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Rapid determination of the applicability of hydrophilic interaction chromatography utilizing ACD Labs Log D Suite: A bioanalytical application

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Abstract

Hydrophilic interaction chromatography (HILIC) is an effective technique for retaining and separating polar compounds. This approach offers several advantages for bioanalytical liquid chromatography/mass spectrometry, considering that a majority of active pharmaceutical ingredients are polar amines. HILIC employs high concentrations of relatively polar organic mobile phase components (i.e. acetonitrile), providing enhanced desolvation and electrospray ionization efficiency, as well as allowing direct injection of many protein precipitation, liquid/liquid, and solid phase extracts. A set of 30 probe compounds was evaluated to demonstrate a relationship between a compound's HILIC capacity factor (k'), and pH dependent distribution coefficient (D), using three sets of generic isocratic conditions. Plots of $\log k'$ versus $\log D_{\mathrm{pH\,3.0}}$ produced correlation coefficients of 0.751, 0.696, and 0.689 at acetonitrile mobile phase concentrations of 85%, 90%, and 95% (v/v), respectively. For bioanalytical applications a $k' > 2$ is typically targeted to ensure adequate retention of a given analyte relative to extracted matrix components. Using $k' \geq 2$ as a measure of HILIC applicability, the linear relationships for each of the three acetonitrile levels predicted whether or not HILIC was able to meet this criterion for at least 90% of the compounds tested. Overall, the relationship between k' and $\log D$ can serve as a valuable tool for identifying the applicability of HILIC and a starting point for the chromatographic conditions, prior to the initiation of any laboratory activities. Additionally, this relationship can assist with the selection of appropriate chemical analog internal standards.

Keywords: Capacity factor, k'; Hydrophilic interaction chromatography, HILIC; Log D relationship

1. Introduction

The term "hydrophilic interaction chromatography" (HILIC) was originally described by Alpert in 1990 [1]. In his work he employed this technique to chromatographically resolve mixtures of proteins, peptides, amino acids, oligonucleotides, and carbohydrates. The elution order of HILIC is from least polar to most polar, which is opposite of traditional reverse-phase chromatography, making it a viable technique for extremely polar compounds [1,2]. HILIC utilizes the same aqueous and organic mobile phases that are used in reversed-phase chromatography, except that water is the "strong" solvent. Alpert speculated that the mechanism of HILIC involves analyte partitioning between the hydrophobic mobile phase and a partially immobilized layer of water on the stationary phase [1]. This partitioning mechanism differentiates HILIC from normal phase chromatography, where the primary mechanism is adsorption to the polar stationary phase [1–4].

HILIC is becoming a more prominent separation technique in the field of bioanalytical liquid chromatography/mass spectrometry [5,6]. The relatively high organic mobile phase concentration provides increased electrospray ionization efficiency through better desolvation and reduced surface tension, compared to reverse-phase chromatography, as well as decreased column back-pressure [5,6]. HILIC coupled with tandem mass spectrometry (HILIC–MS/MS) has been utilized as a quantitation method for a number of bioanalytical applications with complex matrices. Examples include: levosuloiride in human plasma [7], acetylcholine and choline in microdialysis samples from extracelluar fluid of rat and mouse brains

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[8], aminoglycosides in human serum [9], and avoparcin in swine kidney [10]. In addition, many organic solvents commonly used for protein precipitation, liquid/liquid extraction (LLE), and solid phase extraction (SPE) can be directly injected, without evaporation and reconstitution, resulting in a reduction of sample processing steps and simplification of procedures [6]. Hsieh and Chen employed direct injection of methanol protein precipitation supernatant onto HILIC–MS/MS in order to analyze nicotinic acid and its metabolites in dog plasma [11]. Song and Naidong utilized LLE and HILIC–MS/MS for quantitation of omeprazole and 5-OH omeprazole in human plasma, utilizing an extraction which only required a simple dilution of the ethyl acetate extract with acetonitrile prior to injection onto the HILIC–MS/MS system [12]. Li et al. performed direct injection of SPE extracts onto a HILIC–MS/MS system for the analysis of isoniazid and cetirizine in animal (dog, guinea pig, and monkey) and human plasma extracts, respectively [13]. Further benefits of HILIC have been realized with minimal suppression issues from endogenous matrix components [14]. Ionization suppression evaluations performed in our laboratory for several validated HILIC methods have produced minimal ionization suppression issues. Overall, improved signal-to-noise, ease of sample preparation, and reduced matrix suppression effects are transitioning this technique to the primary method of choice over standard reversed-phase systems, when applicable.

Hemström and Irgum recently published a comprehensive review article on HILIC, in which the authors discussed the history, mechanism, column stationary phases, and applications of this technique [4]. HILIC uses a polar stationary phase, such as underivatized silica or silica bonded with polar chemical moieties: aminopropyl, amide, cyanopropyl, poly(succinimide), diol, cyclodextrin, and sulfoalkylbetaine [4,15]. Additionally, HILIC can be performed with polymer-based columns, such as sulfonated styrene-divinylbenzene (S-DVB) [4]. Although a number of stationary phases exist for HILIC, underivatized silica is the most common material employed in recently published methods [4]. This is understandable, considering the relative instability and higher cost of many derivatized stationary phases.

Retention of an analyte on a chromatographic column is described by the term capacity factor (k') and is defined as

$$k' = \frac{t_R - t_0}{t_0} \tag{1}$$

where t_R is the retention time of the analyte and t_0 is the elution time of the void volume or non-retained components [2,16–19]. The Center for Drug Evaluation and Research (CDER) recommends a minimum k' value of >2 [17]. This k' value helps ensure adequate separation of the analyte from un-retained matrix components.

The relationship between the retention of an analyte, k', and the fraction of organic solvent in the mobile phase is described by the following equation for a reversed-phase separation mechanism [2,4,18,19]:

$$\log k' = \log k'_w - S\varphi \tag{2}$$

where k'_w is the theoretical value of k' for when the mobile phase is purely water, φ is the volume fraction of organic in the mobile phase, and S is a constant representing the slope of $\log k'$ versus φ [2,4,18,19]. The reverse-phase separation mechanism is analogous to the extraction of compounds from water into an organic solvent, such as *n*-octanol [16,18–21]. The octanol–water extraction system is a standard tool used to gauge the lipophilicity of a compound and understand partitioning within a biological system. The partitioning between the aqueous and organic layers are represented by the partition coefficient, P, which is defined as [20]:

$$P = \frac{\gamma_O C_O}{\gamma_W C_W} \tag{3}$$

where γ_O is the activity coefficient of the compound in octanol, C_O is the concentration of the compound in the octanol layer, γ_W is the activity coefficient of the compound in water, and C_W is the concentration of the compound in the water phase [20]. In diluted solutions, such as a reversed-phase chromatographic system, this relationship can be simplified to [20]:

$$P = \frac{C_O}{C_W} \tag{4}$$

In the pharmaceutical drug discovery environment the Lipinski "rule of 5" helps provide an indication of a compound's propensity for poor adsorption or permeation [22]. Along with other physicochemical parameters, these guidelines set a "high end" $\log P$ cut-off of 5 for assessing the potential development success of a particular compound. Several researchers have utilized reversed-phase chromatography to accurately predict the $\log P$ of various compounds [18–21,23–25]. A plot of $\log k'$ versus $\log P$ for reference standard compounds produces a linear correlation, in which the $\log P$ of an unknown compound can be predicted. Software packages such as ACD/LogP DB Suite Version 9.0 from Advanced Chemistry Development, Inc. (ACD Labs) are capable of rapidly calculating $\log P$ values from a database of fragmental increments [26]. However, $\log P$ takes into consideration only compounds in the un-ionized form [27,28].

The distribution coefficient, D, is defined as the equilibrium concentration ratio of given compound in both its ionized and un-ionized forms between an aqueous buffer at a specified pH and octanol [27,28]. Log D for monoprotic bases is defined as [27,28]:

$$\log D_{bases} = \log P + \log\left[\frac{1}{1 + 10^{pK_a - pH}}\right] \tag{5}$$

This same equation can be used for a monoprotic acid by replacing the exponent, $pK_a - pH$, with $pH - pK_a$ [27,28]. Lombardo et al. applied the relationship between $\log k'$ and $\log D$, at the physiological pH of 7.4, to predict $\log D$ values of neutral and basic drug compounds [28]. The use of $\log D$, as opposed to the traditional approach of $\log P$, takes into account the compounds pK_a as well as the pH under investigation. The above equations however, are only valid for monoprotic acids and bases. Many of the compounds evaluated in our generic HILIC system are

Table 1
Physicochemical properties of probe compounds

Compound identification no.	Molecular weight (Da)	Acidic pK_a[a]	Basic pK_a[a]	Log $D_{pH\,3.0}$[b]	Log P[b]
1	366	–	9.37, 5.65, 4.25	−8.68	−0.02
2	448	–	8.71, 7.48, 2.98	−7.44	3.09
3[c]	363	11.8	10.1, 4.11	−6.75	1.69
4[c]	377	–	10.1, 3.94	−6.36	1.64
5[d]	439	–	7.67, 5.46	−6.32	0.88
6[d]	453	–	7.67, 5.46	−5.79	1.41
7	227	–	9.95	−4.72	2.23
8[e]	322	–	9.16	−4.38	1.77
9	258	–	8.86, 2.75	−4.20	1.83
10[e]	318	–	9.35	−4.06	2.28
11	355	–	8.17, 4.83	−3.37	3.20
12	510	–	6.99, 5.60	−3.35	2.74
13[f]	378	–	9.43	−2.76	3.39
14[f]	366	–	9.41	−2.44	3.69
15	508	–	4.36, 1.31	−2.10	−0.71
16	470	12.1	8.18, 4.44	−1.08	5.12
17	300	–	9.04	−1.06	4.99
18	335	–	8.49	0.33	5.81
19	448	–	7.67, 2.61	0.67	5.14
20[g]	489	–	4.10	0.85	2.02
21[g]	474	–	4.10	0.94	2.11
22	325	9.25	–	1.51	1.51
23	382	–	5.23, 4.85	1.71	6.07
24	454	3.10	1.44	2.73	3.00
25	559	4.29	–	4.10	4.13
26	469	3.14	2.33	4.46	4.78
27	447	3.33, 11.5	–	5.88	6.08
28	639	10.5, 12.4	1.78	7.14	7.16
29	595	–	–	7.27	7.28
30	600	–	–	8.16	8.16

[a]Calculated using ACD Labs pKa Suite, version 9.0.
[b]Calculated using ACD Labs Log D Suite, version 9.0.
[c–g]Chemical analog pairs.

polyprotic bases. ACD/Log D Suite Version 9.0 is capable of calculating log D values for polyprotic acids and bases over the pH range of 0–14 with increments of 0.1 pH units [29]. This software takes the proposed equilibrium scheme for all of the species in the organic and aqueous phase into account for this calculation [29].

The ability to estimate the pH dependent distribution coefficient of a given compound allowed the following hypothesis to be evaluated: A relationship exists between an analyte's HILIC capacity factor (k') and log $D_{pH\,3.0}$. A mobile phase pH of 3.0 was chosen, considering that a majority of active pharmaceutical ingredients are basic amines that will be protonated under acidic conditions, thereby decreasing their distribution coefficient and potentially their HILIC k'. The following assumptions were made for the evaluation of this hypothesis: (1) Partitioning between the hydrophobic mobile phase and a hydrophilic partially immobilized layer of water on the stationary phase is the primary chromatographic retention mechanism in HILIC, (2) pH of immobilized layer of water on the silica stationary phase is 3.0, (3) ion pairing is insignificant, and (4) ACD Labs Log D Suite can provide reasonable estimate of log $D_{pH\,3.0}$.

2. Materials and methods

2.1. Analytes and reagents

HPLC grade water and acetonitrile were obtained from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Reagent grade ammonium formate was from Sigma–Aldrich Chemical Co. (St. Louis, MO). Reagent grade formic acid was acquired through Acros Organics (Geel, Belgium). Probe compounds were synthesized by Pfizer, Inc. (New York, NY).

A set of 30 probe compounds were selected to cover an expansive log D range at pH 3.0. This set of probe compounds included five chemical analog pairs in order to determine if log $D_{pH\,3.0}$ can be utilized as a tool to screen potential internal standards prior to performing HILIC. Refer to Table 1 for a tabulation of the relevant physicochemical properties of the probe compounds, as well as identification of the chemical analogs. Calculations of log D were made utilizing ACD Labs Log D Suite, version 9.0, at pH 3.0 [29]. Stock solutions of each probe compound were prepared at ca. 100 μg/mL in 50:50 (v/v) water:acetonitrile. Working solutions of each probe compound were prepared at ca. 100 ng/mL in acetonitrile, with the exception of compound no.

1. A working solution of this compound was prepared at ca. 10,000 ng/mL in order to attain an adequate peak response.

2.2. LC–MS conditions

The chromatographic system was comprised of a Shimadzu SCL-10A controller, Shimadzu LC-10AD pumps, and a CTC Analytics (LEAP) HTLS PAL autosampler. The autosampler was equipped with L-Mark syringe from Leap Technologies, P/N LMK.2620719. An Atlantis™ HILIC silica column, 2.1 mm × 50 mm, 5 μm, 100 Å, P/N 186002012 from Waters Corporation (Milford, MA), operated at ambient temperature, was used to achieve separation. A 200 mM ammonium formate stock solution was prepared in water and adjusted to a pH of 3.0 with concentrated formic acid. HPLC grade water and acetonitrile were combined with the 200 mM ammonium formate stock solution to produce three separate mobile phase solutions with acetonitrile concentrations of 85%, 90%, and 95% (v/v) and a total ammonium formate concentration of 10 mM. Previous unpublished work and literature review indicated that these mobile phase compositions were representative HILIC mobile phase systems.

An Applied Biosystems API 4000 tandem quadrupole mass spectrometer equipped with TurboIonspray™ source was employed for selective detection of the probe compounds. The source was operated in the positive-ion electrospray mode with the exception of probe compound nos. 24 and 27, in which negative-ion electrospray was employed. The precursor and product ion pairs were identified via standard mass spectrometer tuning procedures. The probe compounds were prepared in separate solutions and infused independently for this process. The mass spectrometer source parameters were optimized during infusion using the same flow rate and mobile phase composition as the 90% (v/v) acetonitrile isocratic conditions described above. Data acquisition and chromatographic review was performed using Applied Biosystems/MDS SCIEX Analyst, version 1.4.

Probe compound working solutions were injected in triplicate with a run time of 7 min. Prior to injection of these solutions the column was allowed to equilibrate at a flow rate of 1.0 mL/min with each of respective mobile phase conditions. Injections were made by loading a 3 μL sample loop with 10 μL of each probe compound working solution.

3. Results and discussion

3.1. HILIC capacity factor and log $D_{pH\,3.0}$ relationship

The 30 probe compounds selected for this work are representative of the following therapeutic areas: anti-infectives, cancer, cardiovascular and metabolic disease, and central nervous system. These compounds were chosen based on their broad range of log D values at pH 3.0. The k' for each analyte was determined using the retention time of compound no. 30 as the elution time of the void volume, t_0. Compound no. 30 was chosen for the void volume marker due to its high log P and absence of ionizable functional groups. In addition, previous unpublished HILIC experiments evaluating a wider range of acetonitrile compositions (75–95%), produced comparable retention times for compound no. 30 from 0.14 to 0.15 min, indicating that it is not retained and therefore an appropriate t_0 marker. Linear regression analysis of log k' versus log $D_{pH\,3.0}$ values produced correlation coefficients of 0.751, 0.696, and 0.689, for mobile phase acetonitrile concentrations of 85%, 90%, and 95% (v/v), respectively. Fig. 1 provides an illustration of these results.

These direct correlations indicate a relationship between HILIC k' and log D, demonstrating that log D can be utilized as an estimation of analyte partitioning between the mostly organic mobile phase and the stationary water layer in HILIC. The following Eqs. (6)–(8) define the relationship between log k' and log $D_{pH\,3.0}$ for mobile phase acetonitrile concentrations of 85%, 90%, and 95% (v/v), respectively:

$$\log k' = -0.132(\log D) - 0.234 \quad (6)$$

$$\log k' = -0.132(\log D) + 0.034 \quad (7)$$

$$\log k' = -0.139(\log D) - 0.008 \quad (8)$$

These equations can then be used to provide an analyte log $D_{pH\,3.0}$ cut-off point for attaining a minimum k' value of 2 for a given set of isocratic conditions. A k' value of 2, as recommended by CDER [17], was chosen in order to ensure adequate separation of the analyte from un-retained and more hydrophobic matrix components. Log D values of −4.0, −2.0, and −2.2 were determined for mobile phase acetonitrile concentrations of 85%, 90%, and 95% (v/v), respectively, to meet this baseline k' criterion. These values can serve as log $D_{pH\,3.0}$ thresholds for determining the applicability of HILIC, as well as estimating the initial HILIC conditions for method development. Averaged experimental k' values ($n = 3$) produced from the three generic HILIC conditions, along with the predicted k' values utilizing Eqs. (6)–(8) are presented in Table 2. Overall, results indicate that HILIC applicability ($k' \geq 2$) was correctly predicted for approximately 90% of all compounds tested for each of the three acetonitrile concentrations.

3.2. Discussion of assumptions

3.2.1. Partitioning and secondary interactions

Deviation of the correlation coefficients from unity, as well as the bias between experimental and predicted k' values, is believed to be attributed, in part, to the four assumptions that were made when formulating our hypothesis. The first assumption assigns partitioning, between the hydrophobic mobile phase and partially immobilized layer of water on the stationary phase, as the primary HILIC retention mechanism. There are three types of intermolecular forces responsible for chromatographic retention, which include: dispersion forces, polar forces, and ionic forces [30]. The silica stationary phase utilized in this work can exhibit all three of these intermolecular forces to some extent, producing secondary interactions. The influence of excessive secondary interactions may impact the observed k', relative to values that would be expected through partitioning alone. How-

Table 2
Tabulation of experimental k', predicted k', and prediction of HILIC applicability utilizing log D

Compound identification no.	Mobile phase condition								
	85% acetonitrile			90% acetonitrile			95% acetonitrile		
	Mean ($n = 3$) experimental k'	Predicted k'	Correct applicability predictions[a]	Mean ($n = 3$) experimental k'	Predicted k'	Correct applicability predictions[a]	Mean ($n = 3$) experimental k'	Predicted k'	Correct applicability predictions[a]
1	3.56	8.20	Y	7.44	15.13	Y	10.73	15.92	Y
2	12.71	5.62	Y	30.64	10.38	Y	18.09	10.70	Y
3	7.18	4.56	Y	12.51	8.41	Y	18.94	8.57	Y
4	7.62	4.05	Y	11.71	7.47	Y	11.45	7.56	Y
5	4.07	4.00	Y	9.16	7.38	Y	14.67	7.47	Y
6	3.51	3.40	Y	7.89	6.28	Y	12.03	6.30	Y
7	1.36	2.46	N	2.67	4.54	Y	3.18	4.47	Y
8	1.80	2.22	N	3.40	4.09	Y	3.06	4.01	Y
9	7.98	2.10	Y	18.40	3.88	Y	18.52	3.78	Y
10	2.00	2.01	Y	3.80	3.71	Y	2.73	3.61	Y
11	1.04	1.63	Y	2.00	3.01	Y	2.52	2.90	Y
12	0.91	1.62	Y	1.11	2.99	N	0.73	2.88	N
13	1.07	1.35	Y	2.13	2.50	Y	2.06	2.38	Y
14	1.00	1.23	Y	2.04	2.27	Y	1.97	2.15	Y
15	0.20	1.11	Y	0.27	2.05	N	0.55	1.93	Y
16	3.40	0.81	N	7.31	1.50	N	2.91	1.39	N
17	0.80	0.81	Y	1.40	1.49	Y	0.82	1.38	Y
18	0.80	0.53	Y	1.33	0.98	Y	0.64	0.88	Y
19	0.93	0.48	Y	1.67	0.88	Y	0.73	0.79	Y
20	0.33	0.45	Y	0.47	0.83	Y	0.27	0.75	Y
21	0.36	0.44	Y	0.47	0.81	Y	0.30	0.73	Y
22	0.07	0.37	Y	0.09	0.68	Y	0.18	0.60	Y
23	0.40	0.35	Y	0.53	0.64	Y	0.18	0.57	Y
24	0.40	0.25	Y	0.73	0.47	Y	1.27	0.41	Y
25	0.18	0.17	Y	0.40	0.31	Y	0.64	0.26	Y
26	0.38	0.15	Y	0.67	0.28	Y	1.00	0.23	Y
27	0.07	0.10	Y	0.27	0.18	Y	0.27	0.15	Y
28	0.00	0.07	Y	0.00	0.12	Y	0.00	0.10	Y
29	0.07	0.06	Y	0.13	0.12	Y	0.00	0.10	Y
30	0.00	0.05	Y	0.00	0.09	Y	0.00	0.07	Y
% correct applicability predictions			90			90			93

[a] Correctly predicted if a $k' \geq 2$ would be achieved by HILIC.

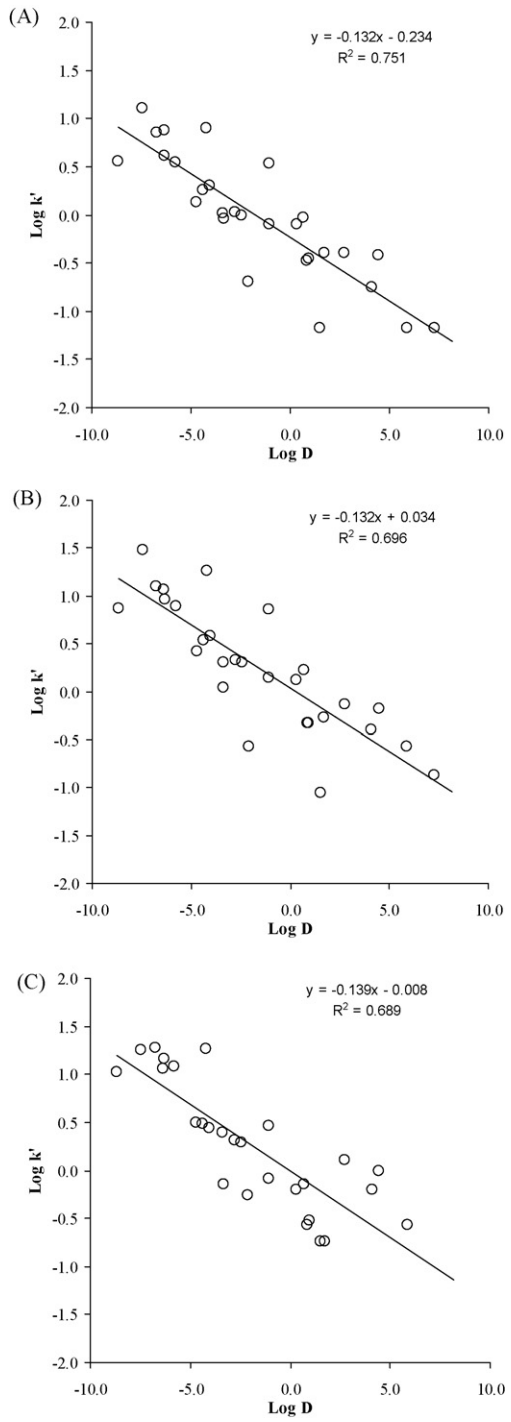

Fig. 1. Log k' vs. log D_{pH} 3.0 obtained utilizing generic HILIC methodology (Atlantis^{TM} HILIC silica column, mobile phase: 5% aqueous 200 mM ammonium formate, pH 3.0 mobile phase containing acetonitrile concentrations of 85% (v/v) (A), 90% (v/v) (B), or 95% (v/v) (C)). Water was utilized for make-up volume. Linear regression analysis produced correlation coefficients (R^2) of 0.751 (A), 0.696 (B), and 0.689 (C).

ever, our generic HILIC systems were designed to minimize possible secondary interactions with the silica stationary phase. Nawrocki provides a comprehensive review article on the silanol group and its role in liquid chromatography [31].

Dispersion forces, also referred to as "London dispersion forces" or hydrophobic forces are related to the polarizability of the molecule, and its propensity to contain fluctuating charges that can interact with the opposite charge on another molecule [30]. The silica columns used for our work contains siloxane bonds, which are considered to be hydrophobic in nature [31]. Considering the relatively high organic solvent concentration of our HILIC systems, dispersive interactions appear to be rather unlikely. Polar forces are due to a dipole or dipoles on a molecule [30]. This type of force has no net charge, considering that there is an equal and opposite charge on the molecule [30]. An example of a polar force is hydrogen bonding [30]. However, direct polar interactions between the solute molecules and the silica stationary phase are assumed to be minimized by the hydration layer.

Ionic forces are caused by molecules that contain a net charge that interact with molecules having the opposite charge [30]. Positively charged analytes can undergo electrostatic interactions with negatively charged silanols [4,31]. This secondary interaction with the stationary phase would increase analyte retention, relative to only the presence of a partitioning mechanism. Conversely, negatively charged analytes may undergo electrostatic repulsion with negatively charged silanols, thereby decreasing retention compared to purely a partitioning mechanism [4]. The majority of the probe compounds investigated for this model were basic amines and are positively charged at the experimental pH of 3.0. This is evident from review of the pK_a values of the probe compounds in Table 1. Previous unpublished experiments, utilizing compound no. 1, indicated that a total buffer concentration of at least 10 mM ammonium formate was required to minimize secondary electrostatic interactions, contributing to band broadening, peak tailing, and excessive retention. Therefore, this relatively high ammonium formate concentration of 10 mM was employed to minimize electrostatic secondary interactions in the HILIC systems under investigation in this study. Ammonium formate was chosen as the buffer species due to its volatility, making it compatible with mass spectrometric ionization. Additionally, the pK_a of formic acid, 3.75, provided adequate buffering capacity at the target pH of 3.0 [32]. Evaluation of Table 2 indicates that the predicted k' of the following compounds (nos. 2–5, 9, and 16) were significantly underestimated. These compounds all contained at least one basic functional group that was fully protonated at the experimental pH of 3.0. This net positive charge would interact with negatively charged silanols of the stationary phase contributing to an elevated experimental k'. These overestimations are not of concern, considering that scope of this work was to provide a tool capable of rapidly determining HILIC applicability ($k' \geq 2$). Capacity factor values of greater than 5 are not typically used for bioanalytical LC/MS/MS assays. For instance, if a k' value of 10 was observed for an active pharmaceutical ingredient at 90% acetonitrile in the mobile phase, the aqueous portion of the mobile phase would be increased to reduce the k' to between 2 and 5. It should be mentioned that the presence of a basic functional group does not ensure HILIC retention if the compound has hydrophobic moieties that contribute to an overall log D of >-2.

Table 3
Chemical analog internal standard selection evaluation results

Compound no.	85% acetonitrile			90 % acetonitrile			95% acetonitrile		
	Experimental k'[a]	Predicted k'[b]	% differential k' ratio[c]	Experimental k'[a]	Predicted k'[b]	% differential k' ratio[c]	Experimental k'[a]	Predicted k'[b]	% differential k' ratio[c]
3	7.18	4.56		12.51	8.41		18.94	8.57	
4	7.62	4.05		11.71	7.47		11.45	7.56	
k' ratio	0.94	1.13	−19.5	1.07	1.13	−5.4	1.65	1.13	31.5
5	4.07	4.00		9.16	7.38		14.67	7.47	
6	3.51	3.40		7.89	6.28		12.03	6.30	
k' ratio	1.16	1.18	−1.5	1.16	1.18	−1.2	1.22	1.19	2.8
8	1.80	2.22		3.40	4.09		3.06	4.01	
10	2.00	2.01		3.80	3.71		2.73	3.60	
k' ratio	0.90	1.10	−22.7	0.89	1.10	−23.2	1.12	1.11	0.6
13	1.07	1.35		2.13	2.50		2.06	2.38	
14	1.00	1.23		2.04	2.27		1.97	2.15	
k' ratio	1.07	1.10	−2.6	1.04	1.10	−5.5	1.05	1.11	−5.9
20	0.33	0.45		0.47	0.83		0.27	0.75	
21	0.36	0.44		0.47	0.81		0.30	0.73	
k' ratio	0.92	1.02	−11.6	1.00	1.02	−2.5	0.90	1.03	−14.2

[a] Experimentally determined k'.
[b] Predicted k' utilizing respective Eqs. (6)–(8).
[c] Percent difference between ratios of experimentally determined and predicted k'.

3.2.2. Mobile phase pH and analyte pK_a

The second assumption in our hypothesis is that the pH of immobilized layer of water on the silica stationary phase is actually 3.0. This pH value is important, considering that the $\log D$ values for each compound are pH specific. Rosés and Bosch provided a review on the influence of mobile phase acid–base equilibria on the chromatographic behavior of protolytic compounds [33]. Although this article was tailored toward reverse-phase chromatography, it can be applied to HILIC. Their findings indicate that an organic solvent, such as acetonitrile, in the mobile phase can change the pK_a of the buffer, as well as that of the analyte. This pK_a deviation was found to be compound specific and can be explained by preferential solvation of the compounds by the components of the solvent mixture [33]. Rosés and Bosch provided an in depth discussion of the relationship between the pK_a of a solute measured in pure water versus the pK_a of a solute in mobile phase containing organic modifier [33]. However, for purpose of this paper, only the following generalities are of concern: (1) Acids generally demonstrate increasing pK_a with increasing organic modifier concentration, thereby increasing the pH of the buffer, (2) the buffer species formate, utilized in this work would relate to this trend, and (3) the pK_a values of the protonated basic compounds, as those investigated in this work, decrease with the addition of organic solvent (minimum pK_a obtained at approximately 60% acetonitrile) and then increases to a pK_a value for pure organic solvent higher than for water [33]. Unfortunately, a thorough literature search failed to locate information on the influence of acetonitrile with respect to the pK_a of formate in the presence of 85–95% (v/v) acetonitrile, as used in our HILIC systems. Therefore, it is difficult to determine the actual effective pH of our HILIC systems, as well as the effective pK_a of our probe compounds, due to the significant amount of acetonitrile in the mobile phase. However, the assumption that the pH of the immobilized water layer is 3.0 is required to establish a relative pH value for $\log D$ value assignment. The relatively high ammonium formate concentration of 10 mM ensures an adequate buffering capacity, corresponding to a constant effective pH.

3.2.3. Ion pairing

The third assumption is that ion pairing is not a significant factor for influencing partitioning under our experimental conditions. Formate has been shown to act as a counter ion for protonated amines in some reversed-phase chromatography applications [34,35]. In our application, the total formate concentration of the mobile phase was approximately 40 mM, taking into account the additional formic acid used to adjust the pH of the 200 mM ammonium formate stock solution to 3.0. The magnitude of possible ion-pair formation or salt precipitation cannot be estimated under HILIC chromatographic conditions, however it can be assumed to be compound specific and dependent on the compound's pK_a [36]. ACD Labs Log D Suite has an ion-partitioning option available; however this option presumes an ionic strength of 0.15 M, in order to simulate physiological conditions. The ionic strength of the mobile phase utilized for our model was appreciably less

(ca. 0.025 M) and when coupled with the fact that formate is a weak counter ion, it is not believed to have any sizeable impact. The ion-pair feature was therefore disabled for the log D predictions.

3.2.4. Accuracy of ACD Labs Log D Suite

The fourth assumption is that ACD Labs Log D Suite can provide reasonable estimates of log D values. Calculation of log D utilizes the compound's calculated log P, calculated aqueous pK_a values, and the aqueous pH (Eq. (5)). ACD Labs states that the error associated with pK_a and log P predictions is ± 0.2 pK_a units and ± 0.3 log P units, respectively [29]. Since the pK_a error corresponds to a purely aqueous environment it is expected to be larger considering the acetonitrile modifier. Additionally, there is an undetermined error in the mobile phase pH due to the acetonitrile modifier, which was explained, in the above discussion of the third assumption. Overall, the estimates of log D appear to be adequate, considering the linear relationship observed between log k' and log D.

3.3. Identification of analog internal standards

Bioanalytical LC/MS/MS requires the use of chemical analog internal standards when stable label internal standards are unavailable. Typically, a similar retention time for the internal standard and analyte are desired in order to compensate for inherent system variability and potential matrix effects, such as ionization suppression or enhancement. The five chemical analog pairs utilized in this work were evaluated in order to determine if $\log D_{\mathrm{pH}\,3.0}$ can be utilized as a tool to screen potential internal standards prior to performing HILIC. Table 3 provides a tabulation of experimental and predicted k' ratios for the chemical analog pairs. Additionally, this table provides percent difference values comparing the experimental and predicted k' ratios in order to provide an evaluation of the utility of using $\log D_{\mathrm{pH}\,3.0}$ as a tool to select possible chemical analog internal standards. Percent difference values in the experimental and predicted k' ratios ranged from -1.5% to -22.7%, -1.2% to -23.2%, and -14.2% to 31.5% for acetonitrile mobile phase concentrations of 85, 90%, and 95%, respectively. These relatively minor differences demonstrate the utility of using $\log D_{\mathrm{pH}\,3.0}$ similarity as an internal standard selection criterion for HILIC.

4. Conclusions

A direct correlation between a compound's HILIC capacity factor k', and pH dependent distribution coefficient, D, was determined. This relationship supports Alpert's HILIC partition mechanism theory. To the best of our knowledge, this is the first time that HILIC retention has been directly related to log D. Although a direct prediction of k' cannot be made from this correlation, due to secondary interactions, this model can serve as a tool to rapidly determine the applicability of HILIC during the method development process, as well as assisting in the identification of appropriate chemical analog internal standards.

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