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A new model to determine lipophilicity of 1,2-ethanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid and 1,3-propanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid derivatives with antiproliferative activity by combining shake flask procedure and UHPLC-MS method

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Fourteen compounds representing ester derivatives of (*S,S*)-1,2-ethanediamine-*N,N'*-di-2-(3-cyclohexyl) propanoic and (*S,S*)-1,3-propanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acids, expressing antiproliferative activity *in vitro* were examined. The objective of this study was to determinate their lipophilicity data, and also to ensure a mathematical model for prediction lipophilicity data of potential *in vivo* metabolites and new derivatives of (*S,S*)-1,2-ethanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid, based on chromatographic parameters. Experimentally, lipophilicity data were obtained by a traditional shake flask procedure and an ultra-high performance liquid chromatographic tandem mass spectrometry (UHPLC-MS) method. A correlation between the partition coefficient *n*-octanol/water ($\log D_{7.4}$) and chromatographic data (CHI, φ_0), and also, between $\log D_{7.4}$ and retention time was investigated. A very good correlation (r^2=0.8969) was found between lipophilicity parameters φ_0 and $\log D_{7.4}$ obtained using UHPLC-MS and shake flask methods: $\log D_{7.4} = (0.11\pm0.01)\times\varphi_0 + (1.25\pm0.20)\times N_c - (9.19\pm1.18)$; statistical parameter F=47.84; significance of F = 3.74×10^{-6}, N_c=number of C atoms between two amino groups (N_c=2 for 1,2-ethanediamine derivatives and N_c=3 for 1,3-propanediamine derivatives). The model predictivity power was determined by cross validation leave one out (LOO) technique, and expressed by the term Q^2, was 0.89. The developed model has good predictivity power for prediction lipophilicity data of potential *in vivo* metabolites of the investigated compounds, such as novel 1,2-ethanediamine and 1,3-propanediamine *N,N'*-di-2-(3-cyclohexyl)propanoic acid derivatives. Also, the lipophilicity data obtained in the present study correlated with the antiproliferative activity of the investigated substances shown previously in *in vitro* studies.

1. Introduction

During the early stages of drug discovery, it is very important to determine lipophilicity and to investigate and predict processes of drug distribution and resorption in human body, *i.e.* their bioavailability. This often involves identification of the compounds more likely to be satisfactorily absorbed and appropriately distributed within the body. Altough there are many routes for absorption of pharmacologically active agents through cell membranes and tissues, passive diffusion represents the most common transport route. To be absorbed by this route, drugs should be sufficiently lipophilic to penetrate cell membrane lipids, while those with poor lipophilicity remain at the membrane surface. Lipophilicity is usually expressed by the *n*-octanol/water partition coefficient (logP) for neutral molecules and the distribution coefficient (logD) for ionized molecules (Xuan et al. 2015).

The logarithm of the *n*-octanol/water partition coefficient (logP) provides information regarding physicochemical properties of drugs and industrial chemicals. The logP value represents the most widely employed descriptor of hydrophobicity, lipophilicity and quantitative structure-activity relationships of biological, pharmaceutical and environmentally relevant compounds (Morikawa et al. 2016; Sangster 1996; Poole et al. 2006; Berthod et al. 2004; Hermens et al. 2013).

The shake flask method represents a classical procedure to directly determine both logP and logD, but it is time-consuming, labor intensive and requires relatively large amounts of pure analytes. Besides, this procedure requires the measurement of the compound concentration in octanol and water phases after equilibration of both phases according to Eq. (1). Thus, Eq. (1) can be written as:

$$\log D = \log (c_{octanol}/c_{water}) \quad (1)$$

(Andres et al. 2015).

where $c_{octanol}$ and c_{water} are the concentrations of a substance in *n*-octanol and aqueous phase of the partition, respectively.

To overcome the above disadvantages of shake flask method, other *in vitro* methods were developed for absorption prediction (Odovic et al. 2012; Escuder–Gilabert et al. 2003; Henchoz et al. 2007). Because of its clear relationship to the partitioning phenomenon, the shake flask method is the benchmark serving to validate other methods.

According to the available literature, a number of authors investigated possibilities of drug absorption prediction based on immobilized chromatographic techniques (Odovic et al. 2012; Kotecha et al. 2008; Liu et al. 2002), with development of artificial membranes (Odovic et al. 2012; Corti et al. 2006) or liposome-water partitioning (Odovic et al. 2012; Plemper van Balen et al. 2004). Nowadays, different chromatographic techniques *e.g.* thin layer (TLC), high performance liquid (HPLC) and ultra-high performance liquid chromatographic (UHPLC) are recognized as high-throughput methods for lipophilicity investigations of neutral, acidic, alkaline and amphoteric drugs or novel potential drugs.

In isocratic liquid chromatographic methods, the lipophilicity index - retention factor *k* - is in correlation with the retention time (t_R) and dead time (t_0):

$$\log k = (t_R - t_0)/t_0 \quad (2)$$

Using log$k$, only a limited range of lipophilicity measurements can be obtained for one mobile phase composition. To cover a range of solutes with different lipophilicity, several mobile phase compositions are required. By plotting the organic phase concentrations and the corresponding log$k$ values for each compound, the extrapolated log k_W values can be calculated and taken as an estimate of log$k$ value of a compound at 0% organic phase.

Two other reversed-phase retention parameters have been introduced, chromatographic hydrophobicity index (φ_0 and CHI). The φ_0 index is the volume percent of organic phase concentration in the mobile phase by which retention time is twice the dead time, meaning that log$k$=0. Compounds with higher hydrophobic character require a much higher organic phase concentration to achieve log$k$=0 at a relatively short time. There is a linear relationship of φ_0 value and lipophilicity of a compound, *i.e.* higher $\varphi_{0,}$ the higher lipophilicity. There is a significant correlation between φ_0 and the traditional *n*-octanol/water partition coefficient, for a large number of structurally unrelated compounds. The φ_0 values can be calculated from the intercept (logk_W) and the slope (S) of the straight line plots of log$k$ values with organic phase concentrations in the mobile phase using Eq. 3. (My Du et al. 1998).

$$\varphi_0 = -\log k_W/S \qquad (3)$$

Chromatographic hydrophobicity index (CHI) puts gradient retention time onto an organic phase concentration (φ_0) scale using a calibration set of compounds.

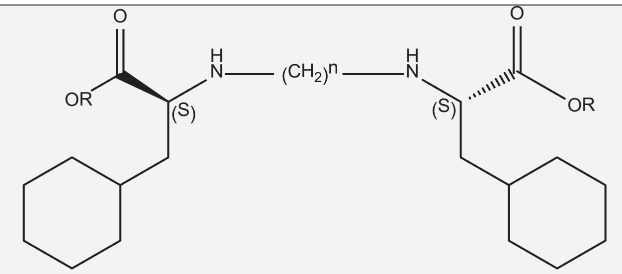
Both φ_0 and CHI are parameters designed to measure lipophilicity of substances. They differ in their method of measurement, *i.e.* φ_0 is derived from a series of isocratic measurements, while CHI is derived from the retention time.

$$\mathrm{CHI} = \mathrm{A}t_R + \mathrm{B} \qquad (4)$$

The data for over 500 compounds (Valko et al. 1993) showed that for organic phase concentration in the mobile phase an approximation can be made using acetonitrile (AcN) and methanol (MeOH) based on calculated octanol/water partition coefficients (logP) of a compound (Valko 2004). Logk_W value is the most widely used chromatographic lipophilicity parameter (My Du et al. 1998).

In the present study we observed a set of 14 compounds representing ester derivatives of (*S,S*)-1,2-ethanediamine-*N,N'*-di-2-

Table 1: Derivatives of (*S,S*)-1,2-ethanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid, and (*S,S*)-1,3-propanediamine-*N,N'*-di-2-(3-cyclohexyl) propanoic acid



N	Name of compound	Empirical formula	Abbreviation	MW (g/mol)*	R	n	m/z
1.	(*S,S*)-1,2-Ethanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid dihydrochlorides	$C_{20}H_{38}O_4N_2Cl_2$	EDCP	441.43	H	2	$369\rightarrow152^{**}$ $369\rightarrow198^{**}$
2.	(*S,S*)-*O,O*-Dimethyl-1,2-ethanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoate dihydrochlorides	$C_{22}H_{42}O_4N_2Cl_2$	DM-EDCP	469.48	CH_3	2	$397\rightarrow212^{**}$
3.	(*S,S*)-*O,O*-Diethyl-1,2-ethanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoate dihydrochlorides	$C_{24}H_{46}O_4N_2Cl_2$	DE-EDCP	497.54	C_2H_5	2	$425\rightarrow198^{**}$ $425\rightarrow226^{**}$
4.	(*S,S*)-*O,O*-Dipropyl-1,2-ethanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoate dihydrochlorides	$C_{26}H_{50}O_4N_2Cl_2$	DP-EDCP	525.59	C_3H_7	2	$453\rightarrow240^{**}$
5.	(*S,S*)-*O,O*-Dibutyl-1,2-ethanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoate dihydrochlorides	$C_{28}H_{54}O_4N_2Cl_2$	DB-EDCP	553.64	C_4H_9	2	$481\rightarrow254^{**}$
6.	(*S,S*)-*O,O*-Diisobutyl-1,2-ethanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoate dihydrochlorides	$C_{28}H_{54}O_4N_2Cl_2$	DIB-EDCP	553.64	C_4H_9	2	$481\rightarrow254^{**}$
7.	(*S,S*)-1,3- Propanediamine-*N,N'*-di-2-(3-cyclohexyl) propanoic acid dihydrochlorides	$C_{21}H_{40}O_4N_2Cl_2$	PDCP	455.46	H	3	383^{***}
8.	(*S,S*)-*O,O*-Dimethyl-1,3- propanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoate dihydrochlorides	$C_{23}H_{44}O_4N_2Cl_2$	DM-PDCP	483.51	CH_3	3	411^{***}
9.	(*S,S*)-*O,O*-Diethyl-1,3- propanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoate dihydrochlorides	$C_{25}H_{48}O_4N_2Cl_2$	DE-PDCP	511.56	C_2H_5	3	438^{***}
10.	(*S,S*)-*O,O*-Dipropyl-1,3- propanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoate dihydrochlorides	$C_{27}H_{52}O_4N_2Cl_2$	DP-PDCP	539.62	C_3H_7	3	467^{***}
11.	(*S,S*)-*O,O*-Dibutyl-1,3- propanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoate dihydrochlorides	$C_{29}H_{56}O_4N_2Cl_2$	DB-PDCP	567.67	C_4H_9	3	495^{***}
12.	(*S,S*)-*O,O*-Diisobutyl-1,3- propanediamine-*N,N'*-di-2-(3-cyclohexyl) propanoate dihydrochlorides	$C_{29}H_{56}O_4N_2Cl_2$	DIB-PDCP	567.67	C_4H_9	3	495^{***}
13.	(*S,S*)-*O,O*-Di-*n*-pentyl-1,3- propanediamine-*N,N'*-di-2-(3-cyclohexyl) propanoate dihydrochlorides	$C_{31}H_{60}O_4N_2Cl_2$	DPE-PDCP	595.72	C_5H_{11}	3	523^{***}
14.	(*S,S*)-*O,O*-Diisopentyl-1,3- propanediamine-*N,N'*-di-2-(3-cyclohexyl) propanoate dihydrochlorides	$C_{31}H_{60}O_4N_2Cl_2$	DIPE-PDCP	595.72	C_5H_{11}	3	523^{***}

*MW=Molecular Weight; **Selected reaction monitoring (SRM); ***Single ion monitoring (SIM)

(3-cyclohexyl)propanoic acid, and (*S,S*)-1,3-propanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid (Table 1). Novel ester derivatives of (*S,S*)-ethylenediamine-*N,N′*-di-2-(3-cyclohexyl) propanoic acid are toxic to leukemic cell lines. It was demonstrated that methyl, ethyl, and *n*-propyl esters were toxic to HL-60, REH, MOLT-4, KG-1, JVM-2, and K-562 leucemic cell lines, while the nonesterified compound and *n*-butyl ester were devoid of cytotoxic action. The ethyl ester exhibited the highest cytotoxic activi-ty on leucemic cell line HL-60 (IC50 in the range of 11 μM – 45 μM) (Misirlić-Denčić et al. 2012). Derivatives of 1,3-propanediamine-*N,N'*-2-(3-cyclohexyl)propanoic acid are toxic to glioma cell lines. *In vitro* antitumour potentials were investigated for methyl, ethyl, n-propyl, and n-butyl esters of (*S,S*)-1,3-propanediamine-*N,N′*-di-2-(3-cyclohexyl)propanoic acid on several tumour cell lines: human (U251), and rat (C6) glioma, HL-60, SHSY-5Y, L929. The *n*-butyl ester was showed the highest cytotoxic activity to glioma cells, with 24-h IC_{50} values lower than those for cisplatin (Savić et al. 2014).

The literature shows no reports about the lipophilicity of the tested substances. The aim of this study was to determine the lipophilicity data of these. Lipophilicity data are needed for prediction bioavailability of these substances in future non-clinical studies. Also, the objective of this work was to ensure a reliable mathematical model for the prediction lipophilicity data of potential *in vivo* metabolites and new derivatives of (*S,S*)-1,2-ethanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid, and (*S,S*)-1,3-propanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid, based on chromatographic parameter φ_0 of these substances.

2. Investigations, results and discussion

In the present study fourteen compounds (Table 1) expressing *in vitro* antiproliferative activities were experimentally investigated. Lipophilicity data of the substances was determined, and the correlation between experimental $\log D_{7,4}$ obtained by traditional shake flask method and chromatographic data (CHI, φ_0) obtained by UHPLC-MS method, and also, between $\log D_{7,4}$ and retention time was examined.

According to the OECD recommendations, the shake flask method is appropriate for the determination of the *n*-octanol/water partition coefficient ranging from -2 to 4, while a HPLC method can be applied when the *n*-octanol/water the partition coefficient spans from 0 to 6 (OECD Guidelines for the Testing of Chemicals, Test No. 107 ana Test No. 117).

2.1. Lipophilicity determination by the shake flask method

n-Octanol-water partition coefficient $\text{Log}D_{7,4}$ values of the investigated compounds were obtained by using the shake flask method. The procedure was mainly based on the current guidelines of the Organization for Economic Cooperation and Development (OECD Guidelines for the Testing of Chemicals 1995). A solution of 0.1 M sodium phosphate buffer (pH 7.4) served as aqueous phase of the system. The pH values of the aqueous phase for the shake flask method were measured with a pH-Meter with an accuracy of ±0.002. As a non-polar phase, *n*-octanol was used. Each compound was dissolved in the aqueous phase to saturation. Thereafter, an appropriate volume of the prepared aqueous phase was transferred into glass vials and mixed with the equal volume of *n*-octanol. Tightly closed vials completely immersed in a horizontal position were heavily shaken at a mechanical shaker for 2 h and then left resting for at least 24 h to ensure complete separation of the two phases. Quantitative analyses of each investigated compound concentration in water and organic phase were done by a UHPLC/MS-MS method (Tubić et al. 2016). Mobile phase A composed of ammonium acetate (5 mM) - trifluoroacetic acid (99.9:0.1, v/v) and mobile phase B composed of methanol - trifluoroacetic acid (99.9:0.1, v/v). Partition coefficient *n*-octanol-water ($\log D_{7,4}$) was determined by the shake flask method for all examined compounds, in the first stage of this study. The obtained $\log D_{7,4}$ values covering a range from 0.03 to 3.40 units (Table 2) indicated their lipophilicity.

Table 2: Values of partition coefficient *n*-octanol-water determined by shake flask method ($\log D_{7,4}$) and calculated values of logP based on chromatographic (UHPLC-MS) data

Abbreviation	$\text{Log}D_{7.4}$ by shake flask method	LogP based on chromatographic data
EDCP	0.03	1.48
DM-EDCP	1.32	2.36
DE-EDCP	1.68	2.64
DP-EDCP	1.77	2.93
DB-EDCP	1.80	3.20
DIB-EDCP	2.95	3.19
PDCP	0.57	1.50
DM-PDCP	1.93	1.88
DE-PDCP	2.90	2.26
DP-PDCP	3.00	2.62
DB-PDCP	3.04	2.85
DIB-PDCP	3.15	2.85
DPE-PDCP	3.29	3.08
DIPE-PDCP	3.40	3.08

2.2. Lipophilicity determination by the UHPLC/MS method

In isocratic mode, calculations of log*k* values were performed with t_R measurements at six different mobile phase compositions (mobile phase A/mobile phase B: 10:90; 20:80; 30:70; 35:65; 40:60 and 50:50 v/v).

Samples of mixed compounds were injected in triplicate for each of the above mobile phases composition and t_R were determined and expressed as the mean values. Finally, $\log k_W$ values were obtained by extrapolation to 0% organic modifier using linear relationships between log*k* values and methanol percentages. An exception in these measurements was made with DP-EDCP, DB-EDCP, DIB-EDCP, DB-PDCP, DPE-PDCP, DIB-PDCP and DIPE-PDCP in sample with mobile phase (50 % methanol). Namely, due to its expected high retention time under the applied conditions (50 % methanol in mobile phase), the run was terminated after 150 min.

In gradient mode the initial and final eluent compositions were programmed to increase linearly from 5 to 95 % organic modifier in first 15 min, and then it was lineary decreased from 17.20 to 20 min to 40 %. In this mode, the total run time was 20 min including column re-equilibration.

From the data obtained in isocratic elution mode, corresponding φ_0 values were calculated (Table 3).

Hydrophobicity index CHI was calculated using gradient retention times (Figs. 1 and 2).

A significant correlation was established in all plots. From each plot and φ_0 values, constants *A* and *B* (Eq. 4) were provided and hydrophobicity index CHI was calculated according to the proposed methodology.

For asterisked compounds (DP-EDCP, DB-EDCP, DIB-EDCP, DB-PDCP, DPE-PDCP, DIB-PDCP and DIPE-PDCP), retention time values were over 120 and 150 min. For these compounds, log*k* values were determined by using 60 % and higher methanol concentrations (60 %, 65 %, 70 %, 80 % and 90 %). In reversed-phase liquid chromatography, the retention (*i.e.* compound distribution between mobile and stationary phases) is governed by hydrophobic forces which can be regarded as a measure of compound lipophilicity. Chromatographic data of the investigated compounds obtained by UHPLC-MS ($\log k_w$, φ_0, CHI) are listed in Table 3.

The lipophilicity data obtained for all the examined compounds by shake flask method and various chromatographic retention parameters, log *k* (isocratic), log k_w, φ_0, and CHI (gradient) were compared.

Values of partition coefficients calculated on the basis of chromatographic data were higher compared to those obtained by the shake

Table 3: Chromatographic data of the investigated compounds obtained by UHPLC-MS

Abbreviation	y=ax + b (Eq.3)	Isocratic elution				Gradient elution	
	Y=log*k*; x=φ**** a=slope (-*S*); b=intercept (logk_w)	logk_w*****	-*s*	r^2	$φ_0$	t_R	CHI
EDCP	Y=-0.0431x+2.639	2.64	0.04	0.9671	61.23	6.06	61.73
DM-EDCP	Y=-0.0562x+4.0832	4.08	0.06	0.9880	72.65	9.34	71.16
DE-EDCP	Y=-0.0664x+5.0671	5.07	0.07	0.9954	76.31	10.80	75.37
DP-EDCP	Y=-0.0763x+6.1152	6.12	0.08	0.9997	80.15	12.35	79.82
DB-EDCPˣ	Y=-0.0855x+7.148	7.15	0.09	0.9986	83.60	13.64	83.53
DIB-EDCPˣ	Y=-0.0845x+7.0567	7.06	0.08	0.9988	83.51	13.60	83.42
PDCP	Y=-0.0486x+2.9408	2.94	0.05	0.9953	60.51	5.99	61.53
DM-PDCP	Y=-0.0503x+3.3426	3.34	0.05	0.9524	66.45	7.69	66.42
DE-PDCP	Y=-0.0612x+4.3702	4.37	0.06	0.9705	71.41	9.28	70.99
DP-PDCP	Y=-0.0729x+5.5447	5.54	0.07	0.9865	76.06	10.87	75.56
DB-PDCPˣ	Y=-0.0832x+6.5805	6.58	0.08	0.9978	79.09	12.23	79.48
DIB-PDCPˣ	Y=-0.0821x+6.4873	6.49	0.08	0.9981	79.02	12.17	79.30
DPE-PDCPˣ	Y=-0.0956x+7.8438	7.84	0.10	0.9989	82.05	13.42	82.90
DIPE-PDCPˣ	Y=-0.095x+7.7955	7.80	0.10	0.9989	82.06	13.42	82.90

****φ=actual organic phase concentration (vol % term);

*****log*k* was determined based on retention time and dead retention time (Eq.2)

flask method for derivatives of 1,2-ethanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid. This difference was the most prominent for non-esterified derivatives of 1,2-ethanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid and 1,3- propanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid (EDCP and PDCP). Significantly higher values of the LogP are obtained by the chromatographic method in comparison to the shake flask method for the EDCP and PDCP, due to the high presence of hydrophobic elements in the structure (per two cyclohexylalkyl groups) that may notably interact with the stationary phase and affect the retention time extension consequently.

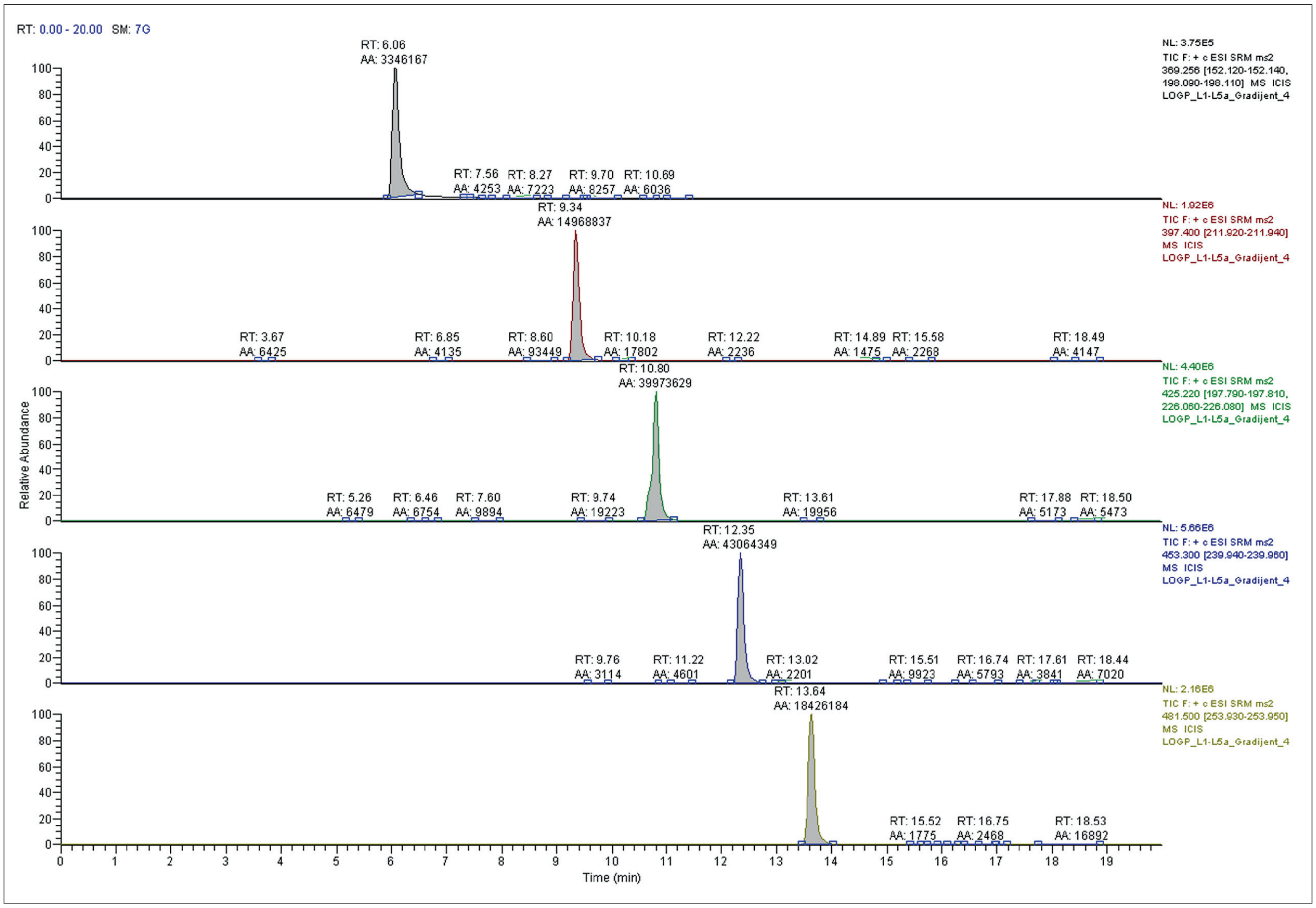


Fig. 1: Chromatogram of (*S,S*)-1,2-ethanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid (gradient)

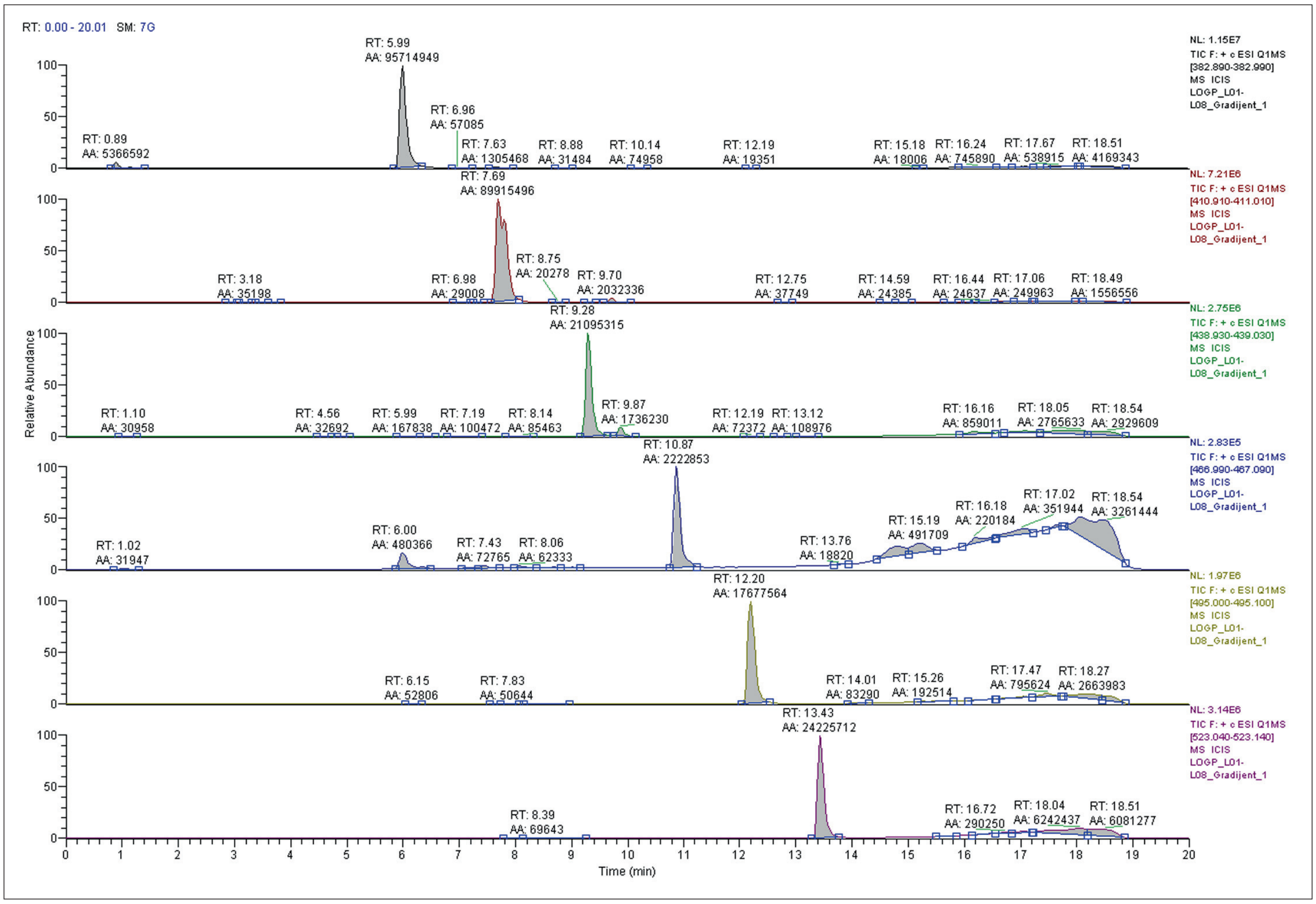


Fig. 2: Chromatogram of (*S,S*)-1,3-propanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid (gradient)

So, the above differences could be ascribed to potentially numerous interactions of 1,2-ethanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid derivatives with the stationary phase and as a result, the retention of the observed substances in the column takes longer time. However, values of partition coefficients calculated based on chromatographic data were somewhat lower than those obtained by the shake flask method for derivatives of 1,3-propanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid, except for PDCP, DPE-PDCP and DIB-PDCP. This difference could be interpreted in terms of differences in the number of established interactions of the compounds with the stationary phase.

It was found that lipophilicity values of dipenthyl ester of 1,3-propanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid and diisopenthyl ester of 1,3-propanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid calculated on the basis of chromatographic data were very similar. Also, lipophilicity values for the dibutyl ester of 1,3-propanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid and the diisobutyl ester of 1,3-propanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid were very similar. The same situation was observed with dibutyl and diisobutyl esters of 1,2-ethanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid. It could be supposed that the alkyl chain branching expressed insignificant influence on retention time, although it expressed the effect on *n*-octanol/water partition coefficient. Isopentyl and isobutyl esters of 1,3-propanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid had lower *n*-octanol/water partition coefficient values determined by shake flask method than *n*-butyl and *n*-penthyl esters of 1,3-propanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid.

Also, looking at chromatographic parameters it can be noticed that CHI and φ_0 values of 1,2-ethanediamine-*N,N'*-di-2-(3-cyclohexyl) propanoic acid derivatives were higher than those of the corresponding 1,3-propanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid derivatives.

In order to explain the observed differences, the influence of numerous descriptors connected to structural differences between 1,2-ethanediamine and 1,3-propanediamine derivatives, *e.g.* volume of the molecule, surface area, molecular mass, *etc.* should be considered. It could be hypothesized that the observed differences might result from the differences in the number of the examined compound interactions with the stationary phase. The distance of 2C atoms between the amino groups (1,2-ethanediamine derivatives) provides an opportunity for more interactions with the stationary phase than when amino groups were separated by 3C atoms (1,3-propanediamine derivatives).

2.3. *Validation of mathematical method*

This hypothesis is supported by the obtained correlations between each of chromatographic parameters (CHI and φ_0) and Log$D_{7.4}$ values, and also, between retention time and Log$D_{7.4}$ values, in all examined compounds when the number of carbon atoms separating amino groups was taken into consideration.

These correlations are shown in the next three developed models: Model 1, Model 2 and Model 3, which were made to predict Log$D_{7.4}$ using UHPLC-MS methods. Also, all three models were validated using cross validation by the leave one out (LOO) technique. This validation technique is used to assess the robustness of a developed model (stability of model parameters), and to make estimates of predictivity. Parameters predictive error sum of squares (PRESS) and cross-validated Q^2 are calculated and observed to justify the thesis that the model is predictable for a presented group of compounds.

Table 4: Calibration and statistical parameters of developed models with parameters of leave-one-out validation of the model's predictive power

Model	Calibration and statistical parameters					Leave-one-out validation	
	Calibration curve ($\log D_{7,4}$ calculated value)	r^2	r	F	Significance F	PRESS	Q^2
Model 1	$\log D_{7,4}=(0.11\pm0.01)\times CHI+(1.17\pm0.22)\times N_c^{******}-(8.84\pm1.28)$	0.8738	0.9348	38.09	$1{,}14x10^{-5}$	3.28	0.78
Model 2	$\log D_{7,4} = (0.11\pm0.01)\times \varphi_0 + (1.25\pm0.20)\times N_c^{******} - (9.19\pm1.18)$	0.8969	0.9470	47.84	$3.74x10^{-6}$	1.62	0.89
Model 3	$\log D_{7,4} = (0.31\pm0.04)\times t_r + (1.17\pm0.22)\times N_c^{******} - (4.11\pm0.76)$	0.8738	0.9348	38.09	$1.14x10^{-5}$	3.28	0.78

******Nc=number of C atoms between two amino groups (Nc=2 for 1,2-ethanediamine and Nc=3 for 1,3-propanediamine derivatives)

The statistical parameters of correlation for Model 1, Model 2 and Model 3, and predictivity power of these models are presented in Table 4.

A positive relationship between retention time in reverse phase high pressure liquid chromatography and partition coefficient *n*-octanol-water has already been known.

It can be shown that the best parameters of correlation are established between *n*-octanol-water partition coefficients obtained by shake flask method ($\log D_{7.4}$) and chromatographic parameter φ_0 obtained by UHPLC-MS method (Fig. 3). Also, this model is shown the best predictivity power, expressed by the term Q^2, was 0.89.

Results of lipophilicity data which were presented in this paper show that the length of alkyl chain on ester groups has influence on the lipophilicity/hydrophobicity of observed investigated compounds. Lipophilicity data obtained by the shake flask method and UHPLC-MS are correlated with results from previously *in vitro* activity studies of investigated compounds. EDCP and PDCP have the lowest values of lipophilicity and the lowest antineoplastic activity (Misirlić-Denčić et al. 2012; Savić et al. 2014). It could be hypothesized that the observed compounds with the most significant antineoplastic activity in previous studies DE-EDCP, and DB-PDCP, have a mechanism of action on cell's membrane level, or on intracellular level. Also, the developed mathematical model has shown good performance and predictivity power. This model can be used for lipophilicity prediction of potential *in vivo* metabolites and for new compounds derived from of (*S,S*)-1,2-ethanediamine-N,N'-di-2-(3-cyclohexyl)propanoic acid, and (*S,S*)-1,3-propanediamine-N,N'-di-2-(3-cyclohexyl)propanoic acid.

3. Experimental

3.1. Chemicals

Investigated compounds (Table 1) were provided by the Faculty of Chemistry, University of Belgrade, Serbia. Methanol and trifluoroacetic acid (HPLC grade), ammonium acetate (CH_3COONH_4) obtained from Fluka (Sigma-Aldrich Co.) and deionized water (Gen Pure Ultrapure, Germany) were used. Phosphoric acid (H_3PO_4), monosodium phosphate (NaH_2PO_4), and disodium phosphate (Na_2HPO_4) of analytical grade of purity were Merck (Darmstadt, Germany) products and dimethyl sulfoxide (DMSO) was produced by Sigma-Aldrich Co. Uracil used for dead time determinations and n-octanol employed as a non-polar phase in the shake flask method were produced by Merck (Darmstadt, Germany).

3.2. Equipment

Analyses were performed on a UHPLC-MS system consisting of Thermo ACCELA (Thermo Scientific, Waltham, Mass., USA) UHPLC system coupled to a triple quad Mass Spectrometer Thermo TSQ Quantum Access Max (Thermo Scientific, Waltham, Mass., USA) with a heated electrospray ionization (HESI) interface. (10 µL) Samples were injected into a reverse-phase Thermo Scientific Hypersil GOLD aQ column (1.9 µm, 100 mm x 2.1 mm) with guard cartridge (SecurityGuard™ ULTRA cartridges for C18 UHPLC, with 2.1 mm internal diameters (ID) Phenomenex (Værløse, Denmark). MS analysis was performed as selected reaction monitoring (SRM) for derivatives of 1,2-ethanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid and single ion monitoring (SIM) for derivatives of 1,3-propanediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid in a positive mode. The monitored ions (m/z) are shown in Table 1. The spray voltage was 4000 V, temperature in the capillary was adjusted to 300 °C and the vaporizer temperature set to 300 °C. Sheet gas pressure was set to 50 units, while the auxiliary valve flow rate to 10 units. MS resolution values were defined to correspond to a mass resolution of 0.7 Da. All data were acquired and processed by Xcalibur software (Thermo Fisher, San Jose, CA, USA). Solids were weighted by 5-digit Mettler analytical balance (Mettler-Toledo International Inc, USA). Mechanical shaker by KINETOR-M (Elektromedicina-Ljubljana, Slovenia) and pH-Meter by pH/Ion meter MeterLab PHM240, Radiometer analytical (France) were used.

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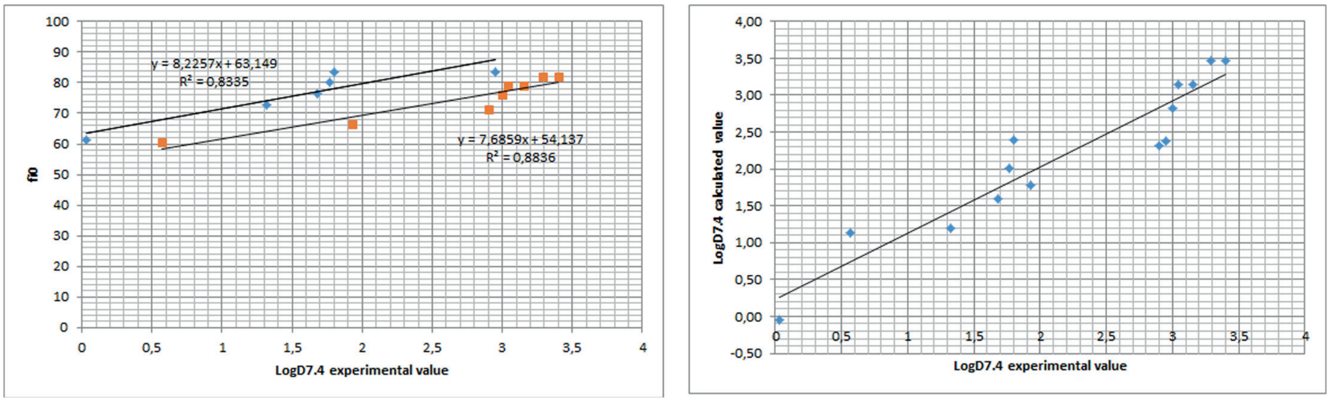

Fig. 3: Plot of $\log D_{7.4}$ determined by shake flask method for the set of 14 investigated compounds *versus* φ_0 values measured by chromatography (left) and Plot of $\log D_{7.4}$ determined by shake flask method for the set of 14 investigated compounds *versus* $\log D_{7.4}$ calculated from φ_0 values measured by chromatography and the number of C atoms (right)

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