.0 mL of 1 mg mL^{-1} ACh solution was added. After that a 0.5 mL accurate portion of ChE was added, then 1.6 mL of 10% hydrogen peroxide solution was added while stirring, shaken up thoroughly and kept for 10 min in a thermostat. Then 0.5 mL of 1% *p*-Ph solution was added, and the mixture was diluted to the volume with double distilled water. The stopwatch was switched on, and the solution was scanned photometrically every minute over the period of 15 min (λ = 358 nm). The solution containing only 0.2 M phosphate buffer (10.0 mL, pH = 8.3) was used as a reference solution. The rate of the reaction

[(ChE + ACh) + H_2O_2 + *p*-Ph] was determined as a slope of the kinetic curve *A vs t*, (tgα, min^{-1}).

The third part: 10.0 mL of 0.2 M of phosphate buffer solution (pH = 8.35) was transferred into a 20 mL graduated test tube with a ground stopper. The accurate volumes (from 0.40 to 3.20 mL) of WSS of PhT derivatives (Inh) were added to the tube. Then 0.5 mL of ChE was added while stirring, the stopwatch was switched on, every solution was shaken thoroughly and thermostated for 10 min. After that 1.0 mL of 1 mg mL^{-1} ACh solution was quickly added, and the stopwatch was switched on, the content was shaken thoroughly and thermostated for 10 min again. Then 1.6 mL of 10% hydrogen peroxide solution was added, the tube was kept for 10 min in a thermostat, 0.5 mL of 1% *p*-Ph solution was added and diluted to the volume with double distilled water. The stopwatch was switched on, and the solution was scanned photometrically every minute over the period of 15 min (λ = 358 nm). The buffer solution with double distilled water was used as a reference solution. The rate of the reaction [(ChE + Inh) + ACh] +H_2O_2 + *p*-Ph] was determined as a slope of the kinetic curve *A vs t* (tgα_i, min^{-1}).

Screening of AChE inhibitors

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

$$IE\ (\%) = \frac{tg\alpha_i - tg\alpha}{tg\alpha_0 - tg\alpha} \times 100\ \%$$

where tgα_i (min^{-1}) is the slope tangent of the linear part of the kinetic curve in the *A vs t* coordinates (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;
tgα_0 is the slope tangent of the linear part of the kinetic curve in the *A vs t* coordinates (a slope of the kinetic curve) for the reaction [(ACh + H_2O_2) + *p*-Ph] in the absence of AChE and an inhibitor;
tgα is the slope tangent of the linear part of the kinetic curve in the *A vs t* coordinates (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.

Results and discussion

Figures 2–4 show the dependence of *IE* on the concentration of PhT derivatives and their S-oxides for the reaction [(ChE + Inh) + ACh] + H_2O_2 + *p*-Ph]. The inhibition ability of CPZ, PMZ and THZ and their metabolites is evaluated by the IC_{50} value, which is the concentration of the inhibitor needed for 50% inhibition of the AChE activity. The IC_{50} value of CPZ, PMZ and THZ and their metabolites in relation to the AChE activity was estimated as 11 ng mL^{-1} (CPM) and 1.8 ng mL^{-1} (CPM S-oxide), 17 ng mL^{-1} (PMZ) and 2.5 ng mL^{-1} (PMZ S-oxide) and 27 ng mL^{-1} (THZ 2S,5S-dioxide) from the plots of *IE* versus the concentration of inhibitors (Figures 2–4), which were similar to the previous reports [27, 28].

Thus, to summarize, a new kinetic spectrophotometric method for determining the activity of AChE and its inhibitors of PhT antipsychotic drugs and their sulfoxides metabolites is attractive because of its convenience, without adding an exogenous catalyst to obtain a chromogenic

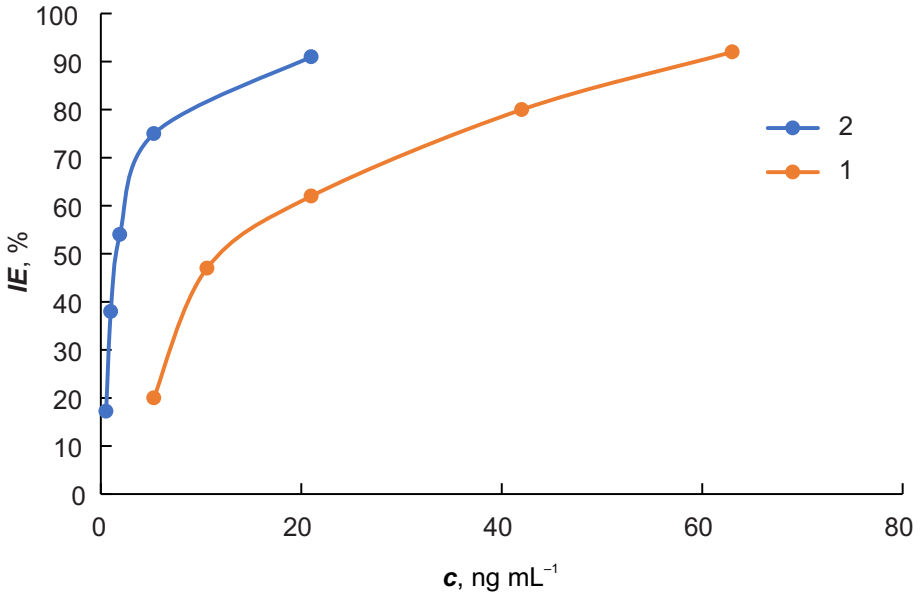

Figure 2. The effect of the CPZ (1) and its S-oxide (2) concentration on the AChE inhibition efficiency

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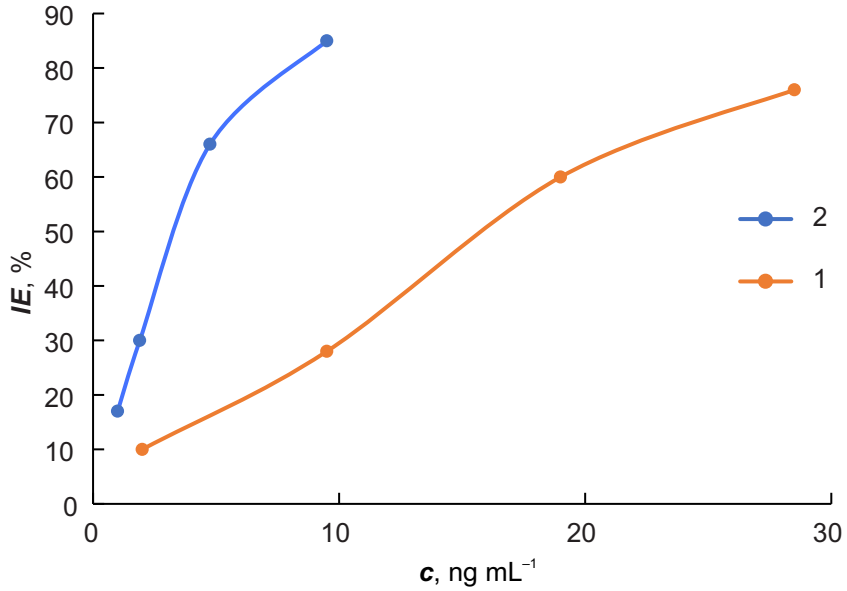

Figure 3. The effect of the PMZ (1) and its S-oxide (2) concentration on the AChE inhibition efficiency

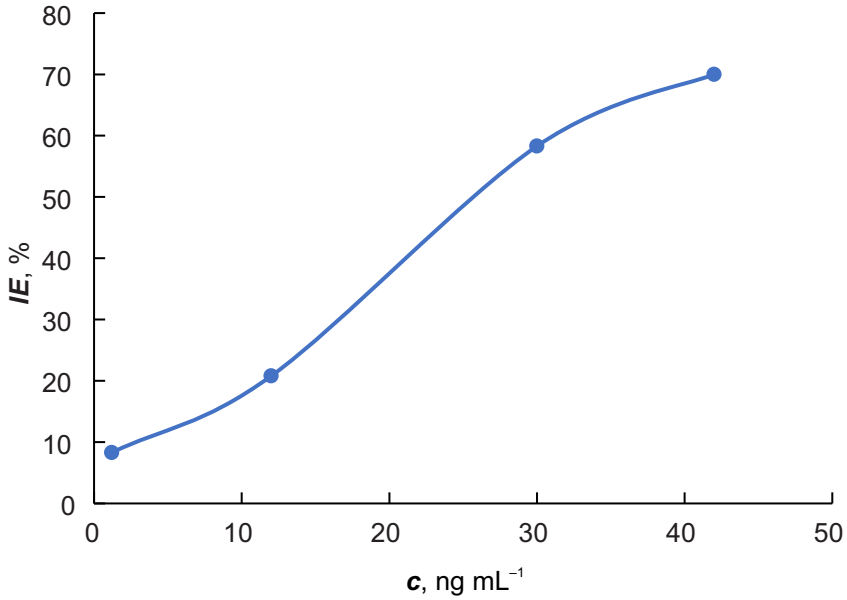

Figure 4. The effect of the THZ 2S,5S-dioxide concentration on the AChE inhibition efficiency

agent, which can lead to complexity and interferences. In this study, for the first time we discovered that ACh itself mimicked the activity of peroxidase, and based on it, a simple and reliable spectrophotometric system containing ACh–H_2O_2–*p*-Ph for a sensitive and selective assessment of the AChE activity and determination of its inhibitors was developed.

PhT derivatives and their S-oxides inhibit the activating action of AChE in the hydrolysis reaction of acetylcholine. As seen, the values of the inhibition efficiency IC_{50} obtained for sulfoxides of the corresponding PhT derivatives were an order of magnitude lower than those of the corresponding PhT derivatives.

This result displays that the detection method proposed provides a sensitive and rapid strategy for screening AChE inhibitors.

It has also been demonstrated that this strategy can be applied to the determination of PhT antipsychotic drugs in real samples. Meanwhile, the sensor platform can also be implemented on test sensors for fast PhT monitoring. Thus, this extremely simple spectrophotometric strategy without the addition of other exogenous catalysts holds promise for the on-site determination of PhT antipsychotics and can be additionally used for sensory applications in areas related to environmental protection and food safety, as well as in the chemical-toxicological analysis.

■ Conclusions

A new kinetic spectrophotometric method for determining AChE inhibitors – phenothiazine antipsychotic drugs and their sulfoxide metabolites without adding an exogenous catalyst to obtain a chromogenic agent has been developed. The ACh itself mimics the activity of peroxidase, and based on it, a simple and reliable spectrophotometric system containing ACh–H_2O_2–*p*-Ph for a sensitive and selective assessment of the AChE activity and determination of its inhibitors has been developed. The values of the inhibition efficiency IC_{50} obtained for sulfoxides of the corresponding PhT derivatives are an order of magnitude lower than those of the corresponding PhT derivatives. It has also been demonstrated that this strategy can be applied to the determination of PhT antipsychotic drugs in real samples.

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The allelopathic activity of water-soluble biologically active substances from *Hyssopus officinalis* L. of Marquis varieties and their effect on the root growth of *Lepidium sativum* L.

Abstract

Aim. To study the effect of secretions of bioactive water-soluble compounds from leaves, stems, flowers, and the root system of *Hyssopus officinalis* L. on the root growth of the test sample (*Lepidium sativum* L.).

Materials and methods. To determine the effect of biologically active water-soluble compounds released by hyssop plants, a series of biotests was conducted; the allelopathic activity of leaves, stems, and flowers of *H. officinalis* L. and its root system were determined. The preparation of extracts of physiologically active substances was carried out according to the method of *A. M. Grodzinsky*. The allelopathic effect of *H. officinalis* L. was assessed by the effect of the water extracts of various concentrations (100 %, 50 % and 10 %) on the root length of seedlings of *L. sativum* L. test objects. The inhibition index was determined by Williamson method.

Results and discussion. According to the results of the research, it was found that the allelopathic activity of water-soluble compounds of common hyssop affected the germination and length of seed roots of *L. sativum* L. as it changed with the age of the plant : in the first year of vegetation, the stimulating effect of aqueous solutions of hyssop on seed germination was observed, in the second year, the stimulating effect decreased, and in the third year of vegetation, the inhibitory effect on seed germination of the test object was observed. Thus, the index of the allelopathic activity also changed.

Conclusions. It was found that the highest allelopathic activity of biologically active water-soluble compounds of root secretions, leaves, stems and flowers of *H. officinalis* L. was during the flowering phase in concentrations of 100 %, 50 % and 10 %, affecting the length of the roots of seedlings of *L. sativum* L. test objects. At the same time, the maximum stimulating effect on the length of the roots of the test object was in the variant using an aqueous extract of plant flowers of the second year of vegetation in a 10 % concentration. The use of aqueous solutions of plants of the third year of vegetation had an inhibitory effect on the root length of *L. sativum* L seedlings.

Keywords **:** *Hyssopus officinalis* L.; *Lepidium sativum* L.; allelopathic activity; water extracts; biologically active water-soluble substances

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Алелопатична активність водорозчинних біологічно активних речовин *Hyssopus officinalis* L. сорту Маркіз та їх вплив на ріст коренів *Lepidium sativum* L.

Мета. Дослідити вплив виділень біологічно активних водорозчинних речовин листків, стебел, квіток і кореневої системи *Hyssopus officinalis* L. на ріст коренів тест-об'єкта (*Lepidium sativum* L.).

Матеріали та методи. Для виявлення впливу біологічно активних водорозчинних речовин, виділюваних рослинами гісопу, було проведено серію біотестів, за якими визначали алелопатичну активність листків, стебел, квіток та кореневої системи *H. officinalis* L. Приготування екстрактів фізіологічно активних речовин проводили за методикою А. М. Гродзінського. Алелопатичний вплив *H. officinalis* L. оцінювали за впливом водних екстрактів різних концентрацій 100 %, 50 % і 10 % на довжину корінців проростків тест-об'єктів *L. sativum* L. Індекс інгібування визначали за методикою Вільямсона.

Результати та їх обговорення. За результатами досліджень з'ясовано, що алелопатична активність водорозчинних речовин гісопу лікарського на проростання та довжину корінців насіння *L. sativum* L. змінюється з віком рослини: на першому році вегетації спостерігалась стимулювальна дія водних розчинів гісопу на схожість насіння, на другому році стимулювальна дія зменшується, а на третій рік вегетації спостерігається гальмувальна дія на проростання насіння тест-об'єкта. Відповідно змінюється і індекс алелопатичної активності.

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Висновки. Виявлено, що найбільша алелопатична активність біологічно активних водорозчинних речовин кореневих виділень, листків, стебел і квіток *H. officinalis* L., яка впливає на довжину корінців проростків тест-об'єктів *L. sativum* L., є за фази цвітіння в концентраціях 100%, 50% та 10%. Водночас максимальний стимулювальний вплив на довжину корінців тест-об'єкта виявлено у варіанті з використанням водного екстракту квіток рослин другого року вегетації за 10% концентрації. Використання водних розчинів рослин третього року вегетації має інгібувальний вплив на довжину корінців проростків *L. sativum* L.

Ключові слова: *Hyssopus officinalis* L.; *Lepidium sativum* L.; алелопатична активність; водні екстракти; біологічно активні водорозчинні речовини

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■ Introduction

One of the most important factors affecting the production of stable and high-quality agricultural yields is soil fertility, which is directly related to the nature of accumulation and transformation of biologically active substances in the soil. Plant waste products, which are a component of this organic soil complex, affect the soil biota and plant communities in different ways, and they are characterized by a certain allelopathic activity. Allelopathic effects of the soil environment include soil fatigue and phytotoxic plant residues [1–3]. Thus, allelopathy is a property of the living phase of soils that affects the level of fertility.

According to *Grümmer* [4], the active substances released by the plant in the process of allelopathy (allelochemistry) are divided into:

1. senility-toxic substances, which source is microorganisms and some types of fungi. Senility suppresses the vital activity of higher plants;

2. phytoncides are substances released by higher plants that inhibit the vital activity of microorganisms;

3. colins are chemical products of the vital activity of higher plants that inhibit the vital activity of other higher plants.

Colins were chosen as the area of our research since they are a combination of active substances formed naturally in the environment of phytocenosis.

The amount of biologically active substances released by a plant depends on its type, variety, phase of development, the organ under study, the age of the plant, its physiological state and soil and climatic conditions of cultivation [5]. The allelopathic effect of one plant on another can have a negative or positive effect, depending on the concentration of secretions [6].

Allelopathic substances play a vital role in regulating the structure of plant communities [7]; they can be used as the raw material for the production of biodegradable herbicides and pesticides, creating mixed plantings, which, according to *A. M. Grodzinsky* [8], can even stimulate the growth of each other in compacted crops. In addition, the determination of allelopathic properties of agricultural crops prevents soil fatigue that occurs during their permanent cultivation as a result of the one-sided development of certain groups of soil microflora to the detriment of other groups [9].

In this regard, the aim of our study was to elucidate the effect of the allelopathic activity of aqueous extracts of leaves, stems and flowers of *Hyssopus officinalis* L. and soil in the rhizosphere zone for the root growth of *Lepidium sativum* L.

To achieve this goal, the following tasks were set:

- to study the allelopathic activity of aboveground organs of the plant *H. officinalis* L. and soils in the zone of its rhizosphere;
- to compare the allelopathic activity of the water extracts obtained in relation to the test object;
- to determine the allelopathic activity of aqueous extracts of aboveground organs of the plant *H. officinalis* L. and the root system in the zone of its rhizosphere in relation to the test object.

Materials and methods

The research was carried out in the model experiments at the premises of laboratories of the Mykolaiv National Agrarian University during 2019-2021. The plant and soil material for the study was collected during the hyssop flowering phase before the experiment began.

The allelopathic activity in biologically active water-soluble plant secretions of *H. officinalis* L. of Marquis varieties were determined by *Grodzinsky* method [8] (the method of test bioassays). Single-day seedlings of *L. sativum* L. were selected as the test culture because of watercress had a high seed germination rate and it was sensitive to external factors.

To determine the allelopathic activity of hyssop plants, extracts of various concentrations were used from flowers, leaves and stems of the plant in the flowering phase and the root layer of the soil, which was the main place of manifestation of allelopathic relationships [1]. The experiment used water extracts from the aboveground organs of hyssop plants and the soil extract with the concentrations of 1:10, 1:50, 1:100.

The experiment was repeated 3 times. In each repetition, 100 watercress seeds were sprouted in Petri dishes at a temperature of 23°C. Control test objects were sprouted when moistened with distilled water. To determine the effect of water extracts of different concentrations on the test object, the root length of *L. sativum* L. was measured. The increase of the root length was calculated as a percentage of the control using mathematical statistics [10].

The response index (RI) or inhibition index, which characterizes the vector and relative value of the effect of allelopathic compounds on the growth rate of watercress seedlings was determined by *Williamson* as follows:

- if $T > C$, then $RI = 1 - (C/T)$;
- if $T < C$, then $RI = (T/C) - 1$

where T – is the morphometric indicator of the embryo in the experiment (treatment response), and C – is the morphometric indicator of the embryo in the control (control response).

The stimulating effect occurs at a value of $RI > 0$, the inhibitory effect occurs at a value of $RI < 0$ [11].

Results and discussion

The active media of allelopathy are allelochemicals formed mainly in the form of secondary plant metabolites or decomposition products of microorganisms [12]. Around each plant, within its phytogenic field, an allelopathic sphere is formed; it is associated with the accumulation of physiologically active substances of colins released by plants into the environment. These substances can be both direct metabolites of the plant and its secondary metabolites, which the plant produces during its growth and development, as well as under the influence of environmental conditions [13].

The task of our experiment was to determine the activity of colins in the allelopathic sphere of common hyssop, which makes it possible to draw conclusions about the possibility of soil fatigue after permanent cultivation of this crop for years exceeding the recommended time frame. According to research, the recommended growing time in one field of common hyssop (*H. officinalis* L.) is 5–6 years [14].

The results showed that the allelopathic effect of *H. officinalis* on the germination of watercress seeds varied depending on the age of the plant. Both the stimulating effect of the extracts on the germination of the test object seeds and the inhibitory effect on the germination of watercress seeds were revealed.

During 2019–2021, there was an accumulation of colins in the rhizosphere of common hyssop of Marquis variety with both a stimulating and inhibitory effect on the germination of watercress seeds (Table 1). According to Table 1, it can be seen that in 2019 the allelopathic activity of the soil in the rhizosphere zone of common hyssop in the flowering phase was more active. It was expressed in the stimulating development of the test object and amounted up to 59.82%, while in 2020 it was up to 56.55% in relation to the control. This is also confirmed by the value of the allelopathic activity index (Figure). The Figure shows that the index of the allelopathic activity of the soil, which expresses the inhibitory effect in the rhizosphere zone of hyssop, increases in accordance with the age of plants. It was the largest in 2021 for plants of the third year of vegetation. The growth of the roots of the test object was inhibited by 11.02%, which was confirmed by the allelopathic activity index, which was a negative number (-0.11).

In the natural range of the Mykolaiv region, *H. officinalis* L. was not found, therefore, the plant was introduced. Any deviation from the ecological conditions of the plant origin can cause more intensive accumulation of biologically active compounds in the plant organs [15, 16].

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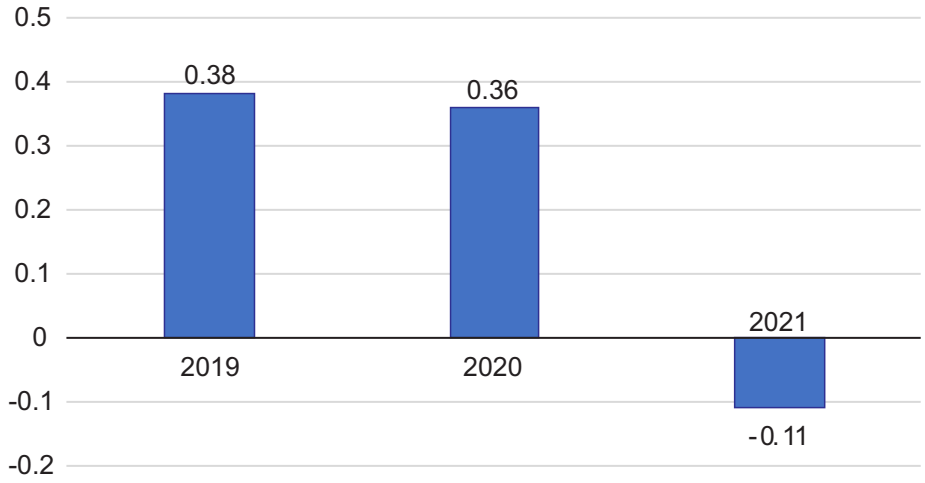

Figure. Changes in the index of the allelopathic activity of the root layer of the soil over the years of research

Table 1. The effect of root secretions of common hyssop plants of different years in the flowering phase on the growth of watercress roots

Vegetation year	Allelopathic activity of the filtrate (dilution 1:100)		
	Root length of the test object (M ± m, mm)	Allelopathic activity, %	Allelopathic activity index (RI)
Control	3.36 ± 0.13	–	–
2019	5.37 ± 0.15	+59.82	+0.38
2020	5.26 ± 0.16	+56.55	+0.36
2021	2.99 ± 0.11	-11.02	-0.11

The results of analysis of the root length of the test object and the allelopathic activity of water-soluble biologically active compounds of aboveground organs of *H. officinalis* L. Marquis varieties are listed in Tables 2 and 3.

The use of hyssop flowers in the concentration of 1:50 in the first year of vegetation caused the growth of the root system of watercress 1.31 times faster compared to the control. Using the (leaf + stem) combination resulted in a slight decrease in the linear growth compared to the flower. All other combinations did not have reliable confirmation.

During the years of research, when studying the allelopathic effect of the age of plants and their aboveground organs on the growth of watercress roots, we found that the highest effect on this indicator was in the variant in which common hyssop flowers were used in the concentration of 1:10 from plants of the second year of vegetation. This concentration contributed to the active growth processes of the watercress root, which was 5.36 mm long and by 2.0 mm longer than the control variant. Decreasing the concentration down to 1:50 and 1:100 reduced this indicator down to 4.56 and 4.73 mm, respectively.

The use of plants in the third year of vegetation led to a sharp decrease in the linear growth

Table 2. The root length of the test object, mm

Plant organ	Year	Solution concentration		
		1:100	1:50	1:10
Leaf	2019	3.64 ± 0.17	3.40 ± 0.17	3.49 ± 0.16
	2020	4.44 ± 0.19	4.54 ± 0.17	4.95 ± 0.14
	2021	2.60 ± 0.07	2.51 ± 0.08	1.02 ± 0.05
Stem	2019	3.84 ± 0.18	3.79 ± 0.15	3.90 ± 0.18
	2020	4.34 ± 0.15	4.83 ± 0.16	4.46 ± 0.15
	2021	2.73 ± 0.09	2.59 ± 0.09	1.46 ± 0.07
Flower	2019	4.04 ± 0.14	4.42 ± 0.16	4.15 ± 0.15
	2020	4.73 ± 0.16	4.56 ± 0.16	5.36 ± 0.15
	2021	2.96 ± 0.11	2.81 ± 0.10	1.85 ± 0.06
Leaf + stem	2019	4.07 ± 0.15	4.37 ± 0.13	3.45 ± 0.13
	2020	4.73 ± 0.18	4.83 ± 0.19	3.80 ± 0.12
	2021	1.39 ± 0.05	1.12 ± 0.05	0.68 ± 0.03
Control	**H_2O**	3.36 ± 0.13	3.36 ± 0.13	3.36 ± 0.13

Table 3. The allelopathic activity, %

Plant organ	Year	Solution concentration		
		1:100	1:50	1:10
Leaf	2019	8.33	1.19	3.87
	2020	32.14	35.12	47.32
	2021	-22.62	-25.30	-69.64
Stem	2019	14.29	12.80	16.07
	2020	29.17	43.85	32.74
	2021	-18.75	-22.92	-56.55
Flower	2019	20.25	31.55	23.51
	2020	40.77	35.71	59.52
	2021	-11.90	-16.37	-44.9
Leaf + stem	2019	21.13	30.06	2.68
	2020	40.77	43.75	13.10
	2021	-58.63	-66.67	-79.76
Control	**H_2O**	–	–	–

of watercress roots. In addition, aqueous solutions of all variants of concentrations inhibited the growth of the roots of the test object, i.e., an inhibitory effect occurred.

The highest allelopathic activity of 23.51 and 31.55% was demonstrated when using an aqueous solution of the plant flowers of the first year of vegetation in the concentration of 1:10 and 1:50, respectively. Reducing the concentration down to 1:100 stimulated the active linear growth of watercress roots in a lesser extent.

The use of all plant organs in the second year of vegetation had a positive effect, but some of the variants had a lesser effect on the allelopathic activity. Thus, the lowest effect (13.10%) was found in the (leaf + stem) variant in the concentration of 1:10, and the highest effect (59.52%) of this indicator was when using the flower infusion in the concentration of 1:50.

It is characteristic to note that when using flowers in concentrations of 1:100 and 1:50 the positive effect was observed, but the allelopathic activity of plants of the third year of vegetation was significantly inferior to plants of the first and, especially, the second year of vegetation.

The results of calculating the index of the allelopathic activity of aboveground organs of hyssop plants of the Marquis variety are shown in Table 4.

Based on the data given in Table 4, it can be argued that the highest indicator of the allelopathic activity index (RI) was the use of flower

Table 4. The allelopathic activity index (RI)

Plant organ	Year	Solution concentration		
		1:100	1:50	1:10
Leaf	2019	0.08	0.01	0.04
	2020	0.24	0.26	0.32
	2021	-0.23	-0.25	-0.70
Stem	2019	0.12	0.11	0.14
	2020	0.23	0.30	0.25
	2021	-0.19	-0.23	-0.57
Flower	2019	0.17	0.24	0.19
	2020	0.29	0.26	0.37
	2021	-0.12	-0.16	-0.45
Leaf + stem	2019	0.17	0.23	0.03
	2020	0.29	0.30	0.12
	2021	-0.59	-0.67	-0.80
Control	**H_2O**	–	–	–
Average	2019	0.14	0.15	0.10
	2020	0.26	0.28	0.27
	2021	-0.28	-0.33	-0.63

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infusion in the concentration of 1:50 from plants of the second year of vegetation (2020 year) in relation to the control.

As we can see, an average over the years of research, the index of the allelopathic activity of hyssop varied depending on the concentration of an aqueous solution of biologically active substances of aboveground plant organs. Thus, the most stimulating effect of the allelopathic activity index was in the solution with the concentration of 1:50. The maximum inhibitory effect was observed when the solution concentration was 1:10.

■ Conclusions

Thus, all parts of common hyssop plants have an allelopathic effect on the linear growth of the root system of watercress. In the first two years of our research, the stimulating effect of aqueous solutions of root secretions and aboveground organs of hyssop plants was observed regardless of their concentrations. We found that the variant using hyssop flowers in the concentration of 1:10 from plants of the second year of vegetation had the highest allelopathic activity. Aqueous solutions of root secretions of common hyssop and its aboveground organs of the third year of vegetation showed an inhibitory effect on the linear growth of watercress roots. It was the largest in the (leaf + stem) variant in the aqueous solution with the concentration of 1:10.

Over the years of research, it was also found that on average, the index of the allelopathic activity of water-soluble biologically active substances of aboveground organs of hyssop plants had a stimulating effect in the solution concentration of 1:50, and an inhibitory effect in the solution concentration of 1:10.

Therefore, the additional research is required for determining the soil fatigue in the conditions of permanent cultivation of common hyssop of Marquis variety during the periods exceeding the recommended terms.

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The quantitative determination of glutathione by the effect of the chemiluminescence inhibition in the catalytic oxidation reaction of luminol with hydrogen peroxide in the presence of hemoglobin

Abstract

Aim. To develop a method for the quantitative determination of reduced glutathione in a lyophilized powder for the preparation of "Hepaval®" solution for intravenous and intramuscular administration by the effect of inhibiting chemiluminescence in the luminol (H_2L)–H_2O_2–hemoglobin (*Hb*) system.

Materials and methods. The study object was the reduced glutathione substance and lyophilized powder for the preparation of "Hepaval®" solution for injection, ampoules of 4 mL No. 10 manufactured by "Valartin pharma" (Italy). The glutathione content in powder was determined using the chemiluminescence method by the effect of inhibiting the luminol oxidation reaction with hydrogen peroxide in the presence of *Hb* as a catalyst. The analysis was performed by the standard addition method.

Results and discussion. As a result of the studies, it has been found that under optimal conditions glutathione shows a noticeable inhibitory effect on chemiluminescence in the H_2L–H_2O_2–*Hb* system. This phenomenon was used to develop a new procedure for the quantitative determination of glutathione in the substance and lyophilized powder for the preparation of "Hepaval®" aqueous solution for injection. The linear dependence of the integral chemiluminescence intencity (*S*) on the molar concentration of glutathione was maintained in the concentration range of $(2-20)\times10^{-7}$ mol L^{-1}. The graph equation was $S = (-1.6\pm0.2)\times10^{7}\times c + (198.9\pm2.0)$, (r = 0.999). The relative standard deviation (RSD) was ±1.82 % (n = 7, P = 0.95).

Conclusions. The method has been developed, and the possibility of the quantitative determination of glutathione in powder for the preparation of "Hepaval®" solution for injection by the method of the chemiluminescence inhibition of the H_2L–H_2O_2–*Hb* system has been shown. The content of glutathione in powder calculated with reference to dried substance was 91.49 % (against 90.8 % by the certificate). The accuracy was +0.76 %.

Keywords: glutathione; chemiluminescence inhibition; quantification; luminol

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Кількісне визначення глутатіону за ефектом інгібування хемілюмінесценції в реакції каталітичного окиснення люмінолу гідроген пероксидом у присутності гемоглобіну

Анотація

Мета. Розробити методику кількісного визначення відновленого глутатіону в ліофілізованому порошку для приготування розчину для внутрішньовенного та внутрішньом'язового введення «Гепавал®» за ефектом інгібування хемілюмінесценції системи люмінол (H_2L)–H_2O_2–гемоглобін (*Hb*).

Матеріали та методи. Об'єктом дослідження були відновлений глутатіон та ліофілізований порошок для приготування розчину для ін'єкцій «Гепавал®», ампули по 4 мл № 10 виробництва «Валартин фарма» (Італія). Визначення вмісту глутатіону в порошку здійснювали методом хемілюмінесценції за ефектом інгібування реакції окиснення люмінолу гідроген пероксидом у присутності *Hb* як каталізатора процесу. Аналіз проводили методом стандартних добавок.

Результати та їх обговорення. У результаті дослідження з'ясовано, що в оптимальних умовах глутатіон виявляє помітну інгібувальну дію на хемілюмінесценцію в системі H_2L–H_2O_2–*Hb*. Це явище було використано для опрацювання нової методики кількісного визначення глутатіону в субстанції та ліофілізованому порошку для приготування водного

розчину для ін'єкцій «Гепавал®». Лінійна залежність сумарного світіння (*S*) від молярної концентрації глутатіону зберігалася в інтервалі концентрацій (2–20)×10^{-7} моль $л^{-1}$. Рівняння графіка має вигляд $S = (-1,6 \pm 0,2) \times 10^7 c + (198,9 \pm 2,0)$, (r = 0,999). Відносне стандартне відхилення (RSD) становило ±1,82 % (*n* = 7, *P* = 0,95).

Висновки. Розроблено методику та продемонстровано можливість кількісного визначення глутатіону в порошку для приготування розчину для ін'єкцій «Гепавал®» методом інгібування хемілюмінесценції системи $H_2L – H_2O_2 – Hb$. Вміст глутатіону в порошку в перерахунку на суху речовину становив 91,49 % (проти 90,8 % за сертифікатом). Правильність становила +0,76 %.

Ключові слова: глутатіон; інгібування хемілюмінесценції; кількісний аналіз; люмінол

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Conflict of interests: The authors have no conflict of interests to declare.

■ Introduction

Glutathione (GSH, *L*-γ-glutamyl-*L*-cysteinyl-glycine) is a biologically active tripeptide found in all organisms. It consists of γ-glutamic acid, cysteine, and glycine residues and can exist in both oxidized (GSSG) and reduced (GSH) forms (Figure 1). The reduced form of GSH protects SH-groups of proteins from oxidation by various oxidants [1].

The protection mechanism consists in the oxidation of the SH-group of GSH with the formation of the oxidized form (disulfide) and the retention of the SH-groups of proteins in the active reduced form. GSH acts as a cofactor of some oxidoreductases like glyoxalase system [2], and glutathione peroxidase [3]. An important role of GSH is binding of free radicals, and the reduction of hydrogen peroxide and other peroxides; it prevents the development of chain free radical processes [4]. Moreover, glutathione facilitates the metabolism of xenobiotics with support of glutathione S-transferase enzymes catalyzing its conjugation to lipophilic xenobiotics [5].

In laboratory practice, glutathione is needed to be determined both in biological fluids (saliva, urine, blood serum) and in pharmaceutical or cosmetic preparations. For this purpose, a variety of instrumental methods of analysis, such as spectrophotometry based on the interaction of glutathione with 5,5'-dithiobis-2-nitrobenzoate acid (Ellman's reagent) [6], high-performance liquid chromatography (HPLC) with various detectors and a precolumn derivative [7], electroanalytical methods [8], as well as the kinetic method of chemiluminescence [9–12], are widely used. The last one is characterized by simplicity of implementation, low cost, and the possibility of miniaturization of the instrument base. According to the British Pharmacopoeia, the reduced glutathione content in a pure substance is determined by iodimetry [13].

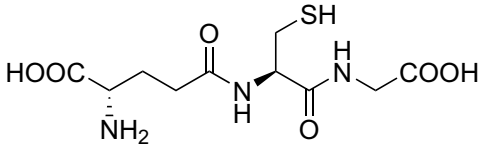

Figure 1. The structure of reduced glutathione

The aim of the study was to develop a method for the quantitative determination of reduced glutathione in a lyophilized powder for the preparation of "Hepaval®" solution for intravenous and intramuscular administration by the effect of inhibiting chemiluminescence in the luminol (H_2L)–H_2O_2–hemoglobin (*Hb*) system.

■ Materials and methods

The study object was the reduced glutathione substance and lyophilized powder for the preparation of "Hepaval®" solution for injection, ampoules of 4 mL No. 10 manufactured by "Valartin pharma" (Italy) (1 ampoule contains 643 mg of glutathione (reduced) sodium salt, which is equivalent to 600 mg of glutathione).

The glutathione content in powder was determined using the chemiluminescence method by the effect of inhibiting the luminol oxidation reaction with hydrogen peroxide in the presence of *Hb* as a catalyst. The analysis was performed by the standard addition method.

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The substance of reduced *L*-glutathione produced by *Bio*Chemica (AppliChem GmbH, Germany) was used in the study. The certificate of analysis: HPLC 98.7%.

The standard 1×10^{-3} mol L^{-1} solution of luminol (5-amino-2,3-dihydrophthalazine-1,4-dione, H_2L, RPF "Synbias", Ukraine): 0.217 g of luminol was placed in a 100 mL volumetric flask, dissolved in 10 mL of 0.01 mol L^{-1} sodium hydroxide solution, and diluted to the volume with double distilled water. The solution was kept in a dark place.

For the medium pH correction, 0.1 mol L^{-1} solution of sodium hydroxide was used; pH of the solutions was controlled by an "Ionomer I-130" laboratory potentiometer with an ESL-43-07 glass electrode, a silver-chloride electrode and an I-130 laboratory ionomer (ZIP, Gomel, Belarus). All solutions were prepared using double distilled water.

Hydrogen peroxide 5.8% solution was prepared from 58% high pure H_2O_2 solution (produced by LTD "Inter-Syntes", Boryslav, Ukraine) by its 10 times dilution with double distilled water: 10 mL of 58% H_2O_2 was transferred into a 100 mL volumetric flask and diluted to the volume at 293 K. This solution was stored at a temperature of +8–10°C. 0.29% working solution of H_2O_2 (8.5×10^{-2} mol L^{-1}) was obtained by diluting 5.8% solution with double distilled water 20 times: 5 mL of the original solution was transferred into a 100 mL volumetric flask and diluted to the volume at 293 K. The working solution can be used throughout the day.

Human blood hemoglobin (*Hb*) produced by "Simko Ltd" (Lviv, Ukraine) was used as a catalyst. 100 μg mL^{-1} hemoglobin solution was prepared by dissolving 10 mg of hemoglobin in 50 mL of double distilled water in a 100 mL volumetric flask under heating and subsequent adding of 1 mL of 1.0 mol L^{-1} sodium hydroxide solution. The volume was diluted with double distilled water at 293 K and stirred. The working solution of hemoglobin was prepared by diluting the initial one with double distilled water 100 times. The working solution can be used throughout a day.

The intensity of chemiluminescence was measured in relative units on a device with a FEU-84-A photoelectric multiplier, using an IMT-0.5 and quick-acting (time constant 0.1 s) automatic potentiometer for measuring low currents.

The reaction accompanied by chemiluminescence was performed in a cylindrical 30 mm diameter quartz cell with a working volume of 10 mL. The following order of mixing the reagents was used in the experiment: to the mixture of the luminol indicator in the alkali solution and H_2O_2, with the presence or absence of the glutathione solution (in the control experiment), 0.50 mL of the *Hb* solution was added using a volumetric pipette. The kinetic curve of the chemiluminescence intensity in relative units against time was registered. The volumetric pipette was built into the removable holder isolating a photocathode of the photoelectric multiplier from outside light, and allowing it to work in the daylight. All experiments were performed at 293 K. The integral chemiluminescence intensity was registered using a "Digital automatic integrator I-02" (Micron, Russia) for 40 seconds.

All other chemicals were of analytical grade. Solutions were prepared with double distilled water produced in a quartz water distiller.

The sample solution of glutathione

0.3618 g (accurate weight) of the glutathione powder was transferred into a 100.0 mL measuring flask, dissolved in double distilled water, and diluted to the volume with the same solvent. Using a pipette, 10.00 mL of the solution prepared was transferred to a 1000 mL volumetric flask, diluted to the volume with double distilled water, and mixed thoroughly.

The standard working solution of glutathione ($c_{st} = 1\times10^{-5}$ mol L^{-1})

It was prepared in the same way as the previous one with the difference that the glutathione powder with the known basic content was used as an addition substance, and then the solution was prepared with the known concentration of glutathione ($C_{10}H_{17}N_3O_6S$) calculated with reference to dried substance. 0.31137 g (accurate weight) of the glutathione powder was transferred into a 100.0 mL measuring flask, dissolved in double distilled water at 393 K, and diluted to the volume with the same solvent. Using a pipette, 10.00 mL of the solution prepared was transferred to a 1000 mL volumetric flask, diluted to the volume with double distilled water, and mixed thoroughly.

The procedure for determining the content of glutathione in "Hepaval®" (by the addition method)

The solutions were sequentially introduced into the quartz cuvette of the chemiluminometer in the following order: 1.00 mL of 1×10^{-3} M solution of H_2L, 5.00 mL of 0.1 M solution of sodium hydroxide, $(10 - x)$ mL of double distilled water (where x is the total volume of all reagents with the sample solution (or sample and addition in another experiment with an addition), in mL), 0.50 mL of 8.5×10^{-2} M of H_2O_2 solution and

0.50 mL of the sample solution (or with 0.50 mL of the addition solution). The cuvette with the resulting mixture was placed in a chemiluminometer light-resistant chamber and using a dispenser, 0.50 mL of 1 μg mL^{-1} working solution of *Hb* was added.

The molar concentration of glutathione in the sample solution c_x (mol L^{-1}) was calculated by the formula:

$$c_x = \frac{c_{st} \times S_x}{S_{x+a} - S_x}$$

where c_{st} – is the molar concentration of glutathione in the standard working solution of glutathione, $C_{10}H_{17}N_3O_6S$, mol L^{-1};
S_x – is the analytical signal of the integral chemiluminescence intensity in the experiment with the sample solution of glutathione for 40 s, rel. units;
S_{x+a} – is the analytical signal of the integral chemiluminescence intencity in the experiment with the sample solution of glutathione and addition for 40 s, rel. units;

The content of glutathione in powder calculated with reference to dried substance X (%) was determined by the formula:

$$X = \frac{c_x \times M \times 1000 \times K \times 100 \times 100\ \%}{m \times (100 - w)}$$

where c_x – is the molar concentration of glutathione in the sample solution, mol L^{-1};
M – is the molar mass of glutathione (reduced) sodium salt, 329.307 g mol^{-1};
K – is the dilution factor of the sample solution, 100;
w – is the mass fraction in loss on drying, % (1.0% by the certificate);
m – is the weight of the glutathione sample taken for analysis, g.

Results and discussion

The dependence of the *chemiluminescence intensity* (I_{CL}) on *time* (s) is shown on the kinetic graph (Figure 2). The experiment was repeated five times. The desired signal was the area under the curve – the integral chemiluminescence intensity over the time period (40 s) (S, rel. units) obtained by averaging the values of five experiments.

The highest intensity of chemiluminescence in the H_2L–H_2O_2–*Hb* system was observed when the *Hb* solution was added last. The optimal conditions of the experiment were determined earlier [14]: $c(NaOH)$ = 0.05 mol L^{-1}, $c(H_2O_2)$ = = 4.25×10^{-3} mol L^{-1}, $c(H_2L)$ = 1×10^{-4} mol L^{-1}, $c(Hb)$ = 5×10^{-2} μg mL^{-1}.

The presence of glutathione in the H_2L–H_2O_2–*Hb* system leads to a decrease in the maximum intensity (Figure 2) and the integral chemiluminescence intensity (S), indicating the chemiluminescent reaction inhibition (Figure 3). This effect increases with the increasing concentration of the inhibitor.

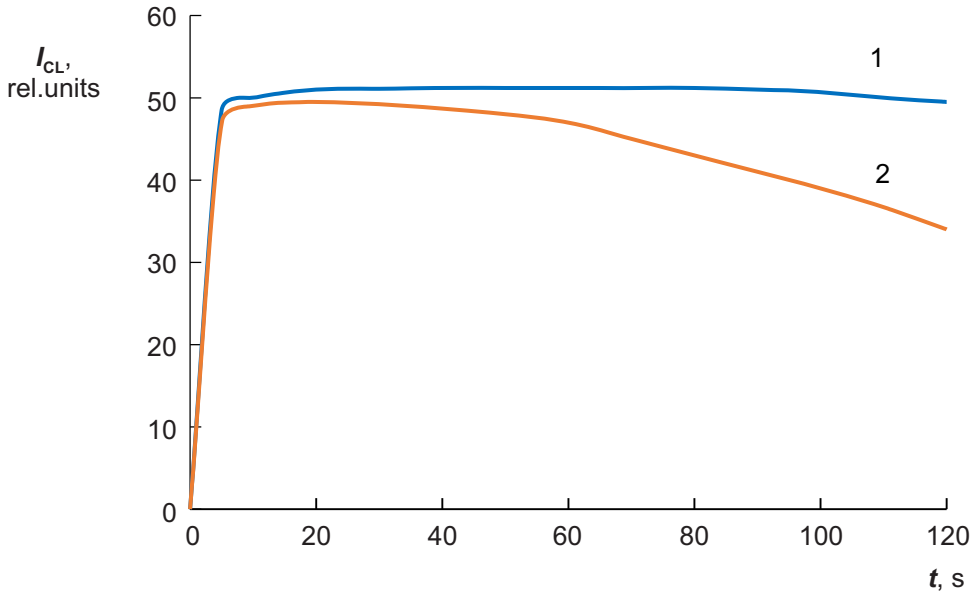

Figure 2. The kinetic curves of the dependence of the chemiluminescence intensity on time in the H_2L–H_2O_2–Hb system in the absence (1) and in the presence (2) of glutathione. c(NaOH) = 0.05 mol L^{-1}, $c(H_2O_2)$ = 4.25×10^{-3} mol L^{-1}, $c(H_2L)$ = 1×10^{-4} mol L^{-1}, $c(Hb)$ = 5×10^{-2} μg mL^{-1}, c(GSH) = 1×10^{-6} mol L^{-1}

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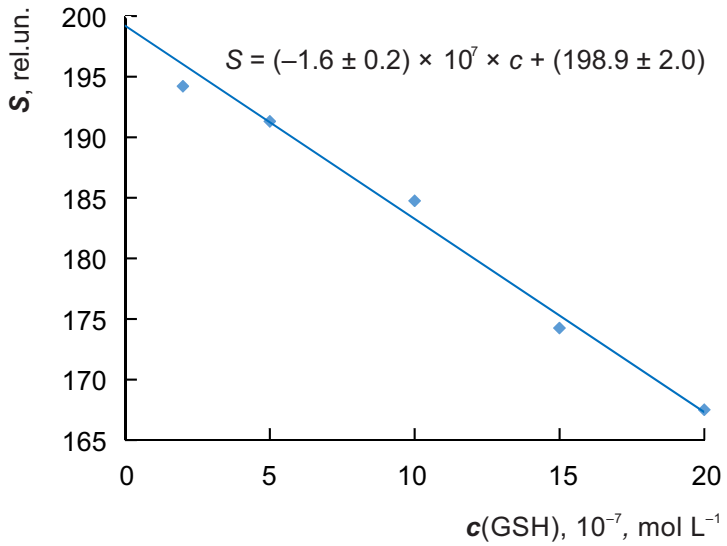

Figure 3. Dependence of *S* (in rel. units) on the molar concentration of glutathione

Table 1. Analytical characteristics of the calibration graph (y = bx + a) for the quantification of glutathione in "Hepaval®"

Characteristics	Parameters
Concentration range (μg mL^{-1})	0.2–30
Correlation coefficient (r)	0.999
Linear regression equation	$S = (-1.6 \pm 0.2) \times 10^7 \times c + (198.9 \pm 2.0)$
Slope (b±Δb)	$(-1.6 \pm 0.2) \times 10^7$
Intercept (a±Δa)	198.9 ± 2.0
S.D. of slope (S_b)	0.06×10^7
S.D. of intercept (S_a)	0.70
LOD (3S) (mol L^{-1})	1.5×10^{-7}
LOQ (10S) (mol L^{-1})	4.4×10^{-7}

Table 2. The results of the quantitative determination of glutathione in powder for the preparation of "Hepaval®" solution for injection

Taken	Found, %	Metrological characteristics (n = 7, P = 0.95)
0.31137 g A lyophilized powder for the preparation of "Hepaval®" solution for injection manufactured by "Valartin pharma" (Italy)	92.1 90.9 89.1 91.2 92.8 90.2 94.1	$\bar{x}$ = 91.49 S = 1.67 $\Delta\bar{x}$ = 1.55 *RSD* = ±1.82% $\delta^{[a]}$ =+0.76%

Note: [a] The calculation is based on the certificate of quality data of the powder for the preparation of "Hepaval®" solution for injection of glutathione ($C_{10}H_{17}N_3O_6S$) calculated with reference to dried substance by the method of Ph. Eur. – 90.8% (μ). $\delta = (\bar{x} - \mu) \times 100\%/\mu$

The linear dependence of S on the molar concentration of glutathione was maintained in the concentration range of $(2–20)\times10^{-7}$ mol L^{-1}. The graph equation was $S = (-1.6\pm0.2)\times10^7\times c + (198.9\pm2.0)$, (r = 0.999) where c was the concentration of glutathione solution in mol L^{-1} (Figure 3). Analytical characteristics of the calibration graph are given in Table 1.

Precision and accuracy of the quantitative determination of glutathione in powder for the preparation of "Hepaval®" solution for injection by the method of the chemiluminescence inhibition of the H_2L–H_2O_2–Hb system were studied by analyzing seven replicates of the sample solutions. The precision of the method developed with reference to the relative standard deviation (RSD) was ±1.82% (n = 7, P = 0.95). The accuracy was +0.76%. The results obtained are summarized in Table 2.

■ Conclusions

The method has been developed, and the possibility of the quantitative determination of glutathione in powder for the preparation of "Hepaval®" solution for injection by the method of the chemiluminescence inhibition of the H_2L–H_2O_2–Hb system has been shown. The content of glutathione in powder calculated with reference to dried substance was 91.49% (against 90.8% by the certificate). The accuracy was +0.76%. The method proposed is promising for the determination of glutathione in substances and drugs in the practice of control and analytical laboratories, as well as in the chemical and pharmaceutical industry.

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The quantitative content determination of main groups of biologically active substances in batches of *Viburnum opulus* fruits

Abstract

Aim. To determine the quantitative content of the main groups of biologically active substances (BAS) in batches of *Viburnum opulus* fruits using pharmacopeial methods for their subsequent use in the standardization of the plant raw material.
Materials and methods. For the study, 6 batches of air-dried *Viburnum opulus* fruits crushed to a particle size of 1–2 mm and harvested in different regions of Ukraine in the mass fruiting phase in October-November 2020 were used. The quantitative determination of polyphenols and tannins was performed using the spectrophotometric method at a wavelength of 760 nm calculated with reference to pyrogallol and dried substance. The total amount of hydroxycinnamic acids was determined according to the method of the SPhU (State Pharmacopoeia of Ukraine) 2.0 described in the monograph "Kidney Tea^{N}" calculated with reference to rosemary acid. The total amount of organic acids was determined by the titrimetric method according to the method of the monograph of the SPhU 2.1 "Rose hips^{N}" calculated with reference to malic acid.
Results and discussion. The quantitative content of polyphenols (calculated with reference to pyrogallol and dried substance) in batches of *Viburnum opulus* fruits varied by about 1.4 times from 1.74 ± 0.01 % to 2.36 ± 0.01 %. According to the results obtained, the quantitative content of tannins (calculated with reference to pyrogallol and dried substance) in batches of *Viburnum opulus* fruits varied by 1.7 times from 0.73 ± 0.01 % to 1.23 ± 0.01 %. The quantitative content of the total amount of hydroxycinnamic acids calculated with reference to rosemary acid in batches of *Viburnum opulus* fruits ranged by 1.2 times from 3.96 ± 0.01 % to 4.73 ± 0.01 %. The quantitative content of the total amount of organic acids calculated with reference to malic acid in batches of *Viburnum opulus* fruits fluctuated more than 1.3 times from 6.80 ± 0.01 % to 9.08 ± 0.01 %.
Conclusions. The quantitative content of the main groups of biologically active substances has been determined in 6 batches of *Viburnum opulus* fruits harvested in different regions of Ukraine using pharmacopoeial methods: polyphenols (varied by 1.4 times), tannins (varied by 1.7 times), the total amount of hydroxycinnamic (varied by 1.2 times), and the total amount of organic acids (varied by 1.3 times) calculated with reference to dried substance. The content of the groups of biologically active substances in batches of *Viburnum opulus* fruits slightly correlates with the place of the raw material harvesting. Thus, the selected methods of the SPhU 2.0 are quite suitable for determining the quantitative content of polyphenols, tannins and of the total amount of hydroxycinnamic and organic acids in batches of *Viburnum opulus* fruits; they can be used in further studies to standardize the raw material.
Keywords: *Viburnum opulus*; fruits; polyphenols; tannins; hydroxycinnamic acids; organic acids; quantitative content

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Визначення кількісного вмісту основаних груп біологічно активних речовин у серіях плодів калини звичайної

Анотація

Мета. Визначити кількісний вміст основних груп біологічно активних речовин (БАР) у серіях плодів калини звичайної з використанням фармакопейних методик для подальшого їх застосування у стандартизації сировини.
Матеріали та методи. Для дослідження використовували 6 серій повітряно сухих подрібнених до розміру частинок 1–2 мм плодів калини звичайної, заготовлених у різних регіонах України у фазі масового плодоносіння в жовтні-листопаді

2020 року. Кількісне визначення поліфенолів і танінів проводили за допомогою спектрофотометричного методу за довжини хвилі 760 нм у перерахунку на пірогалол і суху речовину, суми гідроксикоричних кислот – за методикою монографії Державної фармакопеї України 2.0 «Нирковий чай^N» у перерахунку на розмаринову кислоту, суми органічних кислот проводили титриметричним методом за методикою монографії «Шипшини плоди^N» Державної фармакопеї України 2.1 у перерахунку на яблучну кислоту.

Результати та їх обговорення. Кількісний вміст поліфенолів (у перерахунку на пірогалол та суху речовину) у серіях плодів калини звичайної варіював майже в 1,4 раза – від 1,74 ± 0,01 % до 2,36 ± 0,01 %. Кількісний вміст танінів (у перерахунку на пірогалол та суху речовину) у серіях плодів калини звичайної варіював в 1,7 раза – від 0,73 ± 0,01 % до 1,23 ± 0,01 %. Кількісний вміст суми гідроксикоричних кислот у перерахунку на розмаринову кислоту в серіях плодів калини звичайної коливався в 1,2 раза – від 3,96 ± 0,01 % до 4,73 ± 0,01 %. Кількісний вміст суми органічних кислот у перерахунку на яблучну кислоту в серіях плодів калини звичайної коливався в більше ніж 1,3 раза – від 6,80 ± 0,01 % до 9,08 ± 0,01 %.

Висновки. У 6 серіях плодів калини звичайної, заготовлених у різних регіонах України, з використанням фармакопейних методик визначено кількісний вміст основних груп БАР: поліфенолів (коливався в 1,4 раза), танінів (коливався в 1,7 раза), суми гідроксикоричних кислот (коливався в 1,2 раза) та суми органічних кислот (коливався в 1,3 раза) у перерахунку на суху сировину. Кількісний вміст груп БАР у серіях плодів калини звичайної незначно корелює з місцем заготівлі сировини. Отже, обрані методики ДФУ 2.0 цілком придатні для визначення кількісного вмісту поліфенолів, танінів та суми гідроксикоричних і органічних кислот у серіях плодів калини звичайної, що може бути використано в подальших роботах зі стандартизації сировини.

Ключові слова: калина звичайна; плоди; поліфеноли; таніни; гідроксикоричні кислоти; органічні кислоти; кількісний вміст

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■ Introduction

So far, we have not found information about the drugs obtained from the fruits of *Viburnum opulus* and available at the pharmaceutical market of Ukraine.

As literature data evidence, *Viburnum* fruits are diversely used in ornamental horticulture, food industry and folk medicine. As of October 21, 2021, the State Register of Plant Varieties Suitable for Distribution in Ukraine includes 9 varieties of *Viburnum* (Rubinova, Ukrainochka, Berehynia, Nasoloda, Bagryan, Ulyana, Anya, Koralova, Velykoplidna) [1]. Today, the food industry produces syrups from this type of the raw material [2], there is information about the freezing of semi-finished products from *Viburnum* fruits [3], and the use of *Viburnum* fruit pomace after juices and wines producing in the bakery industry [4]. Additionally, in folk medicine, *Viburnum* juice has been applied to treatment of tumors, ulcer and used for cosmetic purposes; decoction of the seeds has been shown to possess astringent properties and used to cure dyspepsia [5].

Recent studies have revealed bactericidal properties of *Viburnum* fruits, as well as their pronounced inhibitory activity against *Trichomonas* and *Giardia*. It is also known that the extract from this type of the raw material has a cardiotonic effect similar to digitalis, and there is evidence of the prospects of this raw material in prevention and treatment of obesity-related disorders [6].

The high potential of the *Viburnum* raw material is proven by the geography of its studies, which is represented by Europe, Asia, United States and Turkey [7–9]. According to them, the fruits of *Viburnum* can be used in medicine with several purposes. Thus, fruit extracts have shown positive results *in vivo* studies for treating diseases of the endocrine, cardiovascular and genitourinary systems. In particular, such effects as normalization of glucose and fatty acid absorption [7], the vasorelaxant activity [10], reduction of the endometriosis activity [11] and reduction of the reproductive organ damage by chemicals [12] have been identified. *Viburnum* fruit extracts have also shown a significant antioxidant activity [13],

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which is important for maintaining normal functioning of the human body.

Viburnum opulus is widespread in Ukraine [14]. With this, only in the Ivano-Frankivsk region up to 7 tons of fruits are harvested annually for the production of *Viburnum* fruit powder and tea, and with increasing areas occupied by *Viburnum*, annual harvest of up to 100 tons of fruits is possible [15].

The State Pharmacopoeia of Ukraine (SPhU) 2.0 includes the monograph "Viburnum bark" [16]. The pharmacopoeial article "Viburnum fruits" was published in the State Pharmacopoeia of the USSR (11th edition), in which the parameters of standardization were the loss on drying (not more than 15%), total ash (not more than 10%); impurities: crude fruits (not more than 4%), fruits blackened, burnt, affected by pests (not more than 1.5%), other parts of *Viburnum* (peduncles, including those separated during the analysis, twigs, seeds, leaves) (not more than 2.5%), organic impurities (not more than 1.0%), mineral impurities (not more than 0.5%). However, the article did not describe a method for determining the quantitative content of biologically active substances in fruits [17]. Recently, the monograph "*Viburnum* fruits" has been included in the SPhU 2.4 [18]. The article controls the following parameters: the loss on drying – not more than 15.0%, impurities (crude fruits – not more than 4.0%, colored fruits – not more than 1.5%, other *Viburnum* particles (peduncles, in particular separated during the analysis , twigs, seeds, leaves – not more than 2.5%), organic particles – not more than 1.0%, mineral particles – not more than 0.5%, the quantitative content of procyanidins – calculated with reference to cyanidin hydrochloride – not less than 0.2%, the quantitative content of organic acids calculated with reference to citric acid – not less than 7.0%.

The growing popularity of *Viburnum* fruits has been confirmed by a large number of scientific papers, such as reviews [5, 19], coverage of morphological diversity [20, 21], chemical composition [22, 23], and other aspects of the raw material application [24]. Thus, *Viburnum* fruits are a promising type of the plant raw material; therefore, it makes sense to study them in depth to determine the quantitative content of the main groups of biologically active substances. The aim of the work was to determine the content of the main groups of biologically active substances (BAS) in batches of *Viburnum opulus* fruits using pharmacopeial methods to select the optimal ones with their subsequent use in the standardization of the plant raw material.

■ Materials and methods

For the study, 6 batches of *Viburnum opulus* fruits harvested from single specimens of wild plants, were used. Places and terms of harvesting are given in Table 1.

Air-dried *Viburnum opulus* fruits crushed to a particle size of 1–2 mm were used for the study (bringing to a loose state was carried out by drying in a Gorenje FDK24DW fruit and vegetable dryer at a temperature of 40–50°C for 120 min).

The quantitative determination of polyphenols and tannins was performed using the spectrophotometric method at a wavelength of 760 nm in accordance with the requirements of the Supplement of the SPhU 1.2 (2.8.14) [25]. The quantitative content of each group of compounds was calculated with reference to pyrogallol and dried substance.

The quantitative determination of the total amount of hydroxycinnamic acids was performed according to the spectrophotometric method given in the monograph of the SPhU 2.2 "Orthosyphon stamen (kidney tea) leaves^{N}" [26] at a wavelength of 505 nm and calculated with reference to rosemary acid.

The quantitative determination of the total amount of organic acids was performed by the titrimetric method according to the monograph "Rose hips ^{N}" provided by the SPhU 2.1 [16] and calculated with reference to malic acid using 0.1 M solution of sodium hydroxide as a titrant and phenolphthalein as an indicator.

■ Results and discussion

The quantitative content of polyphenols, tannins, the total amount of hydroxycinnamic acids and organic acids in 6 batches of *Viburnum opulus* fruits was determined during the experiment. The results of the quantification are shown in Table 2.

Table 1. Places and terms of harvesting of *Viburnum opulus* fruits

Batch number of the raw material	Harvesting place	Harvesting term
1	Kirovograd region	26.09.2020
2	Lviv region	1.10.2020
3	Luhansk region	26.09.2020
4	Kharkiv region	3.10.2020
5	Ivano-Frankivsk region	27.10.2020
6	Zaporizhia region	20.09.2020

Table 2. The quantitative determination of the main groups of biologically active substances in batches of *Viburnum opulus* fruits calculated with reference to dried substance (n = 5)

Batch number of the raw material	Quantitative content, %			
	polyphenols	tannins	total amount of hydroxycinnamic acids	total amount of organic acids
1	1.88 ± 0.02	0.97 ± 0.01	4.46 ± 0.05	8.42 ± 0.05
2	1.81 ± 0.01	0.92 ± 0.01	4.73 ± 0.04	8.28 ± 0.04
3	1.74 ± 0.02	0.73 ± 0.01	3.96 ± 0.02	6.80 ± 0.03
4	2.24 ± 0.02	1.26 ± 0.02	4.17 ± 0.04	8.88 ± 0.04
5	2.36 ± 0.02	1.23 ± 0.01	4.34 ± 0.03	9.08 ± 0.05
6	1.87 ± 0.01	0.94 ± 0.01	4.18 ± 0.02	7.98 ± 0.04

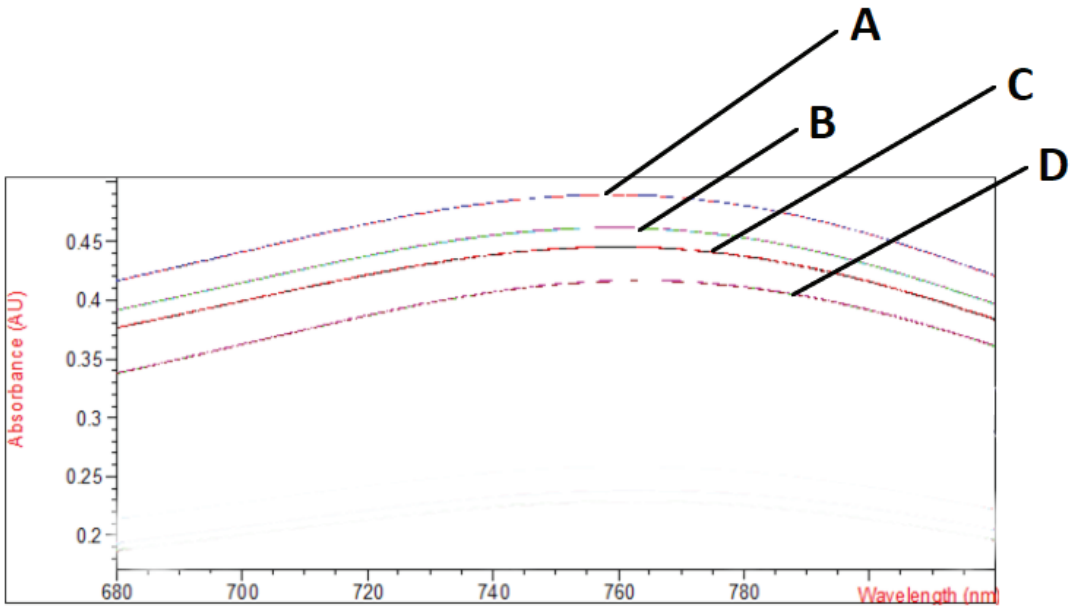

Figure 1. UV spectra of *Viburnum opulus* fruit extracts of batch 1 (A), batch 2 (B), batch 3 (C) and the standard solution of pyrogallol (D) (quantification of polyphenols)

Typical absorption spectra in the UV region of *Viburnum opulus* fruit extracts and the standard solution of pyrogallol with the corresponding reagent are shown in Figure 1. UV absorption spectra of extracts of some *Viburnum opulus* fruit batches treated with the corresponding reagent according to the method of determining the total amount of hydroxycinnamic acids [26] is shown in Figure 2.

From the data of Table 2 it follows that the quantitative content of polyphenols (calculated with reference to pyrogallol and dried substance) in batches of *Viburnum opulus* fruits varied almost by 1.4 times from 1.74±0.01% (batch 3) to 2.36±0.01% (batch 5).

According to the results obtained, the quantitative content of tannins (calculated with reference to pyrogallol and dried substance) in batches of *Viburnum opulus* fruits varied by 1.7 times (from 0.73±0.01% in the raw material of batch 3 to 1.23±0.01% in the raw material of batch 5).

The quantitative content of the total amount of hydroxycinnamic acids calculated with reference to rosemary acid in batches of *Viburnum opulus* fruits varied by 1.2 times: from 3.96±0.01% (batch 3) to 4.73±0.01% (batch 2).

The quantitative content of the total amount of organic acids calculated with reference to malic acid in batches of *Viburnum opulus* fruits varied by more than 1.3 times: from 6.80±0.01% (batch 3) to 9.08±0.01% (batch 5).

During the experiment it was found that the difference between the quantitative content of each of the groups of biologically active substances in batches of *Viburnum opulus* fruits is insignificant. The methods of the SPhU 2.0 selected for our research are quite suitable for determining the quantitative content of polyphenols, tannins and the total amount of hydroxycinnamic and

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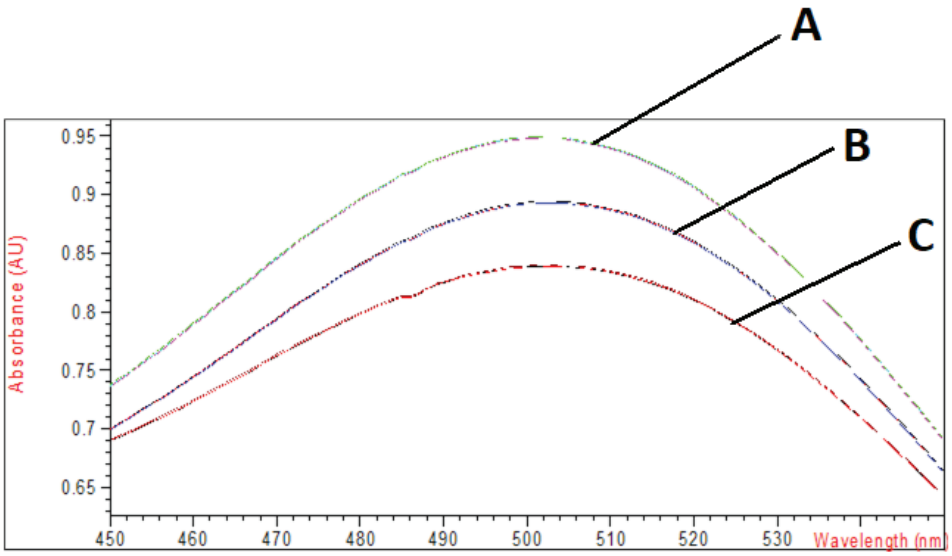

Figure 2. UV spectra of *Viburnum opulus* fruit extracts of batch 1 (A), 2 (B) and 6 (C) (quantification of the total amount of hydroxycinnamic acids)

organic acids in batches of *Viburnum opulus* fruits, They can be used in further studies of the raw material.

■ Conclusions

The quantitative content of the main groups of biologically active substances has been determined in 6 batches of *Viburnum opulus* fruits harvested in different regions of Ukraine using pharmacopoeial methods: polyphenols (varied by 1.4 times), tannins (varied by 1.7 times), the total amount of hydroxycinnamic (varied by 1.2 times), and the total amount of organic acids (varied by 1.3 times) calculated with reference to dried substance.

The content of the groups of biologically active substances in batches of *Viburnum opulus* fruits slightly correlates with the place of the raw material harvesting. Thus, the selected methods of the SPhU 2.0 are suitable for determining the quantitative content of polyphenols, tannins and of the total amount of hydroxycinnamic and organic acids in batches of *Viburnum opulus* fruits; they can be used in further studies to standardize the raw material.

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