Chromatography At a Glance

Chapter 1

Chromatographic Approaches for Physicochemical Characterization of Compounds

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Abstract

It is well recognized that the physicochemical characterization of the compounds has primary importance during the early-stage drug screening, as well as for drug product formulation and drug quality control. The pharmaceutical research and industry require reliable and high-throughput methods for estimation for the critical physicochemical properties of the compounds such as ionization constant, lipophilicity, protein binding and permeability. This book chapter covers different chromatographic approaches that have been used for physicochemical characterization of the compounds. The theoretical principles, different calculation methods, advantages and possible drawback, as well their applications are summarized. In addition, an overview of the most widely used biomimetic chromatographic systems is presented.

Keywords: Reverse-Phase Liquid Chromatography; Ionization Constant; Pka; Lipophilicity; Logp; Immobilized Plasma Protein Chromatography; Immobilized Artificial Membrane Chromatography; Biopartitioning Micellar Chromatography.

1. Background

It is well recognized that the physicochemical properties of the compounds (such as ionization constant, lipophilicity, protein binding and permeability) are of great importance to a wide range of research fields [1]. The ionization constant (expressed by the pKa value) is one of the crucial physicochemical property that has an influence on the solubility, lipophilicity and protein binding. The pKa value of the compound, along with the pH value of the solvent medium, has an impact on the solubility of the charged compounds. Depending on the compound's pKa value and the pH value of the media, the molecule could be present in neutral or ionized form. If this is put in context of pharmaceutical active compounds, then the pKa value plays an important role during the formulation of the dosage form, intrinsic solubility and the permeability through the phospholipid membranes.

The pKa value is also tightly related to the lipophilicity. The lipophilicity, expressed by the logarithm of octanol-water partition coefficient (logP), is an indicator of the hydrophobic character of the compound. The ionized form of the compound has greater solubility, but on the other hand the lipophilicity and the passage through the membranes is correlated with the neutral form of the compound. The interaction with the macromolecules on the receptor sites sometimes correlates with the lipophilicity of the compounds. This indicates that the physicochemical parameters are important for understanding the pharmacokinetic behavior of the compounds in the organism.

The knowledge and the accurate determination of the physicochemical characteristics of the compounds are of great importance during the early stage drug development studies, when a large number of compounds should be screened to find out the promising drug candidate.

The physicochemical characteristics of the compounds are also substantial from analytical point of view. Every skilled analyst knows that the selection of a suitable extraction solvent or the choice of proper chromatographic stationary and mobile phase is governed by the pKa value and the lipophilicity of the compound. The awareness of the physicochemical characteristics during the analytical method development leads to faster method optimization and provides ruggedness [1-3].

2. Traditional Methods for Physicochemical Characterization of Compounds

Throughout the years, various techniques and methods have been used for determination of the physicochemical properties of the compounds. In general, the approaches for the physicochemical characterization of the compounds could be divided in two groups, experimental and theoretical.

The traditional experimental methods for pKa determination are spectrophotometry

[4-8] and potentiometry [6-10]. The spectrophotometry is well established technique with excellent precision for determination of the ionization constant for compounds, but it only works for compounds where the chromophore is close to the ionization site in the molecule. In case the chromophore is further from the ionization site, then the absorptivity will not be affected by the dissociation and the compound will have a pH-independent spectrum. The UV/Vis spectrometric method is applicable only on pure compounds because the presents of impurities that absorb in the UV/Vis range could lead to overlapping spectra [4-7].

The potentiometry is a gold standard for pKa determination. This technique offers great precision, accuracy and fast determination. However, the potentiometric method is suitable for compounds having solubility higher than 0.8 mM in the whole pH interval of titration. Alike the spectrophotometric method, the potentiometric method also requires larger quantity of pure compound and it's also sensitive to impurities [6-10].

The capillary electrophoresis (CE) relies on the different mobility of the ionized and neutral form of the molecule. As a separation technique, CE offers advantages over the traditional methods in terms of sample quantity, solubility and purity [7]. The drawback of the CE is the need of preparation of different buffer solutions for each pH to be measured. Also, the use of unsuitable buffer solution could lead to undesirable interactions between the buffer and the compound.

The traditional experimental methods for determination of the lipophilicity are the shake-flask method (SFM) [11] and slow-stirring method (SSM) [12]. These classical methods are time-consuming and are not suitable for degradable compounds. In addition, the SFM method should not be used for compounds having logP value over 5 due to formation of emulsion during shaking. The dual phase potentiometric titration method is suitable for lipophilicity determination of ionisable compounds, but it requires special equipment.

Two reference methods for plasma protein binging are equilibrium dialysis and ultrafiltration [13]. However, these techniques often involve long analysis time or suffer from errors due to nonspecific interactions between the drugs or proteins and components of the analysis system. The traditional methods for predicting permeability includes use of Caco-2 cell line cultures, intestinal tissue or liposome assays [14,15]. These in-vitro methods are laborious, costly to perform and the lack of standardization in cell culturing and experimental procedures makes impossible to compare inter-laboratory permeability data.

Different theoretical approaches and computer programs (for example Pallas program, ACD/Percepta, Marvin predictor, ADME/Tox WEB) have been used for prediction of the pKa value and the logP value. The accuracy of the predicted values is highly dependable from the choice of the mathematical model and the starting parameters used for the calculation. Although these methods do not require sample, the computed pKa and lipophilicity values are inaccurate

especially for sparingly soluble compounds and compound having several functional groups [6, 7].

This book chapter is focused on more advanced approaches for physicochemical characterization of compounds. Number of chromatographic techniques proved to have several advantages over the traditionally introduced ones. This, along with the possibility for reliable estimation of parameters essential for diverse pharmaceutical and medical applications, have put different types of chromatographic approaches in spotlight for determination of physicochemical characterization of compounds.

3. Chromatographic Determination of Ionization Constant

The methodology for ionization constant determination by reverse-phase high performance liquid chromatography (RP-HPLC) was published nearly 50 years ago [16, 17]. The researchers quickly recognized the benefits offered by this technique such as: ability to apply on complex and impure samples, samples with small solubility and the good level of throughput. For more than twenty years, the RP-HPLC has been intensively used for the physicochemical characterization of the compounds [18, 19]. The increased number of published articles points out the fact that this technique is a method of choice for the pKa and logP determination.

The principle of the chromatographic determination of the ionization constant by RP-HPLC is based on different retention times of ionized and neutral form of the compound. In RP-HPLC, the ionized form is less retained on the nonpolar stationary phase, leading to smaller retention factor (k') compared to the neutral form of the compound [6, 18-20]. According to the present literature data, there are three different RP-HPLC approaches for pKa determination: isocratic, gradient and solely aqueous RP-HPLC method.

3.1. Isocratic RP-HPLC approach for pKa assessment

In order to determine the pKa value of the compound using the isocratic RP-HPLC approach, usually at least three different binary mixtures of organic solvent (acetonitrile or methanol) with suitable aqueous buffer should be prepared. It has been observed that the increase of the organic content in aqueous-organic mobile phase leads to suppression of basic pKa of the compound and an increase in the compound's acidic pKa [21]. Considering that the organic solvent has an influence on the pKa value, its content in the mobile phase should not be more that 40%. Generally, in order to evade too low or too high values of k', preliminary experiments are conducted to define the concentration of the organic modifier in the mobile phase. In addition, the pH value of the aqueous buffer should be predefined in rational range, depending on the preliminary experimental data, knowledge obtained from literature data or the compound's structure. The degree of ionization of the compound in the chromatographic

system is governed by the pH of the buffer used in the composition of the mobile phase. Therefore, the chromatographic retention of the compound is evaluated in each mobile phase at different pH values [21-25].

The various k' values, obtained at different pH values of the mobile phase, are used for the determination of the apparent pKa value ($^{s}_{w}pKa$). The term apparent means that this pKa value is determined in non-aqueous medium and it should be further correlated to the aqueous (with zero organic solvent) pKa value ($^{w}_{w}pKa$). One of the approaches for determination of the $^{s}_{w}pKa$ value is by plotting the obtained k' values versus the pH value of the mobile phase. This graph produces sigmoidal plot with inflection point at pH= $^{s}_{w}pKa$. Considering that the pH value of the mobile phase is directly involved in the pKa determination, it is essential to define the procedure of measuring the pH value of the mobile phase.

The most common procedure for determination of the pH value of the mobile phase is by measuring the pH value of the aqueous buffer before mixing it with the organic solvent. In this case the pH meter is calibrated with aqueous buffer solutions, so the measured pH of the mobile phase is denoted as $^{w}_{w}pH$. The analysts are aware that, due to the organic solvent, the pH of the mobile phase differs from the measured pH of the aqueous buffer. Therefore, this procedure is more appropriate for analytical purposes, providing more robust HPLC method. However, if this procedure is used for the experiments for the pKa determination, then the correlation of k' values versus pH of the mobile phase is not quite accurate.

In case of pKa determination, it is more proper to measure the pH value of the mobile phase after mixing the aqueous buffer with the organic solvent. If the pH meter is calibrated with aqueous buffer solutions, the obtained pH value is denoted as $^{s}_{w}pH$ [26]. The plot k' value versus pH value at a particular organic solvent concentration is fitted to a nonlinear symmetrical sigmoidal model. The inflection point of the curve corresponds to the $^{s}_{w}pKa$ value of the compound. There are several nonlinear regression softwares that could be used to obtain the inflection point of the curve, pointing the $^{s}_{w}pKa$ value [23,24].

The $^{s}_{w}pKa$ value could be obtained without the use of regression software by combining the equation 1 and equation 2 proposed by Angelov [27] and Huo [25], respectively. Angelov et al. [27] estimate the pKa value of parabens by HPLC, using an equation based on the Horvath's theoretical model [16] for studying the pH dependence of chromatographic retention for ionizable solute in liquid chromatography.

$$pK_a = pH - \frac{\log(k_{HA} - k)}{k - k_A} \qquad (1)$$

$$(k_{i+1} - k_i)/(pH_{i+1} - pH_i) \qquad (2)$$

The k_{HA} and k_A, in Equation 1, are the neutral and fully ionized forms of the compound, respectively. The parameter k and the pH value in the same equation are the k' value of the compound obtained at the pH value where a maximum slope of the pH – k' plot is obtained.

The equation 2 could be used to define the pH value and k value, needed for the calculation in eq.1. The expression $(k_{i+1} - k_i)$ denotes the difference between the k' values of the compound obtained at two different next to each other pH values. The pH value and the corresponding k' value that give the maximum value for the expression $(k_{i+1} - k_i)/(pH_{i+1} - pH_i)$, should be defined as the corresponding point with maximum slope and further incorporated in the eq.1. This approach was used for determination of the dissociation constant of sparingly soluble drug mupirocin [28].

However, the determination of the ${}^{s}_{w}pKa$ value is only half of the road. The next step is to estimate the ${}^{s}_{w}pKa$ value. As for the determination of the ${}^{s}_{w}pKa$ value, there are also several approaches for ${}^{s}_{w}pKa$ value estimation. One of the most simple approach is based on the linear relationship between the ${}^{s}_{w}pKa$ values and the volume fraction of the organic solvent used in the applied mobile phases. The extrapolation to zero organic solvent mobile phase (the intercept of the linear equation) will give the ${}^{s}_{w}pKa$ value of the compound.

The approach proposed by Kazakevich & Lobrutto [21] takes into account the type (methanol or acetonitrile as most commonly used organic modifiers) and the concentration of the organic solvent (Equation 3).

$$ {}^{w}_{w}pKa = {}^{w}_{s}pKa - (x\%organic) * A \qquad (3) $$

The parameter A in the equation 3 may have values 0.03 or 0.02, which corresponds to acidic or basic compound's pKa shift per 10% *v/v* acetonitrile, respectively.

A large number of researchers [20, 23] used the Yasuda-Shedlovsky extrapolation [29] for estimation of the ${}^{w}_{w}pKa$ value (equation 4).

$$ {}^{s}_{w}\mathrm{pK_a} + \log[H_2O] = \frac{\alpha}{\varepsilon} + \beta \qquad (4) $$

This approach is based on the linear relationship between the ${}^{s}_{w}pKa$ value and the solvent dielectric constant. The ${}^{s}\varepsilon$ denotes the solvent dielectric constant, [H2O] is the molarity of water in the mobile phase, and α and β are constants. The plot of ${}^{s}_{w}pKa$ value + log [H2O] versus $1/{}^{s}\varepsilon$ versus $1/s\varepsilon$ is extrapolated to a value corresponding to pure water (ε=78.3, [H2O] = 55.5). The ${}^{s}_{w}pKa$ value could be calculated from the equation 4, using the obtained value for slope and intercept as α and β constants, respectively.

The isocratic RP-HPLC methodology for determination of the ionization constant of compounds is well established for more than 20 years. Over the years, this approach has shown

to be very reliable and the obtained pKa values correspond to the pKa values determined by the traditional methods, such as potentiometry and UV/Vis-spectrophotometry [22, 23, 28, 30, 31].

The downside of this approch is that it is not applicable for simultaneous determination of large number of compounds. Also, the situation becomes more complicated if the compound has multiple ionization sites and it belongs to a class of polyprotic basic compounds [32]. In this case more experiments should be conducted, which is time-consuming [6, 20]. In addition, attention should be paid towards the selection of the stationary phase. Namely, the experiments for determination of the pKa value of basic compounds should be conducted using strong basic mobile phases (with pH value over 10). A well-known fact is that the ordinary reverse-phase stationary phases have limited stability in high pH mobile phases. Nowadays, there are a large number of commercially available stationary phases that have wide pH stability range that could fit this purpose. Still these columns commonly have pH stability up to 12, so for extremely basic compounds, the column stability issue for the pKa determination is not overcome yet.

3.2. Gradient RP-HPLC approach for pKa assessment

The concept of the pH gradient RP-HPLC approach was proposed by a group of Polish researches led by Wiczling and Kaliszan [20]. This approach is based on linear pH gradient with a fixed organic solvent concentration. During the experiments, the change of the pH of the mobile phase is aimed towards gradually increase of the degree of ionization of the compound, proving decrease in its retention time. Hence, in case of acidic compounds the pH of the mobile phase, during the chromatographic run, is increased and for basic compounds, it is decreased.

At the start, depending of the sample composition and compound's structure, one or two organic solvent gradients at pH value where the ionization of the compounds is suppressed should be conducted. Commonly, for acidic compounds the organic solvent gradient is performed at pH 3.0 and for basic compounds, at pH 10.5. The purpose of this organic solvent gradients is to define the organic solvent concentration in the mobile phase needed to elute the neutral form of the compound and to obtain the maximum retention time. Once the percentage of the organic solvent in the mobile phase is defined, then the pH gradient experiment is conducted. Mobile phase with universal buffer is used in order to control the pH value of the mobile phase. There are different types of universal buffers, but the authors of this approach used a buffer that contains 0.008 M citric acid, 0.08 M tris(hydroxymethyl)-aminomethane and 0.008 M glycine. The pH of the mobile phase was adjusted over a wide pH region using 1 M HCL or 3 M KOH. At the end, one isocratic run at defined pH value where the compound is fully ionized is performed.

Wiczling and coworkers [20] firstly demonstrated the applicability of this approach on 16 basic and 8 acidic compounds. The obtained ${}^{S}_{W}pKa$ values differ from the literature data up to 0.7 pKa units. Two years later, the same research group [33] verified the reliability of the proposed pH gradient approach on 93 acidic and basic drug analytes. Afterwards Wiczling et al. [34] modified and improved the pH gradient approach with the pH/organic solvent gradient approach. This approach was applied for the pKa determination of polyprotic analytes. In the pH/organic solvent gradient RP-HPLC approach, both the pH and the content of the organic solvent in mobile phase is changed with time. Chiang et al. [35] reported a similar medium throughput pH/organic solvent gradient RP-HPLC method for the pKa determination of more than 40 diverse compounds.

Kaliszan [36] clearly highlighted the advantages of the two gradient RP-HPLC approaches. Namely, the pH gradient approach is faster, but prior information about the compound is needed. The main advantage of the combined pH/organic technique is that there is no need to search for specific conditions that could provide measurable retention times of the compounds. Both methods are suitable for analysis of complex mixtures and generally there is no risk analytes to be extremely retained on the column.

The Wiczling and Kaliszan research group went a step further towards modernization of the method and instead UV detector, they used time-of-flight mass spectrometry (TOF-MS) detector. This enabled to expand the applicability of the pH/organic solvent gradient RP-HPLC approach, firstly on mixture of 40 compounds [37] and later on a diverse group of 161 chemical compounds [38]. The applied methodology achieved a throughput of about 100 compounds per day.

The pKa value, using the gradient RP-HPLC methodology, could be calculated using more complex mathematical models set on ground theoretical basis [33,34,36,37]. Although the equations are well described, the beginners may have difficulties to apply the presented calculations on concrete cases. Also, the reasercher have to bear in mind that the gradient RP-HPLC methodology are not quite accurate and that there is a difference of 0.5 to 0.7 pKa units between the observed pKa values and pKa values from the literature data [20,34,39]. Considering the highthroughput and the accuracy of the data, the combined pH/organic solvent gradient RP-HPLC approach is useful for rough estimation of the pKa value needed for a preliminary evaluation of the compounds in early drug discovery phases.

3.3. Solely-aqueous RP-HPLC approach for pKa assessment

Recently, Volna et al. [40] presented new RP-HPLC methodology for pKa determination which is based on solely aqueous mobile phase. This approach was applied on 15 well-known active pharmaceutical compounds with various structure and relatively wide range of lipophilicity (logP from -2.26 to 2.26). The mobile phase consisted of modification of Britton-

Robinson buffer. The original Britton-Robinson buffer has a pH range from 2 to 12. This universal buffer consists of mixtures of acids of diminishing strength (increasing pKa), so that the change in pH is approximately proportional to the amount of alkali added. The modified Britton-Robinson buffer used for these experiments contains 0.01M phosphoric acid, 0.01M acetic acid and 0.01M boric acid as eluent A and 0,1% *v/v* diethylamine as eluent B. The pH of the mobile phase containing only eluent A has a pH of 2.50. The increase of the concentration of eluent B in the mobile phase leads to higher pH value of the mobile phase, up to pH 10.77.

Generally, the conventional C18 columns shows poor performance (retention and peak shape) when they are used with solely aqueous mobile phase, probably due to the low surface hydration caused by repulsion between aqueous mobile phase and the hydrophobic bonded phase. Considering that the mobile phase in this approach is solely aqueous, the experiments should be conducted using stationary phase that is compatible with 100% aqueous mobile phase. Another fact that has to be considered is the column dimension. The compounds having higher lipophilicity, showed high retention times and/or peak broadening. Preferably columns with shorter length and smaller diameter and particle size should be used.

The authors of this method, for the determination of the pKa value for very lipophilic compounds, used modified Britton-Robinson buffer with 5% methanol. The chromatographic run time for these compounds was up to 120 minutes.

The ${}^{s}_{w}pKa$ value of the tested compounds was determined using the calculation (eq.1) proposed by Angelov et al. [27]. This calculation approach was also found applicable for more lipophilic compounds for which 5% of methanol was added to the mobile phase. According to the Yasuda-Shedlovsky extrapolation studies, the addition of 5% methanol has no significant influence on the pKa value of the compounds [41, 42].

The main advantage of this approach is that there is no need for estimation of the ${}^{s}_{w}pKa$ value from the ${}^{s}_{w}pKa$ value, so more complex calculations (such as Yasuda-Shedlovsky extrapolation) are evaded. In addition, the use of solely aqueous mobile phase avoids the need of pKa determination at different binary mixtures of aqueous-organic solvent mobile phases. Still, for the pKa determination of less complex compounds, at least 8 experiments (at different pH values) should be conducted, whereby the complexity of the compounds is associated with a larger number of chromatographic runs.

Volna et al. [38] demonstrated that this approach encounters good correlation between the obtained chromatographic pKa values and the literature data, providing mean difference of only 0.26. The Volna's solely aqueous RP-HPLC approach appeared very promising, but till date there is no further published application based on this approach. So, the time will tell whether this approach will be more widely accepted for the pKa determination of compounds.

4. Chromatographic Determination of Lipophilicity

The RP-HPLC methodology for determination of the lipophilicity of compounds appeared in late 1980s. The principle, as for pKa determination, is based on the relationship between the retention factor of the compound on RP chromatographic column and its lipophilic character [42-47]. In general, the RP-HPLC methodology belongs to the group of indirect experimental methods for logP determination.

Generally, regardless of the used approach, the estimation of lipophilicity is based on the correllation between the logP values of reference (model) compounds and their logk' values obtained under the same chromatographic conditions as for the tested compounds. Therefore, the selection of the model compounds is crucial. In general, the model compounds should be structurally related to the test compounds. The researches should also bear in mind that the estimation of the logP values of the test compound is strongly dependent on the logP values of the reference compounds used for the correlation. Namely, better correlation is obtained between the chromatographically driven logP values and the logP data obtained with classical methods (such as SFM, SSM), compared to the logP data attained by computational approaches (modeling softwares) [48, 49].

The repeatability of the obtained logP data is also associated to the proper determination of the k' value. The imprecision is related to the shifts of the retention time caused by column aging. In order to reduce this deviation, a marker compound for dead time (t_o) is introduced. Usually, thiourea, formamide or sodium nitrate are used as dead time marker. Large number of isocratic and gradient RP-HPLC chromatographic procedures for lipophilicity assessment used this approach, named as single-point retention time correction (SP-RTC). However, it is well known that the to is usually too low to be measured accurately, thus a large random error could be introduced. Han et al. [48] proposed a dual-point retention time correction (DP-RTC). For that purpose, anisole and hexamethylbenzene are used as "anchor compounds" and a correlation between the Rt on the first and the following day is obtained (Eq.5).

$$Rt_{measured} = mRt_{standard} + n \qquad (5)$$

Where as $Rt_{standard}$ is the retention time obtained at the beginning (the fisrt day) and $Rt_{measured}$ is the retention time in the following experiments every day. These two compounds were used as an external calibration method for the retention time of the test compounds on a particular day. This research group [48] compared the precision between the $logk_w$ value acquired using the SP-RTC and DP-RTC approach. The results have shown that the RSD of the logkw value for DDT-related compounds, measured over several months, is significantly lower than the one obtained with SP-RTC approach (0.02% to 0.50% vs 0.12% to 3.27%, respectively. The proposed DP-RTC approach is particularly applicable for hydrophobic compounds having logP value in the range of 4-7.

The general point of view is that the experiments for the lipophilicity assessment should be conducted on silica-based chromatographic columns having minimal residual silanol activity. The residual silanol groups could interact with the test compounds, thus producing unreal picture of the lipophilic character of the compounds. This is particularly important for basic compounds. Nowadays, there are a large number of commercially available chromatographic columns (such as end-capped, double end-capped, base-deactivated columns, columns with bidentate technology) that are free from silanol residual activity [50]. On the other hand, the polar-embedded stationary phases (for example amide, sulfonamide, carbamate) are not the right choice for logP assessment because these polar groups could also interact with the test compounds and as a result disturbance of the linearity between the logP and $logk_w$ could appear.

Methanol and acetonitrile are the most favored organic solvents used for the mobile phase for the logP determination. Methanol is a polar protic solvent and it has a strong ability of hydrogen bonding with the polar active sites of the surface of the stationary phase. As a consequence, methanol forms a monolayer, which provides a hydrogen bonding capability in better agreement with n-octanol [51]. On the other hand, acetonitrile, as a polar aprotic solvent, is only a proton acceptor and doesn't have the ability for hydrogen bonding, thus better simulates the organic phase [52]. Acetonitrile is a preferred solvent for gradient RP-HPLC methods for logP determination [53, 54].

In the subsequent paragraphs, the methodology for lipophilicity assessment through isocratic and gradient chromatographic experiments will be discussed.

4.1. Isocratic RP-HPLC approach for logP assessment

One of the oldest and classical isocratic RP-HPLC method for logP determination is published by Organisation for Economic Co-operation and Development (OECD) [55]. This guideline describes the procedure for logP determination using methanol/water mobile phase and C18 or C8 stationary phase with minimal silanol activity. This approach is based on single isocratic run. The correlation graph between the logP and logk' values of the model compounds is constructed. Afterwards, the logk' for the tested compounds is calculated and the logP value is obtained from the regression equation of the logP-logk' graph of the model compounds. This approach gives a list of 60 model compounds along with their logP value. The OECD method is applicable for compounds having logP values between 0 and 6, and it's not suitable for strong acids and bases, metal complexes and surface-active agents.

It has been demonstrated [56] that the isocratic logk' values give poor correlation to logP values when structurally unrelated compounds are used. This difficulty could be surpassed if extrapolated $\log k'_w$ (at "pure water") is employed. Therefore, several isocratic runs (at least four) at different organic modifier concentration are conducted. The $\log k'_w$ value could be obtained using the Snyder-Soczewinski equation when the logk' values are plotted against the

volume concentration (φ) of the organic modifier (equation 6) [57, 58].

The intercept of this equation represents the log k'w value and the slope (S) is a solute dependent solvent-strength parameter reflecting the interaction between solute and solvent [59].

$$Logk' = logk'_w - S\varphi \quad (6)$$

Afterwards, the relationship between log k'_w value and the logP is given by the Collander-type equation [52], whereas

$$logP = a\ logk'_w + b \quad (7)$$

The last two equations are the basic for the logP assessment of neutral compounds. As decribed by Valkó [58], different $logk'_w$ values for same compound could be obtained if the organic solvent in the mobile phase is used. The Valkó's research group introduced the isocratic chromatographic hydrophobicity index (φ_0) as a parameter that gives good correlation with the logP values for nearly 500 drug molecules analyzed under chromatographic conditions where the neutral form of the compound is dominant. The φ0 could be calculated using the regression constants from the Snyder-Soczewinski equation (eq.8) [56].

$$\varphi_0 = logk'_w / S \quad (8)$$

Namely, the φ_0 value is a ration between the $logk'_w$ (intercept) and the S value (slope). According to the authors [53] the main drawback of the use of φ_0 for the logP calculation is the low througput because experiments at several organic modifier concentrations should be conducted.

However, for structurally related compounds the process could be speed up. This is due to the fact that linear relationship between the slope (S) and the $logk'_w$ value was observed [55, 56]. The linear relationship between S-logk'w serves as an indicator of the uniformity of the retention mechanism between compounds [52]. This relationship is presented by the following equation

$$S = a + b\ logk'_w \quad (9)$$

By combining this equation to the Snyder-Soczewinski equation, a very useful equation (Eq.10) for fast $logk'_w$ estimation is obtained.

$$logk'_w = (\varphi\ a + logk') / (1 - \varphi\ b) \quad (10)$$

Once more it should be pointed out that this approach is applicable only for structurally related compounds. In this case, where the test compounds are structurally related to the compounds used for the establishment of the linear relationship between the slope (S) and

the $logk'_w$, there is no need for conduction of several isocratic experiments. The logk'w value could be estimated directly from the eq.8, based on data for logk' obtained only from one isocratic experiment.

The aforementioned approaches are more suitable for neutral compounds or compounds that are present in unionized form in the applied mobile phase. The factor that governs the ionization of the compounds in the chromatographic system is the pH value of the mobile phase. By applying mobile phase at appropriate pH value, the acidic and basic compounds could be converted to their neutral form. However, the column stability and buffer capacity should be taking into account. The use of buffers that have large buffering capacity and do not interfere with the active groups on stationary phase (such as morphilinepropanesulfonic acid (MOPS), phosphate buffer and phosphate-buffered saline), could solve the problem. However, these buffers have limited solubility in larger concentration of organic solvents and are not compatible with mass detector [62, 63].

The lipophilicity assessment of the strong acids and bases requires addition of ion suppressors or masking agents in the mobile phase. Literature proposes data for the use of acetic acid and perchloric acid as ion suppressors for the lipophilicity experiments of weak monoprotic and polyprotic acidic compounds [64-67]. In case of basic compounds, a hydrophobic amines (triethylamine, n-decylamine and N,N-dimethyloctylamine) in low concentration (0.15% - 0.20% *v/v*) are usually added as a masking agents in the mobile phase [52, 63, 68]. It is preferable to use methanol as a mobile phase in combination with amine masking agents because acetonitrile reduces the capability of the amine to act as a masking agent.

Pallicer and coworkers proposed a method for lipophilicity assessment of basic drugs with pKa higher than 9 [69, 70]. The experiments are conducted with mobile phase at high pH value; thus, the basic compounds are present in their neutral form. Considering the silica-based column stability at strong basic conditions, the method requires high alkaline-resistant stationary phases such as Gemini NX, XTerra RP-18 and XTerra MS C18. The experiments showed that the method is applicable for logP determination of compounds with diverse structure, just from a single isocratic run. In addition, it offers good estimation (with a precision of 0.4 logarithmic units).

Giaginis et al. for more than 15 years are devoted towards development of methods applicable for lipophilicity assessment, particularly for basic compounds [51, 63, 71]. Recently this research group published two detailed protocols for logP assessment for basic/neutral and acidic ampholytic compounds, respectively [68]. The experiments are conducted on Hypersil BDS C18 column, but in addition authors give recommendation for three other columns that could be also used for the experiments. The assessment is conducted under isocratic conditions

with methanol-MOPS mobile phase. The concentration of the organic solvent, pH value of the buffer and type of additives (such as n-octanol or n-decylamine) depends on the compound's structure, i.e., whether it is basic or acidic.

4.2. Gradient RP-HPLC approach for logP assessment

The gradient RP-HPLC methodology for logP assessment allows lipophilicity determination without the need of searching suitable chromatographic conditions for analyte elution in reasonable time which significantlly speeds up the process. One of the most cited gradient RP-HPLC method for logP determination was described by Valkó's research group [53]. The method was applied on 76 structurally unrelated compounds having logP values between -0.45 to 7.3. This research group introduced the term Chromatographic Hydrophobicity Index (CHI) values. The CHI value is obtained using gradient retention times and it represents the percentage of organic modifier required to achieve equal distribution of the analyte between the mobile and stationary phase [58]. The basis of this approach lies in the calibration of the HPLC system with ten reference compounds with known CHI value. For these compounds, linear relationship between the CHI value and gradient retention times t_R was observed (equation 11).

$$CHI = a\, t_R + b \qquad (11)$$

Knowing the regression coefficients of this equation, the CHI value for the tested compounds could be calculated. The CHI value is well correlated to the logP, allowing estimation of the lipophilicity from a single gradient experiment. This approach is suitable for neutral compounds, but it isn't applicable for hydrophilic compounds that have short retention times in presents of high organic eluent concentration.

Another gradient approach for simultaneous determination of pKa and lipophilicity of 93 monoprotic acids and bases was proposed by Wiczling et al. [33]. The proposed approach showed good correlation between the retention times of neutral form of the compounds and the φ_0 value. Several years later, the same research group showed that this approach is suitable for lipophilicity determination on 26 monoprotic bases (imidazoline drugs) [49]. Considering the needs of the pharmaceutical industry for high-throughput screening of the acidity and lipophilicity of the drug candidates, the same gradient RP-HPLC method was coupled with mass detector, thus allowing fast pKa and logP determination with accuracy of less than 0,5 units [37, 38]. The mathematical calculation is more complex and is based on the relationship between the analyte, the column and the equipment specific parameters with the retention time of the compounds obtained under different mobile phase composition. The proposed gradient RP-HPLC/MS methods are inapplicable for compounds having logP below 0.5, do not ionize in the electrospray ionic source or have identical molar masses [38].

5. Biomimetic Chromatographic Partition Systems

Liquid chromatography has been extensively used for simulation of drug protein binding, drug permeability and drug absorption. Mainly three different types of biomimetic chromatographic systems are used for determination of these pharmacokinetic properties: immobilized plasma protein (IPP) chromatography, immobilized artificial membrane (IAM) chromatography and biopartitioning micellar chromatography (BCM). These biomimetic chromatography systems enable further estimation of complex pharmacokinetic properties such as absorption, volume distribution, blood-brain barrier (BBB) penetration, skin penetration, etc. The prediction of these drug properties is based on the chromatographic retention factor; thus, it is necessary to calibrate the chromatographic system. The calibration is performed with set of model compounds for which data for protein binding or drug permeability is available from other traditional techniques such as ultrafiltration and equilibrium dialysis [72].

5.1. Immobilized plasma protein chromatography

In the last few decades, the IPP chromatography has gained importance for the assessment of the percentage of drug protein binding in human plasma. Human serum albumin (HSA) and α1-acid glycoprotein (AGP) are the most abundant plasma proteins that are capable of binding drugs through hydrophobic, electrostatic and steric interactions. HSA has greater affinity towards neutral and acidic compounds, while AGP dominantly binds basic and neutral drugs. Plasma protein binding (PPB) is important pharmacokinetic property that controls the free concentration of drug, thus governs the drug distributions, pharmacological effect and toxicological profile. The knowledge of this property is crucial for the selection of favorable drug candidate in early drug development studies.

HSA and AGP protein stationary phases belong to the class of enantioselective chiral columns that have wide application for drug-protein binding studies. The chromatographic system applied for PPB studies should mimic physiological environment. Buffers that are commonly used during these experiments are 67 mM potassium phosphate pH 7.4, 0.9% sodium chloride pH 7.0, 50mM ammonium acetate pH7.4 or Sørensen phosphate buffer pH 7.0 [73-75]. Considering the fact that organic modifier may affect the drug-protein binding process, it is important to keep the concentration of the organic modifier low. Therefore, the concentration of the organic modifier (2-propanol, acetonitrile or isopropanol) in some of the mobile phases does not exceeds 20% v/v.

Valko et al. [76] published a fast gradient method with cycle time of 15 minutes for determination of HSA binding of 68 drug molecules. The literature data revealed that most of the described methods for PPB are isocratic.

Sing et al. [68] proposed a method for HSA binding of several antibacterial, anti-retroviral and NSAIDS drugs. The results showed that the method gives good correlation with

the data gained with conventional ultrafiltration technique. The applicability of immobilized HSA column for determination of PPB was also confirmed by the Li's work [77]. The proposed isocratic rapid method was applied on around 30 different compounds having PPB in a range from 11.5% to 98%. This research showed that although ultrafiltration measures binding to all plasma proteins compared to HSA which reflects binding to one particular protein, the method could be applied for fast screening of PPB within 5 min. Mallik et al. [78] used HSA microcolumns to measure the free fraction of several model compounds (warfarin, imipramine and ibuprofen) in drug-protein mixtures.

The AGP column is used for PPB determination of drugs that have low or no affinity to HSA. Recently, AGP microcolumns were develop and tested for measurement of AGP binding of several model compounds with known binding constants [79]. This research showed that AGP microcolumns are complimentary with the HSA microcolumns for the measurement of the free drug concentration. Also, this kind of affinity chromatography can be used for investigation of drug-drug competition for the same plasma protein binding sites [80].

The data presented in numerous research studies [73-80] confirm the benefits of the IPP chromatography over the conventional methods (ultrafiltration and equilibrium dialysis) for PPB determination. The HPLC approach for PPB bears the advantages such as high-throughput, automation, reproducibility and the possibility for work under near physiological conditions.

5.2. Immobilized artificial membrane chromatography

The IAM chromatography enables high-throughput prediction of the drug membrane permeability. The founders of IAM are Pidgeon and Venkataran [81], who patented a method for immobilizing phospholipids on silica stationary phases. The IAM columns are prepared by immobilization of phosphatidylcholine on aminopropyl-silica stationary phase. As phosphatidylcholine is the major phospholipid in the cell membrane, thus the IAM stationary phases mimic the composition of the cell membrane. The IAM chromatography is a better model for drug-membrane partitioning than the n-octanol/water partitioning because of the polar and nonpolar groups of the phosphatidylcholine structure. The retention mechanism of the compounds on IAM columns is linked to lipophilic, electrostatic and hydrogen bonding interactions [82-84]. The only limitation concerning the permeability estimation is that the IAM chromatography only reflects the passive diffusion model [85].

There are several types of IAM columns that are commercially available, but for the permeability estimation the IAM.PG.DD2 column (PG stands for phosphatidylcholine, while DD for drug discovery) is preferred. As for the drug-plasma protein binding studies, the experiments are conducted using mobile phases that reflects the physiological environment. The most commonly used buffers are phosphate buffer saline, phosphate buffer without

addition of KCl or NaCl and ammonium acetate buffer [76, 86]. The IAM stationary phases are compatible with fully aqueous mobile phase. The only limitation is these columns is the pH stability, which ranges from 2.5 to 7.4. However, most of the published methods report mobile phases at pH 7.0 [87, 88].

There are numbers of articles that speak for the applicability of IAM chromatography for prediction of drug absorption and drug bioavailability [89-94]. Nowadays, the IAM chromatography is extensively used for prediction of the blood-brain barrier penetration of the compounds [84, 95, 96]. Although the drug absorption, bioavailability and BBB permeability could be effectively modeled by IAM chromatography, the skin permeation is more similar to octadecyl silica partition than to IAM partition [97, 98].

Tsopelas & Tsantili-Kakoulidou research group published several comprehensive reviews on the IAM retention mechanism, IAM chromatographic indices, relationship of IAM retention to lipophilicity and other molecular factors and the potential and advances of IAM chromatography for drug discovery [82, 83, 85, 99].

5.3. Biopartitioning micellar chromatography

Biopartitioning micellar chromatography (BMC) or also known as micellar liquid chromatography (MLC) is another type of biomimetic chromatography system that enables prediction of oral drug absorption in humans. Unlike IPP or IAM chromatography, the BMC is a type of reverse-phase chromatography.

This chromatographic system consists of universal octadecyl silica stationary phase and a mobile phase with surfactant in concentration above critical micellar concentration (CMC). The most commonly used amphiphile is polioxyethylene (23) lauryl ether (Brij 35), which belongs to the class of non-ionic surfactants. The pervasiveness of the use of Brij 35 as a mobile phase is based on the fact that Brij 35 forms neutral type of micelle which is suitable for determination of all types of compounds (neutral and ionic) [95]. The surfactant is present in the mobile phase in form of micelles and as monomers and it adsorbs onto the stationary phase by hydrophobic interaction, thus modifies its surface. The retention of the compounds in the BMC is governed by two different equilibriums, the primary between the bulk aqueous phase and the surfactant-coated stationary phase as well as between aqueous phase and micellar aggregates [100].

Molero-Monfort et al. [14] reported data for oral absorption of 74 structurally diverse drugs (logP ranged from 0.34 to 5.20 and 16-100% range of oral absorption). The results of this study indicated that BMC could be used as a valuable tool for prediction of whether the compounds have favorable transport properties. BMC was also successfully applied for prediction of blood to brain, blood to lung, blood to liver, blood to fat and blood to skin partition coefficients of drugs [84, 101]. The retention factor in BMC showed to be capable of

describing and predicting the permeability and absorption of β-adrenergic blockers, diuretics, analgesics, NSAID, bronchodilators and antipsychotics [95].

The potential of BMC was highlighted by the recent work of Tsopelas et al [100]. Their research, based on the data obtained from 88 structurally diverse drugs, showed that BMC retention factor is better model for permeability prediction than lipophilicity parameter and IAM retention factor. This partitioning model is also a better model for drug biodistribution than the traditional biphasic water-octanol partitioning system, concerning hydrophobic, electronic, and steric properties of compounds [14].

The BMC proved to be a robust technique that is not affected by the column temperature or electrolyte concentration [100]. Another benefit of this technique is that no organic modifier is needed for the mobile phase, thus the chromatographic conditions are more similar to the physiological environment.

6. Concluding Remarks

The chromatographic approaches for physicochemical characterization of compounds are already well established and widely accepted by the pharma industry. Numerous studies have demonstrated the applicability of these approaches for pKa and logP determination, as well as their prediction capacity for drug protein binding and drug permeability. The shared advantages of the chromatographic approaches over the traditional methods include: high-throughput, reproducibility, lower cost, small sample quantity, and in addition lack of issues due to the sample purity and solubility.

The future perspective is focused toward development of chromatographic methods that use stationary and mobile phases that could better mimic the physiological environment and to increase the throughput of the methods. It is equally important to expand the current theoretical models in order to obtain more reliable estimation parameters and to profound the knowledge of the different interactions and retention mechanisms that take place between the compounds and the chromatographic partitioning system.

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