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# Hydrophobic (lipophilic) Interaction Liquid Chromatography

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## ABSTRACT

The retention mechanism in reversed-phase liquid chromatography was quantitatively described using log *P* (octanol-water partition coefficient). The hydrophobic (lipophilic) interaction liquid chromatography was then used to measure the hydrophobicity. Furthermore, a combination of log *P* and p*K*a (dissociation constant) was used for the optimization of reversed-phase liquid chromatography. However, log *P* values cannot be applied to other chromatographic techniques. Therefore, the direct calculation of molecular interactions was proposed to describe the general retention mechanisms in chromatography. The retention mechanisms in hydrophobic interaction liquid chromatography were quantitatively described *in silico* by using simple model compounds andphases. The hydrophobic interaction was demonstrated as the change in the van der Waals energy values calculated using a molecular mechanics program. In addition, this review presented that chromatography can be used as a purification techniqueas well as an analytical method to determine molecular properties during the drug discovery.

## INTRODUCTION

The term "Reversed-Phase Partition Liquid Chromatography" was originally proposed based on the development of surface modified inorganic materials such as silica gels and glass beads using organic compounds. The organic compounds were considered asa liquid. Later such organic compounds were chemically bonded for continuous operation of liquid chromatography. These packing materials are called as bonded-phases. On the other hand, organic polymers have been also used for reversed-phase partition liquid chromatography. Then "reversed-phase liquid chromatography" becomes a commonly used name.

Liquid chromatography is a popular analytical technique; however, the retention mechanisms were not quantitatively explained due to the difficulty in obtaining molecular properties of the analytes. A quantitative explanation of the retention mechanisms is necessary to understand the chemistry of chromatography. Chromatographic retention is described based on a combination of solubility factors. The selection of chromatographic mode and column is performed based on solubility factors. In hydrophobic interaction (reversed-phase) liquid chromatography, increasing the molecular size increases the hydrophobicity of analytes and results in alonger retention time. This indicates that van der Waals (VW) volume is an important property in optimization. Increasing the number of substituents with π-electrons and hydrogen bonding increasesthe solubility in water by increasing their polarity of the

analytes. This indicates that dipole-dipole and hydrogen bonding interactions contribute to hydrophobicity. Therefore, these properties areimportant factors in controlling the retention time in hydrophobic interaction liquid chromatography. However, the π-electrons of stationary phase materials like polystyrene gel and the hydrogen-bonding of non-end capped bonded silica gels also contribute to the retention [1,2]. Later, the proposed retention mechanism was based on the hydrophobic interaction between an analyte and the hydrophobic surface. Such hydrophobic (lipophilic) interaction occurs in nature. In chromatography, VW interaction is avoidable as generally observed in solvation mechanisms because all molecules have certain molecular size, and every molecule exists independently with own molecular size. However, these molecules repulse or condense each other depending on their properties. In a typical hydrophobic interaction system, these molecules are neutral and are not repulsed and slightly condensed each other.

Since a summary of "Reversed-phase High-performance Liquid Chromatography" was published as a book [3], the related reviews have been published, and the major applications in liquid chromatography belong to hydrophobic interaction liquid chromatography. However, the details of the retention mechanisms were not quantitatively demonstrated. First, the retention in hydrophobic interaction liquid chromatography was proposed as partition [4,5]. Therefore, the partition coefficient between octanol and water was quantitatively applied to explain the retention behavior of various compounds in hydrophobic interaction liquid chromatography [1,2].

The development of computational chemical calculations permitted the prediction of the octanol-water partition coefficient (log *P*) values. The log *P* values were first applied to predict retention times in reversed-phase liquid chromatography [6], even when the molecules were ionized, if the dissociation constants (pKa) were known [7]. However, log *P* values can only be used for the prediction of retention times in reversed-phase liquid chromatography.

The quantitative analysis of molecular interaction (MI) is fundamental interest. Computational chemistry is a tool that has allowed the study MIs. The feasibility was demonstrated using small molecules; then, a direct modeling analysis *in silico* was developed for a general explanation of the retention mechanisms in chromatography [8]. VW energy is related to molecular size which affectsthe contact surface area between an analyte and an adsorbent, and contributes to the MI. Hydrogen Bonding (HB) between an analyte and an adsorbent (if present) also contributes to the MI. When ionic interactions exist, electrostatic (ES) energy contributes to the MI. The measurement of direct MIs reveals the different MI strengths between an analyte and the packing material surface or the liquid phase [9-12].

The retention mechanisms are quantitatively explained using model compounds *in silico* similar to methods used in organic chemistry. Different chromatography methods demonstrate the typical MIs. If we can quantitatively reconstruct the obtained solubility factors, we can quantitatively analyze the chromatographic retention mechanisms. Computational chemical analysis methods provide the MI energy as the sum of primarily VW, HB, and ES energy values. The VW energy is related to molecular size; hence, the contact surface area between an analyte and an adsorbent. When HB exists between an analyte and an adsorbent, the HB energy contributes to the MI energy. When ion-ion interactions exist, ES energy contributes to the MI energy. Here, a simple model experiment was conducted *in silico* to obtain a quantitative explanation of hydrophobic-interaction liquid chromatography, specifically studying the effect of alkyl chain (ligand) length.

On the other hand, hydrophilic interactionsare based on HB and ion-ion interaction (ion-exchange). Hydrophilic interaction liquid chromatography, however, should be classified into two groups, non-aqueous hydrophilic (normal-phase) and aqueous hydrophilic liquid chromatography. The latter is prevalent; however, this technique is often misinterpreted as normal-phase liquid chromatography. Therefore, it is crucial to carefully select the appropriate terminology. While using a model ion-exchanger, the strength of ion-ion interaction values can be obtained as molecular interaction electrostatic energy values (MIES) or as molecular interaction hydrogen bonding energy values (MIHB) depending on the model phases and the experimental conditions.

This review focuses onthe quantitative explanation of retention mechanisms in hydrophobic interaction (reversed-phase) liquid chromatography and the practical applications in drug discovery.

**Retention related to the combination of log P and pKa**

The measurement of the solubility of drugs in polar and non-polar media is very important in the pharmaceutical field. One method proposed to describe the solubility is the partition coefficient between octanol and water. The mathematical calculation of an octanol-water partition coefficient from values for functional groups was first proposed by Hansch *et al*. as Hansch's π constants [13]. Later the partition coefficient log *P* values were measured in hydrophobic interaction liquid chromatography [14]. Since them, the hydrophobic interaction liquid chromatography has been used to measure the hydrophobicity (lipophilicity). The simple and/or modified system is practical to measure the relative hydrophobicity of a variety of compounds such as amphoteric compounds [15], hetero arylamides [16], arylpropionic acid [17], dialkylimidazolium ionic liquids [18], haloscent [19], icariside-II and icaritin [20], naproxen and lidocaine [21], neutral, acidic, and basic compounds [22], phenylacetanilides [23], pyrrolyl-acetic acid derivatives [24], spironolactone [25], steroids [26], *N*1-thiocarbamylamidrazone derivatives [27], thiophene derivatives [28], thioquinoline derivatives [29], and triazole [30].

On the other hand, the relative solubility values, log *P*, was first used to explain the relative retention time, log *k*, in reversed-phase (hydrophobic interaction) liquid chromatography [6]. Later, a combination of log *P* and dissociation constant, pKa, demonstrated the quantitative explanation of log *k* in hydrophobic interaction liquid chromatography [7]. This approach was later commercialized as an automated optimization method in liquid chromatography. The system, however, has a fundamental weakness in that the precision of the predicted log *k* values of ionized compounds are subject torelatively large errors due to lack of log *P* values for ionized compounds. To overcome this shortcoming,the log *k* values of ionized compounds can be considered as zero in eluent withahighly concentrated organic modifier. In this case, the method can be practically used to estimate the elution order of a variety of compounds [1,2].

**Direct calculation of MI energy using model phases**

Development of computational chemical programs permits the calculation ofMI energy between model compounds. The program, MM, was applied for quantitative analysis of a variety of log *k* values [9]. In hydrophobic interaction liquid chromatography, the degree of MI is obtained as the change in VW energy values. Simple molecules can be used to demonstrate the major MI mechanisms [31a]; however, an ideal model phase is required for a practical contact surface area between an analyte and the model phase. This approach can be quantitatively described by MI energy values; but, the desorption mechanisms required a more complex model like a solvation. However, the construction of the latter modelwas still difficult. A molecular dynamics (MD) program is one of the possible computational chemical program; however, the reproducibility of the solvation model was not satisfactorily. Therefore, a possible model solvent phase was constructed and improved the precision of predicted log *k* values [11].

The MI energy values were calculated using the following equations. The HB, ES, and VW energy values were calculated using the CAChe MM program (Fujitsu, japan). The computer was a PC model Prime INWIN BL672-4 with Intel Core i7 from Dospara, Yokohama. The MI energy values (kcal $mol^{-1}$) are the sum of solute and model phase energy values minus a complex energy value, were calculated per the following equations [9]. MIHB, MIES, and MIVW are MI energy of HB, ES, and VW energy values, respectively.

MIHB = HB (molecule A) + HB (molecule B) – HB (molecule A and molecule B complex),

MIES = ES (molecule A) + ES (molecule B) – ES (molecule A and molecule B complex),

and

MIVW = VW (molecule A) + VW (molecule B) – VW (molecule A and moleculeB complex).

The relative MIHB, MIES, and MIVW values indicate their contribution level. The difference of MI value in hydrophobic interaction liquid chromatography is defined as MIVW, and the calculation is performed using model phases and analytes.

**Simple model analyses**

Simple models to study MI were proposed and the structures are shown in Figure 1 [31, 31a]. The silicon dioxiderelated atoms were locked (indicated as L in Figure) as solid silica gels. These model compounds were considered as model phases without silanol effect. Freesilanol groups form strong interactions with amines. This effect is overcome in modern bonded-phase silica gels where pure silica gels are chemically

treated to avoid the silanol effect. However, popular amino-, cyano-, and phenyl-silica gels are not treated using secondary silanization (end-capping), and thus, a long lifetime is not guaranteed in aqueous solution containing buffer components. When the silanol effect is included, the analysis of chromatographic retention mechanisms becomes very complicated, and the basic mechanisms cannot be quantitatively analyzed. In this experiment, the model alkyl chain length was tested from ethyl to octadecyl. The analytes were benzoic acid, aniline, benzene, water, and acetonitrile. The molecular and ionized form of benzoic acid and aniline were used to make clear the effect of HB and ES energy change. The polar group effects for direct interactions with the model phase were also studied, where the polar group of analytes directly faced the model phases or the aromatic ring directly faced the model phases [32].

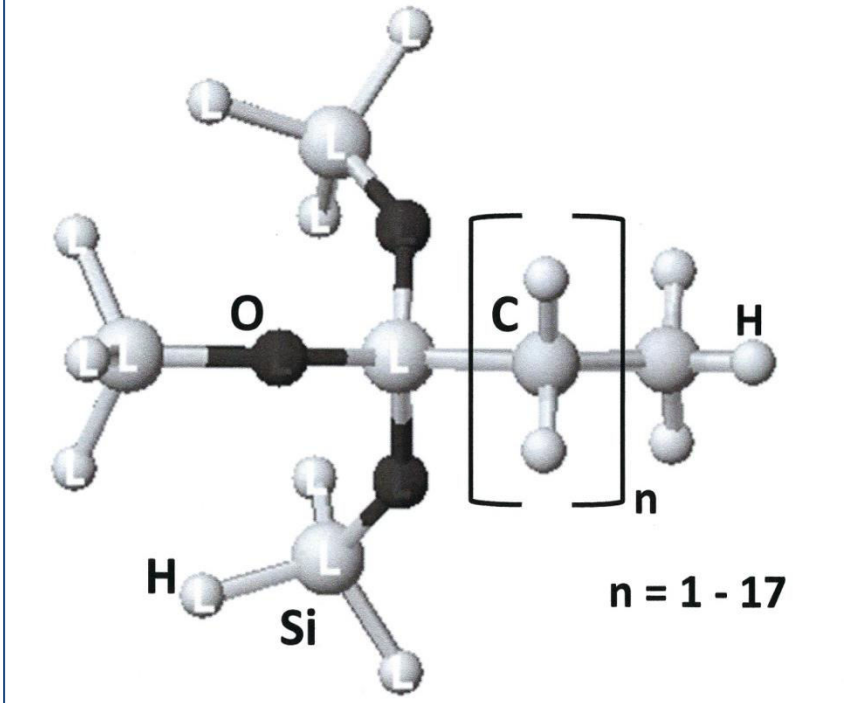


Figure 1: Model alkyl-bonded silicone-dioxide molecule.

The importance of MI in chromatography was described, where the quantitative structure retention relationship was based on molecular properties; however, they did not actually calculate MI [33]. First, the contribution of the effects of the alkyl-chain lengths on the MI were based on the electron localization of alkyl-groups as the alkyl-chain length contributes to the HB of alkyl alcohol. It has been seen that the length of the alkyl chain in alkyl alcohols up to four methylene units can affect the hydrogen bonding based on calorimetric experiments. This result can be explained by the fact that the energy change calculated using MM [9]. The complex conformation was designed to form head-to-head complexes, but not side-to-side complexes because the side-to-side complexes are related to the contact surface area but not direct indications of the electron localization of the head group of in the model phase.

The practical alkyl chain length in reversed-phase liquid chromatography was demonstrated, and the proposed chain length was eleven [34]. Quantitative analysis was achieved using dodecane as the brush for the model bonded phase, and the MI between the dodecane and various analytes like alkanes, alkenes, and alkyl alcohols but not aromatic compounds due to the requirement of steric effects, was quantitatively analyzed using MM calculations. VW energy was found to be the predominant MI energy. Alkyl chain length affected the retention of a variety of compounds. The MIVW of larger compounds was constant when a bonded phase with shorter alkyl chain was used. MIVW energy values of alkenes were smaller than their related alkanes. These resultssupport the idea that the hydrophobic interaction due to MIVW is the

predominant MI in hydrophobic interaction liquid chromatography [9].

**Design model-phases for quantitative explanation of retention**

Silica gel is composed of only two types of atoms, silicon and oxygen.There exist two types of siloxane rings which consist of three $SiO_2$. Half of the surface silicon atoms were used for bonding to improve the stability and selectivity of the stereo structure. Alkyl groups were bonded to silica gels and are used for hydrophobic interaction liquid chromatography [3]. The bonded alkyl chains formed a ridge; therefore, the location of drugs on the surface required chemical institution to determine the initial position on the surface [9].A selection of alkyl-chain length was analyzed, and a dodecyl bonded phase was proposed for general use [34].However, longer alkyl-chain groups were introduced to analyze compounds like carotenoids [35]. Butyl bonded-phasesare used for protein analysis although shorter alkyl-chain bonded phases are unstable in ionic solution. Pentyl-bonded phases and longer are stable and possess at longer life time as evidenced by guaranteed from the manufacturers [36]. The stability of alkyl-bonded phases is explained based on the contribution of carbons onto HB of alkanol oxygen [37].

Once the model bonded-phase was constructed, the retention times of a various compounds were quantitatively analyzed. The simplest model phase was the graphitized carbon phase. It is a flat large polycyclic hydrocarbon constructed using $sp^2$ carbon atom [38-40]. Furthermore, $sp^3$ carbon atoms can construct a honey comb type homogeneous support [37,41]. This model phase was applied to analyze retention time of phenolic compounds [42,43], drug-albumin binding affinity [44], and aromatic acids [45]. Ion-exchanger models were constructed and the chromatographic behavior of acidic drugs on a guanidyl ion-exchanger [46] and basic compounds on a carboxyl ion-exchanger [47] were studied. However, the alkyl-bonded phase is dense and can prefer flat molecules such as phenolic compounds [48]. The alkyl-bonded poly-silicon dioxide support model [43,49] was used to analyze retention times in hydrophobic interaction liquid chromatography of phenolic compounds [50], acidic drugs [51], basic drugs [52] and aromatic acids [53]. Similar studied were also done on normal-phase liquid chromatography [54].

The alkyl-chain length effect was demonstrated using model alkyl-chain bonded poly-silicon dioxide phases and amitriptyline and quinidine were used for the selectivity of bonded-phase silica gels. A model of ethyl-bonded poly-silicon dioxide phase adsorbed quinidine is shown in Figure 2. Especially, quinidine which has the capability for HB demonstrated weak HBon an ethyl-bonded poly-silicon dioxide phase (MIHB 0.285 kcal $mol^{-1}$), and a butyl-bonded poly-silicon dioxide phase (MIHB 0.054 kcal $mol^{-1}$). The MIHB values were negligible with hexy- and octyl-bonded poly-silicon dioxide phases (see Table 1). The weak HBinteractions may a contributing factor to the instability of butyl-bonded silica gels in ionic solution. However such phenomena were not observed usinghomogeneous carbon-based phases.Even MIHB on an ethyl-bonded carbon phase was negligible. However, the distance between alkyl-chains on the carbon phase is too condensed compared to that of the poly-silicon dioxide phase. The polycyclic silicone phase also did not match thepoly-silicon dioxide phase.To overcome this shortfall, a honeycomb type support with lead, a larger atom with$sp^3$atomic orbitals, was further studied.The atomic distance between two lead atoms is slightly shorter than that of thepoly-silicon dioxide phase due to the unavailability of larger atoms having $sp^3$ orbitals in this program; therefore, lead atoms were used to build the polycyclic phase to study *in silico* chromatography based on MI. Various model phases were constructed to analyze the retention mechanisms in hydrophobic interaction liquid chromatography using pentyl- and octyl-bonded silica gel columns. The pentyl-bonded silica gel [36,55] is weak hydrophobic material and requires low concentration of organic modifier compared to the more commonoctadecyl-bonded silica gel, and is used for a combination of isocratic elution and column switching technique [56]. Interestingly, MIHB of quinidine was similar to that of the poly-silicon dioxide phase. However, ionized quinidine has an ability to form a complex with metal atoms. Ionized compounds are usually repulsed from hydrophobic phases, but poly-lead phase demonstrated positive MIES values on ethyl- and butyl-bonded poly-lead phases. This behavior does not allow the use ofthe poly-lead phase to analyze the retention mechanisms of hydrophobic interaction liquid chromatography for certain compounds. In the above experimental, amitriptyline which is

sensitive to the presence of silanol groups did not show such activity as no silanol groups present on these model phases. Improving the construction of homogeneous stereo structure may permit the calculation of MI values to obtain optimized chromatographic retention time. Furthermore, the construction of a model solvent phase should improve the precision of predicted relative retention times [10].

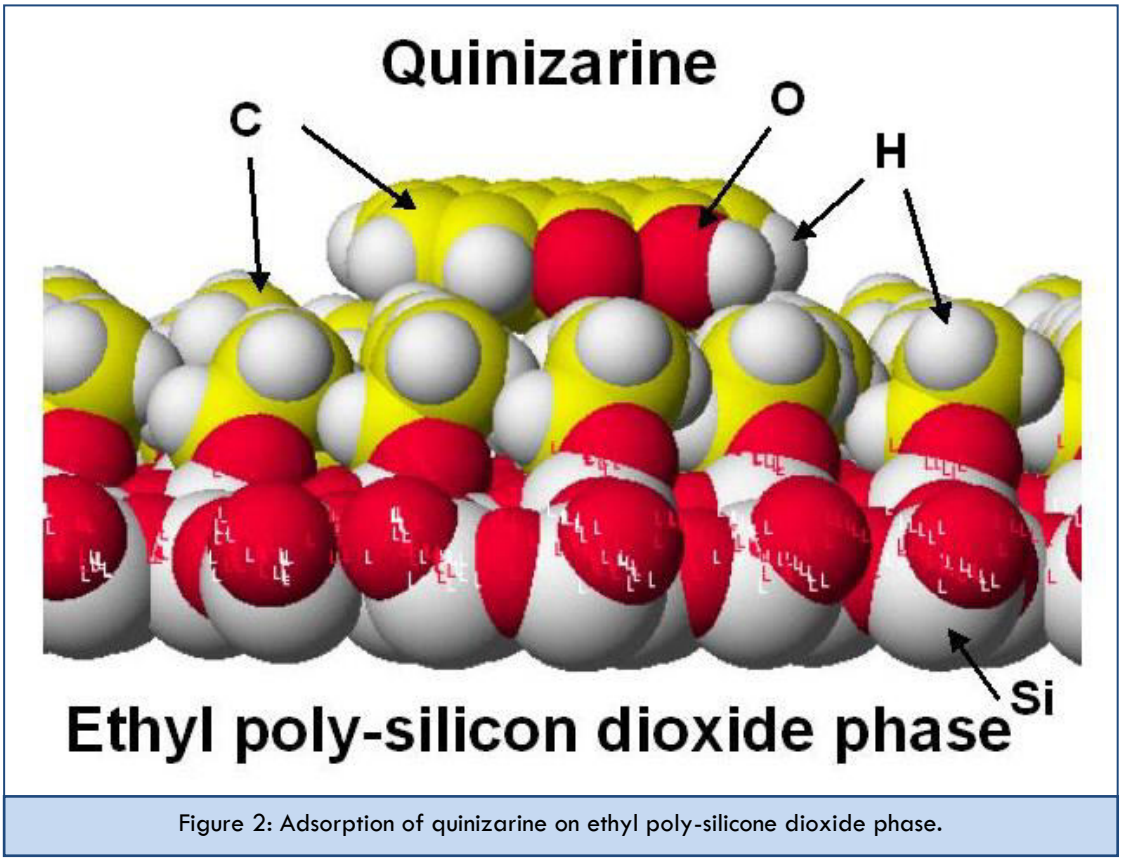


Figure 2: Adsorption of quinizarine on ethyl poly-silicone dioxide phase.

Table 1: Molecular interaction (MI) energy values between alkyl-bonded poly-silicondioxide phase andionized amitriptyline or molecular formquinizarine

| Complex | MIHB | MIES |
|---|---|---|
| C2-SiO2+ionized Amitriptyline | 0.000 | 1.026 |
| C2-SiO2+Quinizarine | 0.285 | -0.013 |
| C4-SiO2+ ionized Amitriptyline | 1.000 | 0.421 |
| C4-SiO2+ Quinizarine | 0.054 | -0.048 |
| C6+SiO2+ ionized Amitriptyline | 0.000 | 0.658 |
| C6+SiO2+ Quinizarine | -0.008 | 0.151 |
| C8+Slo2+ ionized Amitriptyline | 0.000 | 0.231 |
| C8+SiO2+ Quinizarine | 0.000 | 0.015 |

MIHB and MIES: Molecular Interaction Energy Values (kcal $mol^{-1}$) of hydrogen bonding and electrostatic energy; C2 – C8: ethyl – octyl groups.

## CHROMATOGRAPHY FOR DRUG DISCOVERY

The discovery of new drugs has been accelerated by combinatorial chemistry. The fast screening of drug candidates is very important, and the log *P* and the p*K*a are easily measured. Human Serum Albumin (HSA) is a 66 500 Da protein, it is the most common and abundant plasma protein, and is considered to be a multifunctional plasma transport protein. It constitutes approximately 605 of the total serum protein and is a small globular protein with high electrophoretic mobility. It acts to maintain homeostasis in the body, providing a protein reservoir. However, non-enzymatic glycosylation of HSA induced changes in its chemical physical, and ultimately, biological properties, leading eventually to the drug binding affinity (log n*K*) [9,57]. Equilibrium dialysis and ultra-filtration have been used as standard methods to measure protein-drug binding, because of their simplicity and general applicability to

many different systems *in vitro* and *in vivo*. However, containing large amount of glycosylated HSA makes difficult to use HSA, these methods also require a large amount of pure drug candidates.

Compared to conventional techniques, such as equilibrium dialysis and ultra-filtration, the development of chromatographic technology has allowed shorter analysis times, the consumption of fewer chemicals, and higher precision and reproducibility. It also provides the possibility to detect very small differences in the binding affinity of ligands. Several variants of high-performance size-exclusion chromatographic techniques for binding interactions have been developed, *i.e.*, the Hummel-Dreyer method, frontal analysis, the vacancy peak method, retention analysis, and the immobilized protein column method. The measurement of retention time using an immobilized HSA column is seemed simple solution, but the capacity ratios did not correlate well with HSA-drug binding affinity measured by free solution methods. This method required a specific standard for the measurement of the pharmacokinetics of new chemicals [9]. Drug-albumin binding sites have also been studied, but albumin can also function as a scavenger, which indicates that the albumin structure has a dynamic structure with the ability to non-specifically bind to a variety of compounds.For HSA, specific steric effects may not be important and the main binding forces may be hydrophobic interactions and ion-ion interactions. Acidic drug-HSA and basic drug- HSA binding affinities have been successfully determined by a combination of reversed-phase and ion-exchange liquid chromatography [58,59].The system was also quantitatively converted to *in silico* calculation [9,60].

HPLC methods that use chromatographic retention times for gaining information about the properties of compounds for designing drug molecules have previously been reviewed [61]. Properties, such as lipophilicity, protein binding, phospholipid binding, and acid/base character can be incorporated into the design of molecules with the right biological distribution and pharmacokinetic profile to become an effective drug. Therefore, methods to measure thelipophilicity by liquid chromatography were critically reviewed and compared. The chromatographic hydrophobicity index (CHI) was proposed. The fixed CHI values can improve the inter-laboratory comparison and this makes it possible to build large databases. However the utilization of biomimetic stationary phases, such as HSA and alpha-1-acidglycoprotein thosehave the possibility of becoming denaturated or irreversibly binding of these phases. It was also stated that it may be difficult to calibrate immobilized artificial membrane. Crucially, the chromatographic properties measured at the early stages of the drug discovery process provide an easy assessment of lipophilicity, oral absorption, volume of distribution, drug efficiency, and even early dose estimation. The calculated octanol/water partition coefficients were used in various absorption distribution and metabolism (ADME [62]) models, the biomimetic chromatographic properties were also used in other ADME models. The key to success is to generate chromatographic data that are suitable for inter-laboratory comparison that allows the generation of large data bases for further analysis analogous to the log *P* data [63]. The biomimetic gradient retention time measurements on C18, immobilized artificial membrane (HSA, and acid-glycoprotein) stationary phases were used to characterize compounds partitioning into phospholipids and proteins, and these data can be used in equations to estimate the *in vivo* plasma-tissue distribution of the compounds measured. They also demonstrated that the plasma protein binding, brain tissue binding, and *in vivo* drug efficiency can be calculated using the biomimetic chromatographic data [63]. Furthermore, the volume of distribution in the steady state and the volume of distribution unbound are described with experimental data [64].

Beyond the measurement of retention times, chromatographic systems provide useful information on the property of drugs during the discovery process. For example, the intensity of chemiluminescence of a compound was correlated with its toxicity. This behavior is thought to be occurred aschemiluminescencecan be observed upon the production of radical oxygen that may cause damage to our tissue *in vivo*. This specificity was quantitatively analyzed and the mechanism was quantitatively demonstrated *in silico* [65].

## CONCLUSION

The retention mechanisms in the most popular hydrophobic interaction (reversed-phase) liquid chromatography were quantitatively described. The mechanisms were originally proposed as partition; therefore, log *P* was applied to optimize the chromatographic conditions, which was considered

to be a result ofthe hydrophobic interaction. The mechanisms were then quantitatively analyzed *in silico* by using model phases and compounds. The effectiveness of hydrophobic interaction liquid chromatography in drug discovery was also discussed to reveal its further applications in research and development.

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