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Original scientific paper

High throughput determination $\log P_{o/w}/pK_a/\log D_{o/w}$ of drugs by combination of UHPLC and CE methods

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Abstract

In 1997 Valkó et al. developed a generic fast gradient HPLC method, based on the calculation of the Chromatographic Hydrophobicity Index (CHI) from the gradient retention times, in order to measure lipophilicity. We have employed the correlations between CHI and $\log P_{o/w}$ and adapted the rapid gradient HPLC method to UHPLC obtaining excellent resolution and repeatability in a short analysis time (< 4min). $\log P_{o/w}$ values can be easily obtained from these CHI measurements but, unfortunately, these correlations are only valid for non-ionized compounds. Consequently, in order to determine the effective $\log P_{o/w}$ value at a particular pH, a fast high-throughput method for pK_a determination was required. The IS-CE method, based on the use of internal standards (IS) and capillary electrophoresis (CE), is a fast and attractive alternative to other methods for pK_a determination, since it offers multiple advantages compared to them: low amounts of test compounds and reagents are needed, high purity is not required, specific interactions between test compounds and buffers are corrected, etc. In addition, it allows the determination of a pK_a value in less than 5 minutes. Both CHI and IS-CE have been combined in order to describe a high throughput alternative in the determination of the lipophilicity profiles of bioactive compounds.

Keywords: lipophilicity, drug discovery, acidity constants, chromatographic hydrophobicity index, CHI, internal standard

Introduction

The drug discovery and development process requires the high-throughput determination of physicochemical parameters of drug candidates. Parameters measuring lipophilicity, acidity, solubility or protein interaction must be determined in a fast way for a high number of compounds of potential interest to select the most promising compounds for further development.

One of the most important physical properties affecting the biological activity of substances is lipophilicity, traditionally expressed as the logarithm of the octanol-water partition coefficient ($\log P_{o/w}$). In the common case of drug candidates with acid-base properties, the effective partition rate at a particular pH is noted as $\log D_{o/w}$. Although being these the most widely used lipophilicity indexes, experimental reference procedures are usually time consuming and require a high purity and a relatively high amount of sample. In order to decrease the analysis time and overcome these limitations, instrumental analytical

methods have been developed to determine lipophilicity, and the values therefrom obtained in a particular lipophilicity scale have been correlated to $\log P_{o/w}$ or $\log D_{o/w}$ scale.

About twenty year ago Klára Valkó and Péter Slégel [1] proposed a new hydrophobicity index measured by a reversed-phase HPLC method, named φ_0 , defined as the concentration of organic modifier in the mobile phase (methanol or acetonitrile) which is required to obtain a retention time (t_R) double than the dead time (t_0). In these conditions, the logarithm of the retention factor equals to zero ($\log k' = \log ((t_R - t_0)/t_0) = 0$) and, therefore, the compound is equally distributed between the mobile and the stationary phases. The higher the value, the more hydrophobic is the compound. This index is characteristic of a compound and independent of the column dimensions, the mobile phase composition and flow-rate. It only depends on the stationary phase, the particular organic modifier employed, the temperature and, for acidic or basic compounds, the pH. However, φ_0 values were experimentally determined in isocratic mode using several mobile phases containing low fractions of methanol or acetonitrile, which involved long run times for hydrophobic substances. A few years later, Valkó and co-workers [2] proposed a new high-throughput Chromatographic Hydrophobicity Index (CHI), based on φ_0 but derived from retention times observed in a fast gradient reversed-phase HPLC method. Compounds of previously determined CHI values can be used as calibration set, and the retention times of new compounds can be correlated to their corresponding CHI values. In that work the C_{18} column employed was an ODS2-IK5 Inertsil with the dimensions of 150×4.6 mm, and the gradient program lasted for about 15 min. In a paper published in 2001 in collaboration with Michael H. Abraham [3] a much shorter column was used, a 50×4.6 mm Phenomenex Luna C-18(2), and therefore gradient times were reduced to 5 minutes. In addition, correlations between CHI and $\log P_{o/w}$ were improved by means of hydrogen-bond acidity descriptors, since it was found that the major difference between these scales is their sensitivity towards the hydrogen bond acidity of the compounds. However, in the case of compounds with acid/base properties, poor correlations between CHI and $\log D_{o/w}$ have been obtained so far. In 2002 Stephen F. Donovan and Mark C. Pescatore [4] used a 20×4.2 mm octadecyl–poly(vinyl alcohol) cartridge and a fast gradient methanolic elution in order to estimate $\log P_{o/w}$ from chromatographic data. Due to the pH stability of this polymeric column, the pH of the mobile phase could be adjusted to the desired value to ensure the ionizable analyte to be in its neutral form. Nowadays, UHPLC technology presents also an excellent stability of C_{18} columns over a wide range pH, besides higher resolution and sensitivity, and shorter equilibration times within consecutive runs in gradient elution.

Since mostly of the potential drug candidates are acids or bases, $\log D_{o/w}$ is a fundamental parameter to be determined in the drug discovery process in order to estimate the pharmacokinetics of a compound of interest. Especially relevant is the $\log D_{o/w}$ at pH 7.4, the physiological value, which indicates the lipophilicity of a drug in the blood plasma. As long as this effective partition rate at any particular pH can be estimated from the measured $\log P_{o/w}$ for the neutral substance and the pK_a of the compound, we propose a high-throughput methodology based on the combination of UHPLC for CHI measurements and CE for pK_a determination in order to define the $\log D_{o/w}$ profile of drug candidates.

In a series of previous works [5-7], we have proposed a high-throughput method to determine acidity constants of weak acids and bases by capillary zone electrophoresis. This method is based on the use of an internal standard (IS) of similar nature and acidity constant as the test compound, which is injected into the capillary just after the sample compound and analyzed in the same electrophoretic run. This method requires only two electrophoretic runs: a first one at a pH where both the test compound and the IS are totally ionized, and a second one at another pH at which both are partially ionized. Thus, from the mobilities of the compounds at these two pH values and the pK_a value of the IS, the pK_a of the analyte can

be easily calculated [5,8]. Besides its rapidness, the main advantages of this method are the compensation of systematic errors due to simultaneous analysis of analyte and IS, and the unnecessary of an accurate pH measurement.

Experimental

Apparatus

For UHPLC measurements, a Shimadzu (Kyoto, Japan) Nexera UHPLC system was used. The system was equipped with two LC-30AD high-pressure pumps, a DGU-20A₅ online degasser, a CTO-10ASvp oven thermostated at 25°C, a SIL-30AC autosampler, a SPD-M20A diode array detector and a CBM-20Alite controller. Retention data were obtained from a Waters (Milford, MA, USA) Acquity BEH C₁₈ column, 50 mm × 2.1 mm, which allowed the study of basic drugs because of their extended working pH range (up to 12). Injected samples were prepared in DMSO at a concentration of 0.5 mg/mL and thus, due to the melting point of DMSO (19°C), the autosampler temperature was set at 20°C. Aqueous buffers were, in all cases, 50 mM ammonium acetate at the desired pH value. Acidic buffers were prepared from glacial acetic acid and the pH was adjusted with small volumes of concentrated ammonia (25%), and basic buffers were prepared inversely. Medium acidic and basic buffers were obtained by solving the salt and adjusting the pH with concentrated ammonia or glacial acetic acid.

Capillary electrophoresis experiments were performed using a P/ACE MDQ Beckman instrument (Palo Alto, CA, USA), equipped with a diode array detector. Capillary was made of fused silica 50 µm I.D., 375 µm O.D., 35.2 cm length (25 cm to the detector) and purchased from Composite Metal Services Ltd (Shipley, West Yorkshire, UK). The temperature of the capillary was 25.0±0.1°C and the applied voltage during separation was 20 kV. IS and analyte samples were sequentially injected at 0.5 psi for 3 s and analyzed in the same run. Additional 0.5 psi of hydrodynamic flow during separation was applied. The capillary was initially conditioned with 1 M NaOH (2.0 min), water (0.5 min), and running buffer (2.0 min). Between replicates with the same buffer the capillary was not rinsed. 20 psi of pressure was applied during rinsing processes. Running buffers were prepared at different pH values and 50 mM ionic strength as described elsewhere [8]. Injected samples were prepared in methanol/water mixtures at a concentration of 0.1 mg/mL, containing DMSO as neutral marker. Appropriate IS were selected from the set proposed in previous works [8]. All samples and buffers were filtered through a nylon mesh 0.45 µm porous size (Whatman, Maidstone, UK) before use.

pH measurements were performed with a combined Crison (Alella, Spain) 5014 electrode in a Crison GLP22 pH meter. The electrode system was standardized with ordinary aqueous buffers of pH 4.01, 7.00 and 9.21.

Chemicals

Acetonitrile HPLC gradient grade was purchased from VWR (West Chester, PA, USA). Water was purified by the Milli-Q[®] plus system from Millipore (Billerica, MA, USA) with a resistivity of 18.2 MΩ cm. The chemicals used for buffer preparation were anhydrous sodium acetate (>99.6%) and glacial acetic acid from J.T. Baker (Deventer, The Netherlands), 2-(Cyclohexylamino)ethanesulfonic acid (CHES, >99%), and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS, >98%) from Sigma (St. Louis, MO, USA), 2,2-Bis(hydroxymethyl)-2,2',2''-nitrilotriethanol (BisTris, >99.9%) from Fluka (Buchs, Switzerland), sodium hydroxide, hydrochloric acid and ammonia from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO, >99.9%) was purchased from J.T. Baker. The injected compounds were purchased from Merck, Sigma-Aldrich and Fluka, or obtained from Almirall (Barcelona, Spain), all of them in high purity grade.

Results and Discussion

Measurement of CHI_{MeCN} values by UHPLC

The HPLC fast gradient method developed by Valkó and co-workers [3] was transferred to our UHPLC system taking into consideration the column and particle sizes, the flow rate, the injection volume, and the dwell time of the UHPLC instrument. The final UHPLC conditions, together with the original HPLC ones, are shown in Table 1. DMSO was used as sample solvent because its ability to solve hydrophobic compounds sparingly soluble in MeCN/water mixtures. In the present work MeCN was used as organic modifier as long as this solvent allows better correlations between $\log P_{o/w}$ and CHI than methanol.

Several substances covering a wide range of CHI_{MeCN} and $\log P_{o/w}$ values were tested as calibration standards. Finally, the representative compounds shown in Table 2 were selected. They are mainly unionizable substances that can be used at any pH value (acetanilide and phenones), but 4-hydroxybenzyl alcohol is a phenol that should be used in its neutral form below pH 8. When using aqueous buffers above this pH value, caffeine was used instead of 4-hydroxybenzyl alcohol. A representative chromatogram of the calibration set is shown in Figure 1. After plotting the CHI_{MeCN} values against the retention times in the fast gradient elution, a quadratic calibration curve is obtained with a typical coefficient of determination (r^2) higher than 0.998 and a root mean square error (RMSE) lower than 1.5.

Table 1. Comparison between HPLC and UHPLC conditions to measure CHI values

	HPLC [3]	UHPLC (present work)
Column	C18, 50 x 4.6 mm, 5 μ m	BEH C18, 50 x 2.1 mm, 1.7 μ m
Flow rate	2.0 mL/min	0.5 mL/min
Temperature	25°C	25°C
Fast gradient program	% MeCN	% MeCN
	0.0 min -> 0%	0.0 min -> 0%
	0.5 min -> 0%	0.4 min -> 0%
	3.0 min -> 100%	2.5 min -> 100%
	3.5 min -> 100%	2.9 min -> 100%
	3.7 min -> 0%	3.1 min -> 0%
	4.5 min -> 0%	3.8 min -> 0%
Injection volume and solvent	3 μ L - MeCN/aqueous buffer	0.2 μ L - DMSO

Similarly to $\log D_{o/w}$ scale, CHI values of acidic or basic compounds depend on the ionization degree. The lower the ionization, the higher the CHI value [9,10]. Therefore, acidic substances present the highest CHI when aqueous buffers of low pH are used, and consequently basic compounds exhibit the reversed trend. On the other hand, non-ionizable analytes show nearly the same CHI values independently of the pH. In the present work all studied analytes were injected at three different pH values (3.0, 7.4 and 11.0), and a representative collection supporting the above statements is presented in Table 3.

Table 2. Compounds used as calibration standards.

Compound	CHI_{MeCN} [11]	$\log P_{o/w}$ [12]	pK_a [12]
4-hydroxybenzyl alcohol ^a	18.91	0.25	9.83
Caffeine ^b	24.45	-0.07	0.60
Acetanilide	42.38	1.16	-
Acetophenone	63.26	1.58	-
Propiophenone	76.93	2.19	-
Butyrophenone	87.14	2.66	-
Valerophenone	96.53	3.28	-
Hexanophenone	105.13	3.79	-
Heptanophenone	112.79	4.32	-

^a)Used below pH 8. ^b)Used above pH 8.

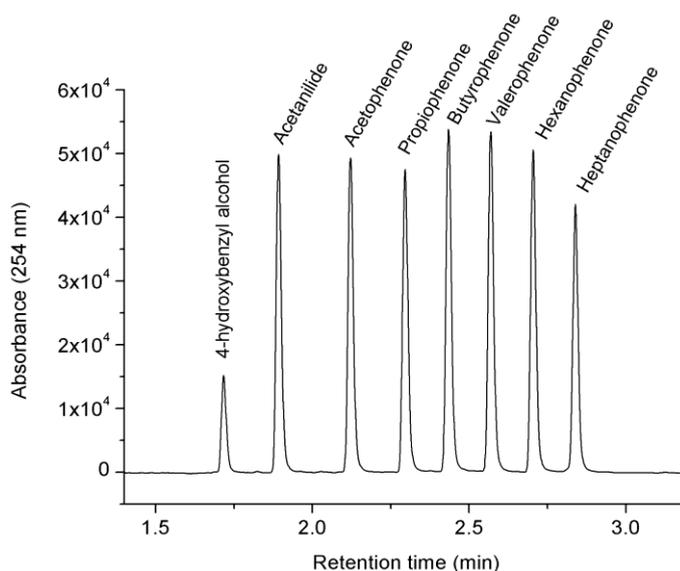


Figure 1. Chromatogram of the calibration set after subtraction of the DMSO blank. UHPLC conditions according to Table 1, 50 mM ammonium acetate pH 7.4 as aqueous buffer.

Table 3. CHI_{MeCN} values obtained at pH 3.0, 7.4 and 11.0.

Compounds	CHI_{MeCN}			Analyte behavior
	pH 3.0	pH 7.4	pH 11.0	
Paracetamol	21.2	20.7	-137.2	Acidic
Theophylline	21.4	20.2	-55.9	Acidic
Atenolol	19.1	18.6	32.1	Basic
Colchicine	43.9	43.4	43.8	-
Salicylic acid	46.0	21.1	-12.5	Acidic
Benzoic acid	48.0	-64.4	-143.2	Acidic
Hydrocortisone	50.2	50.0	50.3	-
Metoprolol	38.1	35.9	61.5	Basic
Procaine	24.9	30.1	62.9	Basic
Propranolol	54.2	51.0	80.9	Basic
Warfarin	82.9	41.8	25.9	Acidic
Ketoconazole	70.8	83.4	83.9	Basic
Lidocaine	35.4	77.9	86.4	Basic
Haloperidol	70.1	66.4	88.9	Basic
Thymol	90.0	89.9	89.5	Acidic
Indomethacin	90.7	54.5	37.7	Acidic
Naphthalene	97.6	97.6	97.5	-
Phenothiazine	98.4	98.4	98.2	-
Reserpine	82.7	95.6	98.7	Basic
Anthracene	111.5	111.7	112.3	-

Determination of $\log P_{o/w}/pK_a/\log D_{o/w}$

As mentioned in the introduction, Valkó and co-workers proposed an equation to correlate CHI_{MeCN} and $\log P_{o/w}$ indexes, which involved the Abraham's solute hydrogen-bond acidity descriptor (A , also noted in the original paper as $\Sigma\alpha_2^H$) [3]:

$$\log P_{o/w} = 0.054 \cdot CHI_{MeCN} + 1.319 \cdot A - 1.877 \quad (n = 86, R = 0.970, s = 0.29, F = 655) \quad (1)$$

Table 4. Validation set used for the determination of log $P_{o/w}$ and log $D_{o/w}$

Behavior	Ref.	Compound	CHI _{MeCN}	A	log $P_{o/w}$ (CHI)	pK _a (IS-CE)	log $D_{7.4}$ (CHI+IS-CE)	log $D_{7.4}$ (reference)
Non-ionizable	1	Acetanilide	40.70	0.41	0.86	-	0.86	1.19 ^a
	2	Acetophenone	62.50	0.00	1.50	-	1.50	1.58 ^a
	3	Anthracene	112.30	0.00	4.19	-	4.19	4.49 ^a
	4	Butyrophenone	87.90	0.00	2.87	-	2.87	2.65 ^a
	5	Caffeine	25.90	0.00	-0.48	-	-0.48	-0.10 ^a
	6	Colchicine	43.90	0.26	0.83	-	0.83	1.09 ^a
	7	Heptanophenone	112.10	0.00	4.18	-	4.18	4.46 ^a
	8	Hexanophenone	105.20	0.00	3.80	-	3.80	3.69 ^a
	9	Hydrocortisone	50.30	0.73	1.80	-	1.80	1.58 ^a
	10	Naphthalene	97.50	0.00	3.39	-	3.39	3.19 ^a
	11	Phenothiazine	98.40	0.13	3.61	-	3.61	4.11 ^a
	12	Propiophenone	77.20	0.00	2.29	-	2.29	2.24 ^a
	13	Valerophenone	97.00	0.00	3.36	-	3.36	3.40 ^a
Basic	14	Papaverine	66.93	0.00	1.74	6.41	1.69	-
	15	Reserpine	98.70	0.31	3.86	6.64	3.79	3.89 ^a
	16	2,4-Lutidine	49.35	0.00	0.79	6.81	0.69	-
	17	Ketoconazole	83.90	0.00	2.65	6.81	2.55	3.42 ^a
	18	Trazodone	76.34	0.00	2.25	6.84	2.14	2.54 ^b
	19	Pilocarpine	20.86	0.00	-0.75	7.08	-0.92	-
	20	2,4,6-Trimethylpyridine	57.17	0.00	1.21	7.51	0.85	-
	21	Lidocaine	86.40	0.26	3.13	7.89	2.52	1.61 ^a
	22	Clonidine	51.30	0.42	1.44	8.10	0.67	0.62 ^b
	23	Bupivacaine	101.75	0.26	3.96	8.19	3.11	-
	24	Quinine	66.42	0.23	2.01	8.45	0.93	-
	25	Haloperidol	88.90	0.31	3.34	8.56	2.15	2.77 ^a
	26	Procaine	62.90	0.23	1.82	9.08	0.14	0.24 ^a
	27	Propranolol	80.90	0.29	2.88	9.48	0.83	1.23 ^a
	28	Metoprolol	61.50	0.29	1.83	9.53	-0.26	-0.25 ^a
	29	Atenolol	32.10	0.78	0.89	9.67	-1.33	-1.81 ^a
Acidic	30	Paracetamol	21.20	0.91	0.47	9.58	0.46	0.35 ^a
	31	2-Chlorophenol	63.64	0.33	2.00	8.50	1.96	-
	32	Theophylline	21.40	0.35	-0.27	8.39	-0.31	-0.07 ^a
	33	Methylparaben	52.33	0.66	1.82	8.35	1.78	-
	34	3,5-Dinitrophenol	85.97	0.83	3.86	8.18	3.80	-
	35	4-Hydroxybenzaldehyde	38.36	0.66	1.07	7.61	0.86	-
	36	Phenobarbital	51.84	0.52	1.61	7.53	1.37	1.51 ^b
	37	Vanillin	41.37	0.44	0.94	7.36	0.61	-
	38	4-Nitrophenol	55.31	0.67	1.99	7.09	1.51	-
	39	Warfarin	82.90	0.31	3.01	5.17	0.83	0.89 ^a
	40	Benzoic acid	48.00	0.57	1.47	4.22	-1.40	-1.27 ^a
	41	Indomethacin	90.70	0.57	3.77	4.16	0.88	-

^a) Shake-flask method, ref. [13]. ^b) Experimental value, ref. [14].

With the aim of validating the UHPLC method proposed, CHI_{MeCN} were measured for several non-ionizable, acidic and basic compounds in the log $P_{o/w}$ range between -0.07 and 4.45. All analytes were injected using aqueous mobile phases of pH 3.0, 7.4, and 11.0, and the highest value was considered for log $P_{o/w}$ determination. These 41 compounds are listed in Table 4, together with the CHI_{MeCN} values of neutral species determined in the present work, the A solute descriptors obtained from the ACD/Percepta platform [15], and the log $P_{o/w}$ determined from CHI_{MeCN} and A according to Eq. (1). There is a good correlation between literature log $P_{o/w}$ values (*Mlog P* values compiled in Bio-Loom database [12]) and the ones measured in the present work (slope: 0.99(±0.06), intercept: -0.15(±0.15), r: 0.940, RMSE: 0.45). Basic

solutes correlate slightly worse than non-ionizable and acidic compounds. Nevertheless, from the 16 studied bases only 6 presented deviations from literature values higher than 0.7 log $P_{o/w}$ units. In four of those cases the determined log $P_{o/w}$ values were lower than the bibliographic ones (compounds 14, 16, 17, and 19 in Table 4), and in two cases (solutes 21 and 29) the reverse trend was shown.

As previously commented, poor correlations are obtained between log $D_{o/w}$ and CHI_{MeCN} values for ionized compounds. However, the effective distribution rate of a drug at a particular pH value can be estimated from its acidity constant and the partition coefficients of the neutral and fully ionized species [16]. The current state of the art does not allow the direct measurement of the partition coefficient of ions but it can be assumed that a typical value is 3.15 units lower than that of the neutral species, according to the work published by Donovan and Pescatore [4]. Thus, log $D_{o/w}$ values of monoprotic compounds can be calculated from the following expressions at any desired pH value:

$$\text{Acidic compound: } \log D_{o/w} = \log \left(P_{o/w} + (P_{o/w} - 3.15) \cdot 10^{\text{pH} - \text{p}K_a} \right) - \log \left(1 + 10^{\text{pH} - \text{p}K_a} \right) \quad (2)$$

$$\text{Basic compound: } \log D_{o/w} = \log \left(P_{o/w} + (P_{o/w} - 3.15) \cdot 10^{\text{p}K_a - \text{pH}} \right) - \log \left(1 + 10^{\text{p}K_a - \text{pH}} \right) \quad (3)$$

Consequently, the measurement of aqueous acidity constants was required in order to estimate log $D_{o/w}$ values and the IS-CE fast method was presented as a very appropriate option. This method allows an accurate determination of acidity constants, which normally present an excellent match with reference values found in literature. In the present work, the correlation between the measured $\text{p}K_a$ values for some of the studied acidic and basic compounds (Table 4) and the ones found in literature was remarkable (slope: $1.01(\pm 0.01)$, intercept: $-0.07(\pm 0.09)$, r : 0.998, RMSE: 0.11).

Table 4 shows the log $D_{o/w}$ values at pH 7.4 resulting from log $P_{o/w}$ determinations and $\text{p}K_a$ measurements (Eqs. (2-3)) and those obtained from reference methods [13,14]. As shown in Figure 2, there is a good agreement between both sets of values, with the only exception of lidocaine, which presented yet one of the worst matches in log $P_{o/w}$ determination.

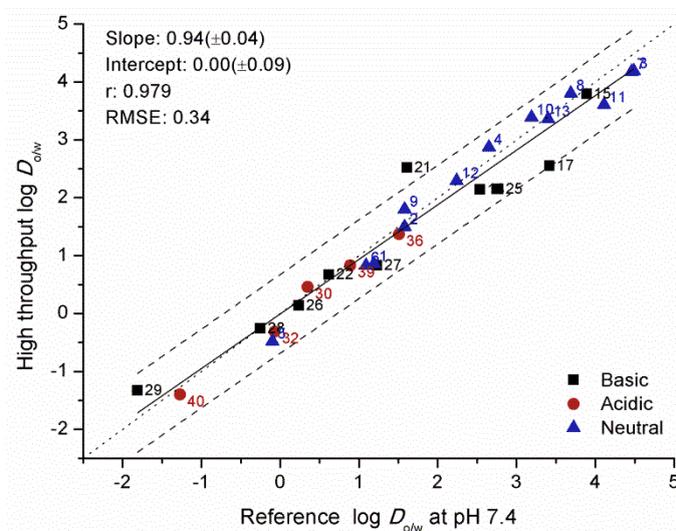


Figure 2. Correlation between reference log $D_{o/w}$ values at pH 7.4 (Table 4) and those obtained from log $P_{o/w}$ and $\text{p}K_a$ measurements (Eqs. (2-3)). Linear regression (continuous lines) and the statistics are also shown, together with the lines corresponding to $\pm 2 \cdot \text{RMSE}$ (dashed lines).

Figure 3 shows the $\log D_{o/w}$ profile obtained in the present work for vanillin and clonidine, which present acidic and basic nature, respectively. For the sake of comparison, the curve calculated by ACD/Labs software is also presented, together with their corresponding calculated and measured pK_a values. In the case of vanillin, the ACD/Labs calculated partition ratio (1.32) is moderately higher than the measured one (0.94), whereas both pK_a values, estimated and experimental, match very well (7.30 and 7.36, respectively). Thus, the ACD/Labs estimated curve shifts up from the experimental one. For clonidine, the ACD/Labs calculated $\log P_{o/w}$ (2.05) is higher than the CHI_{MeCN} measured one (1.44) by 0.6 units, whereas the pK_a (7.90) is lower than the IS-CE determined one (8.10). Therefore, the ACD/Labs estimated curve moves up and left from the measured one, being the values determined in the present work more accurate according to literature (8.05 and 1.43, for pK_a and $\log P_{o/w}$, respectively [12]).

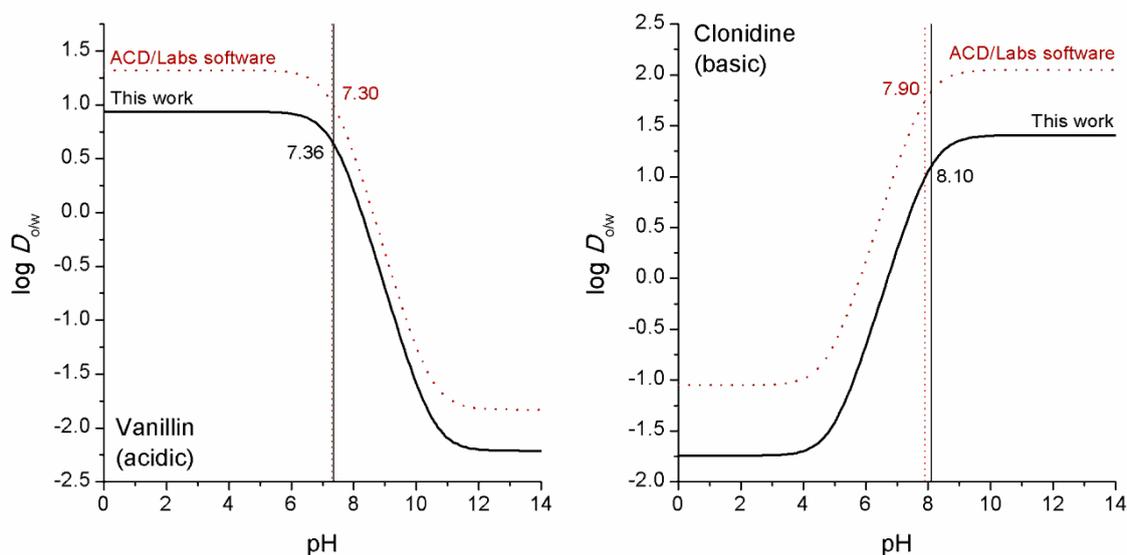


Figure 3. $\log D_{o/w}$ profile for vanillin and clonidine according to Eqs. (2-3) (continuous line) and calculated profile using ACD/Labs software (dotted lines), together with their corresponding measured and calculated pK_a values.

Conclusions

In the present work the combination of two high-throughput methods have been proposed in order to estimate the $\log D_{o/w}$ of an ionizable compound at any desired pH. On the one hand, a fast gradient reversed-phase methodology was successfully transferred into UHPLC in order to determine $\log P_{o/w}$ from chromatographic retention times, based on the formerly developed CHI lipophilicity index. After calibration of the system, a single fast-gradient run performed within 4 minutes is enough to determine the CHI_{MeCN} value of a compound, and then the value can be transformed into $\log P_{o/w}$ lipophilicity scale by means of the established equation. In the case of analytes with acid/base properties, runs performed at 3 different pH values (3.0, 7.4 and 11) are recommended, and the highest CHI_{MeCN} is selected. On the other hand, the IS-CE allows the determination of a pK_a value from only two runs, lasting less than 5 minutes (about 2 min each). Finally, from both the $\log P_{o/w}$ of the neutral species (UHPLC-CHI procedure) and the pK_a value (IS-CE method), the effective distribution rate $\log D_{o/w}$ can be estimated at any particular pH value.

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