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ІНСТИТУТ ОРГАНІЧНОЇ ХІМІЇ НАН УКРАЇНИ
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Features of Nitration of Aromatic Aldehydes with the Difluoromethoxy Group

Abstract

Nitration of aromatic aldehydes with difluoromethoxy group results in the partial *ipso*-substitution of the aldehyde group if difluoromethoxy group is located in the *para*-position to the aldehyde group. The presence of a chlorine atom in the *meta*-position to the aldehyde group increases the contribution of the *ipso*-substitution, while the presence of a chlorine atom in the *ortho*-position to the aldehyde group reduces it. The presence of strong donors (alkoxy groups) in the molecule eliminates the contribution of the *ipso*-substitution.

Keywords: difluorometoxybenzaldehydes; nitration; *ipso*-substitutuion

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Особливості нітрування ароматичних альдегідів, які містять дифлуорометокси-групу

Анотація

Нітрування ароматичних альдегідів з дифлуорометокси-групою призводить до часткового *ipso*-заміщення альдегідної групи, якщо дифлуорометокси-група перебуває в *пара*-положенні до альдегідної групи. Наявність атома хлору у *мета*-положенні до альдегідної групи підвищує внесок *ipso*-заміщення, тоді як наявність атома хлору в *орто*-положенні до альдегідної групи зменшує його. Наявність у молекулі потужних донорів (алкокси-груп) нівелює внесок *ipso*-заміщення.

Ключові слова: дифторометоксибензальдегіди; нітрування; *ipso*-заміщення

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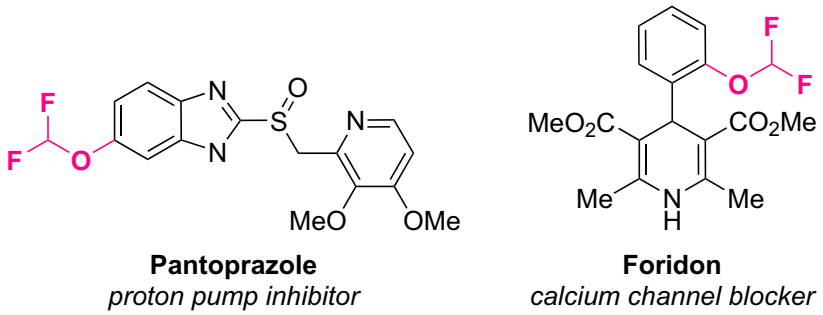
■ Introduction

The difluoromethoxy group has recently become quite readily available, and compounds containing it often exhibit the biological activity [1]. The examples of such prominent $OCHF_2$ bearing drug molecules include a proton pump inhibitor pantoprazole (the brand name Protonix) and a calcium channel blocker riodipine (the brand name Foridon) (**Figure**).

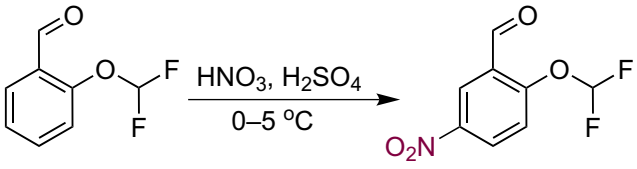
Previously, we obtained a number of biologically active compounds acting as activators of potassium and calcium channels and containing difluoromethoxy group [2]. In particular, starting compounds for our investigations were *o*-difluoromethoxybenzaldehyde (**1**) and its nitration product – 2-difluoromethoxy-5-nitrobenzaldehyde (**2**). Nitration of compound **1** proceeded very easily, even under milder conditions and at lower temperature than nitration of unsubstituted



Pantoprazole
proton pump inhibitor

Foridon
calcium channel blocker

Figure. Examples of bioactive compounds with difluoromethoxy group



1 **2**, *90 %*

Scheme 1. Nitration of *o*-difluoromethoxybenzaldehyde

benzaldehyde, and led to sole product **2** with a high (90%) yield since the substituents in the benzene ring direct nitro group to the same positions (**Scheme 1**).

Nitration of difluoromethoxybenzaldehydes isomeric to **1** and other aromatic aldehydes containing $OCHF_2$ group remained almost unstudied. Only one patent is known on the issue. It describes nitration of *p*-difluorometoxybenzaldehyde in acetic anhydride with a moderate yield [3]. However, such nitration products can become important intermediates for the synthesis of new biologically active substances. This work aims to study the nitration reaction of various benzaldehydes containing difluoromethoxy group, including those with substituent having inconsistent directing influence.

■ Results and discussion

Nitration reactions of aldehydes with the diflouromethoxy group were carried out under conditions close to those of compound **1**, according to the procedure described earlier [2] in a mixture of 96% sulfuric and 100% nitric acids in the ratio of 2:1. We chose *p*-difluorometoxybenzaldehyde (**3**) as the first object of our study. The difluoromethoxy group is a first type director. It activates the *para*-position for electrophilic attacks to a much greater extent than the *ortho*-position. As it was shown earlier, nitration of phenyldifluoromethyl ether under mild conditions leads to the mixture of *p*-nitrophenyldifluoromethyl ether and *o*-nitrophenyldifluoromethyl ether in the ratio of 7:1 [4]. On the other hand, aldehyde group in nitration reactions partially directs the reaction to the *ortho*-position (but never to the *para*-position). Therefore, the described fact that nitration of compound **3** occurred only in the *ortho*-position to the difluoromethoxy group and led to only one product [3] caused us doubt. Nitration at 0–5 °C resulted in a mixture of compounds with the total yield of about 80%. The expected 3-nitro-4-difluoromethoxybenzaldehyde (**4**) was the main reaction product, but the side product was 2,4-dinitrophenyldifluoromethyl ether (**5**). The product of the *ipso*-substitution of aldehyde group **5** was isolated with the aid of chromatography. Compound **5** synthesized according to the method [4] did not give depression of the melting point of the mixed sample with the product obtained according to **Scheme 2**. The ratio of compounds **4** and **5** was 10:1. When carrying out nitration at a higher temperature (15–20°C), a similar mixture was obtained, but with a higher content of the *ipso*-substitution product. The ratio of **4** and **5** in this case was 4:1 (**Scheme 2**).

Previously, only a limited number of cases of the *ipso*-substitution of aldehyde group during nitration of aromatic aldehydes were known. Such a substitution could occur only if the CHO group was in the *para*-position to the alkoxy substituent (OR), and possibly passed through the oxonium intermediate **6** (**Scheme 3**). The content of *ipso*-substitution products in the reaction mixture increased with an increase of the reaction temperature. Thus, nitration of anisaldehyde at 0 °C led only to 3-nitro-4-methoxybenzaldehyde and did not provide an admixture of the *ipso*-substitution product [5], while nitration of anisaldehyde

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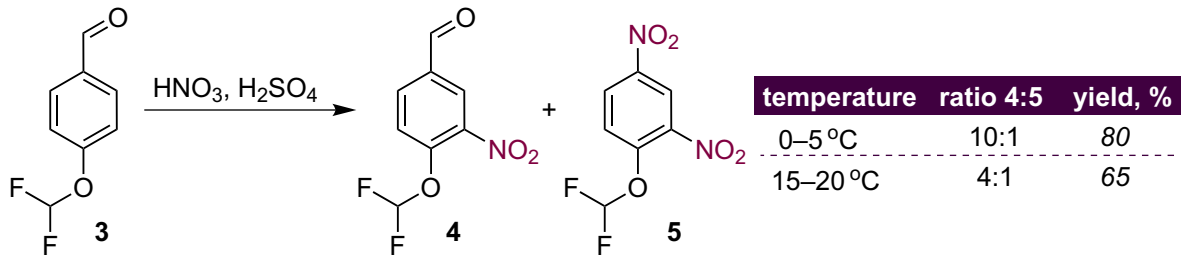
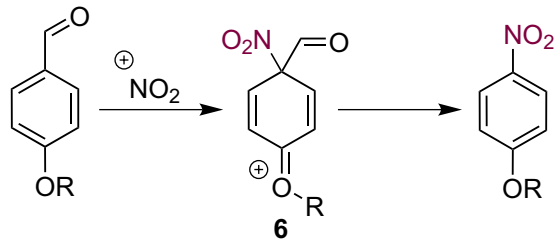


temperature	ratio 4:5	yield, %
0–5 °C	10:1	*80*
15–20 °C	4:1	*65*

Scheme 2. Nitration of *p*-difluoromethoxybenzaldehyde

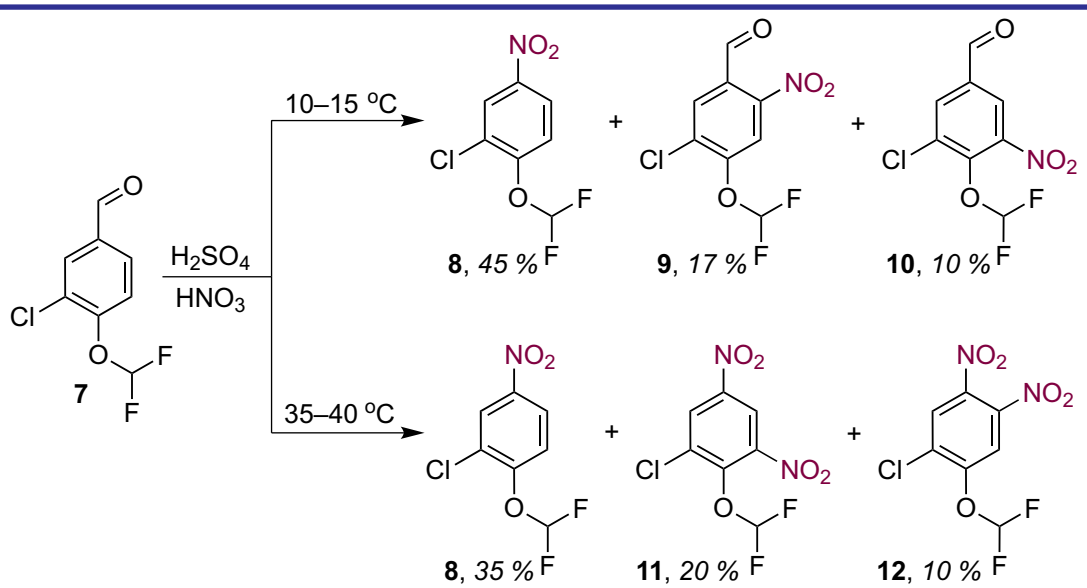


Scheme 3. The possible pathway of the *ipso*-substitutution

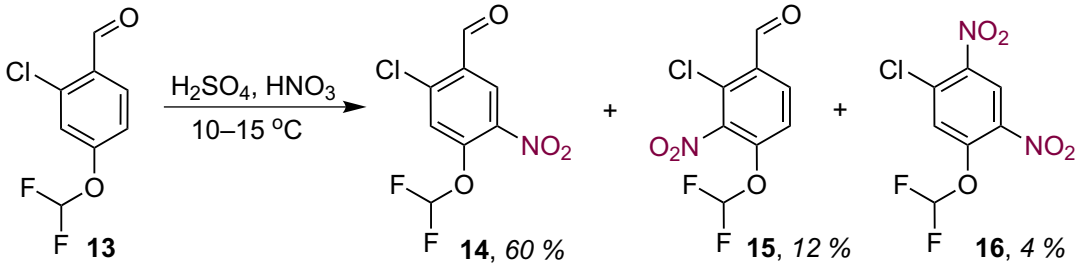
with bismuth nitrate at 80 °C resulted in a mixture of two products containing 30% of *p*-nitroanisole [6]. If nitration to the *ortho*-position of the OR substituent is sterically restricted and the reaction is directed to the *ortho*-position of the aldehyde group, then the content of the *ipso*-substitution product also increases. So, to obtain 6-nitrovaniline, the nitration reaction of *O*-benzylvanillin was used. In this case, even when using mild conditions at least 20% of the *ipso*-substitution product was formed [7].

We studied nitration of other aromatic aldehydes with a difluoromethoxy group in the *para*-position under similar conditions. It turned out that the presence of a chlorine atom in the *meta*-position to the aldehyde group complicated the nitration reaction (the reaction did not proceed at 0 °C) and led to a sharp increase in the content of *ipso*-substitution products. Thus, during nitration of 3-chloro-4-difluoromethoxybenzaldehyde (**7**) at 10–15 °C, the main reaction product was 2-chloro-4-nitrophenyldifluoromethyl ether (**8**) isolated in 45% yield, and the yields of nitroaldehydes **9** and **10** were 17 and 10%, respectively (**Scheme 4**). Compound **8** was described earlier [8], and the product we obtained corresponded to that. The structure of compounds **9** and **10** was unambiguously proven by ^{1}H NMR spectra. Thus, in the case of compound **9**, the signals of the benzene ring protons appear as narrow singlets, which indicates their *para*-arrangement. In the case of compound **10**, the signals of two protons of the benzene ring appear as two doublets with a small spin-spin coupling constant of about 1 Hz, which corresponds to the *meta*-position of the protons.

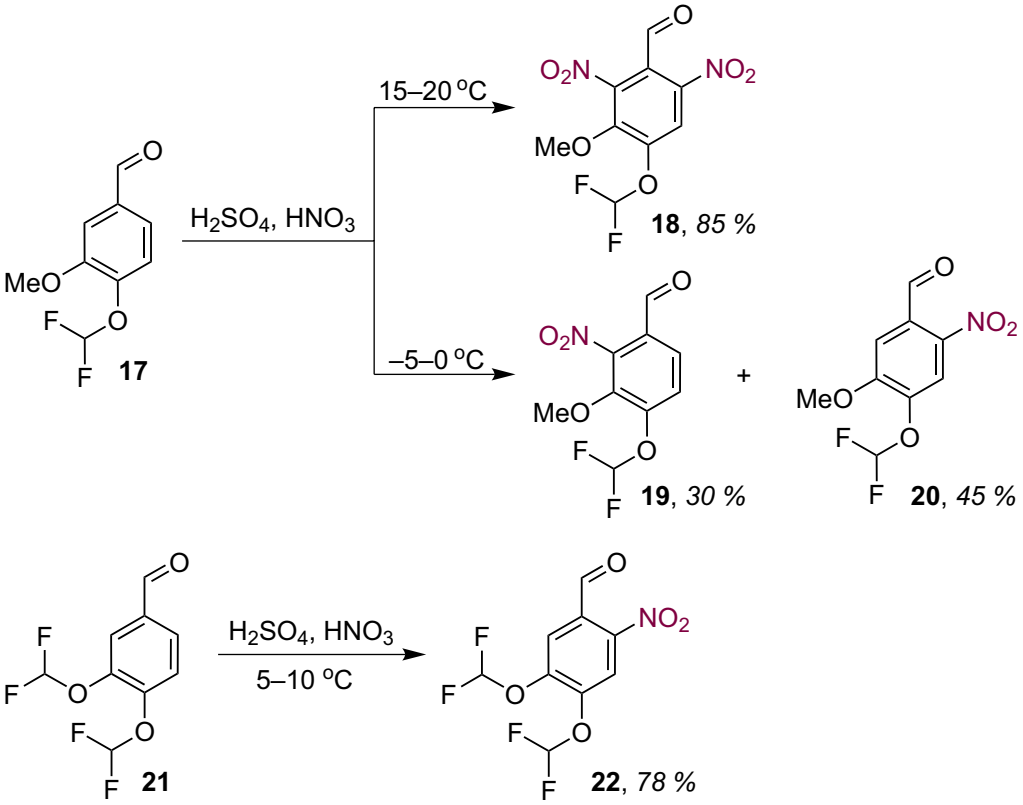
When carrying out nitration at a higher temperature (35–40 °C), aldehydes were not found among the reaction products. A mixture of nitro product **8** and dinitro compounds **11** and **12** were obtained in about 65% overall yield. The structure of compounds **11** and **12** was confirmed similarly to compounds **9** and **10**. The signals of the benzene ring protons in compound **11** appear as



Scheme 4. Nitration of 3-chloro-4-difluoromethoxybenzaldehyde



Scheme 5. Nitration of 2-chloro-4-difluoromethoxybenzaldehyde



Scheme 6. Nitration of *O*-difluoromethylvanillin and 3,4-*bis*(difluoromethoxy)benzaldehyde

two doublets with a small spin-spin coupling constant (about 1 Hz), while in the case of compound **12**, the signals of the benzene ring protons appear as narrow singlets. In compound **12**, two nitro groups are in the *ortho*-position to each other, which is not typical for dinitration products. Nitration of compound **8** leads almost unequivocally to compound **11**, the second nitro group is directed to the *meta*-position relative to the first one. Thus, the formation of compound **12** most likely occurs as a result of the *ipso*-substitution of the aldehyde group in compound **9**.

On the contrary, the presence of a chlorine atom in the *ortho*-position to aldehyde group significantly hinders the formation of *ipso*-substitution products. Thus, we studied nitration of 2-chloro-4-difluoromethoxybenzaldehyde (**13**) (**Scheme 5**). As in the case of aldehyde **7**, the reaction did not proceed at 0 °C; however, at 10–15 °C, nitration led to the formation of a mixture of two aldehydes **14** and **15** in the ratio of 5:1 with a total yield of about 75%, and only an insignificant (2–4%) impurity of the *ipso*-substitution product **16**. Aldehyde **14** was isolated from the reaction mixture by crystallization in about 50% yield. Carrying out the nitration at 20–25 °C resulted in a slight increase in the yield of product **16**, up to 7–9%. Obviously, the decrease in the amount of *ipso*-substitution products is due to steric hindrance created by the chlorine atom, complicating the formation of the intermediate compound type **6**.

The introduction of electron donating methoxy group into the molecule greatly facilitates the nitration reaction and completely excludes the formation of *ipso*-substitution products during nitration under the conditions studied (**Scheme 6**). Thus, during nitration of *O*-difluoromethylvanillin (**17**), two nitro groups were partially introduced into the molecule already at 0–5 °C and during nitration at 5–10 °C *di*nitro compound **18**

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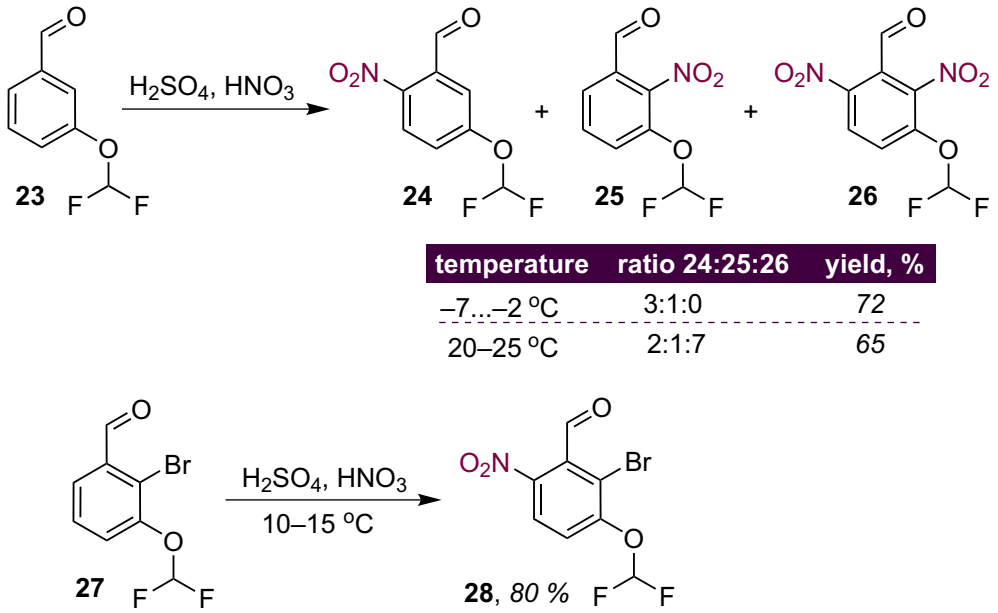

Scheme 7. Nitration of *m*-difluoromethoxybenzaldehyde and 2-bromo-3-difluoromethoxybenzaldehyde

became the main reaction product. At the same time nitration of compound **17** at 15–20 °C led to the formation of only 2,6-*di*nitro-*O*-difluoromethylvanillin (**18**) in a high yield (**Scheme 6**). Carrying out the reaction at a temperature not exceeding 0 °C, with the gradual addition of an equimolar amount of nitric acid, led to the production of two *mono*nitro products – 2-nitro-*O*-difluoromethylvanillin (**19**) and 6-nitro-*O*-difluoromethylvanillin (**20**) in the ratio of 2:3 and the total yield of 75%. Activation of the *ortho*- and *para*-positions by a strong donor completely determined the direction of the reaction. Nitration of 3,4-*bis*(difluoromethoxy)benzaldehyde (**21**) at 5–10 °C was not accompanied by the formation of *ipso*-substitution products as well and proceeded with the formation of only one product – 3,4-*bis*(difluoromethoxy)-6-nitrobenzaldehyde (**22**) since the difluoromoxy group mostly activates the *para*-position.

Nitration of *m*-difluoromethoxybenzaldehyde (**23**) under the studied conditions proceeded at temperatures below 0 °C and led to a mixture of isomers **24** and **25**. Meanwhile, nitration at 20–25 °C provided a significant admixture of the *di*nitro product **26**. The introduction of a bromine atom into position 2 of *m*-difluoromethoxybenzaldehyde made nitration somewhat difficult (the reaction did not proceed at 0 °C), but led to the formation of only single product. Thus, nitration of 2-bromo-3-difluoromethoxybenzaldehyde (**27**) resulted in one nitration product **28**. The structure of compound **28** was unambiguously proven by ^{1}H NMR experiments since proton signals of the aromatic nucleus appeared as two doublets with a spin-spin interaction constant of about 10 Hz corresponding to the *ortho*-position of protons.

In all cases of nitration of *m*-difluoromethoxybenzaldehydes, no signs of *ipso*-substitution were found.

■ Conclusion

In summary, the features of nitration of aromatic aldehydes containing difluoromethyl group have been studied. Some relationships have been found between the structure of the molecule, the reaction conditions, and the contribution of the *ipso*-substitution products of the aldehyde group.

■ Experimental part

Melting points were measured in an open capillary and given uncorrected. ^{1}H NMR (300 MHz, $CDCl_3$), ^{13}C NMR (75 MHz, $CDCl_3$), and ^{19}F NMR (288 MHz, $CDCl_3$) were recorded on a Varian-Mercury-300 spectrometer using TMS and CCl_3F as internal standards. The reaction progress was controlled by TLC on Silufol UV-254 plates. The chromatographic separation of products was carried out on a "Puriflash XS 520 Plus" chromatograph using a "Kieselgel MN 40-60" silica gel. The eluent was hexane/ethyl acetate (0-20% ethyl acetate) with a gradient increase in polarity.

p-Difluorometoxybenzaldehyde (**3**) [9], *O*-difluorometoxyvaniline (**17**) [3], 3,4-*bis*(difluoromethoxy)-benzaldehyde (**21**) [10], and *m*-difluoromethoxybenzaldehide (**23**) [11] were obtained according to the literature procedures.

3-Chloro-4-difluoromethoxybenzaldehyde (7), 2-chloro-4-difluometoxy-benzaldehyde (13) and 2-bromo-3-difluorometoxybenzaldehyde (27). The general procedure of difluoromethylation of corresponding hydroxybenzaldehydes

A solution of a corresponding hydroxybenzaldehyde (0.3 mol) in dioxane (200 mL) was stirred and treated by adding a solution of KOH (90 g, 1.5 mol) in H_2O (180 mL). Freon-22 was bubbled through the vigorously stirred reaction mixture at 45–55 °C until the absorption of gas ceased (the exothermic effect was observed). The reaction was monitored by TLC. If a starting hydroxybenzaldehyde remained, an additional KOH (30 g) was added, and Freon-22 was bubbled until the absorption of gas ceased. The reaction overall time was about 4–5 h. Water (300 mL) was added, the product was extracted by shaking with MTBE (2×300 mL), the organic layer was separated and washed with water (3×300 mL), dried over anhydrous K_2CO_3, and the solvent was evaporated at a reduced pressure. The product was purified by fractional distillation *in vacuo* (**7**, **13**) or crystallization from hexane (**27**).

3-Chloro-4-difluoromethoxybenzaldehyde (**7**)

A colorless liquid. Yield – 78%. B. p. 74–76 °C/0.5 Torr. Anal. Calcd for $C_8H_5ClF_2O_2$, %: C 46.51; H 2.44; Cl 17.16. Found, %: C 46.55; H 2.51; Cl 17.32. ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 6.64 (1H, t, J = 72.0 Hz, O-CHF_2); 7.36 (1H, d, J = 7.0 Hz, ArH); 7.76 (1H, dd, $^3J_{HH}$ = 7.0 Hz, $^4J_{HH}$ = 1.0 Hz, ArH); 7.92 (1H, J = 1.0 Hz, ArH); 9.89 (1H, s, CHO). ^{13}C NMR (75 MHz, $CDCl_3$), δ, ppm: 115.2 (t, J = 287.0 Hz, O-CHF_2); 120.4; 126.9; 129.5; 131.5; 134.0; 151.1; 189.6. ^{19}F NMR (288 MHz, $CDCl_3$), δ, ppm: -82.7 (d, J = 72.0 Hz, O-CHF_2).

2-Chloro-4-difluoromethoxybenzaldehyde (**13**)

A colorless liquid. Yield – 72%. B. p. 76–78 °C/0.5 Torr. Anal. Calcd for $C_8H_5ClF_2O_2$, %: C 46.51; H 2.44; Cl 17.16. Found, %: C 46.71; H 2.52; Cl 17.27. ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 6.65 (1H, t, J = 72.0 Hz, O-CHF_2); 7.12 (1H, dd, $^3J_{HH}$ = 7.0 Hz, $^4J_{HH}$ = 1.0 Hz, ArH); 7.27 (1H, J = 1.0 Hz, ArH); 7.97 (1H, d, J = 7.0 Hz, ArH); 10.44 (1H, s, CHO). ^{13}C NMR (75 MHz, $CDCl_3$), δ, ppm: 115.5 (t, J = 287.0 Hz, O-CHF_2); 120.4; 126.7; 126.9; 128.5; 135.5; 148.4; 191.1. ^{19}F NMR (288 MHz, $CDCl_3$), δ, ppm: -81.4 (d, J = 72.0 Hz, O-CHF_2).

2-Bromo-3-difluorometoxybenzaldehyde (**27**)

A white solid. Yield – 83%. M. p. 63–64 °C. Anal. Calcd for $C_8H_5BrF_2O_2$, %: C 38.28; H 2.01; Br 31.83. Found, %: C 38.48; H 2.11; Br 32.07. ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 6.58 (1H, t, J = 72.0 Hz, O-CHF_2); 7.43–7.48 (2H, m, ArH); 7.77–7.87 (1H, m, ArH); 10.38 (1H, s, CHO). ^{13}C NMR (75 MHz, $CDCl_3$), δ, ppm: 108.9 (C-Br); 115.5 (t, J = 287.0 Hz, O-CHF_2); 121.4; 125.7; 128.5; 132.5; 143.9; 187.9. ^{19}F NMR (288 MHz, $CDCl_3$), δ, ppm: -81.1 (d, J = 72.0 Hz, O-CHF_2).

The general procedure for nitration of aldehydes 3, 7, 13, 17, 21, and 27

An aldehyde (0.02 mol) was added dropwise or in portions to a mixture of 96% sulfuric acid (10 mL) and 100% nitric acid (5 mL) in such a rate that the temperature did not exceed the initially selected temperature by more than 5 °C. After stirring at this temperature for 30 minutes, the reaction mixture was poured onto ice. The product was extracted with MTBE (2×100 mL), washed with a 5% aq soda solution (2×100 mL), the solvent was evaporated off, and the residue was crystallized from hexane in the case of compounds **14**, **18**, and **28**, or distilled in a vacuum in the case of compound **22**. In other cases, the mixture was separated chromatographically. To obtain compounds **19** and **20** nitric acid (1.5 g, 0.022 mol) was added dropwise to the stirred suspension of *O*-difluorometoxyvaniline (**17**) (4.05 g, 0.02 mol) in 10 mL of 96% sulfuric acid.

3-Nitro-4-difluoromethoxybenzaldehyde (**4**)

A yellow solid. Yield – 50–72%. M. p. 33–35 °C (Lit. [3] – oil). Anal. Calcd for $C_8H_5F_2NO_4$, %: C 44.24; H 2.32; N 6.45. Found, %: C 44.55; H 2.28; N 6.42. ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 6.71 (1H, t, J = 72.0 Hz, O-CHF_2); 7.55 (1H, d, J = 7.0 Hz, ArH); 8.12 (1H, dd, $^3J_{HH}$ = 7.0 Hz, $^4J_{HH}$ = 1.0 Hz, ArH); 8.33 (1H, J = 1.0 Hz, ArH); 10.05 (1H, s, CHO). ^{13}C NMR (75 MHz, $CDCl_3$), δ, ppm: 115.2 (t, J = 287.0 Hz, O-CHF_2); 122.4; 133.6; 134.5; 142.5; 147.3; 150.1; 188.6. ^{19}F NMR (288 MHz, $CDCl_3$), δ, ppm: -84.5 (d, J = 72.0 Hz, O-CHF_2).

5-Chloro-4-difluoromethoxy-2-nitrobenzaldehyde (**9**)

A yellow solid. Yield – 17%. M. p. 54–55 °C. Anal. Calcd for $C_8H_4ClF_2NO_4$, %: C 38.20; H 1.60; N 5.57. Found, %: C 38.35; H 1.78; N 5.42. ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 6.78 (1H, t, J = 72.0 Hz, O-CHF_2); 8.02 (1H, s, ArH); 8.04 (1H, s, ArH); 10.36 (1H, s, CHO). ^{13}C NMR (75 MHz, $CDCl_3$), δ, ppm: 114.5 (t, J = 287.0 Hz, O-CHF_2); 115.4; 128.6; 131.7; 132.8; 147.9; 149.5; 185.6. ^{19}F NMR (288 MHz, $CDCl_3$), δ, ppm: -83.2 (d, J = 72.0 Hz, O-CHF_2).

5-Chloro-4-difluoromethoxy-3-nitrobenzaldehyde (**10**)

A yellow oil. Yield – 10%. Anal. Calcd for $C_8H_4ClF_2NO_4$, %: C 38.20; H 1.60; N 5.57. Found, %: C 38.37; H 1.68; N 5.66. ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 6.74 (1H, t, J = 72.0 Hz, O-CHF_2); 8.25 (1H, d, J = 1.0 Hz, ArH); 8.31 (1H, d, J = 1.0 Hz,

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ArH); 10.01 (1H, s, CHO). ^{13}C NMR (75 MHz, $CDCl_3$), δ, ppm: 114.8 (t, J = 287.0 Hz, O-CHF_2); 124.4; 133.1; 134.7; 143.4; 146.0; 149.1; 187.4. ^{19}F NMR (288 MHz, $CDCl_3$), δ, ppm: -81.7 (d, J = 72.0 Hz, O-CHF_2).

2-Chloro-4,6-dinitrophenyldifluoromethyl ether (**11**)

A yellow oil. Yield – 20%. Anal. Calcd for $C_7H_3ClF_2N_2O_5$, %: C 31.31; H 1.13; N 10.43. Found, %: C 31.36; H 1.21; N 10.42. ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 6.77 (1H, t, J = 72.0 Hz, O-CHF_2); 8.60 (1H, d, J = 1.0 Hz, ArH); 8.69 (1H, d, J = 1.0 Hz, ArH). ^{13}C NMR (75 MHz, $CDCl_3$), δ, ppm: 115.4 (t, J = 287.0 Hz, O-CHF_2); 118.9; 129.4; 133.5; 143.9; 144.8; 145.1. ^{19}F NMR (288 MHz, $CDCl_3$), δ, ppm: -85.7 (d, J = 72.0 Hz, O-CHF_2).

2-Chloro-4,5-dinitrophenyldifluoromethyl ether (**12**)

A yellow oil. Yield – 10%. Anal. Calcd for $C_7H_3ClF_2N_2O_5$, %: C 31.31; H 1.13; N 10.43. Found, %: C 31.42; H 1.17; N 10.53. ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 6.75 (1H, t, J = 72.0 Hz, O-CHF_2); 7.82 (1H, s, ArH); 8.07 (1H, s, ArH). ^{13}C NMR (75 MHz, $CDCl_3$), δ, ppm: 114.4 (t, J = 287.0 Hz, O-CHF_2); 116.5; 123.1; 127.7; 140.4; 144.0; 147.1. ^{19}F NMR (288 MHz, $CDCl_3$), δ, ppm: -82.8 (d, J = 72.0 Hz, O-CHF_2).

2-Cloro-4-difluoromethoxy-5-nitrobenzaldehyde (**14**)

A yellow solid. Yield – 60%. M. p. 60–61 °C. Anal. Calcd for $C_8H_4ClF_2NO_4$, %: C 38.20; H 1.60; N 5.57. Found, %: C 38.37; H 1.68; N 5.66. ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 6.70 (1H, t, J = 72.0 Hz, O-CHF_2); 8.25 (1H, s, ArH); 8.31 (1H, s, ArH); 10.37 (1H, s, CHO). ^{13}C NMR (75 MHz, $CDCl_3$), δ, ppm: 114.9 (t, J = 287.0 Hz, O-CHF_2); 124.0; 126.6; 129.9; 141.1; 142.2; 146.7; 186.2. ^{19}F NMR (288 MHz, $CDCl_3$), δ, ppm: -83.1 (d, J = 72.0 Hz, O-CHF_2).

2-Chloro-4-difluoromethoxy-3-nitrobenzaldehyde (**15**)

A yellow solid. Yield – 12%. M. p. 49–50 °C. Anal. Calcd for $C_8H_4ClF_2NO_4$, %: C 38.20; H 1.60; N 5.57. Found, %: C 38.42; H 1.72; N 5.71. ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 6.57 (1H, t, J = 72.0 Hz, O-CHF_2); 7.47 (1H, d, J = 7.0 Hz, ArH); 8.02 (1H, d, J = 7.0 Hz, ArH); 10.39 (1H, s, CHO). ^{13}C NMR (75 MHz, $CDCl_3$), δ, ppm: 114.7 (t, J = 287.0 Hz, O-CHF_2); 118.3; 126.6; 129.9; 131.5; 141.2; 142.5; 186.3. ^{19}F NMR (288 MHz, $CDCl_3$), δ, ppm: -84.4 (d, J = 72.0 Hz, O-CHF_2).

3-Chloro-4,6-dinitrophenyldifluoromethyl ether (**16**)

A yellow oil. Yield – 4%. Anal. Calcd for $C_7H_3ClF_2N_2O_5$, %: C 31.31; H 1.13; N 10.43. Found, %: C 31.42; H 1.17; N 10.54. ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 6.76 (1H, t, J = 72.0 Hz, O-CHF_2); 7.67 (1H, s, ArH); 8.61 (1H, s, ArH). ^{13}C NMR (75 MHz, $CDCl_3$), δ, ppm: 114.8 (t, J = 287.0 Hz, O-CHF_2); 123.6; 124.5; 133.5; 140.0; 143.8; 145.5. ^{19}F NMR (288 MHz, $CDCl_3$), δ, ppm: -86.9 (d, J = 72.0 Hz, O-CHF_2).

2,6-Dinitro-O-difluoromethylvanillin (**18**)

A white solid. Yield – 85%. M. p. 58–59 °C. Anal. Calcd for $C_9H_6F_2N_2O_7$, %: C 37.00; H 2.07; N 9.59. Found, %: C 36.85; H 2.12; N 9.72. ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 4.14 (3H, s, OCH_3); 6. 76 (1H, t, J = 72.0 Hz, O-CHF_2); 8.20 (1H, s, ArH); 10.25 (1H, s, CHO). ^{13}C NMR (75 MHz, $CDCl_3$), δ, ppm: 63.5 (OCH_3); 115.2 (t, J = 287.0 Hz, O-CHF_2); 118.9; 124.7; 142.0; 144.1; 145.7; 148.8; 183.8. ^{19}F NMR (288 MHz, $CDCl_3$), δ, ppm: -82.2 (d, J = 72.0 Hz, O-CHF_2).

2-Nitro-O-difluoromethylvanillin (**19**)

A white solid. Yield – 30%. M. p. 61–62 °C. Anal. Calcd for $C_9H_7F_2N_2O_5$, %: C 43.74; H 2.85; N 5.67. Found, %: C 43.65; H 2.72; N 5.72. ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 4.00 (3H, s, OCH_3); 6.72 (1H, t, J = 72.0 Hz, O-CHF_2); 7.48 (1H, d, J = 7.0 Hz, ArH); 7.71 (1H, d, J = 7.0 Hz, ArH); 9.89 (1H, s, CHO). ^{13}C NMR (75 MHz, $CDCl_3$), δ, ppm: 63.2 (OCH_3); 115.2 (t, J = 287.0 Hz, O-CHF_2); 118.9; 121.6; 124.5; 127.2; 142.2; 149.4; 185.7. ^{19}F NMR (288 MHz, $CDCl_3$), δ, ppm: -82.1 (d, J = 72.0 Hz, O-CHF_2).

6-Nitro-O-difluoromethylvanillin (**20**)

A white solid. Yield – 45%. M. p. 75–76 °C. Anal. Calcd for $C_9H_7F_2N_2O_5$, %: C 43.74; H 2.85; N 5.67. Found, %: C 43.87; H 2.76; N 5.60. ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 4.04 (3H, s, OCH_3); 6.70 (1H, t, J = 72.0 Hz, O-CHF_2); 7.46 (1H, 1H, s, ArH); 7.98 (1H, s, ArH); 10.44 (1H, s, CHO). ^{13}C NMR (75 MHz, $CDCl_3$), δ, ppm: 59.5 (OCH_3); 111.3; 115.2 (t, J = 287.0 Hz, O-CHF_2); 128.5; 124.5; 130.4; 142.4; 155.5; 187.4. ^{19}F NMR (288 MHz, $CDCl_3$), δ, ppm: -82.4 (d, J = 72.0 Hz, O-CHF_2).

3,4-Bis(difluoromethoxy)-6-nitrobenzaldehyde (**22**)

A yellow oil. Yield – 78%. B. p. 112–114 °C/ 0.5 Torr. Anal. Calcd for $C_9H_5F_4NO_5$, %: C 38.18; H 1.78; N 4.95. Found, %: C 38.37; H 2.06; N 5.02. ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 6.71 (1H, t, J = 72.0 Hz, O-CHF_2); 7.80 (1H, s, ArH); 8.05 (1H, s, ArH); 10.37 (1H, s, CHO). ^{13}C NMR (75 MHz, $CDCl_3$), δ, ppm: 114.9 (t, J = 287.0 Hz, O-CHF_2); 115.2 (t, J = 287.0 Hz, O-CHF_2); 118.3; 120.7; 144.5; 145.9; 146.1; 186.1. ^{19}F NMR (288 MHz, $CDCl_3$), δ, ppm: -82.4 (d, J = 72.0 Hz, O-CHF_2).

2-Nitro-5-difluoromethoxybenzaldehyde (**24**)

A white solid. Yield – 25-45%. M. p. 35–37 °C. Anal. Calcd for $C_8H_5F_2NO_4$, %: C 44.24; H 2.32; N 6.45. Found, %: C 44.33; H 2.29; N 6.49. ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 6.69 (1H, t, J = 72.0 Hz, O-CHF_2); 7.85 (1H, d, J = 1.0 Hz,

ArH); 7.90 (1H, d, J = 7.0 Hz, ArH); 8.04 (1H, dd, $^{3}J_{HH}$ = 7.0 Hz, $^{4}J_{HH}$= 1.0 Hz, ArH); 10.09 (1H, s, CHO). ^{13}C NMR (75 MHz, $CDCl_3$), δ, ppm: 115.2 (t, J = 287.0 Hz, O-CHF_2); 122.9; 126.2; 127.2; 139.6; 143.3; 145.9; 189.1. ^{19}F NMR (288 MHz, $CDCl_3$), δ, ppm: -82.7 (d, J = 72.0 Hz, O-CHF_2).

2-Nitro-3-difluoromethoxybenzaldehyde (**25**)

A white solid. Yield – 12-20%. M. p. 25–27 °C. Anal. Calcd for $C_8H_5F_2NO_4$, %: C 44.24; H 2.32; N 6.45. Found, %: C 44.29; H 2.42; N 6.54. ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 6.61 (1H, t, J = 72.0 Hz, O-CHF_2); 7.65 (1H, d, J = 7.0 Hz, ArH); 7.75 (1H, t, J = 7.0 Hz, ArH); 7.84 (1H, d, J = 7.0 Hz, ArH); 9.94 (1H, s, CHO). ^{13}C NMR (75 MHz, $CDCl_3$), δ, ppm: 115.2 (t, J = 287.0 Hz, O-CHF_2); 123.0; 126.8; 127.9; 128.5; 132.0; 142.4; 186.2. ^{19}F NMR (288 MHz, $CDCl_3$), δ, ppm: -82.0 (d, J = 72.0 Hz, O-CHF_2).

2,6-Dinitro-5-difluoromethoxybenzaldehyde (**26**)

A white solid. Yield – 0-50%. M. p. 57–58 °C. Anal. Calcd for $C_8H_4F_2N_2O_6$, %: C 36.66; H 1.54; N 10.69. Found, %: C 36.56; H 1.42; N 10.46. ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 6.71 (1H, t, J = 72.0 Hz, O-CHF_2); 7.69 (1H, d, J = 7.0 Hz, ArH); 8.39 (1H, d, J = 7.0 Hz, ArH); 10.24 (1H, s, CHO). ^{13}C NMR (75 MHz, $CDCl_3$), δ, ppm: 114.7 (t, J = 287.0 Hz, O-CHF_2); 122.7; 127.6; 128.8; 140.7; 143.0; 146.0; 183.7. ^{19}F NMR (288 MHz, $CDCl_3$), δ, ppm: -83.2 (d, J = 72.0 Hz, O-CHF_2).

2-Bromo-3-difluoromethoxy-6-nitrobenzaldehyde (**28**)

A white solid. Yield – 80%. M. p. 63–64 °C. Anal. Calcd for $C_8H_4BrF_2NO_4$, %: C 32.46; H 1.36; Br 26.99. Found, %: C 32.55; H 1.21; Br 27.07. ^{1}H NMR (300 MHz, $CDCl_3$), δ, ppm: 6.71 (1H, t, J = 72.0 Hz, O-CHF_2); 7.45 (1H, d, J = 7.0 Hz, ArH); 8.11 (1H, d, J = 7.0 Hz, ArH); 10.20 (1H, s, CHO). ^{13}C NMR (75 MHz, $CDCl_3$), δ, ppm: 107.3 (C-Br); 114.4 (t, J = 287.0 Hz, O-CHF_2); 120.8; 125.0; 136.4; 143.8; 152.4; 187.0. ^{19}F NMR (288 MHz, $CDCl_3$), δ, ppm: -83.3 (d, J = 72.0 Hz, O-CHF_2).

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Incorporation of *gem*-Difluorocycloalkyl Substituents into Heterocycles *via* the Levin's "Nitrogen Deletion" Strategy

Abstract

A series of compounds containing heterocyclic cores and *gem*-difluorocycloalkyl substituents was obtained under conditions of the parallel synthesis (i.e., simultaneous performance of reaction procedures, treatment of the reaction mixture, and product isolation for a number of similar transformations) using the reductive amination – the "Nitrogen deletion" reaction sequence. The synthesis of the target compounds commenced from heteroaromatic aldehydes and the corresponding *gem*-difluorocycloalkyl or (*gem*-difluorocycloalkyl)methyl amines and included the $NaBH_3CN$-mediated reductive amination and "Nitrogen deletion" upon the action of Levin's anomeric amide. It has been shown that the method can be used to obtain compounds with the aforementioned structural fragments separated by one or two methylene units. The developed protocol allowed for the preparation of a 12-member compound library (67 % synthetic efficiency). Therefore, this novel synthetic methodology is suitable for decorating heterocyclic cores with sp^3-enriched substituents that are attractive for medicinal chemistry.

***Keywords**:* cycloalkanes; fluorine; Levin's anomeric amide; "Nitrogen deletion"; reductive amination

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Введення *гем*-дифлуороциклоалкільних замісників у гетероцикли через стратегію «видалення Нітрогену» Левіна

Анотація

В умовах паралельного синтезу (тобто одночасного виконання реакції, оброблення реакційної суміші та виділення продукту для низки споріднених перетворень) із застосуванням послідовності реакції відновного амінування та «видалення Нітрогену» було одержано серію сполук, що містять гетероциклічні фрагменти та *гем*-дифлуороциклоалкільні замісники. Синтез цільових сполук виходив з гетероароматичних альдегідів і відповідних *гем*-дифлуороциклоалкіл- або (*гем*-дифлуороциклоалкіл)метиламінів та передбачав відновне амінування за участі $NaBH_3CN$ і «видалення Нітрогену» під дією аномерного аміду Левіна. Доведено, що метод застосовний для одержання сполук із вищезгаданими структурними фрагментами, розділеними однією чи двома метиленовими ланками. Розроблений протокол дозволив одержати бібліотеку сполук із 12 представників (синтетична ефективність 67 %). Отже, ця новітня синтетична методологія є придатною для декорування гетероциклічних систем sp^3-збагаченими замісниками, що є привабливими для медичної хімії.

Ключові слова: циклоалкани; Флуор; аномерний амід Левіна; «видалення Нітрогену»; відновне амінування

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Supporting information: Copies of ^{1}H, ^{13}C, ^{19}F NMR spectra of the synthesized compounds.
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■ Introduction

The incorporation of fluorine-containing substituents into organic molecules of interest is a well-known approach to drug design, which can be illustrated by numerous success stories [1–5]. In particular, *gem*-difluorocycloalkyl groups have demonstrated their high relevance to modern drug discovery. For example, they have been incorporated into such marketed pharmaceuticals as Maraviroc, an anti-HIV medication, and Ivosidenib, an anticancer agent, as well as experimental drugs VU6001376, a mGluR4 positive allosteric modulator, IPN60090, a selective glutaminase-1 inhibitor, and RBx 343E48F0, a bronchodilator (**Figure 1**) [6].

To date, most methods for the incorporation of *gem*-difluorocycloalkyl substituents into the molecules of interest were based on the Carbon–heteroatom bond construction. Building blocks and synthetic methodologies for the C–C bond creation involving these moieties are rare and typically based on the multistep transformations. On the other hand, the Levin's "Nitrogen deletion" methodology provides a unique possibility to construct the C–C bond in an unusual manner [7]. Essentially, this approach involves the reaction of secondary amines with the so-called anomeric amides (e.g., **1**), which results in formal extrusion of the NH moiety and combining alkyl radicals attached to it (**Scheme**, *A*).

Recently, we have shown that in combination with reductive amination, this approach can be used for the synthesis of compound libraries by a formal coupling of (hetero)aromatic aldehydes and (het)arylmethylamines (**Scheme**, *B*) [8]. In this work, we sought to extend this methodology to primary amines containing the *gem*-difluorocycloalkyl moiety (**3**) in order to ensure the incorporation of these fluorinated substituents into heterocyclic cores **2** (**Scheme**, *C*).

■ Results and discussion

In this study, a compound numbering system common for combinatorial chemistry was used: the reagents used in the parallel synthesis were denoted as **2**{*i*} and **3**{*j*}, whereas the resulting library members were obtained from **2**{*i*} and **3**{*j*}–**4**{*i*,*j*}.

To confirm the applicability of the *reductive amination – the "Nitrogen deletion"* sequence for the synthesis of compound library **4**, heteroaromatic aldehydes **2**{*1–6*} and primary amines **3**{*1–8*}

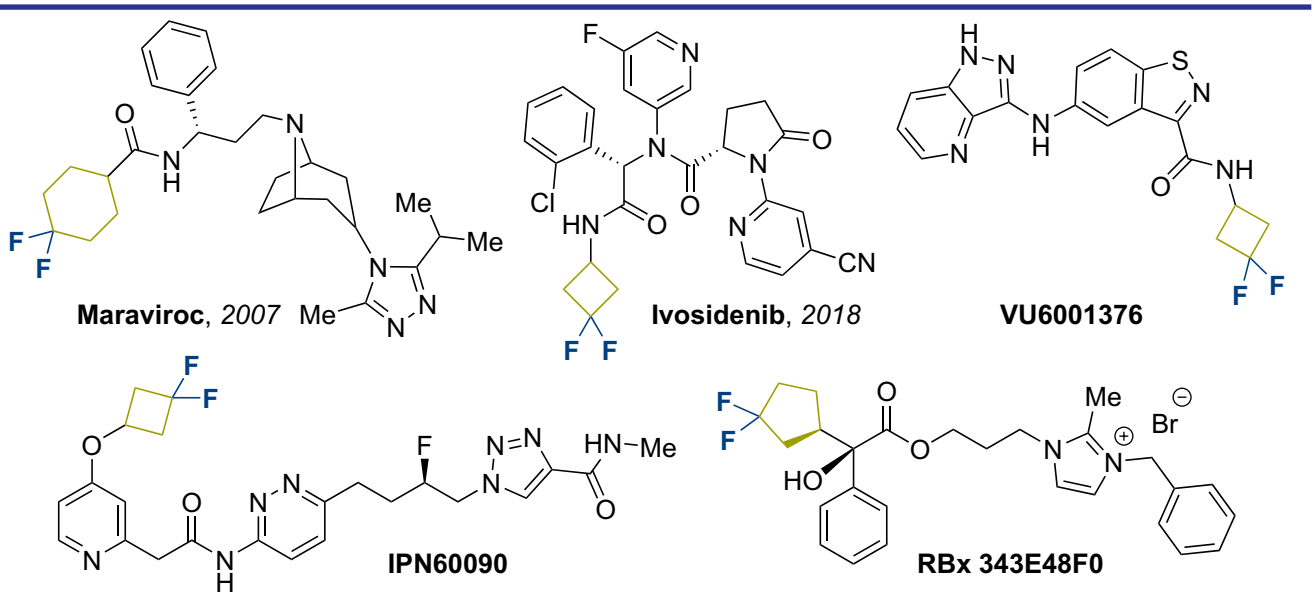

Figure 1. Marketed and experimental drug molecules containing *gem*-difluorocycloalkyl groups

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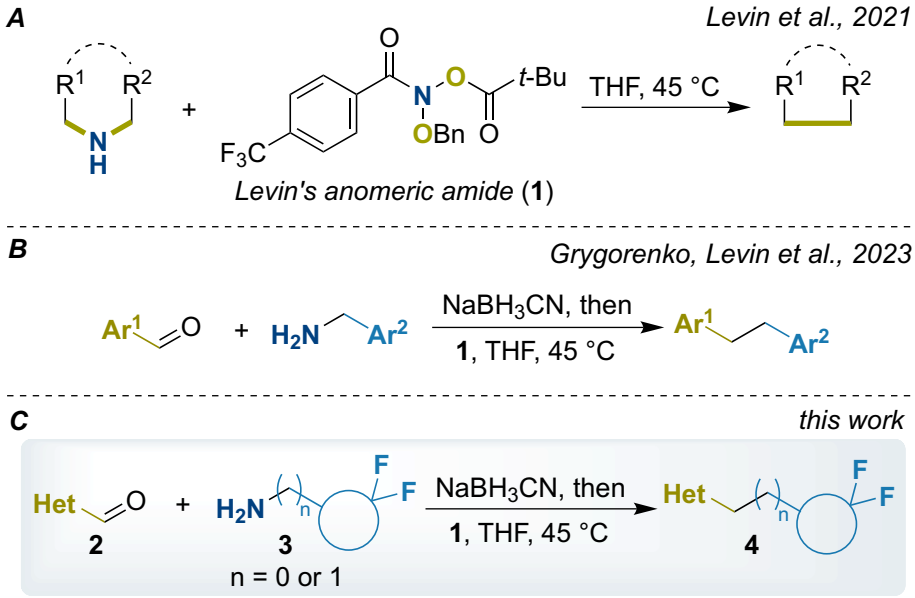

Scheme. Examples of the Levin's "Nitrogen deletion" methodology published before and the outline of the current work

were selected (**Figure 2**). Using the conditions described in our previous work [8], 18 parallel experiments were performed with different combinations of the starting materials, and in 12 of them, the target mini-library members **4**{*1–6,1–8*} were obtained (67% synthesis success rate, **Table 1**; see **Figure 3** for the examples of products obtained).

In general, amines with primary alkyl substituents (e.g., **3**{*8*}) gave somewhat higher yields of the products (17–33%) as compared to the α-branched ones (e.g., **3**{*1*}) (10–18%), which was in accordance with a high sensitivity of the "Nitrogen deletion" step towards steric factors [7]. As for the aldehyde component, compounds that demonstrated somewhat poorer results in our previous study (e.g., pyrimidine **2**{*3*} or pyrazole **2**{*5*} carbaldehydes) did not allow isolating the target products at all. According to LS-MS spectra of the crude reaction mixture, the products were formed, but their purification was not efficient.

■ Conclusions

Reductive amination – the Levin's "Nitrogen deletion" reaction sequence is an efficient approach

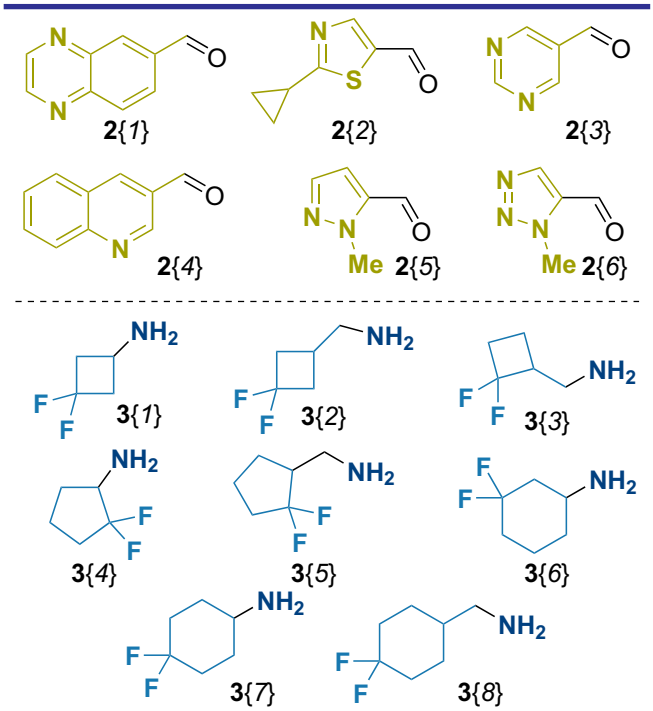

Figure 2. Selected representatives of starting aldehydes **2** and primary amines **3**

Table 1. The parallel synthesis of mini-library **4**{*1–6,1–8*} according to **Scheme** (*C*)

#	Library member	Aldehyde	Amine	Yield of the products	
				mg	%
1	**4**{*1,1*}	**2**{*1*}	**3**{*1*}	134	18
2	**4**{*1,2*}	**2**{*1*}	**3**{*2*}	165	19
3	**4**{*1,3*}	**2**{*1*}	**3**{*3*}	137	17
4	**4**{*1,4*}	**2**{*1*}	**3**{*4*}	–	0
5	**4**{*1,5*}	**2**{*1*}	**3**{*5*}	166	33
6	**4**{*1,6*}	**2**{*1*}	**3**{*6*}	179	28
7	**4**{*1,7*}	**2**{*1*}	**3**{*7*}	100	14
8	**4**{*1,8*}	**2**{*1*}	**3**{*8*}	192	32
9	**4**{*2,1*}	**2**{*2*}	**3**{*1*}	–	0
10	**4**{*2,8*}	**2**{*2*}	**3**{*8*}	115	13
11	**4**{*3,1*}	**2**{*3*}	**3**{*1*}	–	0
12	**4**{*3,8*}	**2**{*3*}	**3**{*8*}	–	0
13	**4**{*4,1*}	**2**{*4*}	**3**{*1*}	71	17
14	**4**{*4,8*}	**2**{*4*}	**3**{*8*}	222	27
15	**4**{*5,1*}	**2**{*5*}	**3**{*1*}	–	0
16	**4**{*5,8*}	**2**{*5*}	**3**{*8*}	–	0
17	**4**{*6,1*}	**2**{*6*}	**3**{*1*}	68	10
18	**4**{*6,8*}	**2**{*6*}	**3**{*8*}	105	21

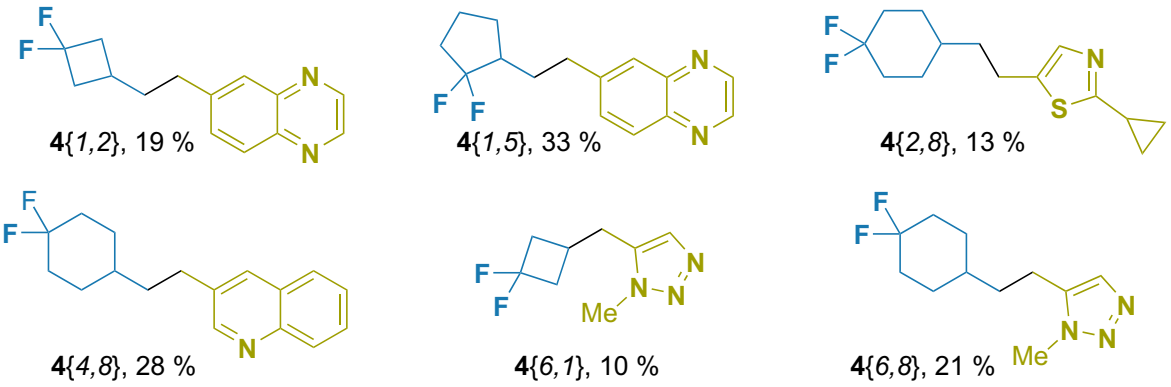

Figure 3. Examples of the products obtained

for introducing *gem*-difluorinated cycloalkyl substituents into heterocyclic systems under conditions of the parallel synthesis; the products having these moieties separated by one or two methylene units can be obtained. The use of α-branched primary amines as the starting materials results in somewhat lower yields of the corresponding library members. Limitations on the aldehyde component correlate with the results obtained in the previous study on (hetero)aromatic series: thus, pyrimidine and pyrazole derivatives were inefficient due to problems with the product isolation. The resulting lead-like fluorinated compound libraries are of special interest to medicinal chemistry; potentially, this method can also be extended to other applications in early drug discovery.

■ Experimental part

All starting materials were available from Enamine Ltd. and Ukrorgsyntez Ltd. Melting points were measured on a MPA100 OptiMelt automated melting point system. ^{1}H and ^{13}C NMR spectra were recorded on an Agilent ProPulse 600 spectrometer (at 600 MHz for ^{1}H NMR and 151 MHz for ^{13}C{^{1}H} NMR). ^{19}F NMR spectra were recorded on a Varian Unity Plus 400 spectrometer at 376 MHz. NMR chemical shifts were reported in ppm (δ scale) downfield from TMS as an internal standard and were referenced using residual NMR solvent peaks at 7.26 and 77.16 ppm for ^{1}H and ^{13}C in $CDCl_3$, 2.50 and 39.52 ppm for ^{1}H and ^{13}C in DMSO-d_6. For ^{19}F{^{1}H} NMR, $CFCl_3$ in $CHCl_3$ was used as an internal standard. Coupling constants (J) were given in Hz. Spectra were reported as follows: chemical shift (δ, ppm), integration, multiplicity, and coupling constants (Hz). LC-MS data were recorded on Agilent 1100 HPLC equipped with a diode-matrix and mass-selective detector Agilent LC/MSD SL instrument, the column: Zorbax SB-C18, 4.6 mm × 15 mm; eluent: (A) acetonitrile – water with 0.1% of TFA (95:5), (B) water with 0.1% of TFA; the flow rate: 1.8 mL min^{-1}. Mass spectra were recorded on an Agilent 1100 LCMSD SL instrument (chemical ionization (CI)).

The parallel synthesis was performed in 20-mL vials or 100-mL flasks; loading of the reagents, as well as treatment of the reaction mixtures was performed manually in a parallel fashion. The reactions were performed in ultrasonic baths or laboratory ovens equipped with a shaker. Centrifugal evaporators were used to remove the solvents from the vials in a parallel fashion. Preparative HPLC was performed on Agilent 1260 Infinity systems equipped with DAD and a mass-detector using a Chromatorex 18 SNB100-5T 100 × 19 mm, 100 Å, 5-μm column with a SunFire C18 Prep Guard Cartridge, 100 Å, 10 μm, 19 mm × 10 mm, with H_2O – MeCN as a gradient, or H_2O – MeOH, or H_2O (with 0.2% HCO_2H) – MeOH as an eluent, with the flow of 30 mL min^{-1}.

For the library members **4**{*1–6,1–8*} synthesized, physical data and mass spectra (**Table 2**), ^{1}H NMR spectra (**Table 3**), ^{13}C NMR spectra (**Table 4**), and ^{19}F NMR spectra (**Table 5**) were given in a tabular format.

Table 2. Physical data and mass spectra for library members **4**{*1–6,1–8*} synthesized

Compound	Appearance	MS (*m/z*, APCI)
4{*1,1*}	Yellowish oil	235 $[M+H]^+$
4{*1,2*}	Yellowish oil	249 $[M+H]^+$
4{*1,3*}	Yellowish oil	249 $[M+H]^+$
4{*1,5*}	Yellowish oil	263 $[M+H]^+$
4{*1,6*}	Yellowish oil	263 $[M+H]^+$
4{*1,7*}	Brownish oil	263 $[M+H]^+$
4{*1,8*}	Yellowish oil	277 $[M+H]^+$
4{*2,8*}	Yellowish oil	272 $[M+H]^+$
4{*4,1*}	Yellowish oil	234 $[M+H]^+$
4{*4,8*}	Colorless oil	276 $[M+H]^+$
4{*6,1*}	Yellowish oil	188 $[M+H]^+$
4{*6,8*}	Brownish oil	230 $[M+H]^+$

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Table 3. ^{1}H NMR spectra data for library members **4**{*1–6,1–8*} synthesized

Compound	^{1}H NMR (600 MHz, DMSO-d_6), δ, ppm
4{*1,1*}	2.32–2.44 (2H, m); 2.52–2.58 (1H, m); 2.59–2.70 (2H, m); 3.05 (2H, d, *J* = 7.6 Hz); 7.75 (1H, dd, *J* = 8.6, 1.9 Hz); 7.92 (1H, s); 8.03 (1H, d, *J* = 8.6 Hz); 8.89 (1H, d, *J* = 1.8 Hz); 8.92 (1H, d, *J* = 1.8 Hz)
4{*1,2*}	1.88 (2H, q, *J* = 7.8 Hz); 2.04–2.14 (1H, m); 2.20–2.31 (2H, m); 2.60–2.70 (2H, m); 2.81 (2H, t, *J* = 7.8 Hz); 7.75 (1H, dd, *J* = 8.5, 1.9 Hz); 7.90 (1H, s); 8.02 (1H, d, *J* = 8.5 Hz); 8.88 (1H, d, *J* = 1.8 Hz); 8.91 (1H, d, *J* = 1.8 Hz)
4{*1,3*}	1.33–1.41 (1H, m); 1.75–1.91 (2H, m); 1.93–2.02 (1H, m); 2.35–2.46 (2H, m); 2.68–2.80 (1H, m); 2.83 (2H, t, *J* = 7.8 Hz); 7.73 (1H, dd, *J* = 8.6, 1.8 Hz); 7.88 (1H, s); 8.01 (1H, d, *J* = 8.6 Hz); 8.87 (1H, d, *J* = 1.8 Hz); 8.89 (1H, d, *J* = 1.8 Hz)
4{*1,5*}	1.36–1.46 (1H, m); 1.59–1.75 (3H, m); 1.88–2.17 (5H, m); 2.83–2.99 (2H, m); 7.76 (1H, dd, *J* = 8.5, 1.8 Hz); 7.90 (1H, s); 8.03 (1H, d, *J* = 8.5 Hz); 8.89 (1H, d, *J* = 1.8 Hz); 8.91 (1H, d, *J* = 1.8 Hz)
4{*1,6*}	1.03–1.11 (1H, m); 1.31–1.41 (1H, m); 1.49–1.75 (4H, m); 1.89–1.99 (3H, m); 2.78–2.89 (2H, m); 7.73 (1H, dd, *J* = 8.5, 1.8 Hz); 8.03 (1H, d, *J* = 8.5 Hz); 7.88 (1H, s); 8.89 (1H, d, *J* = 1.8 Hz); 8.92 (1H, d, *J* = 1.8 Hz)
4{*1,7*}	1.20–1.33 (2H, m); 1.64–1.88 (5H, m); 1.93–2.03 (2H, m); 2.80 (2H, d, *J* = 7.2 Hz); 7.72 (1H, dd, *J* = 8.5, 1.9 Hz); 7.89 (1H, d, *J* = 1.9 Hz); 8.02 (1H, d, *J* = 8.5 Hz); 8.89 (1H, d, *J* = 1.8 Hz); 8.91 (1H, d, *J* = 1.8 Hz)
4{*1,8*}	1.16–1.26 (2H, m); 1.37–1.46 (1H, m); 1.61–1.67 (2H, m); 1.69–1.87 (4H, m); 1.95–2.04 (2H, m); 2.83–2.89 (2H, m); 7.75 (1H, dd, *J* = 8.6, 1.8 Hz); 7.90 (1H, s); 8.01 (1H, d, *J* = 8.6 Hz); 8.88 (1H, d, *J* = 1.8 Hz); 8.90 (1H, d, *J* = 1.8 Hz)
4{*2,8*}	0.84–0.90 (2H, m); 1.01–1.07 (2H, m); 1.10–1.20 (2H, m); 1.34–1.43 (1H, m); 1.51 (2H, q, *J* = 7.5 Hz); 1.67–1.83 (4H, m); 1.92–2.02 (2H, m); 2.26–2.32 (1H, m); 2.76 (2H, t, *J* = 7.5 Hz); 7.28 (1H, s)
4{*4,1*}	2.32–2.44 (2H, m); 2.51–2.57 (1H, m); 2.61–2.72 (2H, m); 2.99 (2H, d, *J* = 7.7 Hz); 7.58 (1H, t, *J* = 7.6 Hz); 7.70 (1H, t, *J* = 7.6 Hz); 7.92 (1H, d, *J* = 8.3 Hz); 7.99 (1H, d, *J* = 8.3 Hz); 8.17 (1H, s); 8.80 (1H, d, *J* = 2.2 Hz)
4{*4,8*}	1.14–1.28 (2H, m); 1.37–1.47 (1H, m); 1.59–1.67 (2H, m); 1.68–1.89 (4H, m); 1.93–2.07 (2H, m); 2.76–2.84 (2H, m); 7.57 (1H, t, *J* = 7.2 Hz); 7.66–7.71 (1H, m); 7.91 (1H, d, *J* = 8.2 Hz); 7.98 (1H, d, *J* = 8.2 Hz); 8.14 (1H, s); 8.80 (1H, d, *J* = 2.2 Hz)
4{*6,1*}	2.26–2.37 (2H, m); 2.41–2.48 (1H, m); 2.68–2.78 (2H, m); 2.88 (2H, d, *J* = 7.7 Hz); 3.92 (3H, s); 7.53 (1H, s)
4{*6,8*}	1.15–1.23 (2H, m); 1.38–1.45 (1H, m); 1.55 (2H, q, *J* = 7.3 Hz); 1.71–1.84 (4H, m); 1.95–2.03 (2H, m); 2.64–2.69 (2H, m); 3.91 (3H, s); 7.50 (1H, s)

Table 4. ^{13}C NMR spectra data for library members **4**{*1–6,1–8*} synthesized

Compound	^{13}C{^{1}H} NMR (151 MHz, DMSO-d_6),δ, ppm
4{*1,1*}	23.7 (dd, *J* = 13.1, 5.9 Hz); 39.7 (dd, *J* = 22.3, 20.9 Hz); 40.4 (dd, *J* = 3.2, 1.4 Hz); 120.7 (dd, *J* = 284, 273 Hz); 127.7; 129.0; 131.6; 141.1; 142.3; 142.4; 145.1; 145.7
4{*1,2*}	22.3 (dd, *J* = 12.7, 6.0 Hz); 33.0; 36.5 (d, *J* = 2.9 Hz); 39.9 (t, *J* = 22.0 Hz); 120.9 (dd, *J* = 284, 274Hz); 127.4; 128.9; 131.6; 141.0; 142.3; 144.3; 144.9; 145.6
4{*1,3*}	16.6 (dd, *J* = 16.5, 3.8 Hz); 29.4 (d, *J* = 4.8 Hz); 32.3 (t, *J* = 22.0 Hz); 32.9; 45.8 (t, *J* = 21.0 Hz); 123.4 (dd, *J* = 288, 277 Hz); 127.9; 129.4; 131.9; 141.5; 142.8; 144.5; 145.4; 146.1
4{*1,5*}	19.5 (dd, *J* = 5.7, 3.3 Hz); 28.7 (dd, *J* = 22.3, 6.6 Hz); 33.0; 34.5 (t, *J* = 24.6 Hz); 39.2; 44.7 (t, *J* = 22.5 Hz); 127.3; 129.0; 131.5; 132.8 (t, *J* = 251 Hz); 141.0; 142.3; 144.4; 145.0; 145.6
4{*1,6*}	21.5 (d, *J* = 9.8 Hz); 29.9; 33.1 (dd, *J* = 24.8, 21.5 Hz); 36.2 (d, *J* = 9.1 Hz); 39.1 (dd, *J* = 24.8, 21.0 Hz); 41.4; 124.4 (dd, *J* = 242, 239 Hz); 128.3; 128.9; 131.9; 141.0; 142.2; 142.4; 145.0; 145.7
4{*1,7*}	28.7 (d, *J* = 9.5 Hz); 33.1 (dd, *J* = 24.8, 22.3 Hz); 36.9; 41.5 (d, *J* = 2.5 Hz); 124.8 (dd, *J* = 241, 239 Hz); 128.7; 129.2; 132.5; 141.5; 142.7; 143.6; 145.4; 146.1
4{*1,8*}	28.4 (d, *J* = 9.4 Hz); 32.6; 32.7 (dd, *J* = 24.8, 22.0 Hz); 34.3 (d, *J* = 1.4 Hz); 36.5 (d, *J* = 2.5 Hz); 124.4 (dd, *J* = 241, 239 Hz); 127.2; 128.9; 131.6; 140.9; 142.4; 144.85; 144.94; 145.6
4{*2,8*}	10.4; 13.9; 23.8; 28.3 (d, *J* = 9.5 Hz); 32.7 (dd, *J* = 24.9, 22.1 Hz); 34.0 (d, *J* = 1.4 Hz); 37.0 (d, *J* = 2.4 Hz); 124.3 (dd, *J* = 241, 239 Hz); 136.7; 138.6; 170.6
4{*4,1*}	23.6 (dd, *J* = 13.0, 6.1 Hz); 37.8 (dd, *J* = 3.2, 1.4 Hz); 39.7; 120.7 (dd, *J* = 284, 273 Hz); 126.6; 127.68; 127.73; 128.6; 128.8; 132.8; 134.2; 146.4; 151.9
4{*4,8*}	28.4 (d, *J* = 9.3 Hz); 29.8; 32.7 (dd, *J* = 24.8, 22.1 Hz); 34.3 (d, *J* = 1.4 Hz); 36.5 (d, *J* = 2.4 Hz); 124.4 (dd, *J* = 241, 239 Hz); 126.5; 127.5; 127.8; 128.5; 128.6; 133.7; 135.1; 146.2; 151.9
4{*6,1*}	21.1 (dd, *J* = 13.6, 6.0 Hz); 28.0 (dd, *J* = 3.3, 1.3 Hz); 34.0; 39.8 (t, *J* = 20.8 Hz); 120.5 (dd, *J* = 284, 273 Hz); 131.6; 135.5
4{*6,8*}	19.9; 28.3 (d, *J* = 9.4 Hz); 32.7 (dd, *J* = 24.9, 22.1 Hz); 33.0 (d, *J* = 2.5 Hz); 33.9; 34.2 (d, *J* = 1.5 Hz); 124.3 (dd, *J* = 241, 239 Hz); 131.4; 137.4

Table 5. ^{19}F NMR spectra data for library members **4**{*1*–*6*,*1*–*8*} synthesized

Compound	$^{19}F\{^1H\}$ NMR (376 MHz, DMSO-d_6), δ, ppm
4{*1*,*1*}	–94.1 (d, *J* = 190 Hz); –81.0 (d, *J* = 190 Hz)
4{*1*,*2*}	–110.7 (d, *J* = 189 Hz); –81.8 (d, *J* = 189Hz)
4{*1*,*3*}	–104.5 (d, *J* = 225 Hz); –96.5 (d, *J* = 224 Hz)
4{*1*,*5*}	–98.4 (d, *J* = 234 Hz); –86.7 (d, *J* = 234 Hz)
4{*1*,*6*}	–99.9 (d, *J* = 232 Hz); –90.1 (d, *J* = 233 Hz)
4{*1*,*7*}	–100.0 (d, *J* = 232 Hz); –89.9 (d, *J* = 232 Hz)
4{*1*,*8*}	–100.1 (d, *J* = 232Hz); –89.9 (d, *J* = 232Hz)
4{*2*,*8*}	–94.0 (d, *J* = 190 Hz); –81.0 (d, *J* = 190 Hz)
4{*4*,*1*}	–94.0 (d, *J* = 190 Hz); –81.0 (d, *J* = 190 Hz)
4{*4*,*8*}	–100.0 (d, *J* = 232 Hz); –89.9 (d, *J* = 232 Hz)
4{*6*,*1*}	–94.2 (d, *J* = 191 Hz); –81.0 (d, *J* = 191 Hz)
4{*6*,*8*}	–100.0 (d, *J* = 232 Hz); –90.0 (d, *J* = 232 Hz)

The general procedure for the synthesis of compound library 4

The procedure from our previous work [8] was followed. Amine **3** (3 mmol) was placed into a 20-mL vial, and MeOH (6 mL), aldehyde **2** (3 mmol), and AcOH (3.6 mmol) were added. The reaction mixture was sonicated at rt for 12 h. Then $NaBH_3CN$ (6 mmol) was added, and the reaction mixture was sonicated at rt for additional 12 h. Volatiles were removed *in vacuo*; the residue was treated with 10% aq Na_2CO_3 (6 mL) and extracted with CH_2Cl_2 (2 × 6 mL). The combined organic layers were placed into a 100-mL flask and concentrated *in vacuo*. The residue was dried thoroughly *in vacuo* and used in the next step without purification. The flask with the crude reductive amination product was filled with argon, and a degassed solution of anomeric amide **1** (0.3 M in dry THF, 15 mL, 4.5 mmol) was added under an argon flow (CAUTION! Compound **1** can be potentially mutagenic). The mixture was shaken in an oven at 45 °C for 16 h (CAUTION! N_2 evolution is observed), then cooled to rt, saturated aq $NaHCO_3$ (30 mL) was added, and the mixture was extracted with Et_2O (2 × 15 mL). The combined extracts were evaporated *in vacuo*, and the residue was subjected to the reverse-phase HPLC purification.

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The synthesis of Novel 2-Hetarylthiazoles *via* the Stille Reaction

Abstract
A preparative approach to the synthesis of 2-hetaryl thiazoles has been developed *via* the interaction of halothiazoles with stannanes according to the Stille reaction. The most effective catalysts and reaction conditions have been found. It has been determined that the formation of by-products occurs due to specific interaction of the corresponding stannanes with the carbonyl group. The by-products have been isolated and characterized. The mechanism of this interaction with the carbonyl group has not been described in literature. The 2-hetaryl thiazoles obtained have great potential as new building blocks for medicinal chemistry and as ligands due to their complexing properties.
***Keywords*:** Stille reaction; heterocycle; thiazole; Buchwald catalysts; chalcone

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Одержання нових 2-гетарилтіазолів за реакцією Стілле

Анотація
Розроблено препаративний підхід до синтезу 2-гетарилтіазолів, що полягає у взаємодії галогенотіазолів зі станнанами за реакцією Стілле. Визначено найбільш ефективні каталізатори та умови проведення реакції. З'ясовано, що за рахунок специфічної взаємодії відповідних станнанів з карбонільною групою відбувається утворення побічних продуктів, які виділили та схарактеризували. Механізм такої взаємодії з карбонільною групою в літературі не описано. Завдяки своїм комплексоутворювальним властивостям отримані 2-гетарилтіазоли мають великий потенціал як нові будівельні блоки для медичної хімії та як ліганди.
***Ключові слова*:** реакція Стілле; гетероцикл; тіазол; каталізатори Бучвальда; халкон

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Supporting information: Copies of ^{1}H and ^{13}C NMR spectra of the synthesized compounds.
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■ Introduction

The chemistry of chalcones and their analogs has been actively developed over the past few decades. However, their selective modification remains an important issue [1]. In previous works, chalcones with 2,4-dichlorothiazole moiety were obtained, and ways for their modification with dialkylamino and methoxy groups were shown [2]. Similar dibromo derivatives were also studied (since in several cases their reactivity was much higher), and other important reagents, thiazolylbutenones, were synthesized and modified in the similar synthetic pathway [3]. This fact allowed us to conclude that such thiazole-containing derivatives of diarylideneacetone deserve closer consideration because of their use both in medicine and as ligands for Pd catalysts in chemistry [4–6]. In addition, some other promising thiazole-containing pyrimidines and benzimidazoles were obtained [7] and their versatile properties were studied.

■ Results and discussion

In this work, we propose various approaches for the thiazole ring modification with a series of different substituents implemented. The first of them was thiazole cyclization based on thio-

urea derivatives with its further modification described previously [8]. However, this way did not allow us to reach high diversity of substrates that constituted significant disadvantage. In addition, restrictions on initial thioureas considerably reduce the number of derivatives that can be obtained by this way.

The most successful way for such transformation is the Suzuki and Stille cross-coupling reactions [9, 10]. Our efforts to put 2,4-dihalogenothiazoles to the Suzuki reaction with boronic acids did not show the expected result. On the other hand, they can react with boronic esters, however conditions of the interactions providing sufficient yields have not been defined yet.

Therefore, the Stille reaction was chosen as our main point of interest. The first study was devoted to finding the most suitable catalyst. It is known that $Pd(PPh)_4$ and $Pd_2(dba)_3$ are commonly used in this reactions. There are also known references to the use of the Buchwald precatalysts Pd G3 and Pd G4 with various ligands in this reaction. Moreover, they showed perfect results in a series of other cross-coupling reactions [11], and this fact turned us to assay them in our cases.

Thus, we chose a model reaction of 3-(2,4-dibromothiazolyl)-1-phenyl-propenone with 2-pyridine-tributylstannane to study regioselectivity of the interaction in the presence of a series of catalysts. The reaction was interesting in terms of comparison the reactivity of C2 and C4 positions of 2,4-dibromothiazoles. As a result, the formation of the product mixture was observed in most cases (**Scheme 1**, **Table 1**).

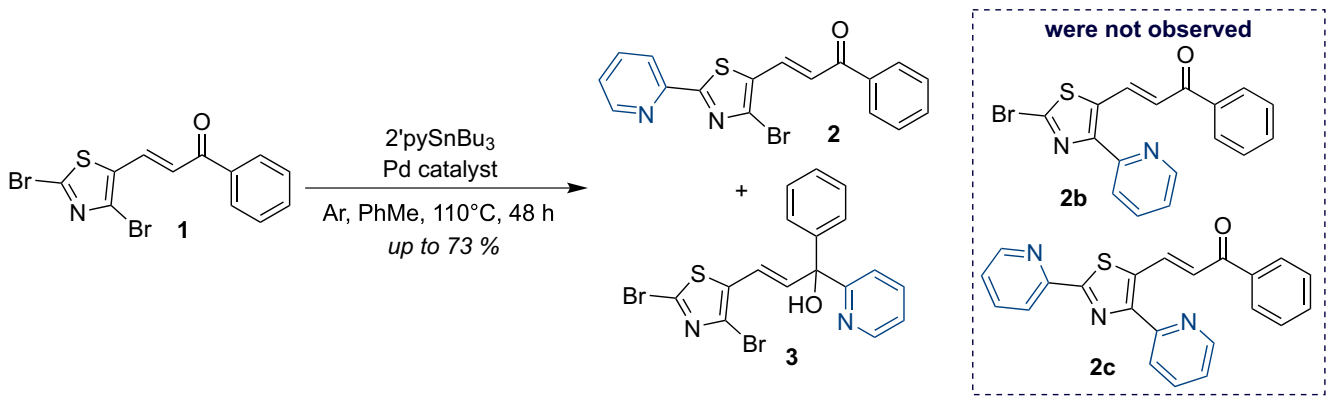
Meanwhile, the assumed products of the reaction in positions C4 (**2b**) or both C2 and C4 (**2c**) were not detected. Nevertheless, we managed to determine the most suitable catalysts for the preparation of both main product **2** (highlighted in green, **Table 1**) and by-product **3** (highlighted in yellow, **Table 1**).

We noted that Pd G3 DavePhos was less suitable for the by-product synthesis than Pd G4 Sphos as evidenced by the *by-product / main product yield ratio*, and with the reaction time variation the second one gave better results. In most cases, a significant number of starting materials remained unreacted in the mixture. In addition, we presumed that catalysts #3 and #4 could be more efficient for compounds without active halogens.

Such a possibility of the carbonyl group in the structure containing reactive bromine atoms to interact first is very promising. In this way, we disclosed a novel reaction type for introducing aryl substituents to carbonyl group of a chalcone fragment. The authors of the ref. [12] reported a similar interaction. However, it was shown only on alkyl derivatives. It should be also noted those chalcones in contrast to the analogs we used did not have an active bromine capable of interacting with tin derivatives. Having obtained such

Table 1. Yields of the products in the Stille reaction of 3-(2,4-dibromothiazolyl)-1-phenyl-propenone with different catalysts (**Scheme 1**)

#	Catalyst	Product **2** yield, %	Product **3** yield, %
1	$Pd(PPh)_4$	40	0
2	Pd G4 XantPhos	0.5	5
3	Pd G4 Sphos	1.3	7.15
4	Pd G3 DavePhos	24.25	14.55
5	Pd G3 CataCXium A	65.2	0
6	Pd G4 *t*-BuXphos	11.1	1.85
7	Pd G3 XantPhos	15.4	7.7
8	Pd G3 AmPhos	73.3	3.8
9	Pd G4 *t*-BuBrettPhos	13.3	3.42
10	$Pd_2(dba)_3$	8.4	4.2
11	Pd G3 SPhos	30.8	5.7
12	Pd G3 Xphos	9.9	1.65
13	Pd G3 *t*-BuBrettPhos	26.4	6.6
14	Pd G4 Xphos	18	10.8
15	Pd G3 *t*-BuXphos	22.2	0



Scheme 1. The Stille reaction of 3-(2,4-dibromothiazolyl)-1-phenyl-propenone with 2-pyridine-tributylstannane

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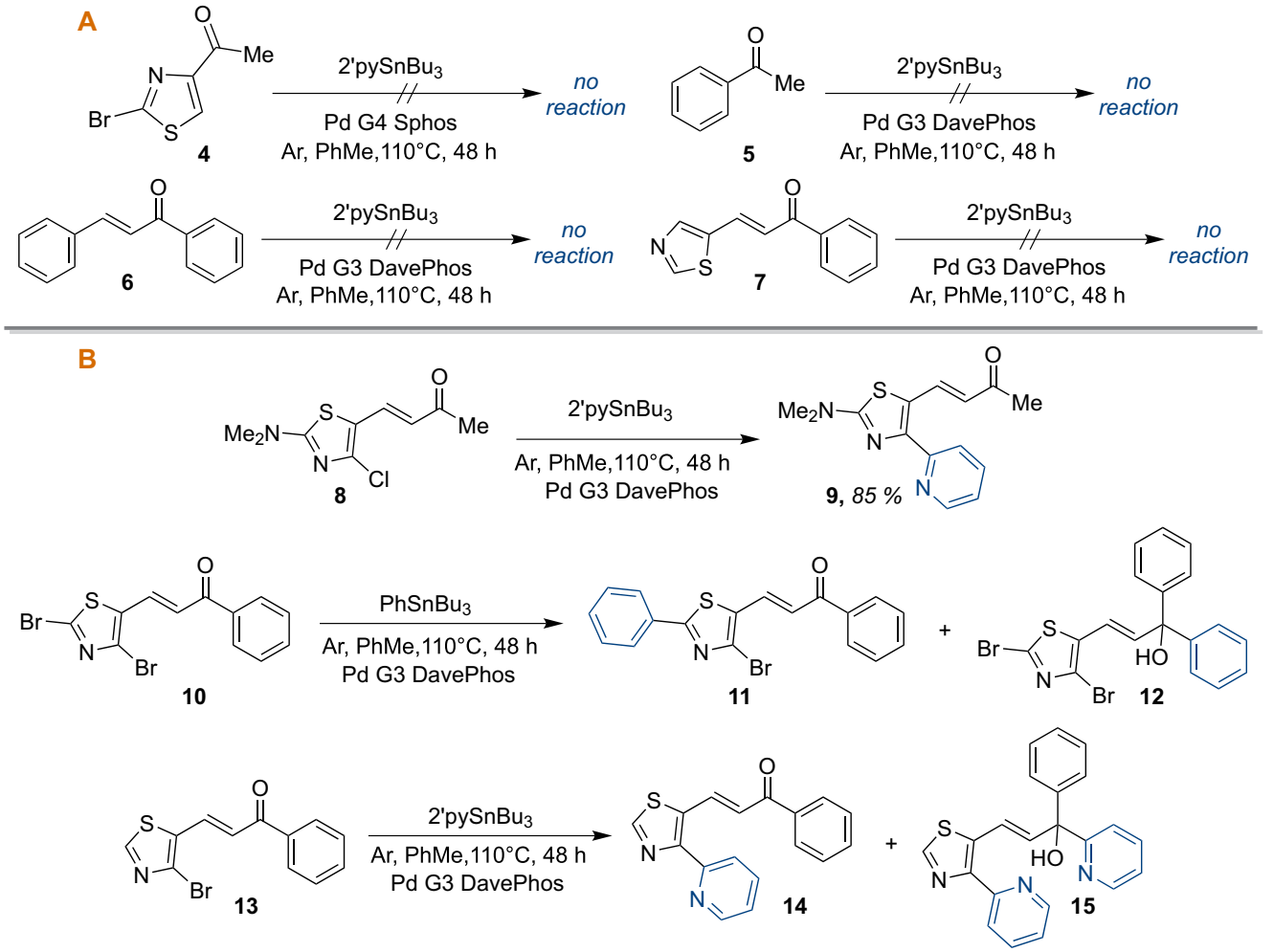


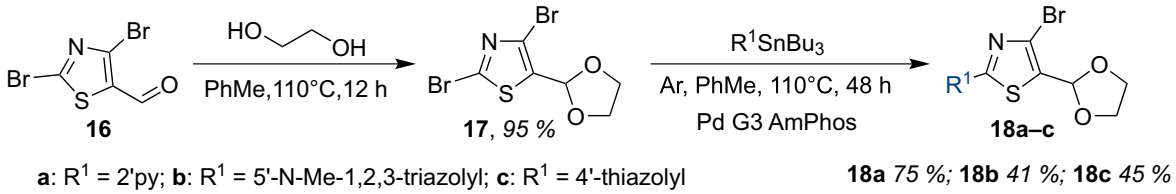
Scheme 2. The study of the reaction scope

an unusual result, we decided to conduct a series of reactions with various carbonyl compounds.

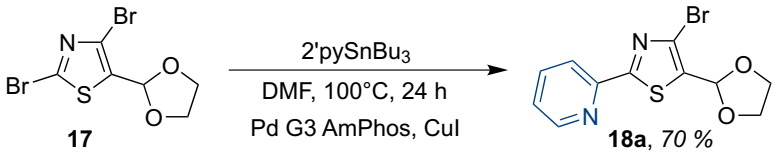
2-Bromo-4-acetylthiazole (**4**) and acetophenone (**5**) were first tested in the reaction. Surprisingly, in the case of Pd G3 DavePhos and Pd G4 Sphos catalysts, we did not observe the product. Taking into account that the conjugated system could lead to another result, chalcone (**6**) and its thiazole analog (**7**) were taken, however, they showed similar result under the same conditions (**Scheme 2**, A). The similar experiment exploiting the same conditions was also carried out for 2'-dimethylamino-4'-chlorothiazol-5'-yl-but-3-en-2-one (**8**). In this case, despite the lower activity of the chlorine as compared to the bromine atom, the reaction proceeded in C4 position giving **9**, and the side product was not observed (**Scheme 2**, B). This fact confirmed the difference in reactivity between 1-methyl and 1-phenyl-3-(thiazol-5-yl)-propenones.

Among other interactions, we studied the reactivity of 4-bromothiazolyl derivative with tributylphenylstannane under the same reaction conditions. The results confirm that 2-pyridile fragment does not play determination role in such interaction. In detail, utilizing 3-(2,4-dibromothiazol-5-yl)-1-phenylprop-2-en-1-one (**10**) under the conditions described above led to two alternative products **11** and **12** (**Scheme 2**, B). In the case of 3-(4-bromothiazol-5-yl)-1-phenylprop-2-en-1-one (**13**), we obtained products of sequential reactions on thiazole ring **14** and carbonyl group **15**. This fact indicates the influence of the bromine atom in position 2 of the ring on the entire thiazolyl-propenone system. Apparently, the results obtained by us are not enough draw convincing conclusions against the background of the observations. Nevertheless, they provide preconditions for further purposeful investigation.

A similar reaction with 2,4-dibromo-5-formylthiazole (**16**) was also carried out, but a mixture of by-products was obtained as a result. For its modification, it was decided to use dioxolane protection of the formyl group. The resulting acetal was introduced into the Stille reaction, which made it possible to diversify a number of products obtained with various stannanes in good yields (**Scheme 3**).



Scheme 3. The pathway to 4-bromo-5-(1,3-dioxolan-2-yl)-2-(hetaryl-2-yl) thiazoles



Scheme 4. Scaling up conditions

Another problem was scaling of the interactions. When 1 g of 3-(2,4-dibromothiazolyl)-1-phenyl-propenone was taken under the same conditions, the yield decreased to 2–7%. To solve this problem, it was proposed to carry out the reaction in DMF with CuI used as a co-catalyst (**Scheme 4**). This method allows conducting such interactions in a bigger scale without the product yields fall.

■ Conclusions

The attractive method of preparative synthesis of the substituted 2-hetaryl thiazoles has been proposed. Buchwald catalysts have shown their effectiveness regardless of the use of the base. The mechanism of the stananne interaction with the carbonyl group is specific and has not been fully studied, which indicates the need to study it. The use of a CuI co-catalyst and the solvent changing to DMF allows scaling of the 2-hetaryl thiazoles synthesis.

■ Experimental part

All chemicals were obtained from Enamine Ltd. and used without further purification. All solvents were purified by standard methods. All procedures were carried out under an open atmosphere with no precautions taken to exclude ambient moisture. ^{1}H NMR spectra were recorded on a Varian MR-400 spectrometer (400 MHz) with TMS as an internal standard. ^{13}C NMR spectra were recorded on a Bruker Avance DRX 500 (126 MHz) spectrometer with TMS as an internal standard. LC-MS spectra were recorded using the chromatography/mass-spectrometric system consisting of a high-performance liquid chromatograph Agilent 1100 LC MSD SL instrument equipped with a diode-matrix and mass-selective detector "Agilent LC/MSD SL". The parameters of chromatography-mass spectrometry analysis were as follows: column – SUPELCO Ascentis Express C18, 2.7 μm 4.6 mm×15 cm. According to the HPLC MS data, all of the compounds synthesized had purity > 95%. The elemental analysis was performed in the Institute of Organic Chemistry of the NASU.

The procedure for the synthesis of 2,4-dibromo-5-(1,3-dioxolan-2-yl)thiazole (17)

2,4-Dibromothiazole-5-carbaldehyde (**16**) (1.0 g, 3.7 mmol) was dissolved in toluene, ethane-1,2-diol (0.69 g, 11.1 mmol), and *p*-toluenesulfonic acid (34 mg, 0.2 mmol) were added. The mixture was refluxed for 24 h and then concentrated under reduced pressure, diluted with 10 mL of water, extracted with *tert*-butyl methyl ether 3×5 mL and washed with brine 3×10 mL. The organic layer was dried over Na_2SO_4, filtered, concentrated under reduced pressure.

A yellow powder. Yield – 1.1 g (95%) Anal. Calcd for $C_6H_5Br_2NO_2S$, %: C 22.88; H 1.60; Br 50.74; N 4.45; O 10.16; S 10.17. Found, %: C 22.87; H 1.60; Br 50.73; N 4.46; S 10.19. ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm: 3.93–4.12 (4H, m, CH_2-CH_2); 5.96 (1H, s, CH). ^{13}C NMR (126 MHz, $CDCl_3$), δ, ppm: 65.09; 97.84; 122.94; 135.21; 136.66. LC-MS (CI, 200 eV), *m/z* (I_{rel}, %): 314 $[M+H]^+$ (30); 316 (60); 318 (30).

The procedure for the synthesis of 4-bromo-5-(1,3-dioxolan-2-yl)-2-(pyridin-2-yl)thiazole (18a)

Method A. 2,4-Dibromo-5-(1,3-dioxolan-2-yl) thiazole (**17**) (100 mg, 0.32 mmol) and 2-(tributylstannyl)pyridine (117 mg, 0.32 mmol) were placed into a 10 mL flask and dissolved in 4 mL of toluene. Then the mixture was bubbled with argon for 15 min, and a catalyst Pd G3 AmPhos (5 mol %) was added. The reaction mixture was stirred for 48 h at 110°C and cooled to room temperature.

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After that it was separated by flash chromatography (hexane/ethyl acetate, a gradient from 100:0 to 20:80) to give the product.

A yellow powder. Yield – 75 mg (75%). Anal. Calcd for $C_{11}H_9BrN_2O_2S$, %: C 42.19; H 2.90; Br 25.51; N 8.95; O 10.22; S 10.23. Found, %: C 42.18; H 2.90; Br 25.52; N 8.96; S 10.23. ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm: 3.97–4.23 (4H, m, CH_2-CH_2); 6.14 (1H, s, CH); 7.34 (1H, t, J = 6.2 Hz, N=CH-CH); 7.79 (1H, t, J = 7.8 Hz, N=CH-CH=CH); 8.16 (1H, d, J = 7.8 Hz, N=CH-CH=CH-CH); 8.58 (1H, d, J = 4.8 Hz, N=CH). ^{13}C NMR (126 MHz, $CDCl_3$), δ, ppm: 65.04; 98.51; 119.06; 124.69; 125.08; 133.69; 136.63; 149.01; 149.61; 168.84. LC-MS (CI, 200 eV), m/z (I_{rel}, %): 313 $[M+H]^+$ (100); 315 (98).

Method B. 2,4-Dibromo-5-(1,3-dioxolan-2-yl)-thiazole (**17**) (1.0 g, 3.2 mmol) and 2-(tributylstannyl)pyridine (1.17 g, 3.2 mmol) were placed into a 50 mL flask, dissolved in 20 mL of DMF, then a catalyst Pd G3 AmPhos (5 mol %) and CuI (12 mg, 0.064 mmol) were added. The reaction mixture was stirred for 24 h at 100°C, cooled to room temperature. After that it was separated by flash chromatography (hexane/ethyl acetate, a gradient from 100:0 to 20:80) to give the product. The method provides the product yield of 0.7 g (70%) with all analytical and spectroscopic data being the same to those obtained in the *Method A*.

4-Bromo-5-(1,3-dioxolan-2-yl)-2-(1-methyl-1*H*-1,2,3-triazol-5-yl)thiazole (18b)

The title product was synthesized according to the procedure used for compound **18a** (*Method A*).

A yellow powder. Yield – 41 mg (41%). Anal. Calcd for $C_9H_8BrN_4O_2S$, %: C 34.08; H 2.86; Br 25.19; N 17.67; O 10.09; S 10.11. Found, %: C 34.09; H 2.85; Br 25.17; N 17.68; S 10.12. ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm: 3.98–4.22 (4H, m, CH_2-CH_2); 4.38 (3H, s, N-CH_3); 6.10 (1H, s, CH); 8.01 (1H, s, CH triazole). ^{13}C NMR (126 MHz, $CDCl_3$), δ, ppm: 13.09; 37.01; 65.19; 98.09; 125.69; 132.42; 133.82; 154.25; 158.54. LC-MS (CI, 200 eV), m/z (I_{rel}, %): 317 $[M+H]^+$ (100); 319 (98).

4-Bromo-5-(1,3-dioxolan-2-yl)-2,4'-bithiazole (18c)

The title product was synthesized according to the procedure used for compound **18a** (*Method A*).

An orange powder. Yield – 46 mg (45%). Anal. Calcd for $C_9H_7BrN_2O_2S_2$, %: C 33.87; H 2.21; Br 25.03; N 8.78; O 10.02; S 20.09. Found, %: C 33.86; H 2.20; Br 25.04; N 8.79; S 20.09. ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm: 3.86–4.29 (4H, m, CH_2-CH_2); 6.13 (1H, s, CH); 8.08 (1H, s, C=CH-S); 8.82 (1H, s, S-CH-N). ^{13}C NMR (126 MHz, $CDCl_3$), δ, ppm: 65.04; 98.49; 116.47; 125.20; 131.65; 148.74; 153.17; 162.53. LC-MS (CI, 200 eV), m/z (I_{rel}, %): 319 $[M+H]^+$ (100); 321 (98).

The procedure for the synthesis of 3-(2,4-dibromothiazol-5-yl)-1-phenylprop-2-en-1-one (1)

2,4-Dibromothiazole-5-carbaldehyde (**16**) (1.0 g, 3.7 mmol) was dissolved in 10 mL AcOH, then acetophenone (0.44 g, 3.7 mmol) and 0.1 mL of conc. H_2SO_4 were added. The mixture was stirred at 60°C for 24 h and then concentrated under reduced pressure, diluted with 10 mL of water and the precipitate formed was filtered off.

An orange powder. Yield – 0.83 g (60%). Anal. Calcd for $C_{12}H_7Br_2NOS$, %: C 38.64; H 1.89; Br 42.84; N 3.75; O 4.29; S 8.59. Found, %: C 38.63; H 1.88; Br 42.85; N 3.75; S 8.60. ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm: 7.27 (1H, d, J = 15.5 Hz, C(O)CH); 7.54 (2H, t, J = 7.6 Hz, m-CH(Ph)); 7.63 (1H, t, J = 7.3 Hz, p-CH(Ph)); 7.85 (1H, d, J = 15.4, C(O)CH=CH); 7.99 (2H, d, J = 7.7 Hz, o-CH(Ph)). ^{13}C NMR (126 MHz, $CDCl_3$), δ, ppm: 125.96; 128.50; 128.83; 131.98; 133.42; 134.62; 137.24; 137.60; 188.62. LC-MS (CI, 200 eV), m/z (I_{rel}, %): 372 $[M+H]^+$ (18); 374 (50); 376 (18).

3-(4-Bromo-2-(pyridin-2-yl)thiazol-5-yl)-1-phenylprop-2-en-1-one (2)

The title product was synthesized according to the procedure used for compound **18a** (*Method A*).

An orange powder. Yield – 72 mg (73%). Anal. Calcd for $C_{17}H_{11}BrN_2OS$, %: C 55.00; H 2.99; Br 21.52; N 7.55; O 4.31; S 8.63. Found, %: C 54.98; H 2.99; Br 21.53; N 7.56; S 8.62. ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm: 7.39 (1H, t, J = 6.2 Hz, N=C-CH=CH); 7.43 (1H, d, J = 15.5, C(O)-CH=CH); 7.51 (2H, t, J = 7.6 Hz, m-CH(Ph)); 7.60 (1H, t, J = 7.3 Hz, p-CH(Ph)); 7.82 (1H, t, J = 7.7 Hz, N=C-CH); 7.93 (1H, d, J = 15.4 Hz, C(O)-CH=CH); 7.99 (2H, d, J = 7.7 Hz, o-CH(Ph)); 8.20 (1H, d, J = 7.9 Hz, N=C-CH=CH-CH); 8.62 (1H, d, J = 4.8 Hz, N-CH). ^{13}C NMR (126 MHz, $CDCl_3$), δ, ppm: 119.72; 124.94; 125.25; 128.03; 128.32; 131.86; 132.59; 132.74; 132.90; 136.82; 137.14; 149.17; 149.30; 168.91; 188.54. LC-MS (CI, 200 eV), m/z (I_{rel}, %): 371 $[M+H]^+$ (100); 373 (90).

3-(4-Bromo-2-(pyridin-2-yl)thiazol-5-yl)-1-phenyl-1-(pyridin-2-yl)prop-2-en-1-ol (3)

The title product was synthesized according to the procedure used for compound **18a** (*Method A*).

An orange powder. Yield – 17 mg (14%). Anal. Calcd for $C_{17}H_{12}Br_2N_2OS$, %: C 45.16; H 2.68; Br 35.34; N 6.20; O 3.54; S 7.08. Found, %: C 45.15; H 2.68; Br 35.32; N 6.21; S 7.09. ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm: 6.69 (1H, d, J = 15.6 Hz,

C(OH)-CH=CH); 6.88 (1H, d, J = 15.6 Hz, C(OH)-CH=CH); 7.33 (2H, d, J = 7.6 Hz, *o*-CH(Ph)); 7.39 (1H, d, J = 7.7 Hz, N=CH-CH=CH-CH); 7.34 (2H, t, J = 7.5 Hz, *m*-CH(Ph)); 7.37–7.46 (3H, m, N=CH-CH, *p*-CH(Ph)); 8.59 (1H, d, J = 4.8 Hz, N-CH). ^{13}C NMR (126 MHz, $CDCl_3$), δ, ppm: 77.60; 118.82; 121.44; 122.50; 123.19; 126.50; 127.50; 128.08; 133.55; 135.22; 137.26; 139.11; 143.79; 146.92; 160.67. LC-MS (CI, 200 eV), *m*/*z* (I_{rel}, %): 451 $[M+H]^+$ (5); 453 (10); 455 (5); 433 (25); 435 (50); 437 (25).

4-(2-(Dimethylamino)-4-(pyridin-2-yl)-thiazol-5-yl)but-3-en-2-one (9)

The title product was synthesized according to the procedure used for compound **18a** (*Method A*).

An orange powder. Yield – 100 mg (85%). Anal. Calcd for $C_{14}H_{15}N_3OS$, %: C 61.52; H 5.53; N 15.37; O 5.85; S 11.73. Found, %: C 61.51; H 5.52; N 15.36; S 11.75. ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm: 2.30 (3H, s, C(O)-CH_3); 3.15 (6H, s, CH_3-N-CH_3); 6.10 (1H, d, J = 15.7 Hz, C(O)-CH); 7.20 (1H, d, J = 6.4 Hz, C(O)CH-CH); 7.72 (1H, d, J = 7.6 Hz, N-CH=CH); 8.02 (1H, d, J = 7.9 Hz, N=CH-CH=CH); 8.63 (1H, d, J = 4.9 Hz, N=C-CH); 8.91 (1H, d, J = 15.4 Hz, N-CH). ^{13}C NMR (126 MHz, $CDCl_3$), δ, ppm: 25.99; 39.53; 121.87; 122.28; 123.69; 124.80; 136.14; 137.21; 148.46; 152.90; 153.46; 168.44; 197.84. LC-MS (CI, 200 eV), *m*/*z* (I_{rel}, %): 274 $[M+H]^+$ (100).

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The Synthesis of Diverse Annulated Pyridines with 6-Membered Functionalized Saturated Cycles for Medical Chemistry Research

Abstract

The article describes a set of pyridines annulated with functionalized 6-membered saturated rings, which are attractive building blocks for the synthesis of diversified compound libraries in medical chemistry. A certain array of compounds includes pyridines with condensed cyclohexane, piperidine and tetrahydropyran cycles containing keto-, amino-, carboxylic groups, as well as fluorinated fragments. The synthesis of the compounds using the procedure previously developed by us *via* $CuCl_2$-catalyzed condensation of propargylamine with ketones was performed. The limits of application of this reaction were further expanded and determined in this work compared to our previous results. Condensed pyridines, which proved problematic or impossible to obtain by this method, were synthesized using other synthetic pathways. Thus, the study offers a number of new building blocks for use in drug discovery.

Keywords: organic synthesis; heterocyclic compounds; pyridines; building blocks; organofluorines; "magic methyl"; scaffold hopping

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Синтез різноманітних піридинів, анельованих функціоналізованими шестичленними насиченими циклами, для досліджень у галузі медичної хімії

Анотація

У статті окреслено набір піридинів, анельованих функціоналізованими шестичленними насиченими кільцями, які є привабливими білдинг-блоками для синтезу диверсифікованих бібліотек у медичній хімії. До визначеного масиву сполук увійшли піридини з конденсованими циклогексановим, піперидиновим та тетрагідропірановим кільцем, що містять кето-, аміно-, карбоксигрупи, фторовмісні фрагменти. Частину сполук було синтезовано за раніше розробленим нами методом – $CuCl_2$-каталізованою конденсацією пропаргіламіну та кетонів. У презентованій роботі було додатково розширено та визначено межі застосування цієї реакції. Конденсовані піридини, одержання яких за цим методом виявилося проблемним або неможливим, синтезовано іншими шляхами. Отже, дослідження пропонує низку нових будівельних блоків для використання в пошуку лікарських засобів.

Ключові слова: органічний синтез; гетероциклічні сполуки; піридини; фторорганічні сполуки; «чарівний метил»; скафолдне прогнозування

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Introduction

Pyridines annulated to saturated cycles (PASCs) are widely used in drug discovery. Among the compounds containing this fragment there are substances demonstrating anti-HIV [1], antiresorptive [2, 3], antibacterial [4] and antimigraine [5] activity (**Figure 1**).

Due to such a wide spectrum of the biological activity demonstrated, chemists need convenient and cost-effective methods for the synthesis of diverse functionalized PASCs in multigram and/or even semi-industrial scales. In this research, we demonstrate our strategy for solving this problem and propose a synthetic strategy for producing a set of bicyclic building blocks containing pyridine and an annelated saturated core with various substituents and functional groups. According to the development of "magic methyl" and "magic fluorine" concepts, along with classical functions, we included compounds bearing methylmethylene (**2**), dimethylmethylene (**3**) and difluoromethylene (**4**) moieties in our short-list. Isomeric conformationally restricted ketones **6a**–**d**, carboxylic acids **7a**–**d**, PASCs with exocyclic amine function **8a**–**d** and those featuring endocyclic one **9a**–**d** were also treated as utility building blocks for modern combinatorial chemistry and drug discovery (**Figure 2**).

Reported approaches towards pyridines annulated with 6-membered saturated cycles include: (**A**) the partial reduction of the corresponding aromatic compounds, (**B**) the construction of the saturated 6-membered ring and (**C**) the construction of the pyridine ring. Viable routes to implement the approaches are illustrated by a retrosynthetic analysis of compound **9c** (**Figure 3**).

Approach **C** can also be illustrated by the intermolecular Diels-Alder reaction with an inverse electron demand [6], intermolecular oxidative cyclization [7, 8] or [4+2]-cyclization. Examples of [4+2]-cyclization include reactions catalyzed by gold [9] and ruthenium [10–13]. Recently, our research group proposed a simple and scalable method *via* the condensation catalyzed by available and cheap $CuCl_2$ [14] (**Figure 4**). Thanks to our research, this approach has become cost-effective and, along with good scalability and diversity, very promising for obtaining such compounds.

In this light, we aimed to extend and determine the scope of the method and perform the synthesis of the set of diverse PASCs. In addition, in some cases of the method, we proposed other approaches for the synthesis of the target molecules.

Results and discussion

In our previous work [9], we reported on the synthesis of the parent core **1**, ketones **6a** and **6b**, carboxylic acid **7b**, amines **8a**, **9a**, **9b** and dihydropyranopyridine **10b**. The scope of the method was successfully expanded to the synthesis of

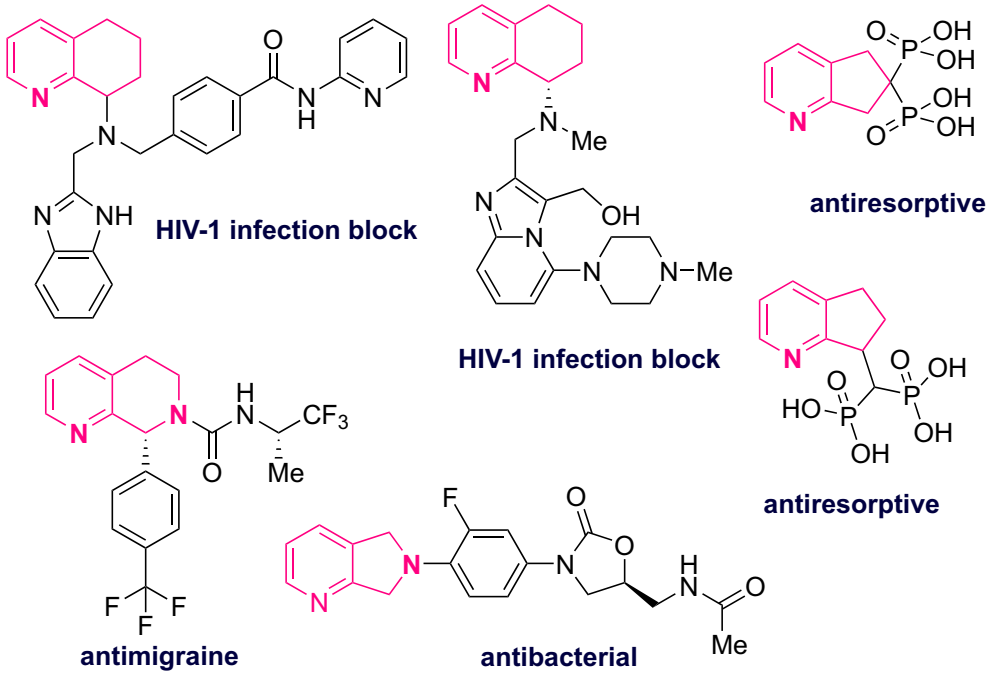

Figure 1. Examples of biologically active compounds containing a PASC moiety

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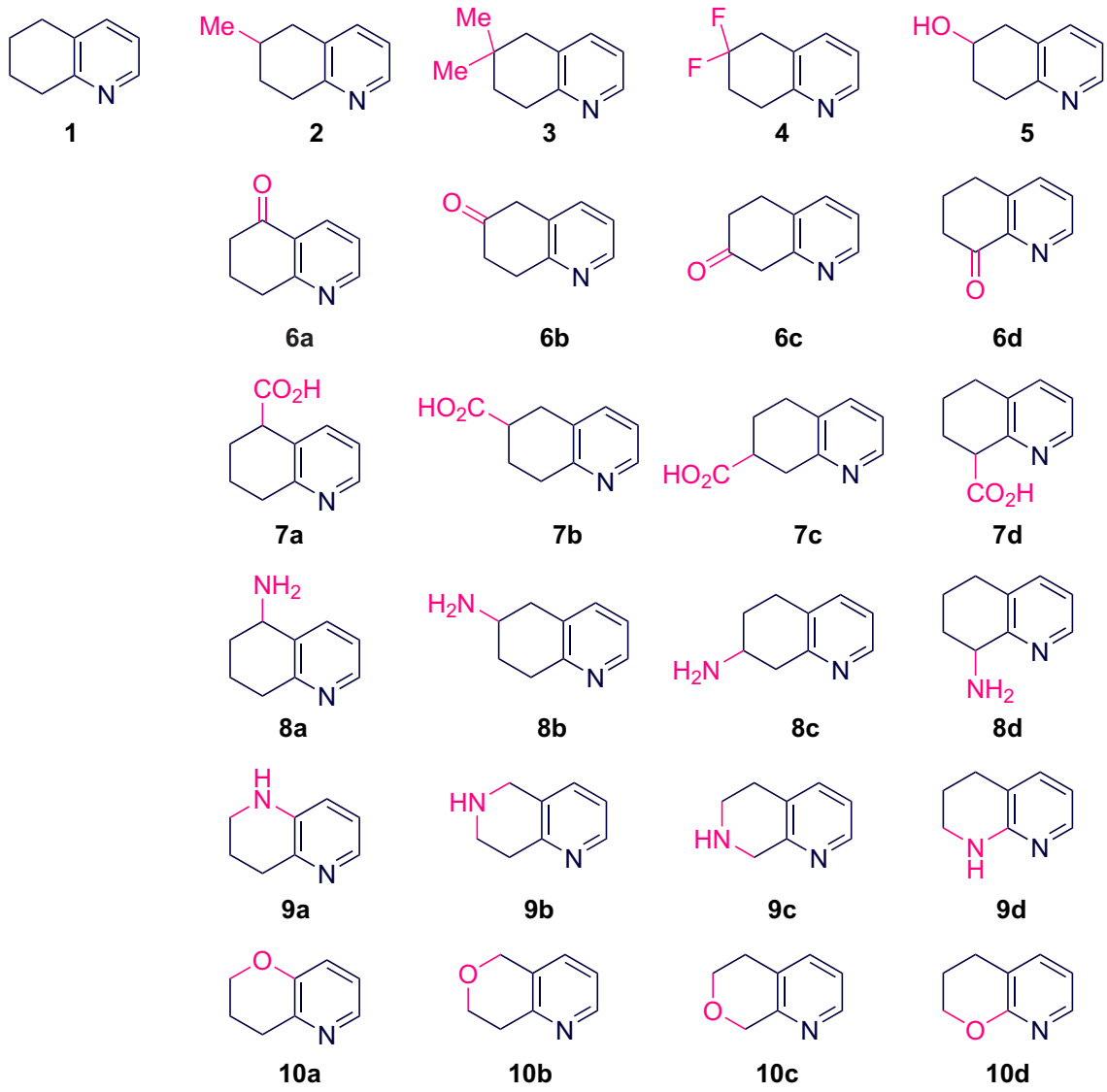


Figure 2. A representative set of PASC building blocks

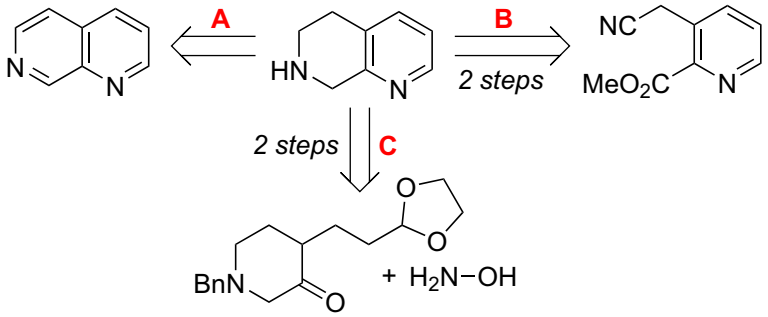


Figure 3. The retrosynthetic analysis of **9c**

pyridines fused with saturated rings bearing CHMe-, CMe_2-, CF_2-, CHOH-moieties. Propargylamine (**11**) was condensed with ketones **12**–**16** in the presence of anhydrous $CuCl_2$ to obtain the desired products (including compounds **3** and **4** previously unknown).

Although alcohol **5** was reported previously, the yield was neither good (e.g., 28%) [15] nor even reported. Our approach works much better – a one-step scalable procedure provides 58% yield of **5**. We also attempted to oxidize alcohol **5** to obtain known [16, 17] ketone **6b** *via* the Dess-Martin oxidation. However, the yields were low (10–15%), so this way needed further optimizations. Notably, ketone **6b** can be involved in self-condensation reactions, and therefore, it should be stored in the freezer.

Ketone **6c** still remains a challenge for synthetic chemists (our attempts were unsuccessful

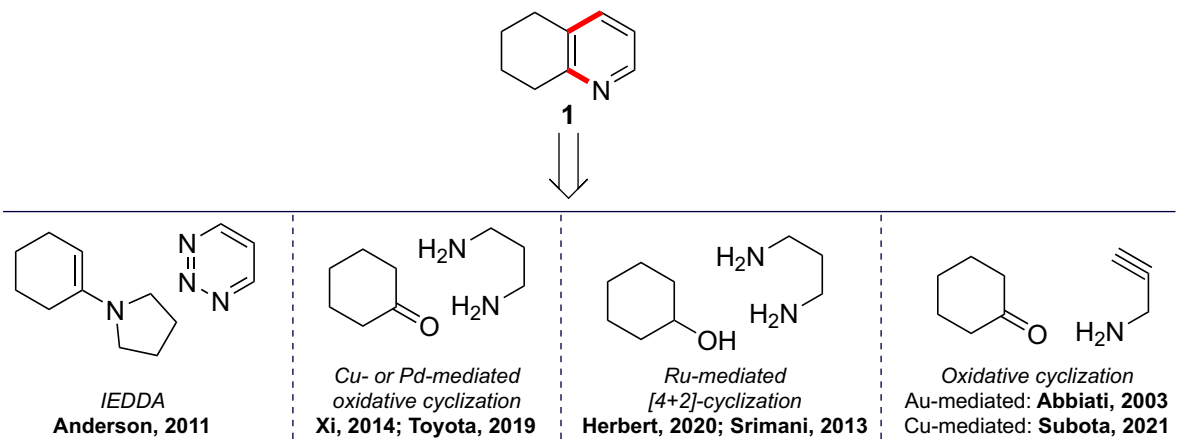
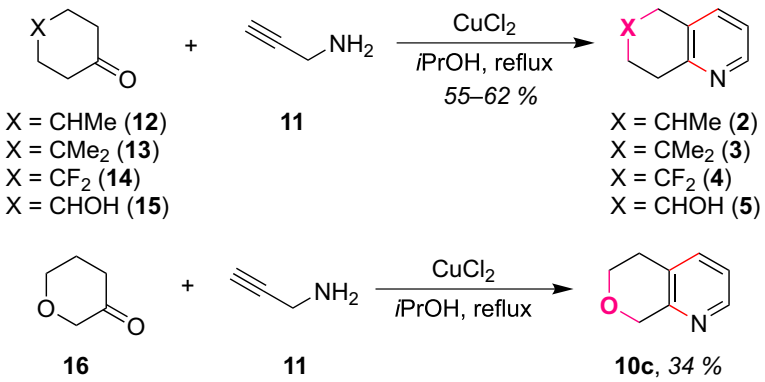


Figure 4. The known approaches for the synthesis of the pyridine ring of PASCs



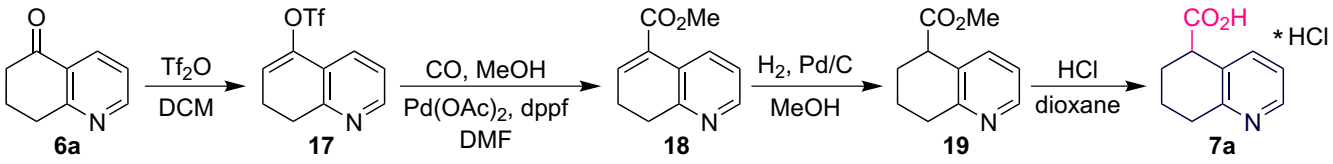
Scheme 1. The synthesis of pyridines **2-5** and **10c**

as well), while more than 20 different reactions for the synthesis of ketone **6d** were reported, even in a kilogram scale [18].

Previously, we obtained ethyl esters of carboxylic acids **7a** and **7c** as an inseparable mixture. The synthesis of **7c** in 75% *via* the Wolff-Kishner reduction was reported in 1974 [19]. Carboxylic acids **7b** and **7d** [20] were also reported. The synthesis of the previously unknown **7a** was carried out starting from ketone **6a**. At the first stage, the ketone was transformed to the enol triflate **17**, and the latter was further carbonylated with CO to form ester **18**. Compound **18** was successfully reduced to saturated acid **19**, the acidic hydrolysis of the latter led to the target acid **7a** (**Scheme 2**).

Ketone **20** reacts with propargylamine yielding pyridine **Boc-9a**, as it was described in our previous work, and isomer **Boc-9c** were detected (**Scheme 3**, A). Therefore, the condensation of ketone **20** with propargylamine is not a suitable method for the synthesis of **9c**. Multistep preparations of **9c** were reported earlier [8, 9]. Alternatively, 1,7-naphtyridine partial reduction gave mixtures of isomers [7]. Hereby, we proposed an alternative 4-step way starting from nitrile **21** (**Scheme 3**, B).

The scalable synthesis of amine **9d** from pyridine **25** was performed through an elegant route based on the construction of the piperidine ring on the b-bond of pyridine in 2-fluoro-4-chloropyridine (**25**) (**Scheme 4**). In contrast to the procedures previously reported [21–23], the proposed reaction sequence leads to a single isomer, consists of common organic procedures and uses available starting materials.



Scheme 2. The synthesis of carboxylic acid **7a**

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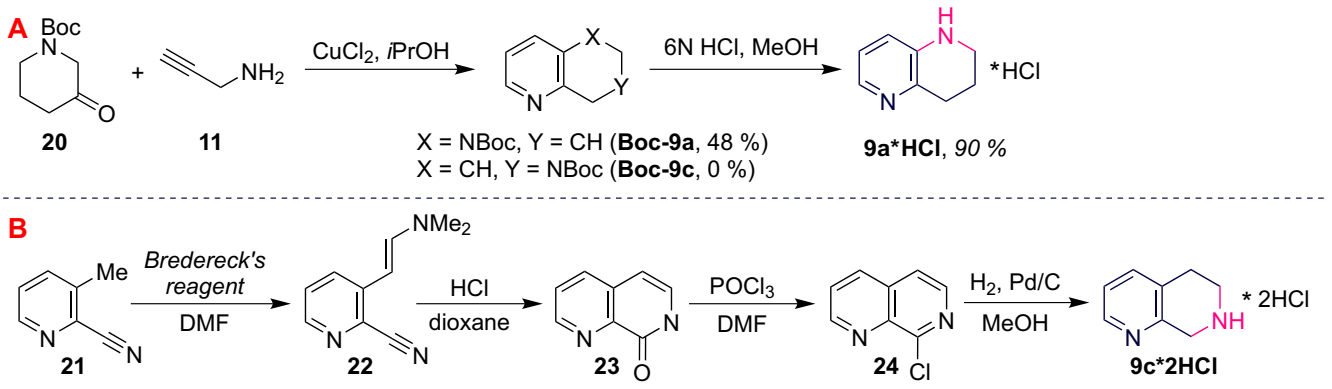
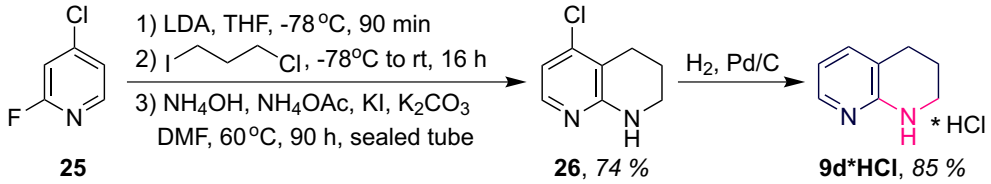


Scheme 3. The synthesis of amine **9c**



Scheme 4. The synthesis of amine **9d**

■ Conclusions

A representative set of pyridines annelated with 6-membered functionalized saturated rings has been synthesized. The scope of $CuCl_2$-catalyzed condensation of propargylamine with ketones has been extended. Other synthetic methods have been proposed for pyridines that cannot be obtained using this procedure. A set of novel building blocks related to medical chemistry has been created for drug development.

■ Experimental part

All solvents were purified according to the standard procedures. Absolute ethanol and isopropanol were used. All starting materials were obtained from Enamine Ltd. Melting points were measured on an automated melting point system. ^{1}H and ^{13}C NMR spectra were measured on a Bruker Avance 500 spectrometer (at 500 MHz for ^{1}H and 126 MHz for ^{13}C nuclei) and a Varian Unity Plus 400 spectrometer (at 400 MHz for ^{1}H and 101 MHz for ^{13}C nuclei). Tetramethylsilane (^{1}H, ^{13}C) was used as an internal standard. Mass spectra were recorded on an Agilent 5890 Series II 5972 GCMS instrument (atmospheric pressure electrospray ionization (ESI)).

The general procedure for the synthesis of pyridines 2–5 and 10c

Pyridines **2–5** and **10c** were obtained according to the procedure previously developed [9].

6-Methyl-5,6,7,8-tetrahydroquinoline (2)

A brownish oil. Yield – 28 g (62%). Anal. Calcd for $C_{10}H_{13}N$, %: C 81.58; H 8.90; N 9.51. Found, %: C 81.38; H 9.01; N 9.59. ^{1}H NMR (400 MHz, Chloroform-*d*), δ, ppm: 1.09 (3H, d, J = 6.5 Hz); 1.43–1.62 (1H, m); 1.86–2.02 (2H, m); 2.43 (1H, dd, J = 16.6, 10.5 Hz); 2.81 (1H, dd, J = 16.5, 5.0 Hz); 2.90–3.04 (2H, m); 7.02 (1H, dd, J = 7.7, 4.8 Hz); 7.34 (1H, d, J = 7.7 Hz); 8.35 (1H, d, J = 4.8 Hz). LC-MS (ESI, positive mode), m/z: 148 $[M+H]^+$.

6,6-Dimethyl-5,6,7,8-tetrahydroquinoline (3)

A yellowish oil. Yield – 38 g (57%). Anal. Calcd for $C_{11}H_{15}N$, %: C 81.94; H 9.38; N 8.69. Found, %: C 81.88; H 9.47; N 8.62. ^{1}H NMR (400 MHz, Chloroform-*d*), δ, ppm: 1.02 (6H, s); 1.69 (2H, t, J = 6.9 Hz); 2.56 (2H, s); 2.96 (2H, t, J = 6.9 Hz); 7.03 (1H, dd, J = 7.6, 4.8 Hz); 7.33 (1H, d, J = 7.6 Hz); 8.38 (1H, d, J = 4.8 Hz). LC-MS (ESI, positive mode), m/z: 162 $[M+H]^+$.

6,6-Difluoro-5,6,7,8-tetrahydroquinoline (4)

A yellowish oil. Yield – 42 g (55%). Anal. Calcd for $C_9H_9F_2N$, %: C 63.90; H 5.36; F 22.46; N 8.28. Found, %: C 63.82; H 5.45; F 22.54; N 8.17. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 2.26–2.41 (1H, m); 3.04 (3H, t, J = 7.1 Hz); 3.29–3.43 (3H, m); 7.22 (1H, dd, J = 7.8, 4.7 Hz); 7.55 (1H, d, J = 7.8 Hz); 8.39 (1H, d, J = 4.7 Hz). ^{19}F NMR (376 MHz, DMSO-d_6) δ, ppm: -95.84. LC-MS (ESI, positive mode), m/z: 170 $[M+H]^+$.

5,6,7,8-Tetrahydroquinolin-6-ol (5)

A brown solid. Yield – 35 g (58%). M. p. 114°C. ^{1}H NMR corresponds to the reported previously [15]. LC-MS (ESI, positive mode), *m/z*: 150 $[M+H]^+$.

5,8-Dihydro-6H-pyrano[3,4-b]pyridine (10c)

A yellowish oil. Yield – 18 g (34%). Anal. Calcd for C_8H_9NO, %: C 71.09; H 6.71; N 10.36. Found, %: C 70.97; H 6.83; N 10.29. ^{1}H NMR (400 MHz, Chloroform-*d*), *δ*, ppm: 2.90 (2H, t, *J* = 5.7 Hz); 4.00 (2H, t, *J* = 5.7 Hz); 4.82 (2H, s); 7.12 (1H, dd, *J* = 7.7, 4.9 Hz); 7.45 (1H, d, *J* = 7.7 Hz); 8.41 (1H, d, *J* = 4.8 Hz). LC-MS (ESI, positive mode), *m/z*: 136 $[M+H]^+$.

The procedure for the synthesis of 5,6,7,8-tetrahydroquinoline-5-carboxylic acid hydrochloride (7a*HCl)

To 6 L round-bottomed flask dried in the oven, 7,8-dihydroquinolin-5(6*H*)-one (**6a**) (147.2 g, 1 mol, 1.0 equiv) was added. The flask was sealed and purged with argon before the addition of CH_2Cl_2 (2.8 L) and Et_3N (208 mL, 1.5 mol, 1.5 equiv). The reaction mixture was cooled to 0°C, and trifluoromethanesulfonic anhydride (242 mL, 6.2 mmol, 1.5 equiv) was added dropwise under argon atmosphere before heating to 40°C and kept at this temperature while stirring for 24 h. Upon completion of the reaction, the solution was washed with water (2×20 mL), and the organic substances were passed through a hydrophobic frit, and concentrated under reduced pressure to give compound **17** quantitatively (~279 g) as a brown oil (85–90% purity), which was used in the next step without purification.

A solution of **17** (279 g, 1 mol) in DMF (2.2 L) was treated with methanol (1.1 L) and N,N-diisopropylethylamine (526 mL, 3 mol), and bubbled with argon for 30 min. The resulting mixture was treated with DPPF (4.5 g, 8 mmol) and palladium (II) acetate (1.8 g, 8 mmol). The resulting solution was bubbled with carbon monoxide for 30 min, and then stirred under a carbon monoxide balloon at 60°C for 6 h. After that, the mixture was cooled to room temperature and diluted with ethyl acetate. The resulting mixture was washed with 1 M aqueous HCl, twice with water, once with the saturated aqueous sodium carbonate, dried over sodium sulfate and then concentrated under vacuum to yield 147.6 g of a residue (78%, ~90% purity) as a yellowish powder. The product was used in the next step without purification.

A solution of **18** (147.6 g, 0.78 mol) in MeOH (2 L) was heated at 50°C under atmospheric pressure and bubbled with H_2 for 2 h in the presence of 10% Pd on charcoal (10 g). After completing the reaction, Pd/C was filtered off, and the residue was evaporated under reduced pressure. The yellowish powder (~149 g, ~90% purity) obtained was used in the next step without purification.

The product **19** (95.6 g, 0.5 mol) was dissolved in the saturated solution of HCl in dioxane (1 L) and boiled until the end of the precipitate formation. Then the solid was filtered off and dried on air. The final product **7a** was obtained as a white powder in 84% yield as hydrochloride (89.8 g).

A white powder. M. p. 164°C. Anal. Calcd for $C_{10}H_{12}ClNO_2$, %: C 62.67; H 4.94; Cl 11.10; N 14.62. Found, %: C 62.60; H 5.03; Cl 10.97; N 14.55. ^{1}H NMR (DMSO-d_6, 500 MHz), *δ*, ppm: 1.55–1.86 (1H, m); 1.86–2.07 (2H, m); 2.07–2.35 (1H, m); 2.92–3.27 (2H, m); 4.06 (1H, t, *J* = 5.0 Hz); 7.81 (1H, dd, *J* = 7.8, 5.6 Hz); 8.37 (1H, d, *J* = 7.8 Hz); 8.69 (1H, d, *J* = 5.6 Hz). LC-MS (ESI, positive mode), *m/z*: 178 $[M-Cl]^+$.

The procedure for the synthesis of 1,7-naphthyridin-8(7*H*)-one (23)

3-Methylpicolinonitrile (**21**) (23.62 g, 0.2 mol) and the Bredereck's reagent (69.6 g, 0.2 mmol) were dissolved in DMF (250 mL). The reaction mixture was heated at 75°C under argon for 72 h. After that, the solvent was removed *in vacuo*. Trituration with MTBE gave a brown oil **21** (~35 g, ~0.2 mol, a quantitative yield, ~85% purity). Further all 35 g of the product was used without additional purification.

The oil from the previous step was dissolved in the saturated solution of HCl in dioxane (200 mL). The reaction mixture was warmed at 45–50°C for 24 h. The reaction solution was filtered, and the filtrate was collected and dried. The light brown solid **23** (24.2 g, 83% yield) obtained was directly used in the next reaction.

A brown solid. M. p. 227°C. Anal. Calcd for $C_8H_6N_2O$, %: C 65.75; H 4.14; N 19.17. Found, %: C 65.64; H 4.19; N 19.28. ^{1}H NMR (DMSO-d_6, 400 MHz), *δ*, ppm: 6.53 (1H, d, *J* = 7.1 Hz); 7.25 (1H, d, *J* = 7.0 Hz); 7.67 (1H, dd, *J* = 8.1, 4.4 Hz); 8.10 (1H, dd, *J* = 8.1, 1.7 Hz); 8.75 (1H, dd, *J* = 4.3, 1.7 Hz); 11.50 (1H, s). LC-MS (ESI, positive mode), *m/z*: 147 $[M+H]^+$.

The procedure for the synthesis of 8-chloro-1,7-naphthyridine (24)

1,7-Naphthyridin-8(7*H*)-one (**23**) (19.1 g, 0.15 mol) was dissolved in 200 mL of toluene. $POCl_3$ (31 g, 0.2 mol) and DIPEA (72 g, 4 mol) were added to the reaction mixture, and then it was

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refluxed for 6 h. After cooling down, the mixture was diluted with EtOAc (15 mL) and washed with ice-cold water, the saturated $NaHCO_3$, brine. The organic layer was dried over Na_2SO_4. The solvent was removed under reduced pressure, and the residue was purified by washing with *i*PrOH to give 20.2 g of a yellow solid in 82% yield.

A yellow solid. M. p. 89°C. Anal. Calcd for $C_8H_5ClN_2$, %: C 58.38; H 3.06; Cl 21.54; N 17.02. Found, %: C 58.27; H 3.11; Cl 21.47; N 16.95. ^{1}H NMR (Chloroform-*d*, 400 MHz), *δ*, ppm: 7.65 (1H, d, *J* = 5.6 Hz); 7.71 (1H, dd, *J* = 8.4, 4.2 Hz); 8.23 (1H, dd, *J* = 8.4, 1.7 Hz); 8.41 (1H, d, *J* = 5.6 Hz); 9.16 (1H, dd, *J* = 4.2, 1.7 Hz). LC-MS (ESI, positive mode), *m*/*z*: 165 $[M+H]^+$.

The procedure for the synthesis of 5,6,7,8-Tetrahydro-1,7-naphthyridine dihydrochloride (9c*2HCl)

A solution of 8-chloro-1,7-naphthyridine (**24**) (16.5 g, 0.1 mol) in MeOH (300 mL) was placed into the autoclave and heated at 50°C under 10 atm pressure of H_2 for 6 h in the presence of 10% Pd on charcoal (5 g). After completing the reaction, Pd/C was filtered off, and the residue was evaporated under reduced pressure. Then the crude substrate was dissolved in the saturated solution of HCl in dioxane. The yellowish powder (~12.3 g, 87% yield) was obtained as a dihydrochloride after simple filtration.

A yellow solid. M. p. 210°C (decomp.). Anal. Calcd for $C_8H_{12}Cl_2N_2$, %: C 46.40; H 5.84; Cl 34.23; N 13.53. Found, %: C 46.34; H 5.93; Cl 34.18; N 13.44. ^{1}H NMR (400 MHz, DMSO-d_6), *δ*, ppm: 3.12 (1H, t, *J* = 6.2 Hz); 3.40 (1H, q, *J* = 6.4 Hz); 4.38 (1H, t, *J* = 4.5 Hz); 7.55 (1H, dd, *J* = 7.8, 5.1 Hz); 8.58 (1H, d, *J* = 5.1 Hz); 10.02 (1H, s). LC-MS (ESI, positive mode), *m*/*z*: 135 $[M-HCl-Cl]^+$.

5-Chloro-1,2,3,4-tetrahydro-1,8-naphthyridine (26)

The synthesis was performed from 4-chloro-2-fluoropyridine (**25**) according to the procedure reported [24].

A yellow solid. Yield – 21 g (74%). M. p. 97°C. Anal. Calcd for $C_8H_9ClN_2$, %: C 56.98; H 5.38; Cl 21.02; N 16.61. Found, %: C 57.05; H 5.33; Cl 20.94; N 16.66. ^{1}H NMR (400 MHz, Chloroform-*d*), *δ*, ppm: 1.94 (3H, pent, *J* = 6.2 Hz); 2.78 (3H, t, *J* = 6.5 Hz); 3.32–3.43 (3H, m); 6.56 (1H, d, *J* = 5.5 Hz); 7.74 (1H, d, *J* = 5.5 Hz). LC-MS (ESI, positive mode), *m*/*z*: 169 $[M+H]^+$.

The procedure for the synthesis of 1,2,3,4-tetrahydro-1,8-naphthyridine (9d*HCl)

The reduction of 5-chloro-1,2,3,4-tetrahydro-1,8-naphthyridine (**26**) was performed by the seminal procedure used for preparation of amine **9c**. Amine **9d** was obtained in 85% yield as a yellow powder in a hydrochloride form (28 g).

A yellow solid. M. p. 71°C. Anal. Calcd for $C_8H_9ClN_2$, %: C 56.98; H 5.38; Cl 21.02; N 16.61. Found, %: C 57.08; H 5.24; Cl 20.93; N 16.66. ^{1}H NMR (500 MHz, Chloroform-*d*), δ, ppm: 1.94–1.87 (2H, pent, *J* = 6.2 Hz); 2.71 (2H, t, *J* = 6.3 Hz); 3.29–3.64 (2H, m); 6.47 (1H, dd, *J* = 7.1, 5.0 Hz); 7.12 (1H, d, *J* = 7.1 Hz); 7.84 (1H, d, *J* = 4.4 Hz). LC-MS (ESI, positive mode), *m*/*z*: 135 $[M+H]^+$.

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Metrological Characteristics of the Potentiometric Assay Developed for Determining the Antioxidant Activity of Ascorbic Acid

Abstract

The potentiometric assay for determining the antioxidant activity of ascorbic acid has been developed and validated according to the following parameters: specificity, linearity, accuracy, repeatability, intermediate precision. The linearity was in the concentration range of 0.002–0.02 mol L^{-1} (r^2 = 0.9993). The percentage of recovery was found to be in the range from 95.38 to 105.00 %. The values of %*RSD* for repeatability and intermediate precision were 1.86 and 1.95 %, respectively. The method is accurate and reliable, with the relative standard deviation of less than 2 %. It has been proven that the method developed is express, rapid, highly sensitive, accurate and sufficiently reliable.

Keywords: antioxidant activity; potentiometric method; validation; ascorbic acid

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Метрологічна характеристика розробленої методики визначення антиоксидантної активності аскорбінової кислоти

Анотація

Потенціометричну методику визначення антиоксидантної активності аскорбінової кислоти було розроблено і валідовано відповідно до таких параметрів: специфічність, лінійність, точність, прецизійність, внутрішня прецизійність. Лінійність зберігалася в діапазоні концентрацій 0,002–0,02 моль $л^{-1}$ (r^2 = 0,9993). Визначено, що відсоток відновлення становить 95,38–105,00 %, прецизійність та внутрішня прецизійність – 1,86 % та 1,95 %, відповідно. Методика характеризується як точна і надійна, має відносне стандартне відхилення менше 2 %. Доведено, що розроблена методика експресна, проста, високочутлива, точна і достатньо надійна.

Ключові слова: антиоксидантна активність; потенціометричний метод; валідація; аскорбінова кислота

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Introduction

Reactive oxygen species are generated endogenously in the mitochondrial oxidative phosphorylation, or they may arise from exogenous sources, such as xenobiotic compounds [1, 2]. Oxidative stress results in indirect or direct damage of nucleic acids, proteins, and lipid, and has been implicated in carcinogenesis, neurodegeneration, atherosclerosis, diabetes and aging [3, 4].

Ascorbic acid is a six-carbon lactone obtained from glucose in the liver. Human is not able to synthesize it due to the lack of *L*-gulonolactone oxidase [5]. Therefore, vitamin C must be obtained from the diet to maintain a normal metabolic functioning of the body. In the body of a human, vitamin C reacts with free radicals as an antioxidant [6]. The ascorbyl radical is formed when ascorbic acid loses one electron, the second form is called dehydroascorbic acid formed with losing an electron by the ascorbyl radical [7, 8].

Most of the physiological functions of vitamin C are related to its reduction properties [9, 10]. Ascorbic acid is involved in the synthesis of collagen, carnitine and neurotransmitters. In addition, ascorbic acid accelerates the healing process, affects the synthesis of a number of hormones, regulates hematopoiesis and normalizes capillary permeability [11–13].

According to Pubmed and ScienceDirect databases, the number of publications dealing with the study of the antioxidant activity of different substances is growing steadily (**Figure 1**). Known assays for determining the antioxidant activity are based on the oxidation of the test sample with oxidizing agents of various nature. Oxidizing agents can be inorganic compounds – $K_3[Fe(CN)_6]$, H_2O_2, $KMnO_4$, and organic compounds – 2,2-diphenyl-1-picrylhydrazyl (in DPPH assay), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (in ABTS assay), Fe(III)-tripyridyltriazine (in FRAP assay) [14]. The antioxidant activity of ascorbic acid has been determined by different assays, such as DPPH [15], ABTS [16], FRAP [17], chemiluscent and potentiometric method [18].

S. Martinez et al. [19] applied the potentiometric assay in determining the antioxidant activity of wine. The potentiometric assay they use is based on titration of test samples with an electrogenerated chlorine. Chlorine has the ability to enter into various reactions (radical, redox, electrophilic substitution and addition to multiple bonds). Due to this, titration with chlorine allows to cover a wide spectrum of biologically active components possessing antioxidant properties. However, in our opinion, this method has some disadvantages. Firstly, in the assay the pH equals 2. At the same time, for studies of the antioxidant activity related to living organisms, the pH value should be maintained in the range of 7.2–7.4 since it is physiological. Secondly, chlorine is rather toxic compound.

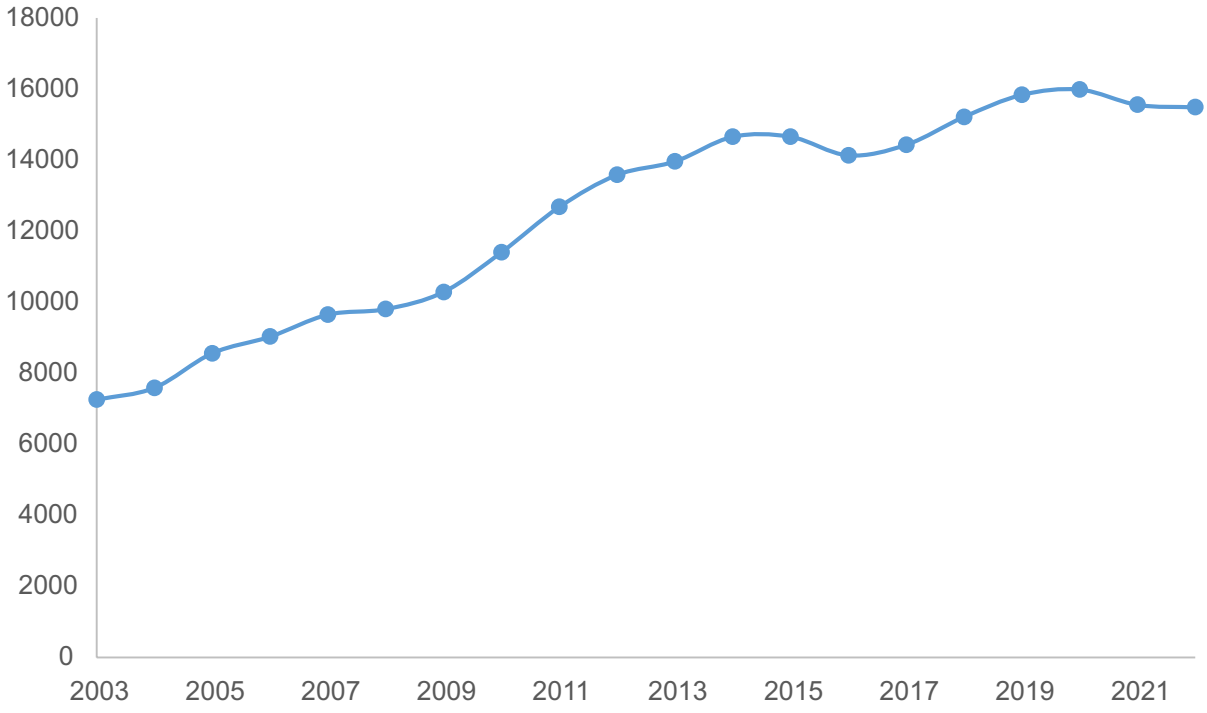

Figure 1. The number of published articles in the period of 2003–2022 years

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Currently, ascorbic acid is found in numerous plants, e.g., rose hips, mountain ash, viburnum, currants, raspberries and citrus fruits. In addition, vitamin C is also an ordinary "guest" in caplets, tablets, capsules, drink mixes, multivitamin, antioxidant formulation and dietary supplements. Hence, the development of methods for determining the antioxidant activity of ascorbic acid is a promising task today. Thus, the aim of the present study was to develop and validate the potentiometric assay for determining the antioxidant activity of ascorbic acid.

■ Materials and methods

Reagents

Ascorbic acid ≥ 98.0% (Sigma-Aldrich). Reagents $K_3[Fe(CN)_6]$, $K_4[Fe(CN)_6]$, $NaHPO_4$, KH_2PO_4 were of analytical grade and purchased from Reakhim (Kharkiv, Ukraine).

Instruments

A pH meter Hanna 2550 (FRG) with a combined platinum electrode EZDO 50 PO (Taiwan) were used during potentiometric measurements. The digital analytical balance AN100 (AXIS, Ukraine) with d = 0.0001 g was used for weighing.

Preparation of standard solutions

A stock solution of ascorbic acid (0.05 mol L^{-1}) was prepared by dissolving ascorbic acid in distilled water.

Model solutions of ascorbic acid were prepared by dilution of the stock solution of ascorbic acid (0.002, 0.006, 0.01, 0.014, 0.02 mol L^{-1}).

Preparation of phosphate buffer solutions

8.00 g of NaCl, 0.20 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 were dissolved in 800 mL of distilled water, and pH to 7.4 was adjusted with hydrochloric acid or sodium hydroxide, and the solution was diluted with distilled water to 1 L.

The procedure for the antioxidant activity determination

2 mmol L^{-1} solution of $K_3[Fe(CN)_6]$ was prepared by weighing 0.8232 g into a 25.0 mL volumetric flask, dissolving the compound in distilled water, and diluted to the volume with the same solvent. 0.02 mmol L^{-1} of $K_4[Fe(CN)_6]$ was prepared by weighing 0.0921 g into a 250.0 mL volumetric flask, dissolving the compound in distilled water, and diluted to the volume with the same solvent. Then 5.00 mL of the aliquot of both prepared solutions was taken and transferred into a 250.0 mL volumetric flask and diluted to the volume with 0.067 mmol L^{-1} phosphate buffer solution. 50.00 mL of the prepared intermediate solution was transferred into an electrochemical cell. The initial potential of the intermediate solution was measured after the initial one was determined, 1.00 mL of the aliquot of the solutions obtained was added, and a final potential was measured. The difference (ΔE) between the initial (E_0) and final (E_1) potentials was found [20].

The antioxidant activity was calculated according to the following equation and expressed as mmol L^{-1}:

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

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 = 96485.333 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 coefficient of dilution.

Validation method

Validation of the potentiometric method for determining the antioxidant activity of ascorbic acid was performed according to the International Conference on Harmonization (ICH) [21]. The method proposed was validated by the following parameters: specificity, linearity, accuracy, repeatability and intermediate precision.

The specificity of the method was studied by the potentiometric titration of the solvent.

The linearity of the method was studied at 5 concentration levels (0.002, 0.006, 0.01, 0.014, 0.02 mol L^{-1}). The antioxidant activity was evaluated by the potentiometric method. The linearity was determined by a linear relationship between the logarithm concentrations of the ascorbic acid solutions prepared. The linear regression was calculated by the method of least squares to obtain the regression equation and determine the correlation coefficient (r^2). According to the requirements of ICH, the value of the correlation coefficient when studying the linearity should not exceed 0.999.

The accuracy was evaluated by calculating recovery of ascorbic acid using the standard addition method. Three levels of ascorbic acid concentration corresponding to 50, 100, 200% of the working concentration of ascorbic acid 0.1 mol L^{-1}

were taken. The acceptance criteria were *RSD* and should not exceed 2%.

The precision of the method was assessed by repeatability and intermediate precision. In the case of repeatability, the working solution of ascorbic acid with the concentration of 0.01 mol L^{-1} was analyzed six times at the same day. The intermediate precision was evaluated with the same concentration of ascorbic acid at different days. The acceptance criteria were *RSD* and should not exceed 2%.

The statistical processing of the experimental data obtained was performed in accordance with the monograph "Statistical analysis of the results of a chemical experiment" of the State Pharmacopeia of Ukraine.

■ Results and discussion

The potentiometric method for determining the antioxidant activity is based on the interaction of a mediator system with an antioxidant. The mediator system consists of an oxidizing agent and a reducing agent. When an antioxidant is added to the mediator system, the ratio of the oxidizing agent and reducing agent changes, which leads to a shift in the potential of the electrochemical cell. After that, the potential difference and the antioxidant activity of the antioxidant studied are calculated.

In order to develop a potentiometric assay for determining the antioxidant activity of ascorbic acid, it was necessary:

1) to choose the optimal redox electrode;

2) to choose the optimal ratio of $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$;

3) the potential of the platinum electrode should correspond to the Nernst dependence of the potential change.

The main criteria for choosing the redox electrode are the range of measuring the potential of the electrochemical cell and the time of establishing the potential.

The potential of the electrochemical cell is measured by platinum, gold and glass-carbon electrodes. Thus, to choose the optimal electrode we analyzed characteristics of three electrodes mentioned above.

Table 1 shows that the glass-carbon electrode has the widest range of measuring the potential of the electrochemical cell, followed by the platinum electrode, whereas the gold one has the narrowest measuring potential of the electrochemical cell. Therefore, the platinum and glass-carbon electrodes meet the requirement of the range of measuring the potential of the electrochemical cell.

Table 1. Measuring ranges of potentials of electrodes

No.	Electrode	Measuring ranges, *V*
1	Platinum electrode	from -0.1 to +0.9
2	Gold electrode	from -0.1 to +0.3
3	Glass-carbon electrode	from -0.9 to +0.8

Table 2. The time of the establishing the potential of the electrochemical cell

No.	Electrode	Time, *sec*
1	Platinum electrode	10±0.3
2	Gold electrode	1800±36.0
3	Glass-carbon electrode	300±6.0

Note: n = 5, p < 0.05

Table 2 demonstrates that the platinum electrode is the fastest (10 sec) in establishing the potential of the electrochemical cell, followed by the glass-carbon electrode (300 sec), and the gold electrode has the longest time (1800 sec).

According to the results obtained, the platinum electrode met all the requirements to the redox electrode and was the most suitable for determining the antioxidant activity of ascorbic acid. Thus, it was used in the study.

The accuracy of determining the redox potential of the system depends on how the Nernst dependence follows the potential change. **Figure 2** shows the dependence of the potential of the platinum electrode EZDO 50 PO in the sodium phosphate buffer with the pH 7.4 and various concentrations of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$. The pre-logarithmic coefficient in this dependence is 58.5 mV (**Figure 2**), which is close to the theoretical value of RT/nF = 59.16 mV in the Nernst equation for a one-electron process at 25°C. From mentioned above, it can be concluded that the platinum electrode is completely suitable according to the criteria set.

To select the optimal concentration and ratio of $K_3[Fe(CN)_6]$ and $K_3[Fe(CN)_6]$, the following criteria were used:

1) the minimum change in the system potential must be at least 20 mV;

2) the high speed of equilibration;

3) the potential of the system must be stable over time.

Table 3 demonstrates the change in the potentials of the system with different ratios of $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ concentrations after the introduction of the test samples (1.0–5.0 mol L^{-1} of ascorbic acid). It is easy to see that in the

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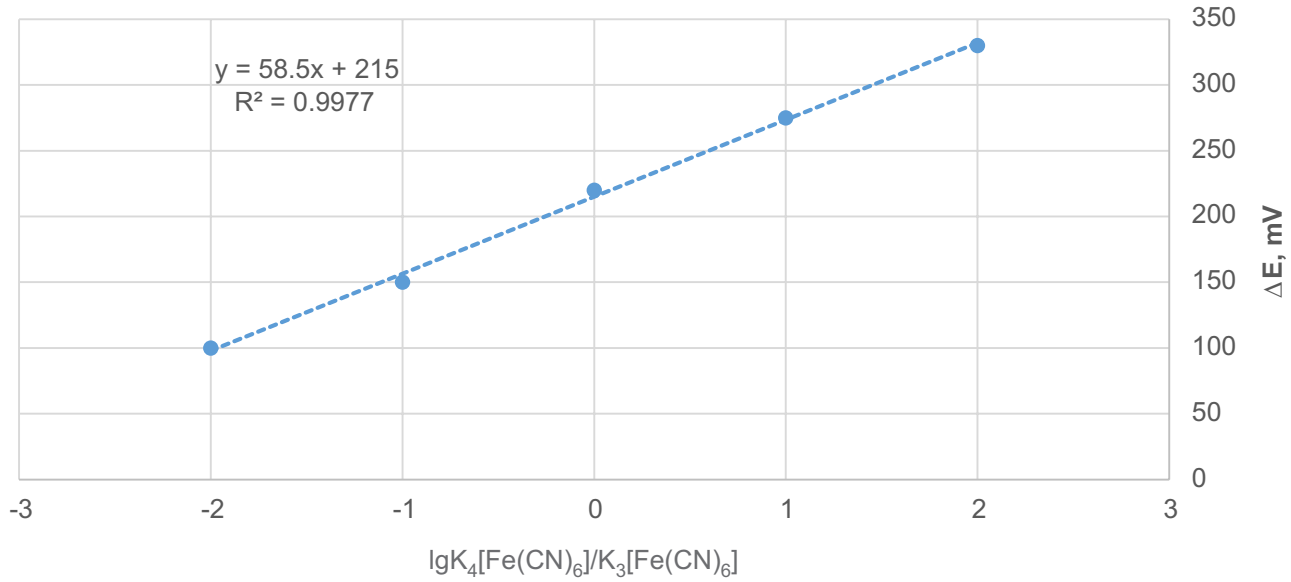

Figure 2. The dependence of the potential of the platinum electrode EZDO 50 PO in the sodium phosphate buffer with the pH 7.4 and various concentrations of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$

Table 3. The change in the potential after the introduction of ascorbic acid (1.0 and 5.0 molL^{-1}) into the mediator system of various compositions

Mediator system	$K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$, mol L^{-1}	ΔE for concentration of 1.0 mol L^{-1}, mV	ΔE for concentration of 5.0 mol L^{-1}, mV
System 1	0.002/0.00002	25.0±0.3	40.0±0.3
System 2	0.001/0.00001	40.0±0.3	100.0±0.3
System 3	0.1/0.001	2.0±0.3	12.0±0.3
System 4	0.05/0.001	10.0±0.3	25.0±0.3

Note: n = 5, p < 0.05

system 1 and 2 the minimum potential change is greater than 20 mV. However, the redox potential of system 2 is not stable for 30 minutes, this may be due to the fact that the concentration of $K_4[Fe(CN)_6]$ is low enough for the potential of the system to be stable over time. System 1 turned out to be the most stable over time, and its potential did not change over the course of an hour. Therefore, this mediator system was chosen to determine the antioxidant activity of the samples under study.

The linearity was proven in the concentration range from 0.002 to 0.02 mol L^{-1}. The regression equation of the curve had the following form: y = 2.5896x + 7.4011. The value of the correlation coefficient (r^2) was equal to 0.9993 (**Figure 3**).

The accuracy of the method was assessed by the percentage of recovery. The percentage of recovery was found to be in the range from 95.38 to 105.00% (**Table 4**).

The precision of the method was confirmed by repeatability and intermediate precision. The values

Table 4. Recovery studies using the standard method of addition

AO	AOA, mmol-eq. L^{-1}	Amount added of AO, mmol-eq.L^{-1}	Amount taken of AO (C_1), mmol-eq.L^{-1}	Amount recovered (C_2), mmol-eq.L^{-1}	%, Recovery $R = \frac{C_2}{C_1} \cdot 100\ \%$
Ascorbic acid	1.95	0.98	2.93	3.00	102.79
				3.02	103.07
				2.80	95.56
		1.95	3.90	4.00	102.39
				4.11	105.00
				3.72	95.38
		2.93	4.88	5.00	102.46
				5.14	105.00
				4.80	98.36

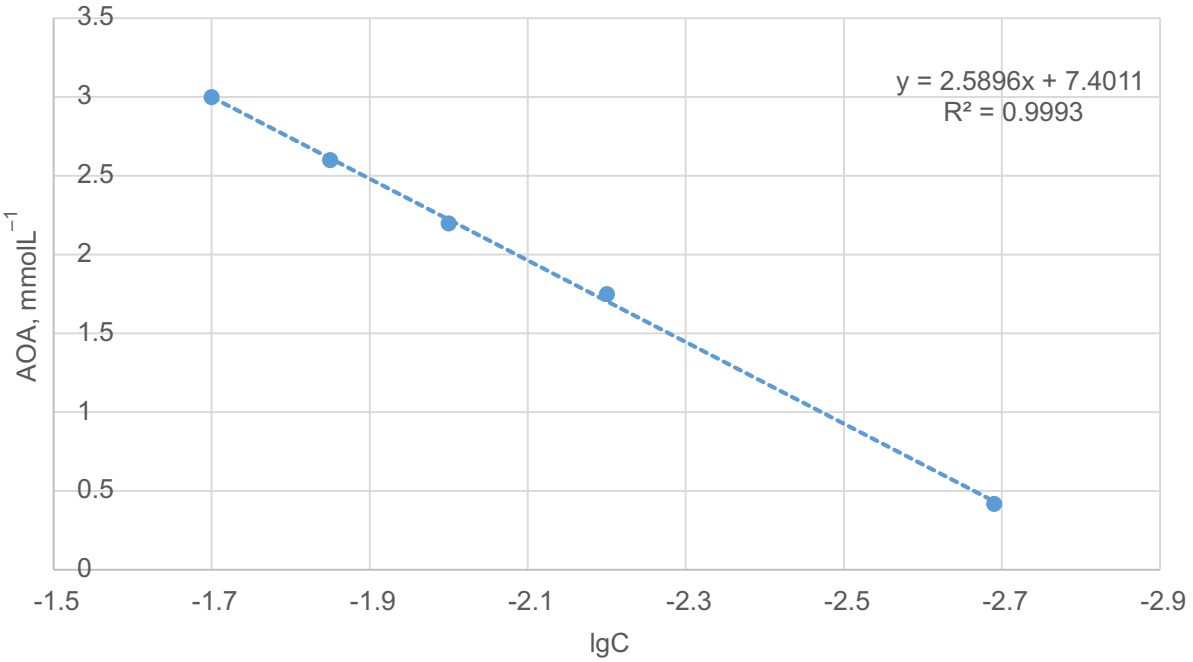

Figure 3. The calibration curve of the concentration logarithm *vs* the antioxidant activity of ascorbic acid

Table 5. Repeatability results for determining the antioxidant activity of ascorbic acid

Number of samples	AOA of ascorbic, mmol-eq. L^{-1}
1	1.95
2	1.99
3	1.91
4	1.91
5	2.00
6	1.95
Mean, mmol-eq. L^{-1}	1.95
SD	0.04045
Confidence interval, mmol-eq. L^{-1}	0.04
RSD	1.95

Table 6. Intermediate precision results for determining the antioxidant activity of ascorbic acid

Number of samples	Ascorbic acid	
	The first day	The second day
1	1.95	1.99
2	1.95	1.98
3	1.97	2.05
4	2.02	1.95
5	1.90	1.97
6	1.98	1.98
Mean, mmol-eq. L^{-1}	1.96	1.99
SD	0.03971	0.03386
Confidence interval, mmol-eq. L^{-1}	0.04	0.04
RSD, %	2.00	1.70
Mean of *RSD*, %	1.86	

of %*RSD* for repeatability and intermediate precision were 1.95 and 1.86%, respectively. The %*RSD* values were less than 2%. It proves that the method is precise (**Tables 5** and **6**).

■ Conclusions

The potentiometric assay for determining the antioxidant activity of ascorbic acid has been developed and validated according to the following parameters: specificity, linearity, accuracy, repeatability, intermediate precision. The method proposed can be used for routine analysis to determine the antioxidant activity of different objects under research and quality control purposes since the method developed is express, rapid, highly sensitive, accurate and sufficiently reliable.

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Obtaining the Enoxaparin Sodium Substance Equivalent to the Original Clexane® and Lovenox®. The Selection of Technological Parameters and Optimization of the "Greenness" of the Purification Stage

Abstract

The aim of the study was to adjust and optimize the purification stage of crude enoxaparin sodium to obtain a substance equivalent to the original drugs Clexane® and Lovenox® according to the criteria specified by the FDA. The purification stage involves the reprecipitation of crude enoxaparin in methanol. Determining the ratio of solvents required for the reprecipitation is important for studying the correlation between the experimental conditions of the technological process and the structural characteristics of enoxaparin samples. In the study, the method of purification of enoxaparin sodium described in the patent was assessed, and the following variations of the MeOH:H_2O solvent ratio were selected – 4:1; 2:1; 1:1. The obtained samples of enoxaparin sodium were analyzed according to the in-house specification developed on the basis of the pharmacopoeial monograph, as well as by non-pharmacopoeial methods, such as two-dimensional NMR spectroscopy (HSQC) and size exclusion chromatography (SEC) for detailed characterization of the molecule. Strategies of greening of the enoxaparin sodium purification stage by reducing the E-factor were also considered in the study. Considering the principles of "green" chemistry, the method of purification of crude enoxaparin sodium was optimized by the solvent regeneration. It was experimentally possible to demonstrate the effect of the solvent ratio at the stage of purification of crude enoxaparin on the composition, as well as on the number and distribution of oligosaccharide fractions in the molecule. Based on the results of the study, it can be concluded that the ratio of MeOH:H_2O=1:1 allows obtaining samples that are closest to Clexane® and Lovenox® in terms of the molecular weight distribution profile and the composition profile. The E-factor was also reduced from 14 to 5.25 by solvent regeneration.

Keywords: enoxaparin sodium; low-molecular-weight heparin; technological parameters; compositional analysis; HSQC; size-exclusion chromatography; green chemistry; E-factor; solvent regeneration

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Одержання субстанції еноксапарину натрію, еквівалентної оригінальним Clexane® та Lovenox®. Підбір технологічних параметрів та оптимізація «зеленості» стадії очищення

Анотація

Метою роботи було налаштувати та оптимізувати стадію очищення технічного еноксапарину натрію для отримання субстанції, еквівалентної оригінальним препаратам Clexane® та Lovenox® за критеріями, окресленими FDA. Стадія очищення передбачає переосадження неочищеного еноксапарину із метанолу. Визначення необхідного співвідношення розчинників для переосадження є важливим для дослідження кореляції між експериментальними умовами технологічного процесу та структурними характеристиками зразків еноксапарину. У дослідженні було оцінено спосіб очищення еноксапарину натрію, описаний у патенті, і обрано такі варіанти співвідношення розчинників MeOH:H_2O – 4:1; 2:1; 1:1. Отримані зразки еноксапарину натрію аналізували відповідно до внутрішньої специфікації, розробленої на основі фармакопейної монографії, а також за допомогою нефармакопейних методів, таких, як двовимірна ЯМР-спектроскопія (HSQC) та ексклюзійна хроматографія (SEC) для детальної характеристики. молекули. У дослідженні також розглядали стратегії екологізації етапу очищення еноксапарину натрію шляхом зниження Е-фактора. З огляду

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на принципи «зеленої» хімії метод очищення неочищеного еноксапарину натрію було оптимізовано шляхом регенерації розчинника. Експериментально вдалося продемонструвати вплив співвідношення розчинників на стадії очищення неочищеного еноксапарину на склад, а також на кількість і розподіл фракцій олігосахаридів у молекулі. За результатами дослідження можна зробити висновок, що співвідношення MeOH:H_2O = 1:1 дозволяє отримати зразки, які за профілем молекулярно-масового розподілу та профілем складу найбільш наближені до Clexane® та Lovenox®. E-коефіцієнт також було знижено з 14 до 5,25 шляхом регенерації розчинника.

Ключові слова: еноксапарин натрію; низькомолекулярний гепарин; технологічні параметри; композиційний аналіз; HSQC; ексклюзійна хроматографія; «зелена» хімія; E-фактор; регенерація розчинника

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■ Introduction

Enoxaparin sodium is a modern low-molecular semi-synthetic anticoagulant, which is a product of the multi-stage conversion of sodium heparin [1]. Enoxaparin sodium has the same properties as its precursor heparin sodium, but due to its improved structure, it does not have the side effects typical of heparin caused by its complex structure and very high molecular weight [2, 3].

Enoxaparin sodium is a heterogeneous mixture of oligosaccharides with a complex structure consisting of repeating units of disaccharide building blocks with one glucuronic acid (GlcA) or iduronic acid (IdoA) residue and one glucosamine (GlcN) residue, which is either *N*-sulfated (GlcNS), or *N*-acetylated (GlcNAc), linked by glycosidic bonds. Enoxaparin sodium is characterized by unique structural elements (fingerprints) that are formed because of modifications during depolymerization, namely, such structures as 4,5-uronates at non-reducing ends and 1,6-anhydro structures at reducing ends [4, 5]. Enoxaparin sodium is a substance of biological origin, i.e., isolated from animal tissues and differs from "normal" substances by its high molecular weight and complex heterogeneous structure, which complicates the development and introduction of similar drugs to the market. Due to the complexity of the structure and the previous experience during the heparin crisis [6], there are serious discussions in the world on the issues of proving the equivalence of generic low-molecular-weight heparins (LMWHs) and establishing permissible fluctuations of the "norm" of biochemical and biological indicators, which potentially affect the safety and effectiveness of the drug. As a result, EMA and FDA have initiated guidelines to confirm the similarity of LMWH [7, 8]. The FDA, for example, introduced a scientific approach to demonstrate the equivalence of generic LMWHs to references, which included compliance not only with biological, but also with chemical characteristics, such as the sequence of disaccharide building blocks, the sequence of oligosaccharide fragments, etc. [9]. Since the aim of our work was the synthesis of the Enoxparin molecule demonstrating the equivalence to the original *Clexane*® and *Lovenox*® (Sanofi-Aventis) according to the specified FDA criteria [10], we conducted a large study to adjust the technological parameters of the process at each stage to obtain a substance as close as possible to the originator [11]. The methods described in the patent [12] were taken as a basis. The analysis of samples for comparison was carried out according to the internal specification developed based on the pharmacopoeial monograph, as well as according to specific methods. Since the structure requires accurate, painstaking analysis of saccharide units and their sequence, additional methods of analysis of similar structures were introduced [13, 14].

One of the steps in the synthesis of enoxaparin is the purification stage, which is a very important in achieving the equivalence with the original *Clexane*® and *Lovenox*® (Sanofi-Aventis). Purification of the substance involves decolorization of enoxaparin sodium, pH correction, elimination of degradation products after depolymerization, and correction of the molecular composition. There are many different methods for the purification of enoxaparin, for example, lyophilization of enoxaparin sodium solution, decolorization of the solution with hydrogen peroxide,

followed by reprecipitation using carbon filters, ion exchange resins, etc. [15]. In the experiment described in this article, the solution reprecipitation was used as a purification method. It is also known that the amount of the solvent for reprecipitation of enoxaparin affects the number and distribution of short and long saccharide chains in the molecule, so we focused on this. Decolorization of the solution is also an important component of obtaining API of proper quality, but it is not a priority of this experiment.

Compliance with the principles of "green" chemistry is no less important in the development of synthetic technologies. "Green" chemistry is a direction in modern chemistry that consists in the improvement of technologies regarding the effective use of the raw material and energy, the avoidance of toxic and poisonous substances, the reduction of waste or the repeated use of chemicals and materials [16]. In this study, the E-factor was chosen as the accent metric for the analysis of "greenness". The E-factor is the ratio of the amount of waste to the amount of a product. All raw materials used, except water, are included in the calculation. The higher the value of the E-factor, the greater the amount of waste [17]. One of the tasks of this work was also to minimize waste at the stage of enoxaparin sodium purification, thereby improving the "greenness" of the synthesis of enoxaparin sodium.

■ The Research Methodology

In the process of planning the experiment for the purification of crude enoxaparin sodium, an analysis of the methods described in the literature was performed.

Thus, the patent [18] describes the following protocol, which it was decided to use as a basis, but with a change in the amount of methanol to observe the effect on the structure of the molecule: "*Suspend crude enoxaparin sodium (5 g) in 50 mL of purified water and dissolve. Add 5 g of sodium chloride and mix. The product is precipitated by adding 150 mL of methanol, filtered, and dried under vacuum at 55°C for 9 hours, yielding 4.39 g of enoxaparin sodium*".

The study included tasks outlined below.

1. To purify the substance according to the parameters selected and to study the effect of the solvent ratio on the product composition in order to obtain a substance equivalent to the original *Clexane*® and *Lovenox*®. The following variants of the ratio of MeOH:H_2O were considered: 4:1; 2:1; 1:1.

1.1. First, it was decided to conduct a test experiment to understand the general trend of the effect of the solvent ratio on the composition and distribution of low- and high-molecular fractions of enoxaparin sodium. Thus, crude enoxaparin sodium synthesized under the so-called "standard conditions" [11] (base/ester ratio – 0.07; the reaction mass temperature – 62°C; the reaction time – 1 hour) was purified in two ways with the following solvent ratios:

- MeOH:H_2O = 4:1 (D475);
- MeOH:H_2O = 1:1 (D478).

1.2. The next step was to analyze the results obtained for more accurate processing of the purification stage, including reprecipitation of samples of crude enoxaparin synthesized according to the optimized parameters of the depolymerization stage [11] (alkali/benzyl ester of the heparin ratio – 0.06; temperature – 57°C, the holding time of the reaction mixture – 1.5 (D492 and D493) and 2 hours (D494 and D495) in the ratio of:

- MeOH:H_2O = 2:1 (D492 and D494);
- MeOH:H_2O = 1:1 (D493 and D495).

2. To optimize the method of synthesis and purification of enoxaparin sodium in view of the principles of "green" chemistry.

The reprecipitation stage is carried out at atmospheric pressure and room temperature, which does not contradict the principles of "green" chemistry. Methanol, which is a poisonous substance, is used as a precipitating agent. However, it is worth noting that according to the in-house guidelines for the selection of solvents of several pharmaceutical companies, methanol belongs to the category "to be confirmed" (**Table 1**) [19].

In addition, the use of methanol is justified by the possibility of obtaining a crystalline precipitate, while the use of solvents with less harmful environmental effects, such as ethanol or isopropanol, provokes the formation of a finely dispersed suspension, which makes it impossible to isolate the precipitate of the substance. One of the most important indicators of "green" chemistry is the E-factor, which is a method of measuring and regulating the amount of waste.

Table 1. Generalized comparison of solvent ratings

Solvent	Astra Zeneka	GCI-PR	GlaxoSmithKlein	Pfizer	Sanofi	Total
MeOH	19	14	14	Preferably	Recommended	To be confirmed

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The E-factor is the actual amount of waste defined as "everything except the desired product" produced per kg of the product, including the loss of solvents and chemicals used in processing [20]. In pharmaceutical production, solvents account for 80–90% of the total mass of non-aqueous material used, most of the waste generated, and 75–80% of the environmental impact of the life cycle, creating the need for solvent regeneration with the subsequent reuse. Therefore, the greening of this stage was carried out due to the regeneration of methanol, which affected the reduction of the amount of waste, and, as a result, a decrease in the E-factor indicator.

3. After analyzing the results of the experimental studies, to make corrections in the method of the enoxaparin sodium synthesis.

■ Results and discussion

As mentioned earlier, the aim of this work was to study the effect of solvents on the composition of the enoxaparin substance. The experiment was conditionally divided into two stages described below.

1. For the study, we chose samples of crude enoxaparin sodium that were processed according to "standard" non-optimized technological parameters using the methodology in the patent [12] and those samples that were processed according to optimized parameters. Crude enoxaparin sodium obtained under the so-called "standard conditions" was purified by reprecipitation of an aqueous solution of enoxaparin in methanol. The ratios of water and methanol for reprecipitation were chosen rather roughly, 4:1 and 1:1, for the initial assessment of the effect of solvents on the distribution of saccharide fractions with different molecular weights:

- MeOH:H_2O = 4:1 (D475);
- MeOH:H_2O = 1:1 (D478).

The samples obtained were analyzed according to the internal specification corresponding to the pharmacopoeial monograph (**Table 2**).

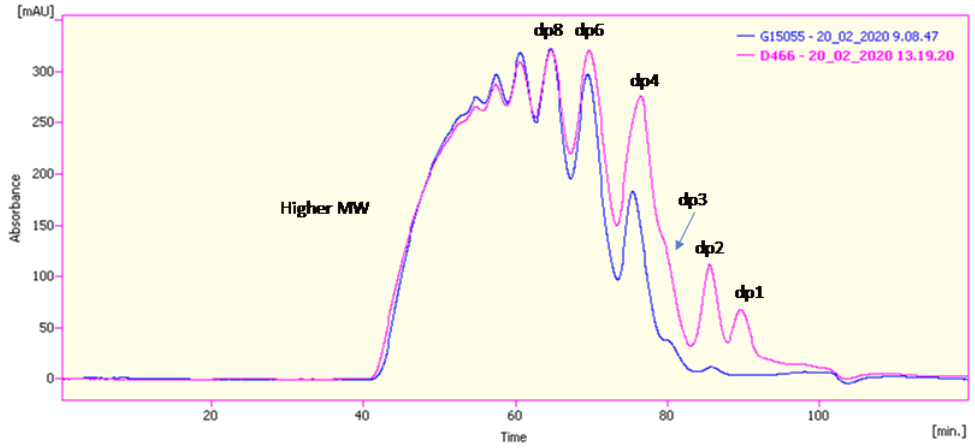
To quantify the effect of the solvent ratio on the composite product, enoxaparin sodium was analyzed by the method of two-dimensional NMR spectroscopy (HSQC) (**Table 3**). In particular, the analysis of the distribution of oligosaccharide fractions was carried out by the SEC method (**Figure 1**).

According to **Table 2**, sample D475 does not meet the requirements of the specification by the "Identification" indicator.

According to a more detailed 2D-NMR analysis, both samples (D475 and D478) represent a high degree of depolymerization, which is evidenced by the low values of normal reduced ANSaRed, MNSaRed residues and high values of 1,6-anhydro ANS/MNS, respectively (**Table 3**). This is the result of the technological parameters of the depolymerization reaction, which obviously needed to be corrected.

Table 2. The results of the analysis of samples of purified enoxaparin sodium with varying solvent ratios according to the specification of JSC Farmak

Parameter	Requirements	D475	D478
Description	A white or almost white powder or crystals	meets	meets
Solubility	Very soluble in water	meets	meets
Loss on drying, %	Not more than 10.0%	8.59	7.52
pH	6.2–7.7	6.31	8.09
Sodium	11.3–13.5	12.4	13.2
Specific absorption	14.0–20.0	18.5	17.1
Residual amounts of organic solvents, ppm	methanol – not more than 0.3% (3000 ppm)	343	13421
	methylene chloride – not more than 0.06% (600 ppm)	0	0
Nitrogen, %	1.5–2.5	1.8	1.8
Molar ratio of sulfate ions to carboxylate ions	not less than 1.8	2.92	5.38
Quantitative analysis	factor Xa activity 90 EU – 125 EU	107.9	104.5
	factor IIa activity 20.0 EU – 35.0 EU	28.1	27.8
	factors Xa/IIa activity ratio 3.3–5.3	3.8	3.8
Identification (the average relative molecular weight and molecular weight distribution)	3800–5000 Da	3978	4239
	<2000 Da 12.0–20.0%	21.9	17.8
	2000–8000 Da 68.0–82.0%	71.1	73.7
Identification (the content of 1,6-anhydro derivatives)	15–25%	23.3	21.8



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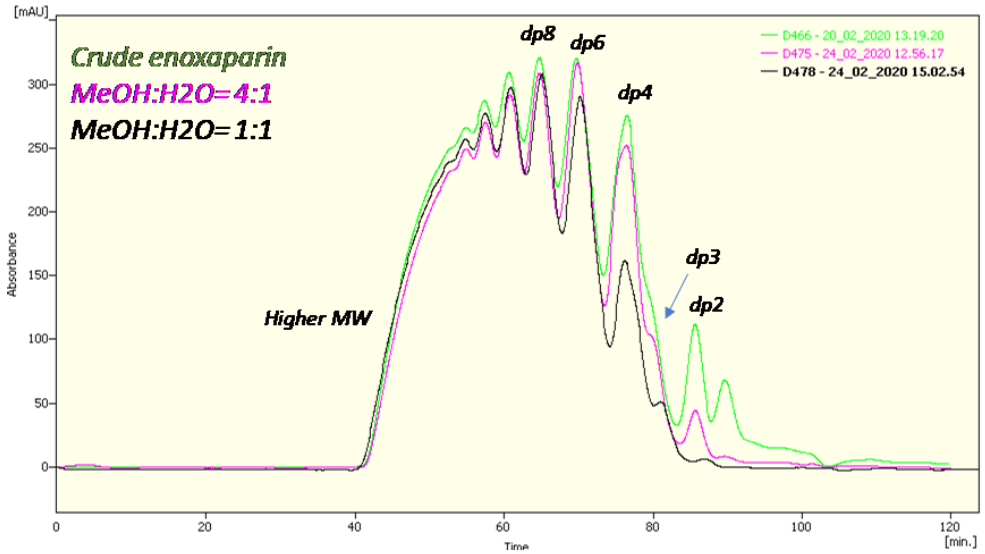

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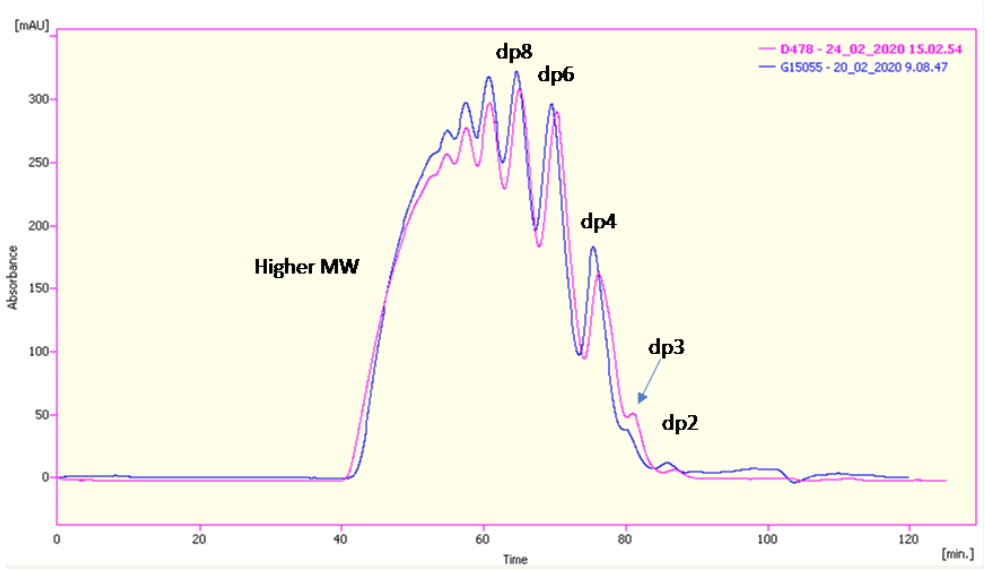

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Figure 1. Distribution profiles of oligosaccharide fractions of treated samples of enoxaparin compared to Clexane®: (**a**) distribution of oligosaccharide fractions of Clexane® (blue) and crude enoxaparin D466 (pink); (**b**) distribution of oligosaccharide fractions of crude enoxaparin (green), purified enoxaparin D475 (pink) and D478 (black); (**c**) distribution of oligosaccharide fractions of Clexane® (blue) and purified enoxaparin D478 (pink)

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Table 3. The results of the analysis of samples of purified enoxaparin sodium with varying amounts of methanol for precipitation by the HSQC method (2D-NMR)

	Crude enoxaparin sodium	MeOH:H_2O 4:1	MeOH:H_2O 1:1	Clexane	
Amines	D466	D475	D478	min	max
ANS,6xaRed	8.4	7.5	7.4	7.8	9.0
ANS,6XbRed	0.9	1.2	0.8	1.0	1.2
ANAc,6xaRed	0.4	0.4	0.4	0.3	0.4
1,6anANS	3.3	3.3	3.0	2.0	2.3
1,6anMNS	3.9	3.5	3.2	2.4	2.5
MNS,6XaRed	2.4	2.2	2.0	2.6	3.0
%A6S	78.7	80.2	80.8	81.8	82.9
Uronic acid					
ΔU42S	20.4	19.0	17.5	17.3	18.1
ΔU4	1.9	1.7	1.5	1.1	1.2
Epox	1.3	1.4	1.4	0.2	0.6
GalA	1.8	1.9	1.9	1.2	1.8
ΔU42S/ΔU	10.7	11.3	11.5	15.7	15.1

Notes:

Abbreviation	Stands for
ANS,6X-αRed	reducing *N*-sulfated-α-*D*-glucosamine
ANS,6XβRed	reducing *N*-sulfated-β-*D*-glucosamine
ANAc,6X-αRed	reducing *N*-acetyl α-*D*-glucosamine
1,6anANS	2-amino-1,6-anhydro-2-deoxy-β-*D*-glucopyranose
1,6anMNS	2-amino-1,6-anhydro-2-deoxy-β-*D*-mannopyranose
MNS,6XαRed	reducing *N*-sulfated-α-*D*-mannosamine
%A6S	*N*-sulfated/acetylated 6-*O*-sulfated α-*D*-glucosamine/mannosamine percent
ΔU42S	2-*O*-sulfo-4-deoxy-α-*L*-threo-hex-4-enopyranosil uronic acid
ΔU4	4-deoxy-α-*L*-threo-hex-4-enopyranosil uronic acid
epox	epoxide residue
GalA	galacturonic acid

The profiles of the distribution of fractions obtained in the samples synthesized demonstrate the dynamics of the distribution of the molecular weight depending on the type of the sample. Thus, crude enoxaparin sodium (D466, **Figure 1a**) coincides with the profile of the originator in the area of high-molecular fragments, while low-molecular residues remain overestimated compared to Clexane®. Reprecipitation with the use of the solvent ratio of MeOH:H_2O = 4:1 (D475, **Figure 1b**) did not give the expected result in reducing low-molecular-weight particles, but the ratio of MeOH:H_2O = 1:1 (D478, **Figure 1c**), on the contrary, showed a significant effect in this area, making this sample as close as possible to the originator.

According to the analyses conducted, the MeOH:H_2O 4:1 ratio option can be immediately excluded from the study, while the 1:1 solvent ratio experiment was repeated after adjusting the parameters of the chemical ß-elimination stage to create a kind of the correct molecular framework.

2. According to the results of the experiment on setting the technological parameters of the depolymerization stage, the optimal parameters of the process were determined:

- the ratio of "alkali/benzyl ester of heparin" – 0.06;
- reaction temperature – 57°C;
- the reaction time – an interval of 1.5–2 hours.

Samples of crude enoxaparin obtained according to these parameters were reprecipitated with the solvent ratio of:

- MeOH:H_2O = 2:1 (D492 and D494);
- MeOH:H_2O = 1:1 (D493 and D495).

The samples obtained were also analyzed according to the specification developed based on the pharmacopoeial monograph. The results and comparison of sample indicators are given in **Table 4**. For these samples, the compositional analysis by the HSQC method (**Table 5**) and the molecular weight distribution by the SEC method (**Figure 2**) were also determined.

The samples of purified enoxaparin obtained were analyzed according to the specifications of JSC Farmak. These samples, as expected, demonstrated compliance with the regulated requirements of the monograph in terms of "Identification" (the average relative molecular weight and molecular weight

Table 4. The results of the analysis of samples of purified enoxaparin sodium with a change in the ratio of solvents according to the specification of JSC Farmak

Parameter	Requirements	MeOH:H_2O 2:1		MeOH:H_2O 1:1	
		D492	D494	D493	D495
Description	A white or almost white powder or crystals	meets	meets	meets	meets
Solubility	Very soluble in water	meets	meets	meets	meets
Loss on drying, %	Not more than 10.0%	6.13	8.71	7.30	6.76
pH	6.2–7.7	7.72	7.23	6.93	7.06
Sodium	11.3–13.5	11.3	11.7	11.8	11.6
Specific absorption	14.0–20.0	17.3	17.1	15.2	15.5
Residual amounts of organic solvents, ppm	methanol – not more than 0.3% (3000 ppm)	583	150	1110	5407
	methylene chloride – not more than 0.06% (600 ppm)	0	0	0	0
Nitrogen, %	1.5–2.5	1.9	2.0	1.9	2.0
Molar ratio of sulfate ions to carboxylate ions	not less than 1.8	2.4	3.5	2.3	2.9
Quantitative analysis	factor Xa activity 90 EU – 125 EU	109.1	101.6	113.7	103.6
	factor IIa activity 20,0 EU – 35,0 EU	29.7	29.7	36.1	35.0
	factors Xa/IIa activity ratio 3.3–5.3	3.7	3.4	3.1	3.0
Identification (the average relative molecular weight and molecular weight distribution)	3800–5000 Da	4553	4625	4880	4905
	< 2000 Da 12.0%–20.0%	18.1	17.0	12.5	12.5
	2000-8000 Da 68.0%–82.0%	70.2	71.0	74.5	74.2
Identification (the content of 1,6-anhydroderivatives)	15–25%	17.2	19.9	15.6	18.7

distribution). According to the results of the compositional analysis, we observe a tendency to decrease the number of residues at the reducing ends of the molecule – ANS/MNSred, 1,6anMNS/ANS and structures at the non-reducing ends of the molecule – ΔU42S, ΔU4, which, however, still does not coincide with the variation ranges of Clexane®.

The analysis of the molecular weight distribution shows that the samples D492 and D494 obtained with the ratio of MeOH:H_2O=2:1 have a larger number of residues with a low-molecular weight than Clexane®. Similarly, high-molecular-weight fragments are more common in D492 and D494 than in Clexane® (**Figure 2**).

Table 5. The results of the analysis of samples of purified enoxaparin sodium with varying amounts of methanol for precipitation by the HSQC method (2D-NMR)

	Crude enoxaparin		MeOH:H_2O 2:1		MeOH:H_2O 1:1		Clexane	
	1.5 h	2 h						
Amines	D484	D485	D492	D494	D493	D495	min	max
ANS,6xaRed	10.1	9.6	9.8	9.0	8.5	8.0	7.8	9.0
ANS,6XbRed	1.3	1.2	1.0	1.2	1.2	1.1	1.0	1.2
ANAc,6xaRed	0.6	0.6	0.5	0.5	0.4	0.4	0.3	0.4
1,6anANS	2.2	2.5	2.0	2.1	1.7	1.9	2.0	2.3
1,6anMNS	2.5	2.7	2.2	2.3	1.9	2.1	2.4	2.5
MNS,6XaRed	2.9	2.7	2.7	2.4	2.4	2.2	2.6	3.0
%A6S	80.4	80.0	80.8	80.3	81.8	81.4	81.8	82.9
Uronic acid								
ΔU42S	19.2	19.2	18.1	17.6	15.7	15.7	17.3	18.1
ΔU4	1.7	1.7	1.5	1.5	1.2	1.2	1.1	1.2
Epox	0.9	0.9	0.9	1.0	0.9	1.0	0.2	0.6
GalA	2.0	1.9	1.9	1.9	1.9	1.8	1.2	1.8
ΔU42S / ΔU	11.3	11.1	11.9	11.8	13.3	12.7	15.7	15.1

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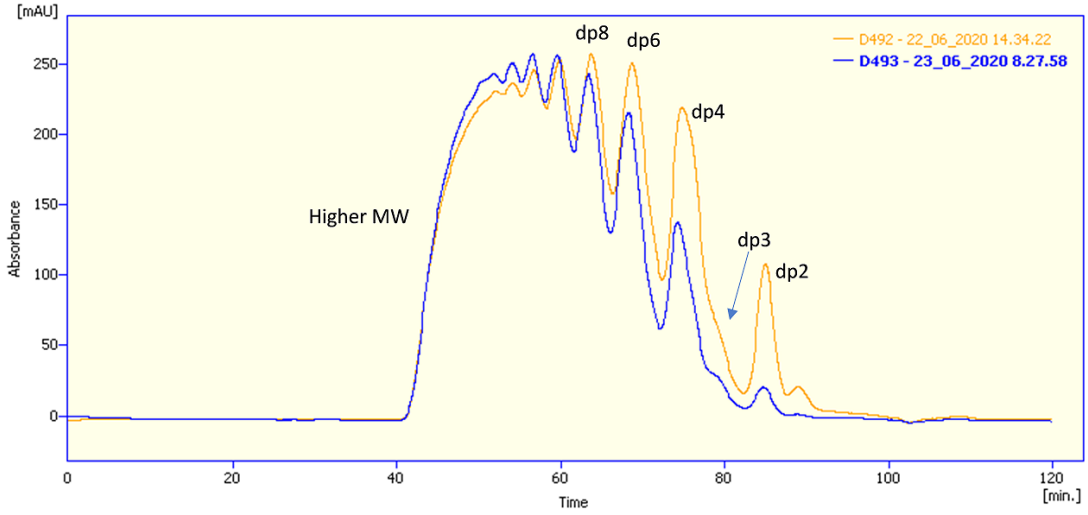

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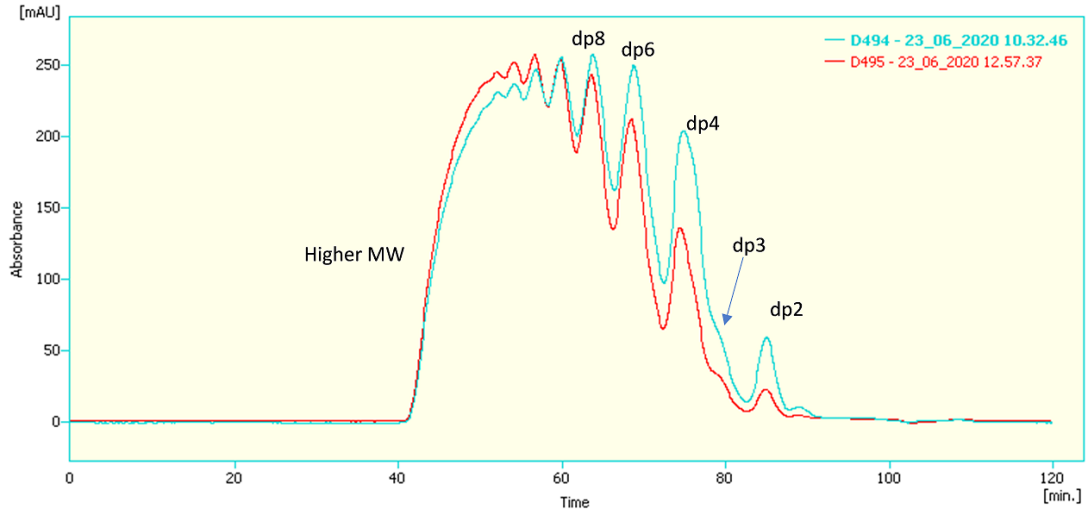

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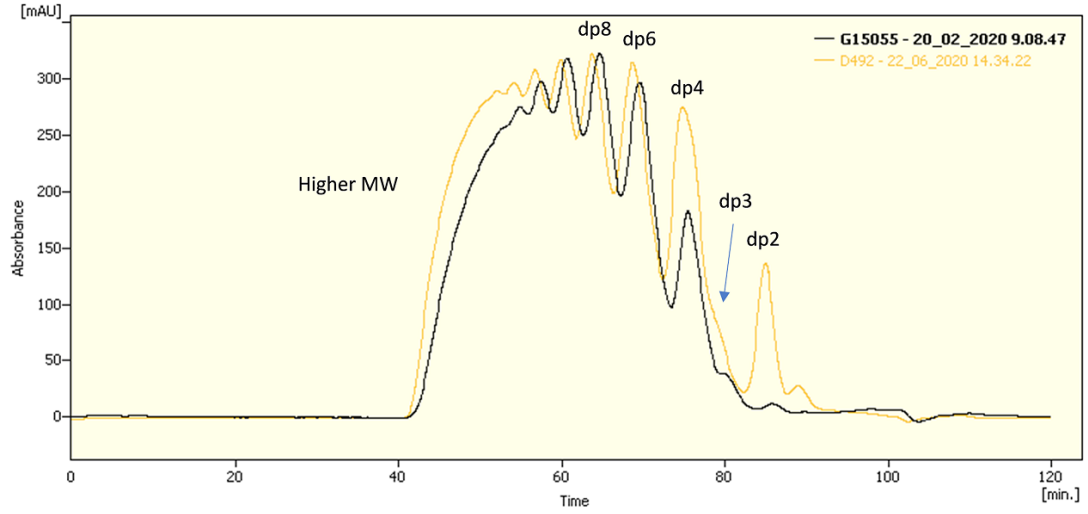

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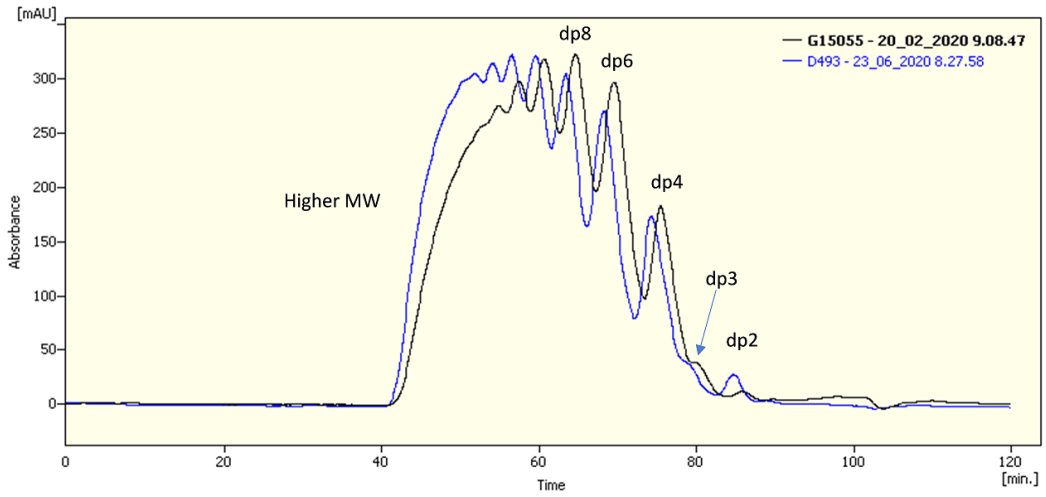
Figure 2. Distribution profiles of oligosaccharide fractions of treated samples of purified enoxaparin compared to Clexane®: (**d**) distribution of oligosaccharide fractions of purified enoxaparin D492 (yellow) and D493 (blue); (**e**) distribution of oligosaccharide fractions of purified enoxaparin D494 (blue) and D495 (red); (**f**) distribution of oligosaccharide fractions of Clexane® (black) and D492 (yellow); (**g**) distribution of oligosaccharide fractions of Clexane® (black) and D493 (blue); (**h**) distribution of oligosaccharide fractions of Clexane® (black) and D494 (blue); (**i**) distribution of oligosaccharide fractions Clexane® (black) and D495 (red) (see on the next page)



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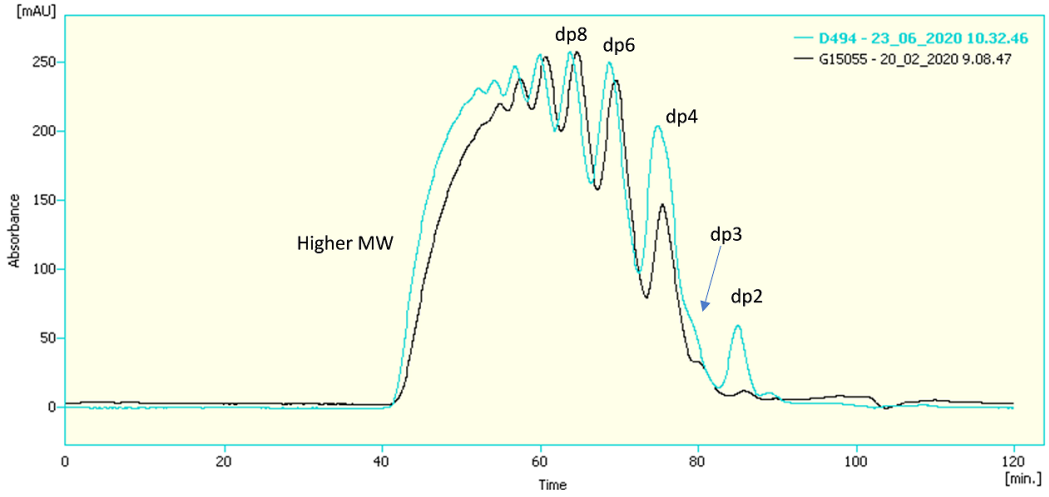

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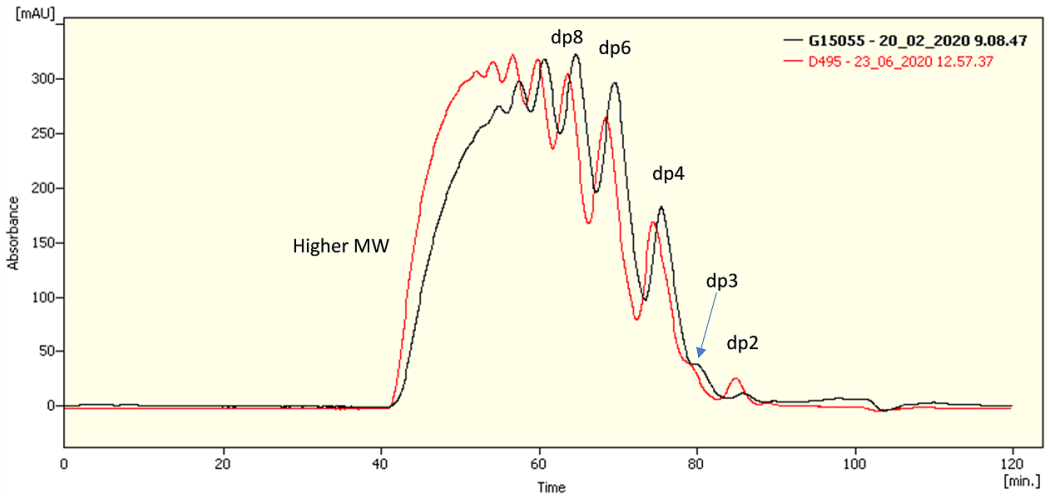

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Figure 2. Distribution profiles of oligosaccharide fractions of treated samples of purified enoxaparin compared to Clexane®: (**d**) distribution of oligosaccharide fractions of purified enoxaparin D492 (yellow) and D493 (blue); (**e**) distribution of oligosaccharide fractions of purified enoxaparin D494 (blue) and D495 (red); (**f**) distribution of oligosaccharide fractions of Clexane® (black) and D492 (yellow); (**g**) distribution of oligosaccharide fractions of Clexane® (black) and D493 (blue); (**h**) distribution of oligosaccharide fractions of Clexane® (black) and D494 (blue); (**i**) distribution of oligosaccharide fractions Clexane® (black) and D495 (red)

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Table 6. Calculation of the E-factor of the purification stage of crude enoxaparin sodium considering the solvent regeneration

Materials	Quantity of materials, kg		Product yield, kg	E-factor	E-factor that takes into account the regeneration
	Without the solvent regeneration	With the solvent regeneration			
Crude enoxaparin sodium	0.1	0.1	0.08	14	5.25
Sodium chloride	0.1	0.1			
Methanol	1.0	0.3			
	1.2	0.5			

Samples D493 and D495 (**Figures 2g,i**) show a profile similar to Clexane® in the range of short oligomers, indicating that the ratio of MeOH:H_2O=1:1 allows better control of the number of low molecular weight oligomers. However, the intensity of the high-molecular range is higher than that of Clexane®. A decrease in the number of short particles, depending on the amount of methanol, shifts the molecular weight distribution towards high-molecular weight.

The methanol regeneration was envisaged as the greening stage of the synthesis. The regeneration yield was 70%. The calculation of the E-factor considering the regeneration is shown in **Table 6**. The E-factor value obtained without the methanol regeneration is 14. Recalculation of the E-factor considering the methanol regeneration is 5.25.

■ Conclusions

In this experimental study, it was possible to clearly demonstrate the effect of the ratio of solvents at the stage of purification of crude enoxaparin on the number and distribution of oligosaccharide fractions in the molecule. Thus, it has been found that an increase in the amount of methanol for the reprecipitation of enoxaparin provokes a shift in the profile of the molecular weight distribution towards low molecular weight oligosaccharides, respectively, a smaller amount of methanol allows obtaining the profile closest to the originator in the area of low-molecular-weight residues. The ratio of MeOH:H_2O = 1:1 makes it possible to obtain samples that are better comparable in terms of the composition to the ranges of Clexane®, except for the terminal residues. However, during the study, it was found that with the reduction of low-molecular-weight residues, the intensity in the area of high-molecular-weight oligosaccharides increased. Summarizing the results obtained, it can be concluded that the ratio of MeOH:H_2O = 1:1 is acceptable for obtaining a substance close to the original one. As an indicator of the effectiveness of the method of the purification stage of enoxaparin sodium in view of the principles of "green" chemistry, there is an E-factor reduced from 14 to 5.25 by the methanol regeneration.

■ Experimental part

This study was conducted during 2019–2021.

The treated samples of purified enoxaparin sodium were analyzed according to the internal specification developed based on the pharmacopoeial monograph. For detailed structural characterization of enoxaparin sodium samples obtained under different conditions, the analysis was performed by specialists of the Ronzoni Institute (Italy) using the methods of 2D-NMR (heteronuclear single quantum coherence spectroscopy) and size exclusion chromatography (SEC). The results of the analysis were compared with the results of the analysis of the original Clexane® referring to the database formed by the Ronzoni Institute.

Clexane® from Sanofi-Aventis was obtained from commercial suppliers.

All samples were analyzed before the expiration date.

The pH test was determined on a Mettler Toledo Seven compact S220 pH meter (Switzerland) (*Ph. Eur. 2.2.3*), the analysis of loss on drying was performed on a Pol-Eko Aparatura slw 53 (*Ph. Eur. 2.2.32*); nitrogen was analyzed on a Vapodest VAP 30s Gerhardt GmbH Distillation System (*Ph. Eur. 2.5.9*); the analysis of residual amounts of organic solvents was carried out by the head-space gas chromatography method on an Agilent GC 7890B chromatograph (USA), column DB-624, 60m×0.32mm, with a layer thickness of 1.8 µm (*Ph. Eur. 2.2.28, 2.2.46*); specific absorption was measured on a Mettler Toledo UV-5 spectrophotometer (*Ph. Eur. 2.2.25*); identification (the average relative molecular weight and

molecular weight distribution) was performed on a Shimadzu chromatograph (Japan), column X_TSKgel G2000SW (300mm×7.8mm×5μm) with a Viscotec 305 detector, Malvern Instruments LTD (England) (*Ph. Eur. 2.2.30*). The content of 1,6-anhydro derivatives was measured by the LC method on a Metrohm chromatograph (*Ph. Eur. 2.2.29*). The molar ratio of sulfate ions to carboxylate ions was measured on a Seven Compact S230 conductometer (Switzerland) (*Ph. Eur. 2.2.38*).

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A New Method for Studying the Kinetics of the Release of Poorly Soluble API from Solid Oral Dosage Forms on the Example of Quertin®

Abstract

In this paper, it is proposed to consider a new method developed for studying the kinetics of release of substances that are poorly soluble in aqueous media on the example of quercetin. The study object was the drug containing plant bioactive components – Quertin® chewable tablets, 40 mg, 3 blisters, 10 pcs – produced by PJSC SIC "Borshchahivskiy CPP". An Agilent 1290 Infinity II LC System liquid chromatograph with an Agilent 6530 mass selective detector (Agilent Technologies) was used for the analysis. Solubility profiles were studied in accordance with the requirements of the Biopharmaceutical Classification System (BCS). The solubility limit of the substance in the media studied has been determined. A method for the quantitative determination of quercetin in test media in the range of specified concentrations with high sensitivity and selectivity has been developed. The dissolution of Quertin® chewable tablets in 3 different aqueous dissolution media with pH 1.2, pH 4.5 and pH 6.8 was studied, the dissolution profiles were compared, and the f_2 factor was calculated. This factor is a criterion for evaluating the study by comparing dissolution kinetics with *in vivo* results. The results obtained indicate that the approach proposed to studying the kinetics of the release of substances that are sparingly soluble in aqueous solutions allows us to correctly assess the release of such substances in accordance with the requirements of the BCS. The method developed has been validated.

Keywords: quercetin; Biopharmaceutical Classification System; quantitative determination; method development; bioequivalence; bioavailability; solubility; dissolution test

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Новий метод дослідження кінетики вивільнення важкорозчинної АФІ з твердих пероральних лікарських форм на прикладі Квертину®

Анотація

У статті запропоновано розглянути новий розроблений метод дослідження кінетики вивільнення речовин, що погано розчиняються у водних середовищах, на прикладі кверцетину. Об'єктом дослідження був препарат з рослинними біоактивними компонентами Квертин® жувальні таблетки, 40 мг, 3 блістери по 10 шт., виробництва ПАТ НВЦ «Борщагівський ХФЗ». Для аналізу використовували рідинний хроматограф Agilent 1290 Infinity II LC System з мас-селективним детектором Agilent 6530 (Agilent Technologies). Профілі розчинності вивчали відповідно до вимог біофармацевтичної системи класифікації. Визначено межу розчинності речовини в досліджуваних середовищах. Розроблено методику кількісного визначення кверцетину в досліджуваних середовищах у діапазоні заданих концентрацій, яка має високу чутливість і селективність. Досліджено розчинення жувальних таблеток Квертин® у 3 різних водних середовищах з pH 1,2, pH 4,5 та pH 6,8, порівняно профілі розчинення та розраховано коефіцієнт f_2, який є критерієм для оцінки дослідження шляхом порівняння кінетики розчинення з *in vivo* результатами. Отримані результати свідчать про те, що запропонований підхід дозволяє правильно оцінити вивільнення важкорозчинних у воді речовин відповідно до вимог біофармацевтичної системи класифікації. Розроблений метод було валідовано.

Ключові слова: кверцетин; біофармацевтична система класифікації; кількісне визначення; розробка методу; біоеквівалентність; біовейвер; розчинність; випробування на розчинення

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■ Introduction

In vitro studies can be conducted to confirm the equivalence of medicinal products in solid dosage forms of systemic action for oral administration. The decision to register a generic medicinal product without conducting *in vivo* bioequivalence studies based on *in vitro* studies following the international practice is called the "biowaiver" procedure [1, 2].

The biowaiver procedure methodology based on the Biopharmaceutical Classification System (BCS) is intended to reduce *in vivo* bioequivalence studies, i.e., it can be considered as a surrogate for *in vivo* bioequivalence studies. *In vivo* bioequivalence studies may not be conducted if the assumption of *in vivo* equivalence can be justified by satisfactory *in vitro* data [3, 4].

The bioavailability procedure is based on the BCS, which allows all active substances to be divided into four classes according to their solubility in aqueous solutions and their permeability. When the aforementioned indicators are combined with the drug dissolution, the system takes into account three main factors: drug dissolution, biopharmaceutical solubility, and the degree of permeation of the active substance [1].

The study of quercetin solubility is a matter of particular interest since the available sources do not provide an unambiguous and uniform value for the solubility limit of this substance. As a part of the study of the quercetin bioavailability, we developed methods for determining the exact solubility limit [5, 6]. The exact value of this physicochemical parameter appears to be extremely important as it is the basis for further studies of bioequivalence and bioavailability of the drug.

Studies must guarantee immediate release properties and confirm comparability between the drugs tested, i.e., the drug under research and the reference drug must show the similar *in vitro* dissolution under physiologically relevant experimental pH conditions. However, this does not establish an *in vitro*/*in vivo* correlation. The *in vitro* dissolution should be studied within the pH range of 1–6.8 (at least pH 1.2, 4.5, and 6.8). Additional studies may be necessary at pH values, at which the active substance has minimal solubility. The use of any surfactants is not acceptable [1, 3].

Comparative *in vitro* dissolution studies must comply with the current Pharmacopoeial standards. Thus, a detailed description of the experimental conditions and analytical methods, including validation data, should be provided [3].

The development of a new methodology and implementation of a modified procedure for studying the release kinetics within *in vitro* studies for poorly soluble substances may affect the following aspects of the pharmaceutical development:

- reduction of cases of biological and pharmacodynamic non-bioequivalence of generic and branded drugs;
- ensuring a higher level of quality for new and existing generic drugs;
- ensuring a higher level of safety through the in-depth study of adverse effects of existing and new generic drugs;
- reducing the cost of the development of new drugs containing low-soluble substances by partially or entirely eliminating the *in vivo* stage;
- the possibility of creating new generic drugs.

The drug Quertin® ("Research and Production Center "Borshchahivskiy CPP") is an example of the above problem since it contains quercetin, which is characterized by very low solubility in the aqueous medium, and, considering the fact that it belongs to the 4th class according to the BCS classification, the study of its dissolution and release kinetics is a very complex process. We have studied the dissolution limit of quercetin, which now provides the basis for continuing research and implementing the next stage, which includes the study of the drug itself.

The aim of the work is to develop a new approach to the study of the release kinetics of

substances that are poorly soluble in aqueous media, for instance, quercetin, which is a component of Quertin® chewable tablets, 40 mg, and further confirm the results obtained by *in vivo* data.

■ Materials and methods

Materials

One batch of Quertin®, chewable tablets, 40 mg, 3 blisters of 10 pcs manufactured by the "Research and Production Center "Borshchahivskiy CPP" was used as the study object. The quercetin substance (CAS registration number 117-39-5) was used as a standard sample – lemon-yellow crystals, slightly soluble in water, diethyl ether, ethanol, chloroform, soluble in acetic acid and alkalis. A laboratory electronic balance (ABT 120-5DM), a pH meter (Starter ST2100, Ohaus), a semi-automatic dissolution testing system "Pharma Test" type PT-DT70 (meets the requirements of the State Pharmacopoeia of Ukraine (SPhU) General Article 2.9.3. "Dissolution Test for Solid Dosage Forms"), an Agilent 1290 Infinity II LC System liquid chromatograph with an Agilent 6530 mass-selective detector (Agilent Technologies), measuring glassware of accuracy class A were applied in the study.

Conditions for the assay by HPLC-MS

For the measurements, an Agilent 1290 liquid chromatograph with an Agilent 6530 TOF mass spectrometry detector, a 50×4.6 mm column filled with a sorbent with a grafted octyl silica gel phase (L1), particle size – 1.7 µm; with thermostatic control (30°C) was used. *Mobile phase A*: 0.1 M trifluoroacetic acid solution degassed in an ultrasonic bath; *mobile phase B*: acetonitrile P; the injection volume – 10.0 µL. The highly selective time-of-flight mass spectrometer had the following settings: the ionization type – electrospray ionization in the positive mode (ESI+); measurement mode – scanning in the mass range of 50–1500; the voltage at the fragmenter – 10 V; the nitrogen temperature – 350°C; the nitrogen flow rate – 10 mL min^{-1}; the nebulizer pressure – 35 psi; the capillary voltage – 4 kV; the elution mode – gradient (**Table 1**).

Table 1. The gradient program

Time, *min*	Mobile phase A, *%*	Mobile phase B, *%*
0	100	0
5	100	0
10	50	50
15	50	50
16	100	0
20	100	0

Preparation of the reference solution

0.6 mg (accurate weight) of thoroughly grounded quercetin was placed in a 100 mL flask, 50 mL of acetonitrile *R* was added, and the mass was dissolved in an ultrasonic bath for 10 min, after which it was diluted to the volume with water *R* and stirred.

The preparation of test solutions is described below in the following section.

The reference solution was prepared simultaneously with test solutions. All solutions were used immediately after preparation.

The methodology for studying the release of quercetin according to the requirements of the SPhU dissolution test.

The *in vitro* dissolution of the tablets was studied in the pH range of 1–6.8 (pH 1.2, 4.5, and 6.8). Additional studies that might be required at pH values at which the active substance had minimal solubility were not conducted. No surfactants were used in the experiment.

For statistical evaluation, we used 6 units of the medicinal product in each study.

The number of test samples for these studies was 6 samples, 1 batch in total. The number of measurements was 156 concentration determinations for two aliquots of each of the three media and 13 sampling time points.

The standard conditions of the study were:

- the medium volume – 1000 mL;
- the rotation speed of the blade – 100 rpm;
- the temperature – 37+0.5°C;
- the dissolution media – (1) buffer solution, pH 1.2; (2) buffer solution, pH 4.5; (3) buffer solution, pH 6.8;
- the sampling timetable – 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39 min.

The sampling was performed with the volume compensation for a dissolution medium.

Methods

2 Tablets of Quertin® chewable tablets (40 mg) were placed in two reservoirs of the Pharma Test dissolution test system type PT-DT70, 1000 mL of an appropriate buffer was added, and the dissolution test was performed under the given conditions. When the sample time was reached, 900 mL of the corresponding buffer was replaced, using an aliquot of the selected 900 mL of the sample for further quantification. The replacement of 900 mL was performed 6 times for each reservoir for even and non-even points of the sampling schedule. The sampling was performed from the center of the reservoir. The volume of

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samples taken was 900.0 mL. This volume was selected experimentally to avoid the sample loss during the analysis.

An aliquot was centrifuged for 10 min at 10,000 rpm. After centrifugation, 1.0 mL of the upper layer was carefully removed, avoiding contact with the agglomerates of the lower layer, and transferred to chromatography vials.

The sample preparation methodology

In compliance with Guideline 42-7.4:2022 [1], the similarity of dissolution profiles was studied in dissolution media, which were buffer solutions with pH 1.2, 4.5, and 6.8. As buffer solutions for the test, the solutions recommended by the SPhU for the dissolution test in accordance with Article 2.9.3 were used [3].

Buffer solution – pH 1.2 (SPhU): 250.0 mL of 0.2 M sodium chloride solution *P* (11.69 g in 1000.0 mL of water *R*) was placed in a 1000 mL volumetric flask, 425.0 mL of 0.2 M hydrochloric acid solution was added, and the solution was diluted to the volume with water *R*.

Buffer solution – pH 4.5 (SPhU): 2.99 g of sodium acetate *R* was dissolved in water *R*; 14.0 mL of 2 M acetic acid solution *R* was added, and the solution was diluted to the volume of 1000.0 mL with water *R*.

Buffer solution – pH 6.8 (SPhU): 250 mL of 0.2 M potassium dihydrogen phosphate solution *R* and 112 mL of 0.1 M sodium hydroxide solution *R* were mixed.

Degassing of all dissolution media was carried out in accordance with the degassing procedure described in Section 2.9.3 of the SPhU [3].

For the analysis, the required volume of the solvent was preliminarily calculated by dividing the dose of quercetin in one tablet – 40 mg – by the value of the solubility limit of quercetin. The solubility limit of quercetin was previously found using the methodology developed by us earlier and then published [5, 6]. This value was 0.0031 mg mL^{-1}. Thus, to determine the release of quercetin in each of the media, 13 L of the solvent were required for 13 solvent changes in the dissolution beakers.

Processing of the data obtained

Excel program (Microsoft Office 2021) was used to calculate the parameters of the classification equations and to draw graphs.

Based on the primary data (peak areas) and calculated parameters (concentrations), graphical dependences of the quercetin concentration values obtained on time were plotted and tabulated.

Results and discussion

Selection of chromatography conditions

The substance quercetin is described in the monograph of the European Pharmacopoeia. This monograph does not provide a chromatographic method for the quantitative determination, or a method for determining the content of impurities in the substance. In connection with the above, we have developed a method for the chromatographic quantitative determination of quercetin in the finished dosage form for the dissolution test since the presence of a complex matrix of excipients can affect the result of quantification. This method uses a gradient type of elution to increase the degree of separation of the substance components with excipients contained in the drug. In addition, the method uses mass-spectrometric detection of the substances, which significantly increases the sensitivity and selectivity of the method. Another advantage of the new method was the use of a chromatographic column with a particle size of 1.7 µm, which made it possible to increase the efficiency of the separated peaks along with sensitivity. All of this allowed determining rather low concentrations of the substances of interest (approximately 0.1 ppm – 1 ppm) without the loss of accuracy, precision, and reproducibility.

Under the conditions we chose, we obtained full ion current chromatograms for samples in three media with pH values of 1.2, 4.5, and 6.8, which were planned to use for the dissolution test (**Figures 1–4**). We used the quercetin substance as a standard sample since the uncertainty of the analysis did not exceed 3%.

The effect of the pH on the dissolution test

The pH-dependent solubility profile of the active substance should be determined and discussed. The active substance is considered highly soluble if the highest single dose of the immediate-release formulation is completely soluble in 250 mL of buffer solutions in the pH range of 1–6.8 at 37±1°C. To prove this, at least three buffer solutions within this range (preferably at pH 1.2, 4.5, and 6.8) and an additional pK_a value if it is within the above pH range should be used. Repeated determinations at each pH value may be necessary to achieve a clear classification of the solubility of the active substance (e.g., by flask shaking or other reasonable method). The pH of each buffer solution should be checked before and after the introduction of the active substance into the buffer solution [1, 3].

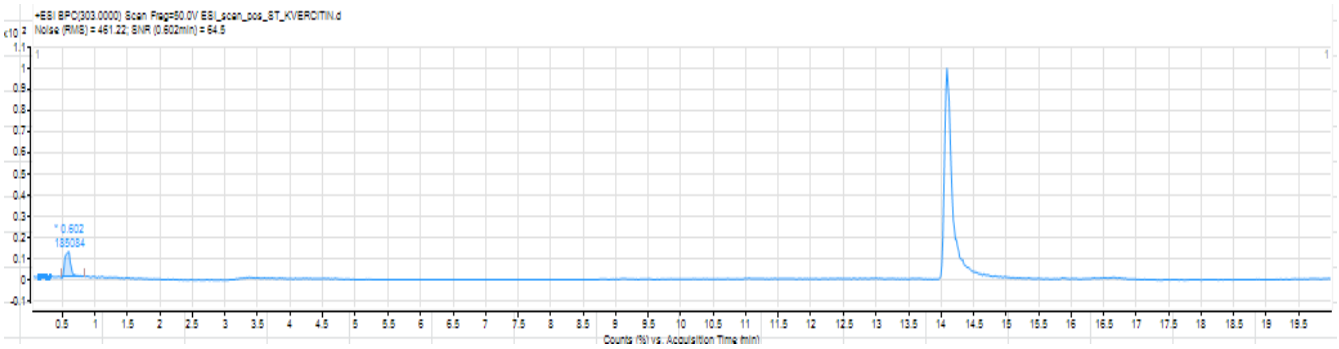

Figure 1. A typical chromatogram of the solution of a standard quercetin sample

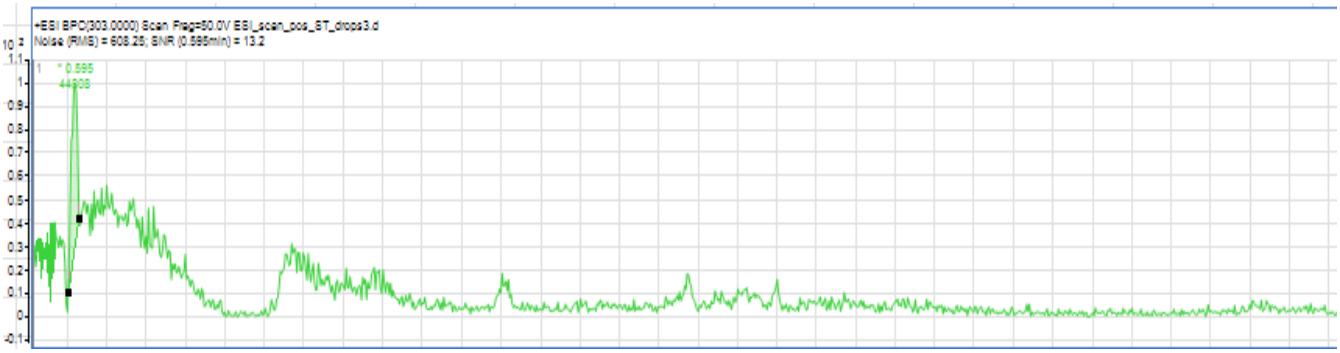

Figure 2. A typical chromatogram of the test solution of Quertin® drug

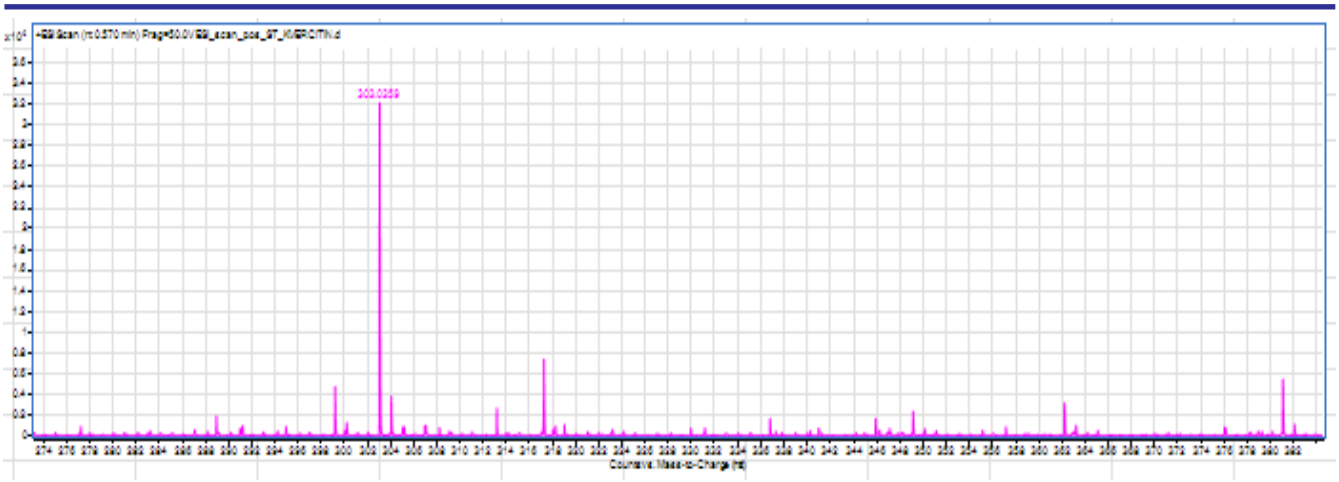

Figure 3. A typical mass-spectrum of the solution of a standard quercetin sample

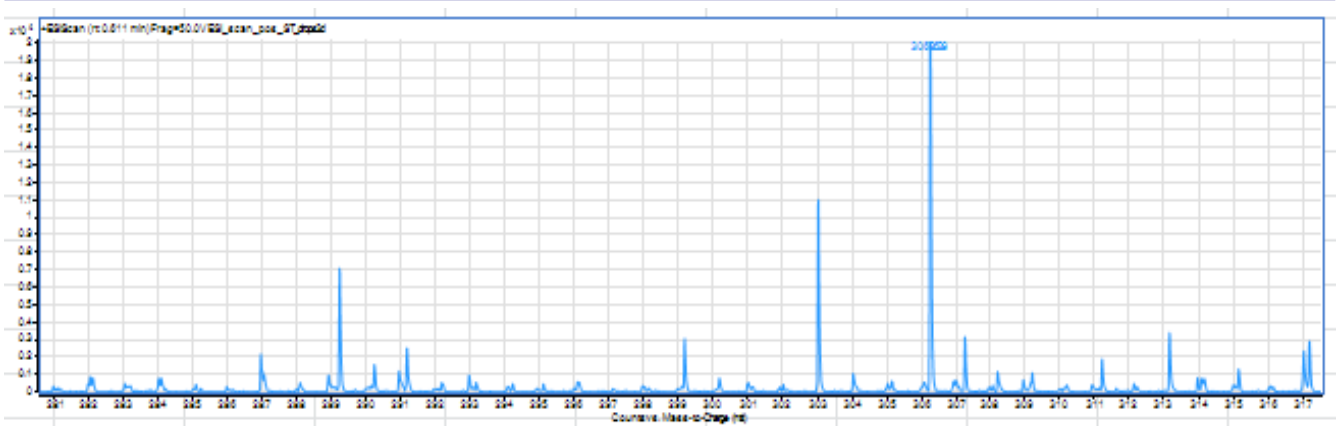

Figure 4. A typical mass spectrum of quercetin in Quertin® drug

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Before studying the dissolution profiles, the pH of the dissolution media to be used for the dissolution test was investigated. The study was conducted for the pH of three buffer solutions under the above conditions for the dissolution test.

The effect of the components of the drugs under study on the pH of the dissolution medium is given below in **Table 2**.

Since the test drug did not significantly affect the pH of the dissolution medium (the deviation of the pH value was less than 0.05), a positive conclusion was made that these dissolution media could be used for the dissolution test.

Results of the dissolution profiles study

After studying the dissolution profiles, the following data were obtained for Quertin® chewable tablets in 3 different aqueous dissolution media with pH 1.2, pH 4.5, and pH 6.8 (**Table 3**).

The degree of the quercetin release into the solution was calculated as the ratio of the amount of quercetin transferred to the solution to the initial sample taken for the study and measured in relative units.

The graphical dependencies (**Figures 5–7**) allow us to conclude that the results obtained meet the BCS requirements for biowaiver results, the relative standard deviation of the first point does not exceed 20%, and the subsequent ones do not exceed 10%. The quercetin content is 85% and is achieved for all media studied in 30 min, which correlates well with the data obtained in the previous studies for the quercetin substance [5, 6] where the content was also achieved in 30 min.

Validation of the methodology developed

To use this methodology for quercetin solubility tests, according to the bioassay scheme for three dissolution media with pH 1.2, 4.5, and 6.8, the method was validated as a quantitative determination method with a maximum uncertainty value of 1.6%. The method was validated by individual validation characteristics: specificity, linearity, convergence, precision, accuracy, and intra-laboratory precision [3].

Table 2. The effect of the components of the drugs under study on the pH of the dissolution medium

Sample No.	pH to dissolution	pH after dissolution	pH to dissolution	pH after dissolution	pH to dissolution	pH after dissolution
1	1.244	1.244	4.540	4.542	6.771	6.782
2		1.246		4.543		6.780
3		1.243		4.542		6.773
4		1.235		4.540		6.779
5		1.245		4.542		6.775
6		1.245		4.540		6.777
7		1.243		4.543		6.780
8		1.244		4.542		6.765
9		1.243		4.544		6.760
10		1.242		4.545		6.760
11		1.243		4.546		6.775
12		1.240		4.546		6.780

Table 3. The concentrations of quercetin obtained in media with pH 1.2, 4.5, and 6.8

Dissolution time, *min*	Dissolution medium – pH 1.2		Dissolution medium – pH 4.5		Dissolution medium – pH 6.8	
	Concentration of quercetin, *%*	RSD, *%*	Concentration of quercetin, *%*	RSD, *%*	Concentration of quercetin, *%*	RSD, *%*
3	7.5	11.4	4.1	17.3	2.7	18.8
6	22.5	6.6	13.8	9.6	10.7	9.8
9	32.5	4.9	22.3	8.2	25	9.4
12	45	7.8	31.2	9.8	33.2	9.2
15	55	9.1	58.0	9.9	51.2	8.9
18	65	6.8	63.9	8.8	63.9	8.9
21	72.5	5	74.7	7.5	71	8.1
24	80	7.1	83.9	7.7	78.5	7.9
27	87.5	4.2	88.6	6.9	82	7.3
30	92.5	5.7	92.2	6.1	88.5	6.9
33	95	4.99	96.1	5.8	95.2	6.1
36	97.5	4.8	98.4	5.5	97.2	6.5
39	100	4.2	100.0	5.1	100	6.1

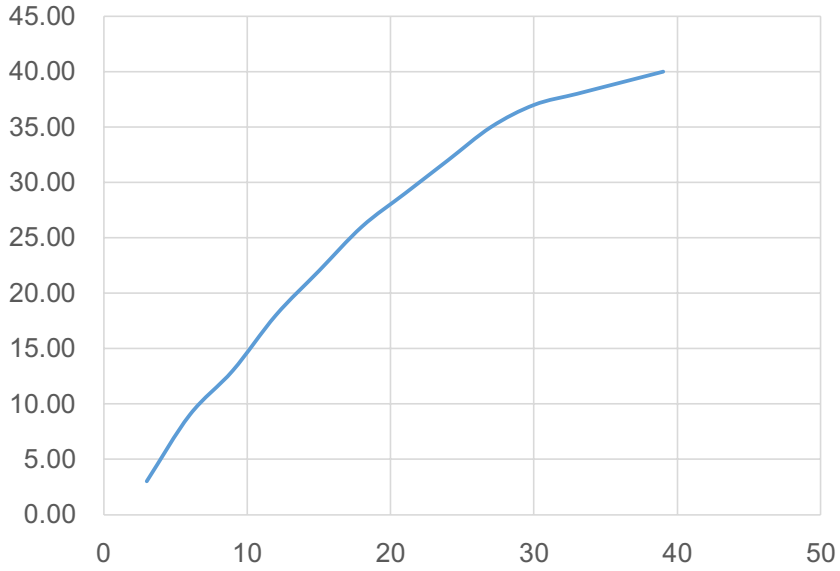

Figure 5. The release graph of quercetin with pH 1.2 for Quertin® chewable tablets

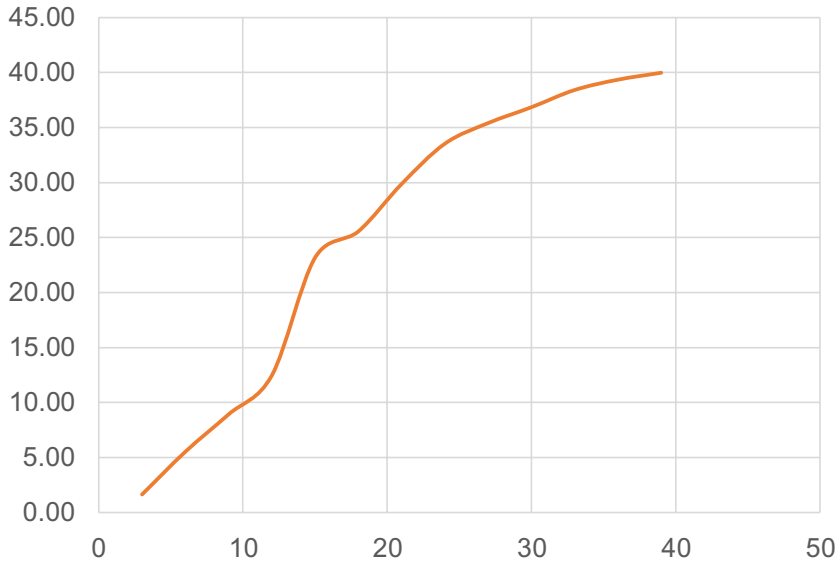

Figure 6. The release graph of quercetin with pH 4.5 for Quertin® chewable tablets

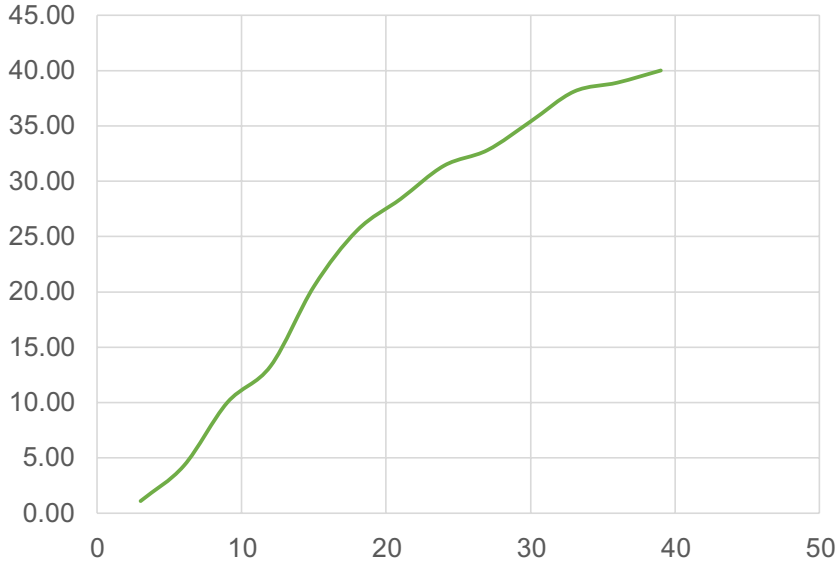

Figure 7. The release graph of quercetin with pH 6.8 for Quertin® chewable tablets

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Since the high-performance liquid chromatography (HPLC) method used in the methodology is specific, it is sufficient to prove that the methodology is specific if all the requirements for the criteria of linearity, accuracy, precision, and intra-laboratory precision are met.

The model solutions for chromatography were prepared according to the analytical procedure described above. The blank solution was prepared similarly to the test solution of the drug.

The linearity was assessed in the range (80–120%) of the method according to the standard method. The nature of the signal dependence on the concentration was studied using 9 model solutions for the analysis with accurate concentration weights: 80, 85, 90, 95, 100, 105, 110, 115 i 120%. At the same time, the concentration taken as 100% was the quercetin concentration, which was in the middle of the range covering the minimum and maximum quercetin concentrations [3].

The results were statistically processed by the least squares method according to the requirements of the SPhU [1]. The calibration graph was constructed in the normalized coordinates (**Figure 8**). The average values of the peak area (S_i) were calculated for each of nine solution samples. The results were processed by the least squares method for the line Y = b*x + a. The calculated statistical values of b, S_b, a, S_a, S_r (final standard deviation) and r (correlation coefficient) are given in **Table 4**.

In our case, the requirements for the linear regression parameters are met over the entire range of the methodology (80–120%).

To measure and calculate the metrological assessment of the accuracy and precision of the method, three peak area values were obtained for the reference solution and 27 peak area values for the model solutions. The actual values, the ratio of the average peak area values for each of 27 solutions to the average peak area value of the reference solution were calculated, obtaining the values $X_i = (S_i/S_{st}) \times 100\%$, $Y_i = (S_i/S_{st}) \times 100\%$, and the value $Z_i = (Y_i/X_i) \times 100\%$ (it is the concentration found as a percentage of the input material). The calculation results are shown in **Tables 5–7**.

Requirements for the maximum allowable RSDP are 1.7%. The calculated value is stored until the measurement results match.

To assess the intra-laboratory precision, the relative confidence interval for 5 parallel determinations of the quantitative content of substances was used, which should be less than the maximum permissible uncertainty of the analysis results: $\Delta z \leq 1.6\%$. Tests were performed using the same batches of the drug by different analysts on the same chromatograph on different days using different measuring dishes.

The intra-laboratory precision was confirmed by the fact that the value of the relative confidence interval for five parallel determinations of one batch of the drug meets the acceptance criterion ($\Delta z = 0.22\% \leq 1.6\%$).

Comparison of the results with the results of *in vivo* studies

To fulfill the task formulated for this study, it was necessary to study the following aspects:

- the first step was to conduct the solubility study and classify the active bioactive substance – quercetin – according to the biopharmaceutical classification system (BCS class); study the solubility limit of a substance in test media;

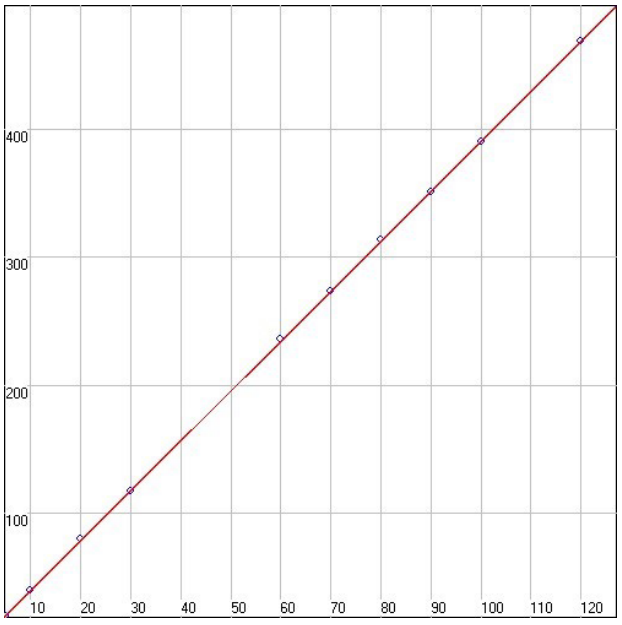

Figure 8. The linear regression of the peak area against the quercetin concentration in the normalized coordinates

Table 4. Linear regression characteristics

Parameter	Value		
b	4		
S_b	0.006		
a	0.8		
S_a	0.4		
S_0	0.7		
S_0/b	| 0.19 |		
S_Y	157.1		
r	| 0.99999 |		
Reference solution	Average S_{St}	C_{St}	RSD_{St}, %
Reference	391.4	100	0.18

Table 5. Variance values

Test solutions	Name	Average S_i	C_i	RSD_i, %
1	5	19.5	5	0.4
2	10	39.8	10	0.2
3	20	79.7	20	0.09
4	30	117.7	30	0.06
5	60	236.3	60	0.03
6	70	273.6	70	0.026
7	80	313.7	80	0.02
8	90	351.5	90	0.02
9	100	390.9	100	0.018
10	120	469.2	120	0.015

Note: Student t-test (95, 1, 11) = 1.7956

Table 6. Results of the analysis of model solutions and statistical processing

Test solutions	Name	Average S_i	C_i	Y_i	X_i	RSD_i, %	Z_i, %
1	5	19.5	5	4.9	5.00	0.4	99.4
2	10	39.8	10	10.2	10.00	0.18	101.5
3	20	79.7	20	20.3	20.00	0.09	101.7
4	30	117.7	30	30.1	30.00	0.06	100.2
5	60	236.3	60	60.3	60.00	0.03	100.6
6	70	273.6	70	69.9	70.00	0.03	99.8
7	80	313.7	80	80.11	80.00	0.02	100.1
8	90	351.5	90	89.8	90.00	0.02	99.8
9	100	390.9	100	99.8	100.00	0.02	99.8
10	120	469.2	120	119.8	120.00	0.02	99.9

Table 7. The accuracy and correctness parameters obtained

Parameter	Name	Value	Requirements 1	Requirements 2	Conclusions
Precision	Δ_Z	1.4	≤ 3		meets
Accuracy	$\lvert Z_{cp} - 100 \rvert$	| 0.29 |	≤ 0.45	≤ 0.96	meet for the 1st criteria

Note: S_z (%) = 0.78103; Student t-test (95, 1, 9) = 1.83310

- as the second step, the development of a method for the quantitative determination of quercetin in test media in a range of specified concentrations with a high sensitivity and selectivity;
- the study of the dissolution of Quertin® chewable tablets in 3 different aqueous dissolution media with pH 1.2, pH 4.5 and pH 6.8, comparison of dissolution profiles and calculation of the f_2 factor, which is the criterion for evaluating the study by comparing dissolution kinetics with *in vivo* results as a finalizing step.

The chemical formula shows that quercetin is an aglycone without a carbohydrate group, which determines its chemical and pharmaceutical properties. A quercetin glycoside is formed by the addition of glucose, rhamnose, or rutinose moieties, which replace one of the hydroxyl groups in its structure, usually in position 3, thus forming a glycosidic bond [7]. This significantly affects the solubility and absorption of quercetin *in vivo* [8]. Hence, the regularity is that the presence of a carbohydrate molecule in the structure of a quercetin glycoside contributes to its better solubility in water compared to a quercetin aglycone [9]. Usually, the term "quercetin" refers only to the aglycone; in medical research, this term is used to define the glycoside molecule of quercetin.

The study of pharmacokinetic properties of quercetin *in vivo* and its complex with pectin (Quertin® chewable tablets, 40 mg) was performed in 8 outbred rabbits of both sexes weighing 2500-3000 g. The rabbits were divided into 2 experimental groups of 4 animals each: *Group 1* – rabbits receiving oral quercetin in the dose of 10.0 mg kg^{-1}; *Group 2* – rabbits receiving an oral combination of quercetin and pectin in the dose of 10.0 mg kg^{-1} for quercetin. As a bioanalytical method for determining the concentration of quercetin and its metabolites (methoxy-, sulfate- and/or glucuronic conjugates) in biological samples, ultra-performance liquid chromatography

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Table 8. Average values of the quercetin (*Q*) and isorhamnetin (*Ir*) content

The study object		The time of blood sampling, *hours*						
		0	0.25	0.5	1.0	2.0	4.0	8.0
Quercetin (substance)	*Q*	<25.0	324.3	279.3	257.8	176.9	121.1	104.3
	Ir	<25.0	122.8	103.4	92.2	85.1	66.8	36.6
	Sum	<25.0	441.7	378.1	345.9	258.3	184.9	139.3

with a mass-selective detector was used. The total concentration of quercetin and its metabolites in the blood plasma was expressed as a pure quercetin (**Table 8**).

The results of the study illustrate that the intragastric administration of quercetin contributed to the appearance and fluctuation of the concentration of the active substance in the blood of experimental animals of both groups.

When analyzing the average values, it was found that the peak concentrations of quercetin and its metabolites were formed in the plasma of animals during the first 25 min and then gradually decreased. Based on the calculations of the mean values of the quercetin concentration, pharmacokinetic curves were constructed, reflecting the dependence of the total content of quercetin and its metabolites (isorhamnetin) in the blood serum of experimental animals on time.

Thus, the results obtained allow us to characterize the degree of the quercetin release within *in vivo* tests by the nature of pharmacokinetic dependence on time.

Using the similarity factor f_2 calculated as described below, the similarity of the dissolution profiles was found from the *in vivo* studies and *in vitro* dissolution profiles obtained for Quertin® chewable tablets, 40 mg, in this study:

$$f_2 = 50 \cdot \log\left[\frac{100}{\sqrt{1+\frac{\sum_{t=1}^{t=n}[R(t)-T(t)]^2}{n}}}\right]$$

Since the calculated value of the similarity factor f_2 amounted to 58, the dissolution profiles were considered similar since the similarity factor $f_2 \geq 50$.

The HPLC method with mass-spectrometric detection will always have limitations related to the design features of the equipment used, such as the sensitivity of the detector, the linear range of concentrations measured, and its ability to detect the substance under research in the sample. Such studies also require the use of a standard sample of the test substance, which can complicate and increase the cost of the study.

First, there is an interest in studying the dissolution profiles, according to the conditions of the method, for other substances that are limitedly soluble. The implementation of the method developed will allow us to study the *in vitro* kinetics when studying the bioavailability of drugs that the BSC classifies as *Class 4*. This will necessarily lead to a reduction in the cost of the process of developing new drugs due to the partial or complete exclusion of the *in vivo* stage.

■ Conclusions

The solubility profiles of quercetin in 3 different aqueous dissolution media with pH 1.2, pH 4.5 and pH 6.8 were obtained for the first time for the drug Quertin® chewable tablets, 40 mg, in compliance with the BCS requirements.

The results indicate that the approach proposed to studying the kinetics of the release of substances that are sparingly soluble in aqueous solutions allows us to correctly assess the release of such substances in accordance with the BCS requirements.

The studies have shown that the *in vitro* dissolution profiles are in good agreement with the results of pharmacokinetic studies of quercetin release *in vivo*. Since the same drug is used for the studies, this confirms the correctness of the *in vitro* results obtained by the methodology developed.

In the process of validation of the method for the quantitative determination of quercetin in Quertin® chewable tablets, 40 mg, the variational characteristics of the method by the standard method were studied: accuracy, linearity, precision, specificity, and intra-laboratory precision. The variational characteristics of the method do not exceed the critical error value (1.6%) and are characterized by qualitative analytical parameters. This method can be correctly reproduced in laboratories and does not depend on excipients.

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Original Research

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