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Application of liquid chromatography in defining the interaction of newly synthesized chalcones and related compounds with human serum albumin

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Abstract: Defining the interaction of newly synthesized compounds with plasma proteins is an important step in the drug development process. Chromatographic techniques can be successfully used in predicting the biopharmaceutical and pharmacokinetic properties of newly synthesized compounds. The aim of this study is to investigate and isolate the most important molecular properties that affect the interaction of 20 newly synthesized chalcones and commercial compounds (lopinavir, ritonavir, darunavir and ivermectin) with human serum albumin (HSA). The retention behaviour of the selected compounds was tested on a CHIRALPAK®HSA column. A mixture of phosphate buffer (pH 7.0) and isopropanol (80:20 volume ratio) was used as the mobile phase, and the support vector method was used to form the quantitative structure retention relationship (QSRR) model. Based on the obtained values of retention parameters, it was observed that halogenated derivatives show the strongest, and methylated chalcone derivatives the weakest interaction with HSA. By correlating the retention and physicochemical properties of the tested compounds, it was shown that the structural (SDSCH) and electronic properties (MAXQ, EEM_F1) groups have the greatest influence on the retention behaviour and the interaction of the tested compounds with HSA. The obtained QSRR model can be applied in the prediction of the retention characteristics of new, structurally related chalcone derivatives on HSA stationary phase.

Keywords: high performance affinity chromatography; support vector method; quantitative structure retention relationship; chalcone; human serum albumin.

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INTRODUCTION

Chromatographic techniques can be successfully applied in predicting and defining basic biopharmaceutical and pharmacokinetic properties in the first stages of discovery and development of new medicinal substances. Chromatographic techniques can be used along with *in silico* predictions and other *in vitro* studies. High performance affinity chromatography (HPAC) is often used in order to more precisely define the interaction of drugs with plasma proteins. HPAC enables precise and less time-consuming analysis of the sample. For these purposes, a phase modified with human serum albumin (HSA) is used as a stationary phase. The mobile phase simulates the physiological composition of blood plasma and enables satisfactory retention behaviour of the tested compounds.[1–3]

Serum albumins are the main soluble proteins of the blood. They make up 55 % of total plasma proteins and have numerous physiological functions: maintenance of osmotic pressure in the blood, transport of various endogenous molecules, various xenobiotics (medicines), *etc*. It is known that the interaction of drugs with HSA significantly affects the volume of distribution and the rate of their elimination. The volume of distribution, free concentration and metabolism of medicinal substances can be significantly changed depending on the value of the binding constant for HSA, which is important from the aspect of drug safety and efficacy. Therefore, the characterization of the drug-HSA interaction in the first stages of the development of new drugs affects the further optimization of the structural and physicochemical characteristics of the newly synthesized compounds. HSA is a protein consisting of three structurally similar domains I–III. Each domain of this protein consists of two subdomains (A and B) stabilized by 17 disulfide bridges.[1,4]

Chalcones are occurring in natural plant products, but they are today mostly obtained by semi- or total synthesis using the aldol condensation reaction (Claisen-Schmidt). Chemically, chalcones are 1,3-diphenylprop-2-en-1-ones, in which two aromatic rings are connected by α,β-unsaturated carbonyl system. In nature, chalcones are precursors in the biosynthesis of flavones, flavanones and chromanones and play an important role in protecting plants from ultraviolet radiation, pathogens and insects. Chalcones have been shown to have a wide range of biological activities such as: antituberculotic, antityrosine kinase, antiproliferative, anti-HIV-1-protease, antimicrobial and antioxidant activities.[5]

Previous studies have shown that chalcones, thanks to the presence of α,β--unsaturated carbonyl chain in the molecule, react with amino groups of enzymes and other proteins (Michael's addition). In this reaction, the enone functional group is as an electron acceptor. Michael's addition is facilitated by the presence of electron acceptor groups in the B ring of the chalcone, while the presence of electron donor groups at the same position hinders this reaction. This could be one of the assumed mechanisms of interaction with plasma proteins.[6,7]

A literature review showed that more detailed studies of the interaction of chalcone with plasma proteins at the molecular level have not been done so far.

In this regard, the aim of this study was to examine the retention behaviour of selected newly synthesized chalcones and related compounds, which show anti-HIV-1-protease activity, on a stationary phase modified with HSA and to use the quantitative structure (chromatographic) retention relationships (QSRR) method to extract the most important structural features that affect the binding of the tested compounds to HSA.[8]

EXPERIMENTAL

Chemicals and reagents

The tested chalcones were synthesized at the Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Belgrade, Serbia, while the commercial compounds are lopinavir, ritonavir, darunavir and ivermectin produced by Sigma Aldrich.[8] The chemical structures of the tested compounds are shown in Figs. 1 and 2.

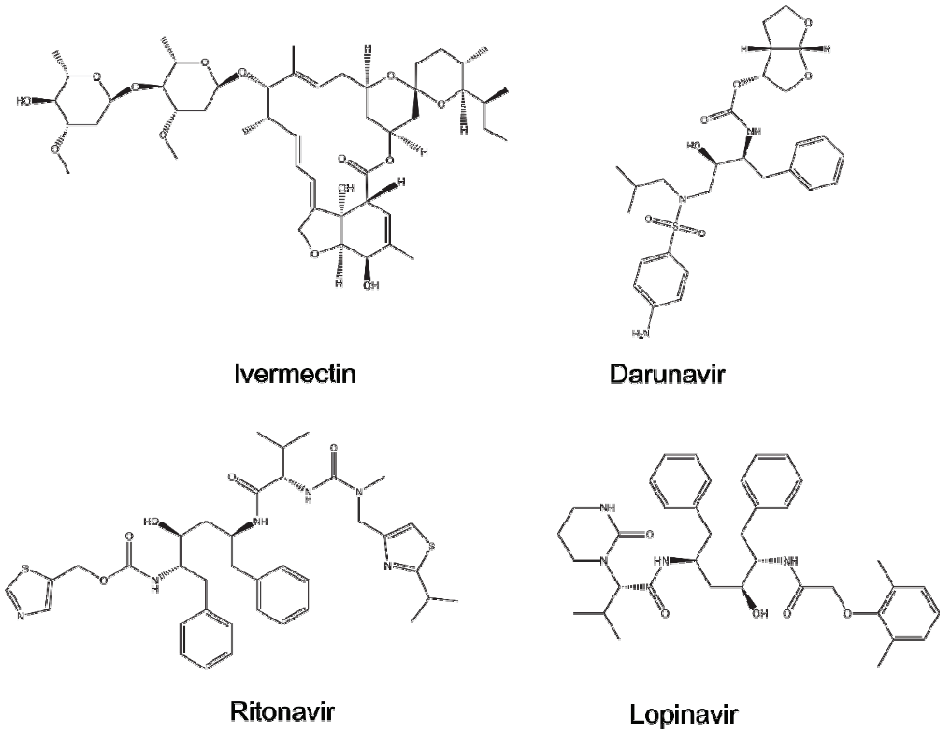

Fig. 1. Chemical structures of tested commercial compounds.

The following chemicals were used during this study: 2-propanol (J.T. Baker, Deventer, Netherlands) HPLC grade; deionized water (TKA water purification system, Niederelbert, Germany); potassium dihydrogen phosphate (Merck); disodium hydrogen phosphate (Merck).

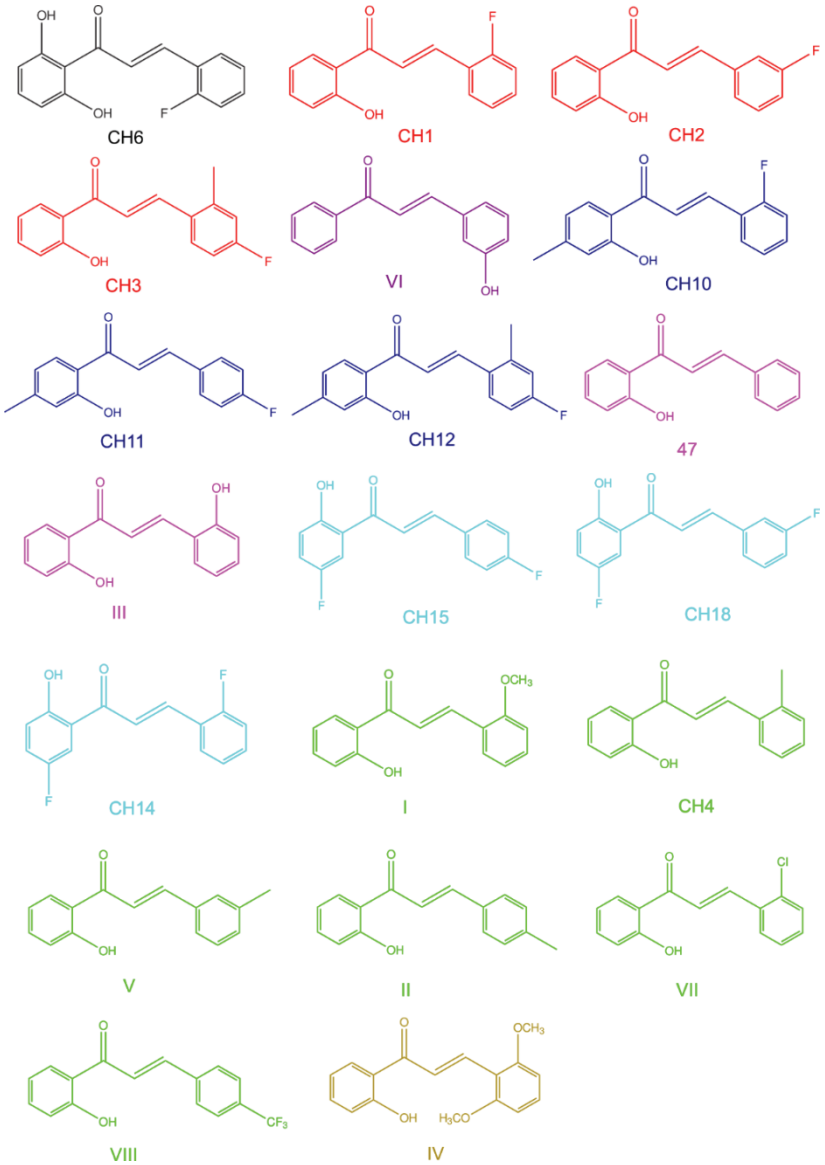

Fig. 2. Chemical structures of tested chalcones. Different colors of the compounds represent different structural groups.

Chromatographic conditions

HPLC analysis was performed on Agilent Technologies 1200 HPLC chromatograph (Santa Clara, CA, USA) at 25 °C. The retention behaviour of selected compounds was examined on CHIRALPAK®HSA column 150 mm×4 mm I.D. packed with HSA chemically bound to silica particles size of 5 µm (Daicel corporation, Illkirch-Graffenstaden, France). The mobile phase consisted of 80 vol. % phosphate buffer (pH 7.0; 0.01 M) and 20 vol. % isopropyl

alcohol. The flow rate was 0.5 mL min^{-1}. UV detection was performed at 254 nm. Standard solutions of the investigated compounds were prepared in methanol at a concentration of 1 mg mL^{-1} and then diluted with the mobile phase to the final concentration of 0.1 mg mL^{-1}. The retention characteristics of the selected chalcones are defined based on the value of the logarithm of the retention factor (log k), log k = log $((t_r–t_0)/t_0)$, where t_0 is the dead time of the column and t_r is retention time.

The HPLC conditions were selected with the aim of achieving satisfactory retention characteristics of all tested compounds which show a strong interaction with HSA column. At low proportions of modifiers in the mobile phase (φ < 20 %), the elution of all tested compounds was not achieved. The analysis is qualitative with the aim of indirect evaluation of the interaction of the tested compounds with HSA through the obtained retention time (t_r) values. Through the QSRR modelling approach, the impact of differences in the structural characteristics of the tested compounds on their HSA interaction was also evaluated. Each compound was analysed individually to clearly define its t_r value. Due to the individual qualitative interpretation of the HSA-interaction for each compound, the simultaneous chromatographic analysis of all 24 compounds was not performed. Common chromatographic conditions were defined, in order to use QSRR analysis to select the common physicochemical properties of compounds that affect their chromatographic behaviour, as well as to determine a reliable correlation with *in silico* binding to HSA. The defined conditions could be applied for the further analysis of newly synthesized chalcones and related compounds.

Calculation of molecular descriptors

Using the Marvin Sketch 6.1.0, (Chem Axon) the dominant molecular and tautomeric forms of the investigated compounds were selected at pH 7.00.[9] The tested molecular forms were brought to the minimum energy state by the optimization process using the semi empirical PM3 and HartreeFock/3-21G methods, in the Gaussian 09 (ChemBio3D Ultra 13.0).[10,11]

For all tested compounds, the molecular descriptors were calculated using the ADMET Predictor 9.5 (Simulation Plus Inc., 2019) and the SWISS ADME database.[12,13] Descriptors with the greatest influence on the dependent variable (log k) were used in Support Vector Machine (SVM)-QSRR modelling performed in the Statistica (StatSoft Inc., version 13.6, 2019).[14] In SVM-QSRR modelling, log k was used as a dependent variable, while the extracted descriptors were used as independent variables.

QSRR analysis included 21 compounds. Due to strong interaction with the stationary phase, compounds VII, VIII and CH6 (Fig. 2) were not included in the QSRR modelling (log k > 1.80). The final training set for QSRR modelling included 16 compounds (47, CH10, CH12, CH14, CH18, CH2, CH3, CH4, darunavir, I, III, IV, ivermectin, ritonavir, V and VI), while the test set contained 5 most important representative compounds (CH1, CH11, CH15, II and lopinavir).

Model validation

The quality of the obtained models was assessed by calculating internal validation parameters (correlation coefficient (r), root mean square error of estimation (*RMSEE*), squared regression coefficient of cross-validation (Q^2_{LOO})) and external validation parameters (r^2(pred), root mean square error of prediction (*RMSEP*)). QSRR models with Q^2_{LOO} and r^2(pred) ≥ 0.5 are considered to have good predictive ability. Additionally, $r^2_{metrics}$ (r^2_m, r'^2_m, $\bar{r}^2_m$, and Δr^2_m) evaluation was applied. This metric is an additional external validation parameter, calculated based on the correlations between the observed and predicted values with

(r^2) and without (r^2_0) intercept for the least squares regression lines, as shown in the following equation (1):

$$r^2_m = r^2\left(1-\sqrt{r^2-r^2_0}\right) \quad (1)$$

The metric r^2_m does not consider the differences between individual responses and the training set mean and thus avoids overestimation of the quality of prediction due to a wide response range (*Y*-range). For a good predictive QSRR model, $\overline{r^2_m}$, r^2_m and $r'^2_m \geq 0.50$, while $\Delta r^2_m \leq 0.20$.[15-17]

RESULTS AND DISCUSSION

The degree of binding of the investigated compounds (chalcones and related compounds) to HSA was evaluated based on their retention behaviour on the stationary phase modified with HSA. The calculated logarithmic values of retention factors (log*k*) were used to indirectly evaluate and compare the drug-HAS interaction.

Classification of interaction of investigated compounds with HSA

The results of the retention behaviour of the tested compounds are shown graphically in Fig. 3.

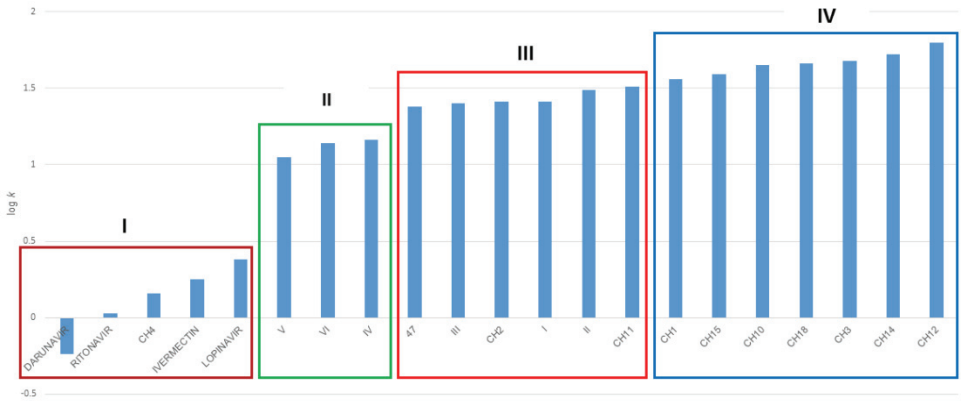

Fig. 3. Obtained values of log *k* of the tested compounds – Group I (log *k* < 0.50), Group II (log*k* 1.00–1.20); Group III (log*k* 1.30–1.50); Group IV (log *k* 1.50–1.50).

The strongest interaction with HSA is achieved by compounds CH6, VII and VIII (log*k* > 1.80), while the rest of the compounds are divided into four categories I–IV according to the length of retention on HSA (based on the value of the log *k* parameter), Fig. 3.

In Group IV, there are compounds with higher values of retention factors and higher binding affinity for HSA (CH1, CH15, CH10, CH18, CH3, CH14 and CH12). Compounds belonging to group I (commercially available antivirals) have the lowest values of retention factors, where the interaction ratio with HSA

(log *k*(lopinavir) > log *k*(ivermectin) > log *k*(ritonavir) > log*k* (darunavir)) is in agreement with the experimentally available data on their total binding to plasma proteins.[18–20]

Based on the obtained values of the retention factors, it is observed that there is a difference in the interaction of the examined compounds with HSA depending on the structural characteristics, *i.e.*, types of substituents in rings A and B. Halogenated derivatives show the strongest HSA-interaction, phenolic and methoxy derivatives show a weaker interaction, while methylated derivatives show the weakest interaction with HSA. From a chemical point of view, this would mean that compounds containing groups with electronegative atoms (fluorine, chlorine, oxygen) show better interaction compared to compounds containing alkyl groups.

The way in which the different type of substituent affects the retention behaviour can be seen on the example of compounds that are *ortho* substituted in ring B (Fig. 4, marked in blue), *e.g.*, log *k*(CH4, methyl substituted) < log *k*(III, hydroxyl (phenolic group) substituted) < log *k*(I, methoxy substituted) < log *k*(CH1, fluoro substituted) < log *k*(VII, chloro substituted) (Fig. 5a).

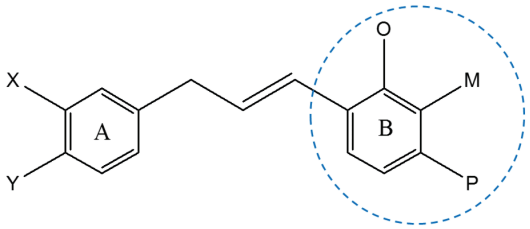

Fig. 4. Basic chemical structure of newly synthesized chalcones.

A similar sequence is also observed with *meta* and *para* structurally related derivatives:

– log *k*(V, methyl substituted) < log *k*(VI, substituted with a phenolic group) < log *k*(CH2, fluoro substituted);

– log *k*(II, methyl substituted) < log *k*(VIII, trifluoromethyl substituted).

Differences in retention behaviour can also be observed in positional isomers, which indicates the importance of geometry and steric effects of molecules during interaction with HSA (Fig. 5b):

– in the case of isomers that have a methyl substituent in ring B, the order is *ortho* < *meta* < *para*, *e.g.*, log *k*(CH4) < log *k*(V) < log *k*(II) (Fig. 5b-1);

– in the case of isomers that have a fluoro substituent in ring B, the order is *para* < *meta* < *ortho*, *e.g.*, log *k* (CH15) < log *k* (CH18) < log *k*(CH14) (Fig. 5b-2).

Compounds CH1 and CH2 differ from each other in the position of the fluoro group in ring B. By introducing an additional methyl group into ring B, (compound CH3), the interaction with HSA increases, so that log *k*(CH2, *meta*-

-fluoro) < log *k*(CH1, *ortho*-fluoro) < log *k*(CH3, *para*-fluoro, *ortho*-methyl) (Fig. 6a).

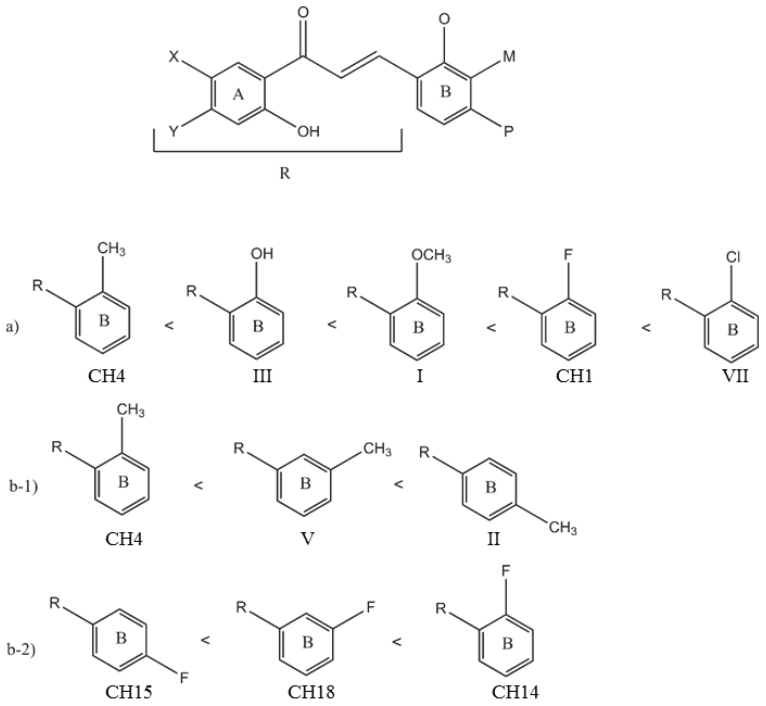

Fig. 5. Comparative presentation of compounds with the influence of: a) different substituent on retention behavior and b) different position of the same substituent (b-1 methylated derivatives, b-2 fluorinated derivatives).

For all investigated compounds from the chalcone group, it was shown that the introduction of an additional X/Y substituent in ring A leads to an increase in the interaction with HSA:

– log *k*(CH3) < log *k*(CH12, methyl substituent in position Y);

– log *k*(CH2) < log *k*(CH18, fluorine atom in position X);

– log *k*(CH11, methyl substituent in position Y) < log *k*(CH15, fluorine atom in position X);

– log *k*(CH1) < log *k*(CH10, methyl substituent in position Y) < log *k*(CH14, fluorine atom in position X) (Fig. 6b).

There is a satisfactory correlation between the obtained log *k* values and the *in silico* (ADMET Predictor 9.5 programme (Simulation Plus Inc, 2019)) estimated binding to total plasma proteins ($r = 0.86$).

For darunavir and ritonavir, the chromatographically estimated interaction with HSA showed a deviation from their *in silico* estimated binding to total

plasma proteins including alpha-1-acid glycoprotein (AGP). It was found that the HIV protease inhibitors such as darunavir and ritonavir bind primarily to AGP.[21]

The obtained *in silico* estimated binding to total plasma proteins (through the percent of the unbound fraction to human plasma protein – %(hum_fup)) is available from corresponding author upon request.

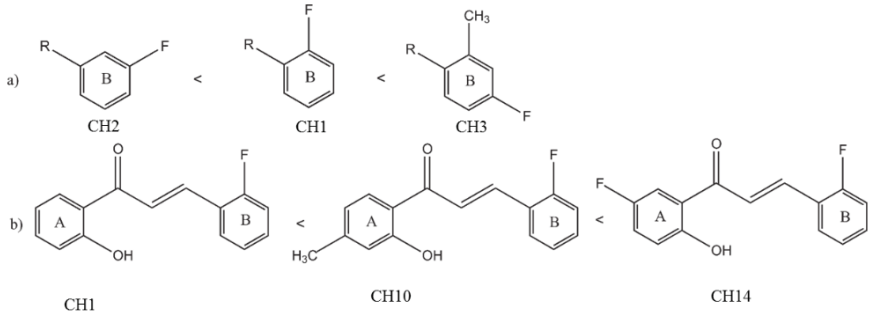

Fig. 6. Comparative presentation of compounds with the influence of an additional substituent on retention behaviour.

Physicochemical characterization of the mechanism of interaction of the examined compounds with HSA

Using the SVM method, a QSRR model was created that defines the relationship between log *k*, as the dependent variable and the molecular properties of the investigated compounds, as independent variables. Based on the correlation with the values of the log *k* parameter, the molecular features with the greatest influence on the HSA-interaction were selected and then used in SVM modelling (Fig. 7).

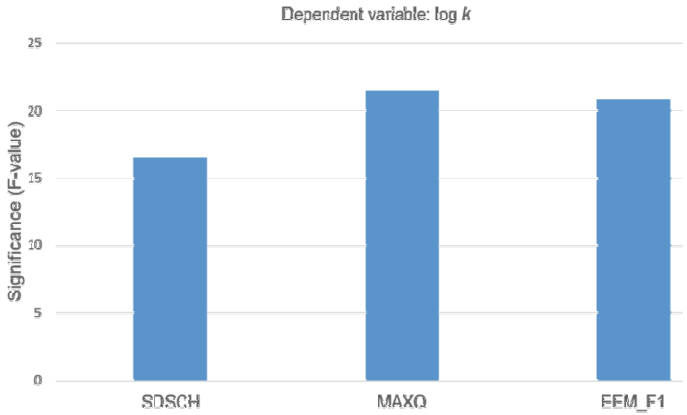

Fig. 7. Selected descriptors in relation to the significance of the influence on the log *k* value.

The most important statistical characteristics of the obtained SVM-QSRR model are shown in Table I, where r(train) is the correlation coefficient for training set and rr^2_m is reverse r^2_m.

TABLE I. The most important statistical characteristics of the obtained SVM-QSRR model

Model	Parameter	Descriptors	r(train)	Q^2_{LOO}	*RMSEE*
SVM (sigmoid function)	log k	SDSCH, MAXQ, EEM_F1	0.76	0.99	0.25
r^2(pred)	*RMSEP*	r^2_m	r'^2_m	$\bar{r}^2_m$	$(rr^2_m-\bar{r}^2_m)/2$
0.95	0.12	0.82	0.77	0.80	0.04

Based on the parameters of internal (r = 0.76, Q^2_{LOO} = 0.99, *RMSEE* = 0.25) and external validation (r^2(pred) = 0.95, *RMSEP* = 0.12), as well as $r^2_{metrics}$ parameters (r^2_m = 0.82, r'^2_m = 0.77, $\bar{r}^2_m$ = 0.80 and $\Delta r^2_m = ((rr^2_m-\bar{r}^2_m)/2)$ = 0.04), it can be observed that the obtained SVM-QSRR model has satisfactory predictive properties.

Structural characteristics (SDSCH) and electronic properties (maximum partial atomic charge (MAXQ) and electron density in the frontier orbital (EEM_F1)) have the greatest influence on the interaction of the tested compounds with HSA.

The descriptor SDSCH defines the total number of CH groups that are connected by double or single bonds in the molecule. As the increase in CH groups correlates with the increase in lipophilicity of the investigated compounds based on the obtained results, it can be concluded that the increase in lipophilicity leads to longer retention (interaction) with HSA. Accordingly, for example:

−for the compound CH12 (which contains an additional methyl group in the A ring) in relation to the compound CH3, log k(CH3) < log k(CH12);

−for the compound CH10 (which has an additional methyl group in the A ring) in relation to the compound CH1, log k(CH1) < log k(CH10).

Also, the interaction of the examined compounds with HSA depends on MAXQ and EEM_F1. Compounds with a larger number of electronegative groups showed a greater affinity for HSA, which can be explained by a greater influence of electrostatic interactions or the formation of hydrogen bonds. Accordingly, for example:

−in the A ring, compound CH18 has an additional fluorine atom compared to the compound CH2, which is why log k(CH2) < log k(CH18);

−compound CH14 has an additional fluorine atom in the A ring compared to the compound CH1, which is why log k(CH1) < log k(CH14).

CONCLUSION

The defined chromatographic conditions can be successfully applied to the investigation of the interaction of chalcone-like compounds and HSA. The formed SVM-QSRR model can be reliably applied for the prediction of retention behaviour of newly synthesized chalcone derivatives on the HSA-stationary

phase. Electrostatic interactions (MAXQ, EEM_F1) and structural characteristics (SDSCH) showed the greatest influence on the interaction of the investigated compounds with HSA. Increasing lipophilicity and introducing electronegative substituents into the structure of the tested compounds strengthens their interaction with HSA.

ИЗВОД

ПРИМЕНА ТЕЧНЕ ХРОМАТОГРАФИЈЕ У ДЕФИНИСАЊУ ИНТЕРАКЦИЈА НОВОСИНТЕТИСАНИХ ХАЛКОНА И СРОДНИХ СУПСТАНЦИ СА ХУМАНИМ СЕРУМСКИМ АЛБУМИНИМА

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Дефинисање интеракције новосинтетисаних једињења са протеинима плазме је важан корак у процесу развоја лекова. Хроматографске технике се могу успешно користити у предикцији биофармацеутских и фармакокинетичких особина новосинтетисаних једињења. Циљ овог рада је испитивање и издвајање најзначајнијих молекулских особина које утичу на интеракцију 24 новосинтетисаних халкона и њима сродних једињења са хуманим серумским албумином (HSA). Ретенционо понашање одабраних једињења је испитано на CHIRALPAK®HSA колони. Као мобилна фаза коришћена је смеша фосфатног пуфера (pH 7,0) и изопропанола (запр. однос 80:20). У формирању QSRR модела коришћена је метода вектора подршке. На основу добијених вредности ретенционих параметара уочено је да халогеновани деривати показују најјачу, а метиловани деривати халкона најслабију интеракцију са HSA. Доводећи у корелацију ретенцију и физичко-хемијска својства испитиваних једињења показало се да структурне (SDSCH) и електронске особине (MAXQ,EEM_F1) група највише утичу на ретенционо понашање и интеракцију испитиваних једињења са HSA. Добијени QSRR модел се може применити у предикцији ретенционих карактеристика нових, структурно-сродних деривата халкона на HSA стационарној фази.

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