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APPLICATION OF THIN LAYER CHROMATOGRAPHY IN EXAMINATION OF THE BIOLOGICAL ACTIVITY PARAMETERS OF AZO DYES

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Abstract: Thanks to the wide range of applications, azo dyes have a special place among synthetic dyes. Also, the application of azo dyes is conditioned by their impact on humans and the environment. Lipophilicity as one of the most important molecular descriptors indicating the potential biological activity of the compound is determined experimentally for selected derivatives of azo dyes using reversed phase thin-layer chromatography (RP TLC18 F_{254s}) in the mixtures of water and two organic modifiers. By using the relevant software packages for the studied azo dyes, the values of partition coefficient (log *P*) were calculated as standard measure of lipophilicity, important pharamacokinetic predictors and the values of the effective concetration (EC_{50}) as the criteria of acute toxicity for different test organisms. The relationships between chromatographic parameters (R_M^0 and *m*) and calculated biological activity parameters were studied by linear regression analysis.

Keywords: azo dyes, lipophilicity, farmacokinetic predictors, toxicity.

1. INTRODUCTION

Azo dyes represent the largest and the most diverse group of synthetic colors, with annual production in the world of over 600,000 tons [1]. The characteristic azo group (–N=N–) is stable at different temperatures, light and pH values, giving the azo dyes extraordinary stability and intensity [2]. Thanks to this, they have been widely used in the textile, pharmaceutical, food and paper industries [3]. Since the amines produced by their reduction are severely degradable, neurotoxic and carcinogenic, the production and the use of azo dyes is increasingly controlled [4–6].

Establishing a balance between the market needs, rational production and the adequate treatment for the removal of azo dyes and their degradation products from the environment is a complex task. The first step is not to plan the synthesis of the new azo dye, but to form a mathematical model that can establish qualitative and quantitative dependencies between its structure, physical and chemical properties and biological activity [7]. Lipophilicity represents the most used molecular descriptor which indicates the potential biological activity of the compound [8]. Usually, lipophilicity is defined by partition coefficient, log *P*, which represents the concentration ratio of the compound in both phases of the saturated system 1-octanol/water [9,10]. Chromatographic parameters, R_M^0 and *m*, obtained by simple and efficient thin layer chromatography on reverse phase (RPTLC) are often applied as alternative reliable measures of the compounds' lipophilicity [11–14]. In addition to lipophilicity, a rational prediction of the biological activity of the compound includes an assessment of its pharmacokinetics, ie. absorption, distribution, metabolism and elimination. Also, the use of the compound is largerly conditioned by its effects on the ecosystem.

In order to study the lipophilicity of the selected azo dyes, their values of the partition coefficient, log *P* were determined by using the relevant software packages. Their chromatographic parameters (R_M^0, *m*) were determined by applying RPTLC in water-formamide and water-acetic acid mixtures. Relationship between the experimentally obtained lipophilic parameters (R_M^0, *m*) and the mathematically calculated values of the log *P*, respectively as the corresponding pharmacokinetic predictors and the parameters of the toxicity of the

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studied azo dyes were examined by using the linear regression analysis.

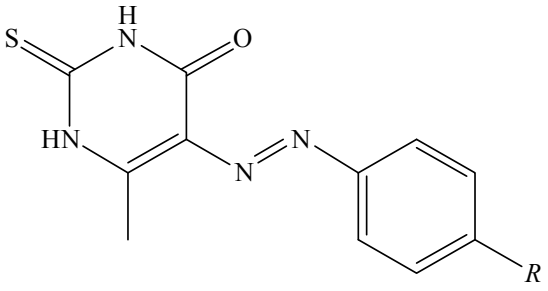
2. EXPERIMENTAL

Structures of the studied derivatives are shown in Table 1. Their synthesis and characterization are described in the literature [15].

The solutions of the studied azo dyes were made in ethanol (J.T. Backer, Deventer, Netherlands), in the concentrations of 2mgml^{-1}. The chromatographic plates RPTLC C18 / UV254s (Macherey-Nagel, Germany) were used as a stationary phase. After application of about 0.2 μl of the prepared azo dyes solutions, the plates were developed in the mixtures: water-foramide (J.T. Backer, Deventer, Netherlands) and water-acetic acid (J.T. Backer, Deventer, Netherlands). The volume fraction of organic modifier was varied in the range φ = 0.36-0.52. Chromatograms were developed by a one-dimensional ascending technique at room temperature, without prior saturation of atmosphere of the chromatographic chamber by the solvent vapor.

Table 1. Structures of the studied azo dyes

Compound	–*R*
1.	–H
2.	–Cl
3.	–Br
4.	–F
5.	–NO_2
6.	–OH
7.	–COOH
8.	–$COCH_3$
9.	–CH_3
10.	–OCH_3



Identification of the developed compounds was carried out under UV light wavelength λ = 254 nm. Three chromatograms have been developed for each compound, and then the average R_f values were calculated. Based on them, R_M values were calculated [16]. The linear dependence of the obtained R_M values on the volume fraction of organic modifier, φ, as the intercept gave the chromatographic retention constant R_M^0, while the slope represented the value of the parameter *m* [17] (equation 1):

$$R_M = R_M^0 + m\varphi \quad (1)$$

Obtained experimental results were processed by the Origin 6.1 software. The values of partition coefficient, log *P*, selected pharmacokinetic parameters and toxicity parameters of examined ayo dyes were calculated by software packages VCCLAB 2007, SimulationPlus and PreADMET [18-20].

3. RESULTS AND DISCUSSION

3.1. Experimental and software determination of the lipophilicity of azo dyes

The mathematically calculated values of the partition coefficient, log *P*, for the examined azo dyes are given in Table 2.

Table 2. Values of log P of studied azo dyes

–*R*	Alog*Ps*	AClog*P*	Alog*P*	*kowwin*	XlogP_2	XlogP_3
–H	2.53	2.19	2.47	2.11	2.06	1.99
–Cl	3.23	2.81	3.14	2.76	2.68	2.61
–Br	3.30	2.89	3.22	3.00	2.85	2.68
–F	2.68	2.25	2.68	2.31	2.22	2.09
–NO_2	2.58	2.20	2.37	2.51	1.95	1.82
–OH	2.30	1.89	2.21	1.63	1.65	1.63
–COOH	2.02	1.71	2.08	1.99	1.67	1.51
–$COCH_3$	2.42	2.12	2.21	1.79	1.90	1.67
–CH_3	2.89	2.51	2.96	2.66	2.49	2.35
–OCH_3	2.62	2.09	2.46	2.19	1.97	1.96

Data from Table 2 show that the values of log *P* of the examined azo dyes depend on their chemical structure and on the applied mathematical calculation method. However, the highest values of log *P* were obtained for the compound with –Br as the substituent, and the lowest for the derivative with the –COOH group, independently of the calculation method.

In addition to mathematical calculations, the lipophilicity of examined azo dyes was determined by using RPTLC in the presence of the protic acetic acid and an aprotic formamide. Table 3 shows calculated R_f values of the studied derivatives in the mixtures of 40% organic modifier and 60% water.

Table 3. R_f values of the studied azo dyes in the mixtures of 40% organic modifier and 60% water

–*R*	R_f	
	formamide	acetic acid
–H	0.273	0.255
–Cl	0.209	0.172
–Br	0.195	0.150
–F	0.235	0.227
$–NO_2$	0.307	0.320
–OH	0.299	0.311
–COOH	0.342	0.331
$–COCH_3$	0.317	0.324
$–CH_3$	0.213	0.181
$–OCH_3$	0.279	0.267

Based on the Table 3, it can be noticed that the retention behavior of the test compounds is conditioned by the selected organic modifier, but also by the nature of the substituent. Comparing the R_f values of the same compound applied in the two organic modifier, it can be seen that there is no significant difference in retention. Stronger retention of the derivatives was observed in the protic acetic acid.

It is also noticeable that the obtained R_f values in one modifier differ from each other. This could be explained by the influence of the nature of the substituent on the ability to form intermolecular interactions with the mobile or stationary phase. As expected, the presence of non-polar $–CH_3$ group generally results in a stronger retention in relation to the unsubstituted molecule. In the case of halogen substituents, there is a stronger retention in the range –Br > –Cl > –F. It is assumed that a derivative with -F has the shortest retention, due to substitents' high polarizaton power. In contrast, in the presence of most polar substituents, the reduction in the retention of the tested derivatives relative to the unsubstituted molecule was observed in both applied organic modifiers. It was expected that R_f values of derivatives with polar substituents will increase in the series **–COOH** > –OH > $–COCH_3$ > **$–NO_2$ >** $–OCH_3$, which was not the case. Namely, the R_f values obtained for derivatives with –OH и $–OCH_3$ group in both organic modifiers are lower than expected. The reason for retention behavior of these derivatives can be explained by the fact that, in contrast to other polar substituents, –OH и $–OCH_3$ group are electron donors- they have a negative Hamamet constant value, σ (Table 4). As a result, these groups favor the formation of an azo tautomer (Figure 1), which achieves stronger interactions with the stationary phase.

Table 4. Values of the Hammet constant, σ

–*R*	$\sigma_{m/p}$
–H	0.00
–Cl	0.23
–Br	0.23
–F	0.06
$–NO_2$	0.78
–OH	-0.37
–COOH	0.55
$–COCH_3$	0.50
$–CH_3$	-0.17
$–OCH_3$	-0.27

It has also been found that the strongest retention in both organic modifiers, obtained for the derivative with –Br as a substituent, and the weakest for the derivative with the most polar –COOH group, which is in accordance with the results obtained by the computational method. Further, using the equation 1, the values of chromatographic parameters R_M^0 and *m* were calculated (Table 5).

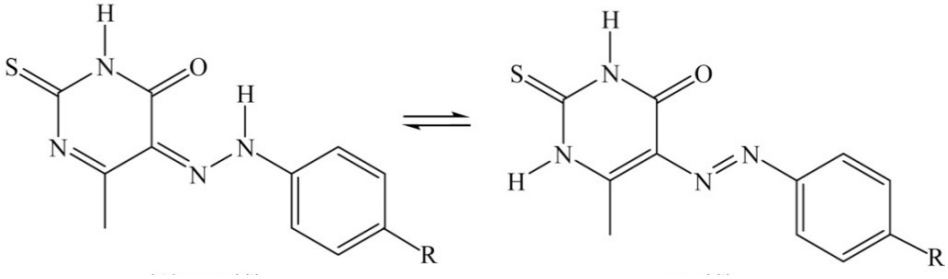


Figure 1. Azo-hydrazone tautomerism of the tested azo dyes

Table 5. Parameters of chromatographic equations R_M^0, m and r for studied azo dyes

–R	modifier					
	formamide			acetic acid		
	R_M^0	*m*	r	R_M^0	*m*	r
–H	1.013	-1.462	0.995	1.443	-2.442	0.999
–Cl	1.215	-1.619	0.992	1.719	-2.619	0.999
–Br	1.265	-1.638	0.998	1.813	-2.655	0.998
–F	1.125	-1.537	0.999	1.534	-2.525	0.998
$–NO_2$	0.607	-1.105	0.996	1.148	-2.263	0.998
–OH	0.932	-1.420	0.998	1.285	-2.363	0.998
–COOH	1.542	-1.805	0.999	1.635	-2.553	0.998
$–COCH_3$	0.862	-1.322	0.995	1.250	-2.327	0.998
$–CH_3$	1.205	-1.601	0.995	1.686	-2.555	0.999
$–OCH_3$	1.043	-1.501	0.992	1.514	-2.469	0.999

The results presented in Table 5 show that the values of the chromatographic retention constants of the tested azo dyes, R_M^0, do not have the same value in the applied modifiers, although it reflects the retention behavior of the compound and depends only on its chemical structure. This phenomenon is often noted during experimental work [22]. The value of the chromatographic parameter *m* except by the applied organic modifier is influenced by the size of the dissolved substance, the type and number of functional groups in the molecule, the specific hydrophobic surface [23].

Based on the data in Table 5, it can be seen that for the tested azo dyes, *m* values change the same as R_M^0 values, so it was assumed that they depend on the same physico-chemical factors. With this idea, the chromatographic retention constant R_M^0 was correlated with the chromatographic parameter, *m* using the linear regression. Linear dependence was obtained in both organic modifiers (Table 6).

Table 6. R_M^0-m relationships for the tested azo dyes in the applied modifiers

modifier	Equation	r
formamide	$R_M^0 = -0.886-1.310m$	0.991
acetic acid	$R_M^0 = -2.705-1.699m$	0.992

3.2. Correlation of the lipophilicity parameters of azo dyes obtained by calculation and experimentally

In order to confirm that the chromatographic parameters (R_M^0, *m*) can be used as reliable criteria of lipophilicity of the studied azo dyes, they were correlated with the software derived partition coefficient, log *P*, as a standard criterion of lipophilicity by the linear regression. Figure 2 and Figure 3 show the linear R_M^0– Xlog P_2 and *m*– Xlog P_2 dependences determined in acetic acid, respectively.

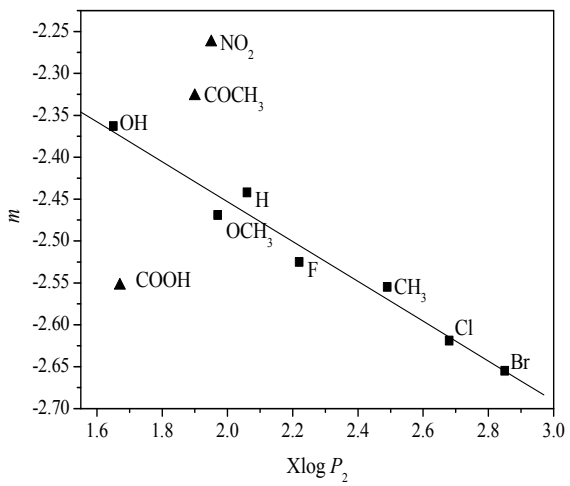


Figure 2. R_M^0 – Xlog P_2 in acetic acid

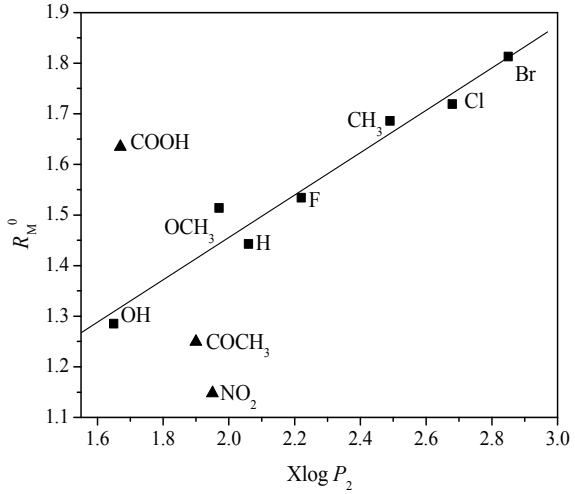


Figure 3. m – Xlog P_2 in acetic acid

Figure 2 and Figure 3 show that derivatives with polar electron-acceptor group as substituent deviated from the established linear dependence. The correlation matrix obtained as a result of linear regression between experimental and computational lipophilicity of the studied azo dyes is given in Table 7.

Table 7. Correlation matrix for R_M^0 – log P and m – log P

log *P*	formamide		acetic acid	
	R_M^0	*m*	R_M^0	*m*
Alog *Ps*	0.951	0.962	0.969	0.981
AClog *P*	0.947	0.949	0.955	0.968
Alog *P*	0.985	0.985	0.977	0.984
kowwin	0.984	0.981	0.998	0.985
XlogP_2	0.978	0.972	0.979	0.985
Xlog P_3	0.966	0.969	0.978	0.984

* excluded derivatives with –NO_2, –COOH and –$COCH_3$

Presented data show that better relationship of all the partition coefficients of the studied azo dyes is obtained in correlation with the chromatographic parameters determined in acetic acid. The best agreement with the R_M^0 and *m* values was obtained in the case of partition coefficient *kowwin*, and the weakest is noted for AClog *P*.

3.3. Correlation of chromatographic parameters R_M^0 and *m* with important pharmacokinetic predictors

The knowledge of the pharmacokinetic properties of the compounds significantly contributes to the prediction and assessment of its possible biological activity. The effect and metabolytic pathway of an oral biologically active substance is predominantly determined by its intestinal absorption. One of the predictors that can be described is human effective permeability in jejunum, P_{eff} [24].

In general, lipophilic compounds have better absorption, and therefore a higher P_{eff} values. The ability to bind to plasma proteins, *PPB*, indicates the tendency of the compound to reach the site of action through the blood [25]. The possibility of passing the compound through the blood-brain barrier can be expressed by a pharmacokinetic predictor *BBB* [26]. The *BBB* values greater than 0.4 indicate a neurological active compound, while *BBB* values lower than -1 indicate that the passage of the compound through the blood-brain barrier is not possible [27].

The calculated values of these pharmacokinetic predictors for the studied azo dyes are given in Table 8.

Table 8. Values of the selected pharmacokinetic predictors of azo dyes

–*R*	P_{eff}	*PPB*	*BBB*
–H	2.020	81.126	0.075
–Cl	2.703	83.948	0.317
–Br	2.877	83.908	0.346
–F	2.558	81.934	0.189
–NO_2	2.394	85.040	0.135
–OH	1.204	78.538	0.032
–COOH	1.410	78.493	0.149
–$COCH_3$	2.163	80.728	0.099
–CH_3	2.380	83.327	0.527
–OCH_3	1.924	82.112	0.019

Data shown in Table 8 indicate that among the examined azo dyes, the best predisposition for good intestinal absorption has the most lipophilic derivative, with –Br as a substituent (the largest P_{eff} value), and the weakest derivative with –OH group. The most pronounced binding tendency for plasma proteins shows the compound with –NO_2, while the –OH compound is largely unbound. The greatest influence on the central nervous system would, according to the *BBB* values have derivatives with –Cl, –Br and –CH_3 group, respectively.

Dependences between experimentally determined lipophilicity parameters of studied the azo dyes and their pharmacokinetic predictors were investigated using the linear regression. The values of the regression coefficients for the obtained linear dependences are shown in Table 9.

Table 9. Correlation matrix obtained for chromatographic parameters–pharmacokinetic predictors relationships

pharmacokinetic predictor	r			
	formamide		acetic acid	
	R_M^0	*m*	R_M^0	*m*
P_{eff}	0.935	0.912	0.917	0.954
PPB	0.939	0.946	0.966	0.944
log *BBB*	0.857	0.848	-	-

* excluded derivatives with –NO_2, –COOH and –$COCH_3$

From Table 9 it can be seen that better relationships were obtained in formamide.

3.4. Correlation of chromatographic parameters R_M^0 and *m* with parameters of toxicity

Risk assessment is of the utmost importance during the planning the properties of a future compound [28]. For azo dyes as the main industrial pollutants, it is necessary to examine the level of toxicity, especially in aquatic ecosystems. In Table 10, the values of effective concentration, EC_{50},

$mgkg^{-1}$ as acute toxicity criteria for selected organisms: *Algae*, *Daphnia*, *Medaka* and *Minnow* are presented.

By comparing the obtained EC_{50} values, it can be noticed that the highest toxicity among the tested azo dyes for all applied test organisms shows the derivative with –Br as a substituent. Also, all investigated compounds are the most toxic to *Minnow* species.

The relationships between chromatographic parameters (R_M^0, m) of the tested azo dyes and their calculated values of EC_{50} for different test organism were investigated using the linear regression. The correlation matrix of the obtained linear dependences is given in Table 11.

Table 10. Software calculated values of EC50 of studied azo dyes for the selected test organisms

–*R*	*Algae*	*Daphnia*	*Medaka*	*Minnow*
–H	0.0488	0.0615	0.0075	0.0068
–Cl	0.0247	0.0308	0.0021	0.0020
–Br	0.0217	0.0251	0.0015	0.0016
–F	0.0384	0.0544	0.0059	0.0033
$–NO_2$	0.0450	0.0533	0.0060	0.0045
–OH	0.0389	0.0668	0.0091	0.0071
–COOH	0.0352	0.0639	0.0087	0.0073
$–COCH_3$	0.0382	0.0659	0.0092	0.0091
$–CH_3$	0.0266	0.0371	0.0028	0.0024
$–OCH_3$	0.0366	0.0611	0.0076	0.0071

Table 11. Correlation matrix obtained for chromatographic parameters – EC_{50} values relationships

toxicity parameter	r			
	formamide		acetic acid	
	R_M^0	*m*	R_M^0	*m*
Algae	0.844	0.879	-	-
Daphnia	0.961	0.968	0.957	0.955
Medaka	0.981	0.983	0.967	0.964
Minnow	0.954	0.943	0.889	0.924

* excluded derivatives with $–NO_2$, –COOH and $–COCH_3$

The results from Table 11 confirm that the chromatographic parameters R_M^0 and m can be successfully applied to predict the toxicity of the examined azo dyes.

4. CONCLUSION

In this paper, lipophilicity as a key indicator of potential biological activity of selected azo dyes, was determined using relevant software packages and by RPTLC in the presence of protic and aprotic organic modifier. It was found that the retention behavior of studied azo dyes depends to a small extent on the applied organic modifier, and is largely conditioned by the nature of the substituent. It has also been observed that the polarity of the substituent has the dominant influence on the lipophilicity of the tested derivatives, and less its electronic effects. The linear dependence between the chromatographic parameters (R_M^0, m) of the examined azo dyes and their software obtained values of the partition coefficient, log P, as a standard measure of lipophilicity, was established. In addition, the values of the obtained regression coefficients indicated the linear dependence between chromatographic parameters of azo dyes and their important pharmacokinetic and toxicity parameters. All obtained results indicate that RPTLC can be reliably applied to describe lipophilicity, and therefore the assessment of the biological activity of studied azo dyes.

5. ACKNOWLEDGEMENTS

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ПРИМЕНА ТАНКОСЛОЈНЕ ХРОМАТОГРАФИЈЕ У ПРОУЧАВАЊУ ПАРАМЕТАРА БИОЛОШКЕ АКТИВНОСТИ АЗО БОЈА

Сажетак: Захваљујући широком спектру примене, азо боје заузимају значајно место међу синтетским бојама. Примена азо боја условљена је и њиховим утицајем на човека и животну средину. Липофилност као један од најзначајнијих молекулских дескриптора који указује на потенцијалну биолошку активност једињења је за одабране деривате азо боја одређена експериментално, применом танкослојне хроматографије на обрнутим фазама (RP TLC18 F_{254s}), у смешама воде и два органска модификатора. Применом релевантних софтверских пакета за испитиване деривата азо боја су израчунате вредности подеоног коефицијента (log *P*) као стандардног мерила липофилности, важних фармакокинетичих предиктора и ефективне концентрације (EC_{50}) као мерила акутне токсичности за различите тест организме. Постојање зависности између хроматографских параметара (R_M^0 и *m*) и израчунатих параметара биолошке активности је испитана применом методе линеарне регресије.

Кључне речи: азо боје, липофилност, фармакокинетички предиктори, токсичност.