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Hydrophilic Interaction Liquid Chromatography for LC-MS

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Abstract

The retention mechanisms in hydrophilic interaction liquid chromatography (HILIC) are quantitatively described in *in silico* analysis. Among a variety of polar bonded-phase silica gels, hexylamine-bonded silica gel is stable and can be used in both aqueous HILIC and ion-exchange liquid chromatography with high reproducibility. Hexynyl and phenylhexyl bonded silica gels are also used in HILIC with high reproducibility. The retention mechanisms in aqueous hydrophilic and non-aqueous hydrophilic (normal-phase) liquid chromatography are similar. Both hydrogen-bonding and weak electrostatic interaction contributes to retention. Furthermore, strong electrostatic interaction is dominant in ion-exchange liquid chromatography. A suitable liquid chromatographic system for liquid chromatography (LC)-mass spectrophotometer (MS) is proposed.

Keywords: Liquid chromatography; Mass spectrophotometer; Electrostatic interaction; Hydrogen bonding, *In silico*

Introduction

Applications of LC-MS

A MS is a powerful detection instrument for identifying analytic structures. LC is an effective method for the purification (separation) of target compounds in crude samples. For target compounds that are volatile and thermo-stable, a combination of gas chromatography (GC) and MS is ideal. GC-MS has been mainly used for environmental and industrial analyses. The analysis of polar compounds commonly found in bio-organisms is of interest. However, bio-related compounds are generally non-volatile and thermally unstable. Generally, pre-derivative reactions are avoided because unspecified reaction products contaminate the target compounds and hinder their identification. Furthermore, large molecules are purified using thin-layer liquid chromatography and then subjected to atomic bombardment and laser desorption techniques for their identification.

The MS is an expensive instrument, especially for LC application, but it has attracted much interest recently. According to Scopus, the number of publications on LC-MS had dramatically increased since 2006. LC-MS has a variety of applications and can be extended to wide a range of target molecules such as metabolites [1-7] and biological polymers [7-13]. This technique was also used to identify unknown compounds in natural products, e.g. herbs, natural medicines and polyphenols [14-16], drugs [17-20], and enzymology [21,22]. Carbohydrates and other related compounds, which are difficult to detect with high sensitivity using other analytical instruments, have been subjected to LC-MS analysis [13,23-27]. Furthermore, LC-MS analysis has been applied for diagnosis [12,28-30] including biological amines [31-34], endogenous carboxylic acids [35,36], and lipids [13,37-39]. LC-MS is also applied to toxicological analysis in the environment [40-46].

Combining mass spectrophotometer and liquid chromatograph

A free connection between LC and a MS was not permitted. At the very least, inorganic salt should be eliminated prior to the use of a MS. One idea is to collect the target fraction in a small trapped column, then wash inconvenient components prior to introduction of the MS. Even if one uses a small column with volatile salt, one has to wash the mass chamber very so often to maintain the sensitivity.

Polar compounds are separated using ion-exchange liquid chromatography; however, the inorganic salt should be eliminated before the mass spectroscopic analysis. The targeted fraction is collected on a small trap column, then salt removed with volatile ion-pair reagent, and the compounds are introduced into the MS. A miniaturized separation column with a volatile eluent and a desalting column are applied when using a MS as a detector [47].

One simplified LC-MS system for bio-related compounds combines aqueous eluent containing volatile buffer components with polar stationary phase. However, silica-gel based polar stationary phases are generally unstable in aqueous solution. Organic-polymer based packing materials are stable in aqueous solution but exhibit inadequate purification (resolution). On the other hand, while unstable in aqueous solution, polar silica-based packing materials are visible. Here, HILIC was used. This technique has been gaining attention even though it difficult to reproduce. In addition, the life time of its columns is shorter than usual as with in reversed-phase liquid chromatography. The reproducibility of the latter has improved since 1988 when high purity silica gels were introduced to support bonded phase silica gels [48]. Considering the retention mechanisms in HILIC, new polar chemically modified silica gels are necessary. Here, HILIC is demonstrated and its retention mechanisms are quantitatively described *in silico*.

Retention mechanisms in HILIC

The name HILIC was proposed in 1990 based on the chromatographic analysis of polar compounds using an ion-exchanger with an amine salt buffer containing acetonitrile, and the retention

mechanism was suggested to involve partition [49]. However, the first HILIC involved the separation of saccharides using ion-exchanger with aqueous ethanol in 1965 [50-52]. The experimental conditions in HILIC were proposed while employing polar stationary phases with aqueous-organic (or polar organic) eluents. This was performed independent of solute distribution, adsorption, partition, or a combination of both, between the stationary and the eluents [53-55]. The chromatographic behavior of the selected compounds using silica-based stationary phases illustrated either partition or adsorption mechanisms with Van Deemter plots of different columns. The inconclusive results for the retention mechanisms in HILIC might be due to the existence of free silanol groups on the surface of the bonded phase silica gels [56,57]. The adsorbed water measured from 14 different stationary phases was affected by free silanol groups. The specific surface areas of the commercialized stationary phases were unknown. Moreover, the estimation of silica mass in the packed columns was difficult. Therefore, evaluating the contribution of the bonded-phases was difficult [58,59].

Furthermore, the physico-chemical properties of 22 hydrophilic and polar stationary phases were analyzed to describe the hydrophilic interaction mechanisms as a combination of hydrophilic, hydrophobic, electrostatic, hydrogen bonding, dipole-dipole, interaction, and shape-selectivity [60]. However, these parameters cannot be independently used because some have overlapping physico-chemical-properties. Electrostatic interaction strongly affects hydrophilic interaction [61,62]. A universal model that is simultaneously applicable to acidic, basic, and neutral analytes could not be obtained even after examining the retention mechanism based on hydrogen bonding, Coulombic interactions and phase ratio using the linear solvation energy relationships of 23 packing materials. A significant improvement in the HILIC systems was observed when the pH and buffer counter ions were changed. This indicated that the HILIC selectivity is based on the additive of a multiplicative phenomenon. HILIC columns were classified by their physico-chemical parameters and their retention mechanisms were intensely discussed. However, the results were inconclusive and applicable only to specific test settings, and varied under different elution conditions [63]. Sample pre-treatment using HILIC was proposed and possible correction mechanisms were proposed [64]. The behavior of pyridine in both reversed-phase and hydrophilic interaction chromatography indicated that the active sites of silica gels became inactive since after being filled with excess acetonitrile, which prevents hydrophobic interactions between silica and pyridine. The adsorption mechanism in HILIC is nearly uniform. The sample sizes may be large before the column is significantly overloaded and peaks tailing is observed [65,66]. However, the aged columns lead to different conclusions as compared with those with reversed-phase silica gel columns. The structure retention relationship was also quantitatively investigated to study the retention time in HILIC based on the physico-chemical properties of the solutes [67-69]. However, the lack of molecular interaction between the analytes and packing materials made the generalization of this type of approach difficult.

in general, the retention mechanisms in reversed-phase liquid chromatography are attributed to hydrophobic interaction. Reversed-phase liquid chromatography is the inverse of normal-phase liquid chromatography. However, it seems that HILIC cannot be regarded as the latter. If hydrophobic interaction is eliminated from the retention mechanisms in liquid chromatography, hydrogen-bonding and Coulombic interaction remain. Coulombic interaction dominates in ion-exchange liquid chromatography, while hydrogen bonding prevalent in of normal-phase liquid chromatography [49]. The difference between HILIC and normal-phase liquid chromatography exists in the properties of solvent used as components of the eluent. In general, only organic solvents are used in normal-phase liquid chromatography and water-saturated organic solvent is often used to improve separation. Since hydrophilic is the opposite of hydrophobic, hydrophilic interaction includes hydrogen bonding and Coulombic interaction. Ion-exchange liquid chromatography is independent from HILIC.

Theoretical demonstration of retention mechanisms in liquid chromatography

The retention mechanism of HILIC mode liquid chromatography is a combination of hydrophilic interaction and ion-exchange. Hydrophilic interaction is based on the hydrogen bonding and electrostatic force. Ion-exchange is also due to the electrostatic force. The difference is in the degree of electrostatic force. The electrostatic force in the HILIC mechanism is like the contact charge transfer: the energy level is smaller than the hydrogen bonding energy. Dipole-dipole and π-π interactions are due to electron localization; therefore, the degree of these interactions was also explained using the electrostatic energy values. However, the electrostatic energy level is higher in the ion-exchange mechanism. Such a difference was quantitatively described by using hydrogen bonding and electrostatic energy values calculated using the molecular mechanics (MM) program. Furthermore, the molecular interaction site was identified by the atomic partial charge calculated using the MOPAC PM5 program. Chromatographic retention mechanisms can mainly be described *in silico* as a combination of van der Waals force, hydrogen bonding and electrostatic energy values. Steric hindrance must be considered for chiral and affinity liquid chromatography [70].

These molecular interaction (MI) energy values (kcal mol^{-1}) are the sum of a solute and model phase energy values minus a complex energy value, calculated as per the following equations [70]. MIHB, MIES, and MIVW are MI energy of hydrogen bonding (HB), electrostatic (ES), and van der Waals (VW) energy values.

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),

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

The relative MIHB, MIES, and MIVW values indicate the contribution level.

A simple quantitative explanation method for the retention mechanisms in HILIC mode liquid chromatography is proposed based on the calculated MIHB, MIES, and MIVW energy values. The relative retention times (k) of acidic and neutral compounds were measured using reversed-phase mode liquid chromatography, an acidic eluent, and different bonded-phase silica gels. The k values measured using the octyl-bonded phase were used as the standard and compared with the k values measured in polar phases.

The measured k values were used for *in silico* analysis using model phases and analytes [71,72]. The model phases were hexenyl-, hexylamine-, and ionized hexylamine-bonded silicon trioxides. Both hexenyl- and the hexylamine demonstrated weak electrostatic

interaction and hydrogen bonding in both normal-phase (non-aqueous) liquid chromatography and (aqueous) HILIC. The ionized amino group worked as an ion exchanger in both ion-exchange and HILIC mode liquid chromatography. The model phases and analytes are shown in Figure 1. Toluene as well as molecular and ionized benzoic acids, were used to demonstrate hydrophobic interaction and weak hydrogen bonding, hydrogen bonding and strong electrostatic interaction, respectively. The complex forms were analyzed based on the direction of analytes toward these model phases.

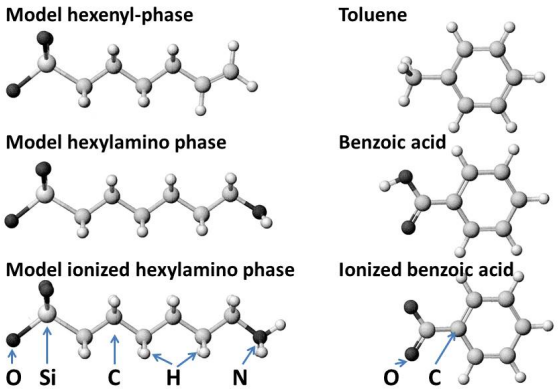

Figure 1: Structure of model phases and analytes.

The carboxyl group of benzoic acid formed hydrogen bonding with the hexynyl group as shown in Figure 2a. Toluene interacting with hexylamine via hydrogen bonding is shown in Figures 2b, 2c and 2d. The hexylamino phase formed hydrogen bonding with ionized benzoic acid as shown Figures 3a and 3b. Moreover, ionized benzoic acid formed a strong ion pair with the ionized amino group from both direct head-to-head and head-to-back conformations as shown in Figures 3c and 3d. The strength of molecular interactions is summarized in Table 1 as MIHB, MIES, and MIVW energy values.

Complex	MIHB	MIES	MIVW
Pentyl+Toluene b	0.000	-0.001	2.258
Pentyl+Toluene f	0.000	0.001	0.831
Pentyl+mBA b	0.000	0.000	0.543
Pentyl+mBA f	0.001	-0.004	0.774
Pentyl+iBA b	0.000	-0.003	0.137
Pentyl+iBA f	0.000	-0.024	0.657
Hexenyl+Toluene b	0.000	-0.002	3.346
Hexenyl+Toluene f	0.000	-0.014	3.306
Hexenyl+mBA b	0.000	0.000	0.127
Hexenyl+mBA f	2.327	0.012	1.175
Hexenyl+iBA b	0.000	-0.011	0.188
Hexenyl+iBA f	0.000	-0.180	0.598
Hexylamine+Toluene b	3.043	0.013	2.413
Hexylamine+Toluene f	0.260	0.029	1.313
Hexylamine+mBA b	0.032	0.013	0.129
Hexylamine+mBA f	0.252	0.573	0.369
Hexylamine+iBA b	3.092	-0.054	2.355
Hexylamine+iBA f	3.071	0.157	1.690
iHexylamine+Toluene b	0.000	0.059	0.632
iHexylamine+Toluene f	0.000	-0.188	0.685
iHexylamine+mBA b	0.000	-0.106	0.137
iHexylamine+mBA f	-0.005	2.599	0.796
iHexylamine+iBA b	0.000	6.212	0.984
iHexylamine+iBA f	0.000	6.770	0.416

Table 1: Molecular interaction energy values between analytes and model phases.

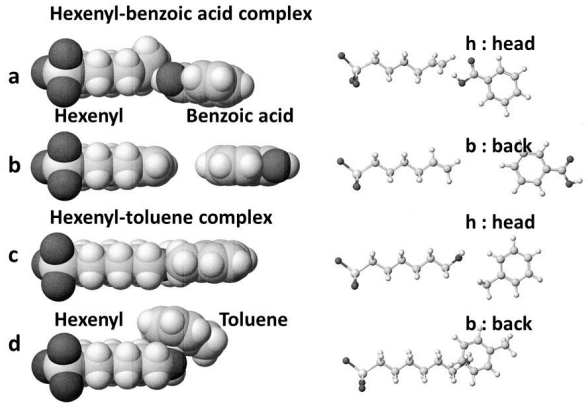

Figure 2: Conformation of complexes between hexenyl-bonded phase and molecular form benzoic acid, and hexenyl-bonded phase and toluene; Symbols: see Figure 1.

Bonded-phase silica gels are chemically stable when they bind with the polar group of long alkyl ligands. Pentyl-, octyl-, and hexyl-phenyl bonded silica gels are all stable. Therefore, hexenyl- and hexylamine-bonded silica gels were synthesized. Their performances were studied by reversed-phase mode liquid chromatography. Increasing the polarity of bonded phases decreases the retention time of non-polar compounds like benzene, toluene, and ethylbenzene. However, the retention time of benzoic acid increased with the polarity of bonded phases. This phenomenon is opposite from those observed in reversed phase liquid chromatography. Computational chemical analysis revealed that the HB energy value for the retention of benzoic acid was high. For instance, the carboxyl group of benzoic acid interacted with the hexenyl group (MIHB: 2.3 kcal mol^{-1}), and the hexenyl group exhibited hydrophobic interaction with toluene (MIVW: 3.3 kcal mol^{-1}), but neither with the phenyl group of benzoic acid. The hexenyl

group did not show strong interaction with the ionized benzoic acid. The hexylamino group formed strong hydrogen bonding with the phenyl-group of toluene, ionized carboxyl-group of benzoic acid, and phenyl-group of ionized benzoic acid (MIHB: 3.1 kcal mol^{-1}). The ionized hexylamino group manifested strong electrostatic interaction with ionized benzoic acid (MIES: 6.5 kcal mol^{-1}), typical in ion-exchange liquid chromatography. The analysis of weak interactions between these model phases and analytes produced positive MI energy values (Table 1). MIVW demonstrated the degree of hydrophobic interactions. The pentyl group interacted with the phenyl group of toluene (MIVW: 2.3 kcal mol^{-1}) creating a weak hydrophobic phase.

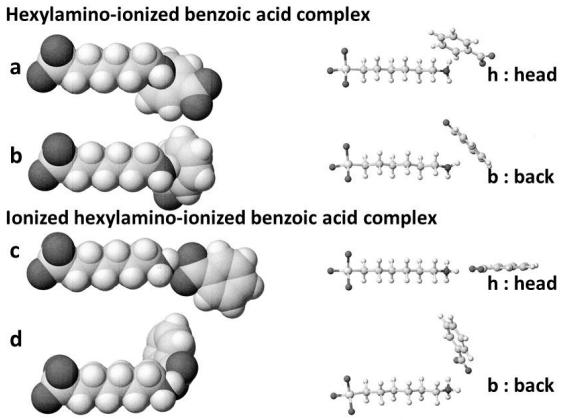

Figure 3: Conformation of complexes between hexylamino-bonded phase and ionized benzoic acid, and ionized hexylamino-bonded phase and ionized benzoic acid; Symbols: see Figure 1.

These compounds were retained at the alkyl-ligands of these bonded-phases by VW force, and the polar groups by HB. The difference in the MI strengths was quantitatively analyzed using the calculated energy values. A simple chromatographic experiment performed using theoretically stable and inert bonded-phase silica gels and MI energy values calculated using a MM calculation make permit quantitative explanation of HILIC retention mechanisms. Polar groups of analytes contact with polar groups of bonded phases. The interaction of MI and removable of analytes from the bonded-phases depend on properties of eluent components in chromatography. The interaction can be quantitatively analyze from the calculated HB and ES energy values.

Results of the study suggest that HILIC should be indicated as aqueous, and the ion exchanger used in aqueous eluent that contains buffer components for ionizable analytes should be classified as ion-exchange liquid chromatography. Hydrophilic interaction can be observed in a variety of chromatography. Therefore the terms pertaining to different methods should be carefully selected. For instance, while ion-exchange mechanism involves separation, it should be addresses as 'ion-exchange liquid chromatography'. These simple model analyses helped classify chromatographic mechanisms and conditions.

Basic concept of stability of bonded-phase silica gels

The HB of alkyl alcohols depends on the alkyl chain length, and up to four methylene units can affect the HB. The fact was calorimetrically analyzed. Alkyl alcohols from methanol to nonylalcohol have been constructed, and individual and identical pair energy values obtained after optimizing the structures using MM2 calculations. The MI energy values are plotted against the carbon number of the alkyl alcohol chain, along with the results for alkanes. The final energy of a pair of identical alcohols was smaller than twice the energy of one alcohol. This indicates that the energy values calculated using the MM2 program can explain the degree of MI. Methanol, ethanol, and propanol had similar values; their HB energy value was about –2.9 kcal mol–1. The HB energy values of n-butanol, n-pentanol and n-hexanol were of the order of 0.1 kcal mol–1. Their corrected final structure energy values increased dramatically with increasing alkyl chain length. Then, the value leveled off to a constant. Up to three carbons, there was a linear increase in the HB. This means HB contributes to molecular interactions for alcohols up to and including three carbon atoms and VW energy is the main energy for alkyl alcohols with longer chains. It is explained by the fact that the energy change for a chain of five or more carbon atoms was almost parallel to the energy change of alkanes [72].

Above fundamental results suggested the chemical stability of pentyl-bonded silica gel. Indeed, pentyl-bonded silica gel were stable over 1300 h immersion in pH 1.5 solution (1 vol. % trifluoroacetic acid in 50 vol. % aqueous acetonitrile) and after over 1900 h immersion in pH 10 solution (10 mM Na_2HPO_4 in 50% aqueous acetonitrile). Hexylphenyl bonded silica gel were stable over 2700 h immersion in both pH 1.5 solution (1 vol. % trifluoroacetic acid in 50 vol. % aqueous acetonitrile and pH 10 solution (10 mM Na_2HPO_4 in 50% aqueous acetonitirile). Furthermore, an anion-exchanger with longer alkyl ligand was stable over 47000 column volume flushing using pH 10 solution (50 mM Na_2HPO_4 in 50 vol. % aqueous methanol). A cation-exchanger silica gel was also stable over 85000 column volumes flushing using pH 10 solution (50 mM Na_2HPO_4 in 50 vol. % aqueous methanol). The stability was analyzed by retention time and peak symmetry of toluene, pyridine, and phenol [48].

A simple model analysis demonstrates the differences of retention mechanisms in both reversed-phase and hydrophilic mode liquid chromatography, and we found that, as the alkyl chain (ligand) length of the bonded phase decreases, the electrostatic energy effect increases. This is the same phenomenon observed for alkyl chain length contribution to aliphatic alcohol hydrogen bonding.

Summary

The reports described the problems for the quantitative explanation of retention mechanisms of aqueous HILIC, and concluded that the retention mechanisms were inconclusive or mixed mechanisms [50-66]. Because their experiments related with HILIC have been performed using bare silica gel, polar bonded-silica gels, and ion-exchangers in aqueous eluents. The surface concentration of bonded phases was not guaranteed and the surface area of the bare silica gel varied; therefore, it was difficult to fit the retention data to the equation for the quantitative explanation. Further, the undefined amount of water in polar phase is a fundamental problem in obtaining reproducible results. Therefore, their conclusions may reflect that the specificity and life time of the bare silica gel and polar bonded-silica gels. The life time of polar bonded phases are not guaranteed by

manufacturers, and the free silanol groups remain in the polar bonded-silica gels. Hydrophilic interaction and ion-exchange should be clearly explained from the nature of analytes and the used packing materials. HILIC mode and HILIC retention mechanism should be carefully described and selected.

Furthermore, a simple *in silico* analysis was performed to explain the retention mechanisms of aqueous HILIC based on the retention time of benzoic acid and toluene in hexenyl- and hexylamine-bonded silica gels. Hydrogen bonding and weak electrostatic interaction were the main retention mechanism in HILIC, while strong electrostatic interaction dominant in ion-exchange liquid chromatography. The experimental results and theoretical proposal suggested that if the stability of the packing materials is guaranteed, such chemically bonded-phase silica gels with polar groups and long alkyl ligand can be practically used in LC-MS analysis with good reproducibility. Otherwise, the soluble silica would contaminate the MS chamber and affect the sensitivity of the instrument; alternatively, a trap column must be used to keep the eluent clean. The definitions of the HILIC retention mechanisms were quantitatively explained for *in silico* analysis.

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