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HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY (HILIC) AND ITS APPLICATION IN PHARMACEUTICAL ANALYSIS

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ABSTRACT

Alpert coined the term,'Hydrophilic interaction chromatography" in 1990. This mode of chromatography dates back to earliest days of liquid chromatography. It happened when two scientists namely, Martin and Synge separated amino acids. They separated amino acids on a silica column. Water saturated chloroform as mobile phase was used by them. These scientists explained the separation mechanism as partition of the solutes between a water layer held onto the column surface and chloroform. HILIC as a complex system involves partition, polar as well as ion-exchange interactions.Partitioning occurs due to phase transfer of polar analytes from an organic rich mobile phase into an adsorbed layer of water on the stationary phase.Polar interactions occur between the active surface or ligands of the stationary phase.Ionic interactions occur between charged analytes and oppositely charged moieties on the stationary phase.Based on these factors, HILIC is a preferred technique for polar substances. Therefore, HILIC mode of LC is gaining popularity in the analysis of polar compounds of Pharmaceutical interest and also in the study of metabolomics. Therefore, HILIC mode of LC with its advantages over RP chromatography in the analysis polar compounds of pharmaceutical interest is presented.

KEYWORDS: 'Hydrophilic interaction chromatography".

INTRODUCTION

This is a successful approach for retention and separation of polar compounds. An increase in the development of polar drugs has caused a substantial increase in popularity of HILIC. Development of polar drugs in pharmaceutical industry and an emerging field of

metabolomics which needs analysis of polar compounds.The technique of HILIC is called as, "reversed reversed phase". In HILIC, stationary phase is polar and aqueous part of mobile phase acts as a strong solvent.[1] This is in opposite to conventional reversed phase chromatography.

Early History

HILIC mode of separation started around 1951. Gregor et.al.[2] described a water enriched layer on an ion-exchange resin surface. Samuelson and sjostoim.[3] analyzed monosaccharides on an ion – exchange column. Ruckert and Samuelson.[4] observed that a stagnant water layer could be responsible for uptake of analytes. In 1975, amino columns were used for analysis of sugars, the dominant HILIC mechanism involves the existence of a semi – immobilized water – enriched layer on the polar stationary phase.[5] This is combined with a partitioning equilibrium of analytes into the mobile phase,[6] In 1990, acronym HILIC was first introduced by Alpert.[7]

Dr. Andrew Alpert suggested the name in 1990.He described HILIC as liquid – liquid partition chromatography. Here analytes elute in order of increasing polarity.

HILIC (Hydrophilic Interaction Chromatography) term was coined in 1990 in order to distinguish it from normal phase chromatography. HILIC can be regarded as a variation of Normal phase chromatography without the disadvantage of use of solvents that are water immiscible. HILIC is also called as "reverse reversed phase" or "aqueous normal phase chromatography". Stationary phase used in HILIC is a polar material made up of Cyano, diol, Silica and amino functionalities. The mobile phase used in HILIC is highly organic (>80%) in nature. This contains a small amount of aqueous / polar solvent. Water (or the polar solvent) is the strong, eluting solvent in this mode of separation. Hence, it can be concluded that HILIC is NP Chromatography of polar and ionic compounds under reversed phase(RP) conditions.

HILIC mechanism on silica

Polar analytes get partitioned into and out of adsorbed water layer. Cation exchange takes place between charged polar analyte and charged silanol groups. Combination of above two mechanism results in enhanced polar retention. If there is an absence of either of these mechanisms, there occurs no polar retention. For very polar bases, HILIC offers greater retention than reversed phase chromatography. The main separation mechanism is due to an

aqueous layer built on the stationary phase.[8] Analytes get partitioned between the very polar stationary phase and the less polar mobile phase. This causes retardation of polar and hydrophilic compounds like Uracil. HILIC has an elution order which is inverse compared to reversed phase separations. Relative solvent strength in HILIC mode is: Acetone<Acetonitrile<Isopropanol<Ethanol<Methanol<Water.[9]

The main mechanism of retention in HILIC is partitioning between a water-rich layer on the surface and the organic enriched mobile phase.Specific adsorption of polar functional groups on the stationary phases, ionic retention on ionized groups or on ionized silanols of the base silica also occur. Reversed phase retention on hydrophobic portion of bonded ligands also occur.[10]

HILIC helps in retaining highly polar analytes, which cannot be retained by reverse phase chromatography. Since the use of high organic mobile phase (>80%) causes enhancement of ESI-MS response.

HILIC stationary phases

HILIC had its early beginning but it was not recognized as a district chromatographic mode of separation. It was rediscovered by the scientific community in the early 2000's.[11] Specifically designed HILIC stationary phase with diverse function offers different selectivity and higher retention of polar compounds. Silica columns intended for HILIC have been developed, these are packed and stored in aqueous / organic phase.[12] This is in contrast to the conventional use of silica in normal phase chromatography. The latter uses mobile phase which could be a mixture of hexane and IPA solvents. Silica materials are also available in sub – 2 micrometer fully porous particles, in superficially porous particles and as monolithic columns.

Apart from bare / hybrid silica, the next most widely used type of HILIC material are the Zwitterionic phases. Sulfobetaine phase is a type of Zwitterionic phase. Sulfobetaine Zwitterion has both positive (Quaternary ammonium) and negative (Sulfonic acid) groups in a 1:1 ratio. Therefore, net surface charge of sulfobetaine Zwitterion is zero.

Irgum et.al. at first introduced the sulfoalkylbetaine Zwitterionic functionality into polymeric supports. This was done in order to prepare ion-exchange materials for the analysis of inorganic compound and proteins. Later, a similar type of substance was immobilized on

silica substrates. Here, the net surface charge is Zero. The negative charge of the sulfonic acid group at the distal end of the phase causes some quantity of electrostatic interaction with charged analytes. Irgum et.al., later introduced a new type of zwitterionic phase, with phosphorylcholine which is grafted onto a polymeric substrate it has got a positively charged ammonium group at its distal end. This type of Zwitterionic phases are used for the analysis of charged and neutral species.This analysis is independent of possible electrostatic interactions that can arise.

Amide – bonded silica phases are another widely used type in HILIC.[13] Amide function is attached to the silica surface via a propyl or proprietary linker. This cannot be charged in the pH range commonly used for HILIC mobile phases. Retention is less affected by ion exchange interaction.Unlike aminopropyl phases, amide phases are less prone to irreversible adsorption. Amide columns possess good reproducibility and stability.

Phase used in HILIC.Aminopropyl materials are used in HILIC separations for carbohydrate analysis. They show irreversible adsorption of analytes esp. for reducting sugars. This occurs due to reactive nature of amino functional group. Cyanomaterials are not used much in HILIC. They have limited scope in this type of chromatography. This is due to insufficient retention of most polar compounds. Cyano groups do not have hydrogen bond donor capacity and hence are not much hydrophilic.

HILIC finds its suitability in study of various bioanalytical compounds used by pharmaceutical industries, biotechnological applications include in the field of proteomics, metabonomics and glycomics.

Any polar chromatographic surface can be used for HILIC separations. Non – Polar bonded silicas can be used with extremely high organic solvent composition. In case silica used for chromatographic media was polar; HILIC phases can be grouped into five categories of neutral, polar or ionic surfaces. That is,

a. simple unbonded silica silanol or diol bonded phases.
b. amino or ionic bonded phases
c. amide bonded phases
d. catonic bonded phases
e. Zwitterionic bonded phases.

Primarily, a HILIC stationary phase consists of classical bare silica or silica gels, modified with many polar functional groups. Polymer based stationary phases are also used.[14]

In 1975, the first generation HILIC mode of separation was used. Amino silica phase, Bondapak (Waters, USA) was used for separation of carbohydrates by Linden et.al. They used a mixture of acetonitrile and water (75:25% V/V). The next generation of stationary phase for HILIC was made by using DIOL- and amide – silica. It is used mainly for Protein Separation. Amide silica columns are available since 1985. This consists of nonionic carbamoyl groups that are chemically bonded to silica gel. It is also known as amide bonded silica. Buszewski et.al. used chemically bonded stationary phases with specific structural properties. They include aminopropyl ligands bonded to silica (SG-NG2) alkylamide packing phase (SG-AP) and mixed phase (SG-MIX) .The latter contains different types of ligands (-NH21-CN, -PH, -C8,-C8) which are bonded to the support.[15]

HILIC columns are distinguished on whether they depend on adsorption and H-bonding or on hydrophilic portioning and multipoint interaction. Plain silica columns exhibit absorption selectivity. But zwitterionic columns exhibit a selectivity pattern which is due to partitioning effect.

Retentions Mechanism involved in HILIC

HILIC can be discussed as a variation of Reversed phase liquid chromatography (RPLC) it is performed using a polar stationary phase and the mobile phase used is highly organic in nature. It also contains a small percentage of aqueous solvent / buffer or other solvent. The water percent in the mobile phase forms an aqueous rich layer adsorbed to the polar surface of the stationary phase.[16]

Commonly uses mobile phase for HILIC includes acetonitrile with some amount of water. Any aprotic solvent miscible with water such as THF or Dioxane can be used. Alcohols can also be used but concentration adjustment is needed in order to achieve the same degree of retention for an analyte in comparison to an aprotic solvent – water combination.[17]

It is assumed that the mobile phase used in HILIC forms a water rich layer on the surface of the polar stationary phase VS the water deficit mobile phase. This creates a liquid / liquid extraction system. The analyte gets distributed in between these two layers. HILIC is more than a simple partitioning as it includes hydrogen donor interaction. This interaction occurs

between neutral polar species and weak electrostatic mechanism under the high organic solvent condition used for the purpose of retention of the more polar compounds will have a strong interaction with the stationary aqueous layer as compared to less polar compounds.[18]

Polar analytes partition into this aqueous rich layer. It is observed that they are retained through a complex. This is a combination of hydrophilic partitioning of the analyte between the aqueous rich layer and the bulk of the mobile phase.

Hydrogen bonding occurs between polar functional group and the stationary phase. Electrostatic interactions are observed on ionized functional groups Vander waals interactions between the hydrophobic portions of the bonded ligands of the stationary phase (or the siloxane groups, at very low organic solvent percentage) and the non – polar part of the analyte are also observed.

Ammonium acetate and ammonium formate are used as ionic additives in HILIC.[19] They are used to control mobile phase pH and ion strength. These ionic additives contribute to the polarity of the analyte. This causes differential changes in retention. For highly polar analytes like aminoglycoside antibiotics or ATP, high buffer concentration such as 100 mM are needed for keeping the analyte in a single ionic form. If this is not done, then asymmetric peak shape, chromatographic tailing as well as poor recovery from the stationary phase will be seen. For separation of neutral polar analytes like carbohydrates, not buffer is needed.

Other salts in concentration of 100-300 mM sodium perchlorate can be used. These salts are soluble in high organic solvent mixture. This can be used to increase the mobile phase polarity to effect elution. As these salts are not volatile, so this technique is not suitable to use with mass spectrometer as the detector. Only a gradient (with increasing amount of water) can help in elution. There is partitioning of all ions in the stationary phase to some degree. Hence an occasional 'Wash' with water is needed for reproducibility of stationary phase.

Advantages of HILIC

HILIC has advantage over RPLC in the retention of hydrophilic compounds. The latter are difficult to be retained in RPLC without the use of an ion pairing agent. Elution order in HILIC is primarily based on analyte hydrophilicity. This is in contrary to hydrophobicity. So, selectivity pattern of HILIC is complementary to RPLC. Mobile phases contain high organic substances. This allows improved signal / noise ratio, (S/N) with MS detection.

Hydrophilic interaction liquid chromatography is a mode of choice for separation of uncharged highly hydrophilic and amphiphilic compounds. These combounds are too polar to be retained in RP-LC. But these compounds have insufficient charge to allow effective electrostatic retention in ion-exchange chromatography. Hence this mode of separation is widely used to analyze carbohydrates, peptides and polar pharmaceuticals.[20]

HILIC method is better to use with ESI-MS. It is because highly organic mobile phase result in desolvation and hence lower detection limits. For example, retention of nicotine and continine by RP chromatography require a highly aqueous mobile phase i.e. around 98%. This causes low S/N ratio even after proper adjustment of electrospray conditions. When nicotine and continine are examimed under HILIC, the S/N ratio even after proper adjustment of electro spary conditions. When nicotine and continine are examined under HILIC, the S/N ratio for nicotine is 15 times higher as compared to RPLC21. Even S/N ratio for continine becomes 5 times higher in HILIC as compared to RPLC. It is shown in the following diagram Organic rich mobile phases provide low viscosity and hence low back pressure, low back pressure enables use of high flow rates for fast analysis and sub – 2micrometers particles can be used. It has been found that high efficiency of HILIC is achieved at low flow rates as compared to RPLC and column overloading with less peak tailing is seen in HILIC. It occurs when peaks for very polar analytes eluted under HILIC conditions are compared to the peaks that can be obtained for these compounds on silica columns using high aqueous conditions in per aqueous liquid chromatography, PALC. Good retention factor is observed under PALC , due to the hydrophobic character of siloxane groups at the surface of the silica. But the adsorption mechanism is heterogenous so involves active adsorption sites. This causes column overloading when analysis of strongly retained samples (K>2) is done.

HILIC is compatible with SPE extracts in 100% aprotic organic solvent. They can be directly injected without the need to dry down and reconstitute the mobile phase. As the organic solvents are weak eluents under HILIC conditions, polar analytes are accumulated on a narrow zone near the column inlet. This is the phenomenon of “sample on – column “focusing.

So it can be said Hydrophilic interaction chromatography (or hydrophilic interaction liquid chromatography, HILIC) is a variant of normal phase liquid chromatography. It also overlaps with other chromatographic applications such as ion chromatography and reversed phase

liquid chromatography. That is, HILIC method uses hydrophilic stationary phase with reversed phase type eluents.

HILIC mode of separation is basically used for separation of biomolecules, inorganic as well as organic molecules. This is done by difference in polarity. Sample preparation for this mode of separation is simple for biological samples. Metabolic process results in the addition of polar groups for increased elimination from body tissues. So metabolites can also be studied for their chromatographic analysis. HILIC technique of separation is also applied for glycosylation analysis and quality assurance of glycoproteins and glycoforms in biologic medical products. For detection of polar compounds with Electrospray ionizations, mass spectrometry as chromatographic detector, HILIC is a better method of choice. This is because HILIC can afford a ten fold increase in sensitivity over reversed phase chromatography. This is because organic solvent is much more volatile.

HILIC Method Development

HILIC is selected to meet analytical challenges in pharmaceutical and biomedical fields.HILIC method development is a process of establishing the analytical procedure for quantitative and qualitative analysis of analytes using chromatographic technique. Dajaegher et.al. reviewed many assay methods based on HILIC and concluded that they employed trial-and error approach.[21] Method development in HILIC include sample preparation, detector selection, system suitability, and quantitative calculation. In order to evaluate these parameters, it is necessary to build a good understanding of properties of target analytes. This include structure, mol.wt.,pk_a,, log P and solubility. A proper understanding of sample characteristics and sample preparation is to be performed. Core of method development include selection of column and mobile phase.

Methods for bioanalytical and pharmaceutical applications require validation. International conference on harmonization(ICH) and FDA guidelines on method validation provide a general framework such as accuracy, precision, linearity, robustness, limit of detection(LOD) and limit of Quantitation(LOQ).Other method attributes are also considered. They are desired resolution between critical pairs, required sensitivity for impurities, and run time.

Development analysts consider factors that have practical applications. This include suitability for routine application in QC labs and adaptability for automation(for high sample volume application).[22]

As already mentioned, HILIC is a chromatographic technique suitable for polar compounds due to stronger retention provided by HILIC as compared to RPLC.

Compounds separated by HILIC include sugars, water-soluble vitamins, metabolites, amino acids, nucleic acids, organic acids, peptides and proteins. Compounds suitable for HILIC separation are those with small positive or negative logP, low mol.wt.(except peptides and proteins). Relative possibility of a polar compound retained in HILIC mode of analysis is assessed by a web-based prediction model. This model is developed by Merck SeQuant (www.sequant.com), which is based on retention data of 40 polar compounds on a ZIC-HILIC column through quantitative-structure-retention relation(QSRR) and multivariate modeling.

The structure of a particular compound in SMILES(Simplified molecular-input line entry – specification) format is entered into web application. This prediction model generates a retention factor in the mobile phase. Mobile phase contains 70% acetonitrile and 30% 100nM ammonium acetate (pH 5.6 or 6.7). The database on which this prediction model is based applies such mobile phase which has 30% aqueous buffer. It provides basic information in developing method for compound retention in HILIC.[23]

The systemic approach to method development is related to quality-by-design (QbD) concept. QbD is a "systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management".

Pharmaceutical applications of HILIC

HILIC is a powerful tool for estimation of polar compounds of Pharmaceutical interest.

The development of ion-pairing mode of RP-HPLC was previously a better response. To overcome the problem of poor retention/separation of charged polar analytes. Here, pH of the mobile phase is buffered to maintain a positive or negative charge on the analyte. A salt containing a counterion which can form an ion-pair with the analyte is added. This action causes charge neutralization. It increases hydrophobicity of the ion pair and hence the greater retention of the analytes. Hence the type of ion-pair reagent and concentration parameters are optimized to achieve the desired result. But this method has its demerits like the impurities present in the ion-pair reagent can form artifacts in the analysis. In addition, many ion-pairing

reagents are not volatile. So it precludes the use of mass Spectrometric(MS), evaporative light scattering(ELSD), or charged aerosol detection(CAD) method.

Totally aqueous(no organic modifier) mobile phases have been used but problems with reproducibility and robustness have been encountered.

HILIC is one mode for analysis of positive and negative counterions of pharmaceutical salts as these analytes have no retention or very short retention times in the RP mode.

CONCLUSION

A general interest among Chromatographers on HILIC mode of separation has raised since the last decade. HILIC has advantages over RPLC in the separation and study of polar compounds, ionic as well as hydrophilic compounds. A better understanding of its mechanism of action is due to partitioning, adsorption, ionic interactions as well as hydrophobic retention which is due to the experimental conditions. HILIC as a method is establishing itself as a complementary to RPLC. This review presents the retention mechanism of HILIC, different stationary phases used in HILIC as well as the method development parameters for HILIC mode of chromatography. Further, Pharmaceutical applications of HILIC is well described as this method is suitable for analysis of polar compounds as well as metabolites of biological and toxicological interest.

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