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REVIEW ARTICLE

Hydrophilic Interaction Liquid Chromatography for the Analysis of Pharmaceutical Formulations

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Abstract: For a long time, Reversed-Phase Liquid Chromatography (RPLC) was the most dominant technique for the analysis of pharmaceutical compounds, but with poor efficiency in the separation of small polar molecules. From the efforts to solve the problem of insufficient retention of these molecules, during the last decades, a mode of liquid chromatography named Hydrophilic Interaction Liquid Chromatography (HILIC) has experienced vast expansion. It is based on the use of a highly hydrophilic stationary phase along with an aqueous mobile phase with high organic modifier content. In this review, the characteristics of stationary and mobile phases used in HILIC are described, and corresponding separation mechanisms are discussed. An overview of recently published papers dealing with the application of HILIC in analyzing pharmaceuticals in biological and non-biological samples is provided. Besides, the application of HILIC systems in the determination of the physicochemical properties of compounds is described.

Keywords: Hydrophilic interaction liquid chromatography, pharmaceutical application, polar compounds, polar stationary phase, hydrophilic partitioning, hydrogen bonding, electrostatic interactions.

1. INTRODUCTION

Liquid Chromatography (LC) is an essential analytical method in the pharmaceutical industry. It is incorporated in all stages of the complex drug development process, followed by pharmacokinetic studies, biological assays, testing of the quality of the final product, and determination of its metabolites [1].

Reversed-phase liquid chromatography, which implies using a non-polar stationary and a polar mobile phase, is generally the most dominant separation technique, but not efficient in the analysis of polar and ionic compounds, which have very weak retention. Besides, a mobile phase with a high water content can cause numerous problems, such as the deterioration of the stationary phase and the reduction of separation sensitivity [2]. On the other hand, the main drawback of normal-phase liquid chromatography, which implies the use of a polar stationary phase in combination with a non-polar mobile phase, is the low solubility of polar and hydrophilic compounds in the non-aqueous chromatographic solvent. Therefore, in order to achieve adequate retention and selectivity of polar and hydrophilic compounds, it was necessary to develop a method that combines a polar stationary phase and a low water content mobile phase.

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In 1990, Alpert [3] described the special mode of normal-phase (NP) liquid chromatography, hydrophilic interaction liquid chromatography known under the acronym HILIC, which implies the use of polar stationary phases, such as bare, amino or cyano silica, but the mobile phase used is similar to those employed in the reversed-phase liquid chromatography (RPLC) mode.

Before the formalization of hydrophilic interaction liquid chromatography (HILIC) by Alpert, the principle was already known in the 1970s for the analysis of sugars on aminopropyl silica with mobile phases rich in acetonitrile content [4]. However, these methods may not have been explicitly referred to as HILIC at the time.

The main advantage of HILIC is that it provides excellent solubility of polar and ionic compounds in the aqueous HILIC mobile phase. However, HILIC does not need the use of expensive ion-pair agents, and it is suitable for coupling with Mass Spectrometry (MS), especially in the electrospray ionisation (ESI) mode [5]. It can be applied to a variety of sample types, including biological fluids [6], pharmaceuticals [7], and environmental samples [8]. Besides, HILIC can reduce matrix effects compared to other chromatographic techniques, making it useful for complex sample matrices [9].

However, there are some disadvantages of HILIC. Optimal mobile phase composition can be challenging to determine, and it may require careful optimization to achieve the

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desired separation. Besides, HILIC is sensitive to changes in mobile phase composition, and small variations can significantly affect separation and peak shapes. Some samples may have solubility issues in the highly organic mobile phases commonly used in HILIC [10].

The mechanism of separation under HILIC conditions is still under debate, but it is generally accepted as a concept of partition-driven phenomena and adsorption (hydrogen bonding, dipole-dipole, attractive or repulsive electrostatic interactions with charged sites on the stationary phase) [11-14].

Since RPLC and HILIC are mutually orthogonal modes [15-17], it is possible to combine them in two independent dimensions, which differ in their separation mechanisms. This significantly affects the increase in the capacity of the system for the separation of complex samples. In this way, it is possible to overcome the disadvantage of the HILIC mode, which concerns the reduced solubility of hydrophobic compounds and the possibility of their precipitation [5].

The HILIC method experienced an expansion in the early 2000s and was used in the analysis of a wide variety of compounds, such as small polar molecules, pharmaceutical compounds, food metabolites [18], drugs of abuse, toxins, carbohydrates, oligosaccharides, amino acids, peptides, proteins, plant extracts, *etc*. [19, 20].

According to our knowledge, only a few review articles provide an overview of the application of HILIC in the analysis of pharmaceutical compounds [2, 21-23].

In the current review, we have focused on the discussion about the characteristics of stationary and mobile phases, as well as the mechanisms governing retention in HILIC systems and their applications for the analysis of polar pharmaceutical compounds, such as drugs compounds, their impurities and metabolites in both biological and non-biological materials for the period of 2019–2023.

2. HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY (HILIC)

2.1. Stationary Phases

HILIC phases can be divided into neutral, charged and zwitterionic. The surface of HILIC stationary phases can be acidic, neutral or basic, and accordingly, the retention mechanism can be different [24, 25]. A detailed overview of the chromatographic systems, and especially the stationary phases, used in HILIC was given by Jandera [26]. In his review paper, Guo recently presented a review of currently available polar stationary phases in the market, as well as novel stationary phases utilized in HILIC [27]. Guarducci and colleagues provided a comprehensive summary of the commonly employed stationary phases in HILIC. They categorized achiral stationary phases for various applications, distinguishing between commercially and non-commercially available options. Additionally, they highlighted chiral stationary phases for enantioseparations, with a specific emphasis on developments within the past 15 years [5].

Polar stationary phases commonly used in HILIC separations (Fig. **1**) are silica gel and modified silica gels with diol-, amino-, amido-, cyano-, and zwitterionic functional groups.

Silica-based and amino-based stationary phases undeniably represent some of the initial and widely employed options in HILIC.

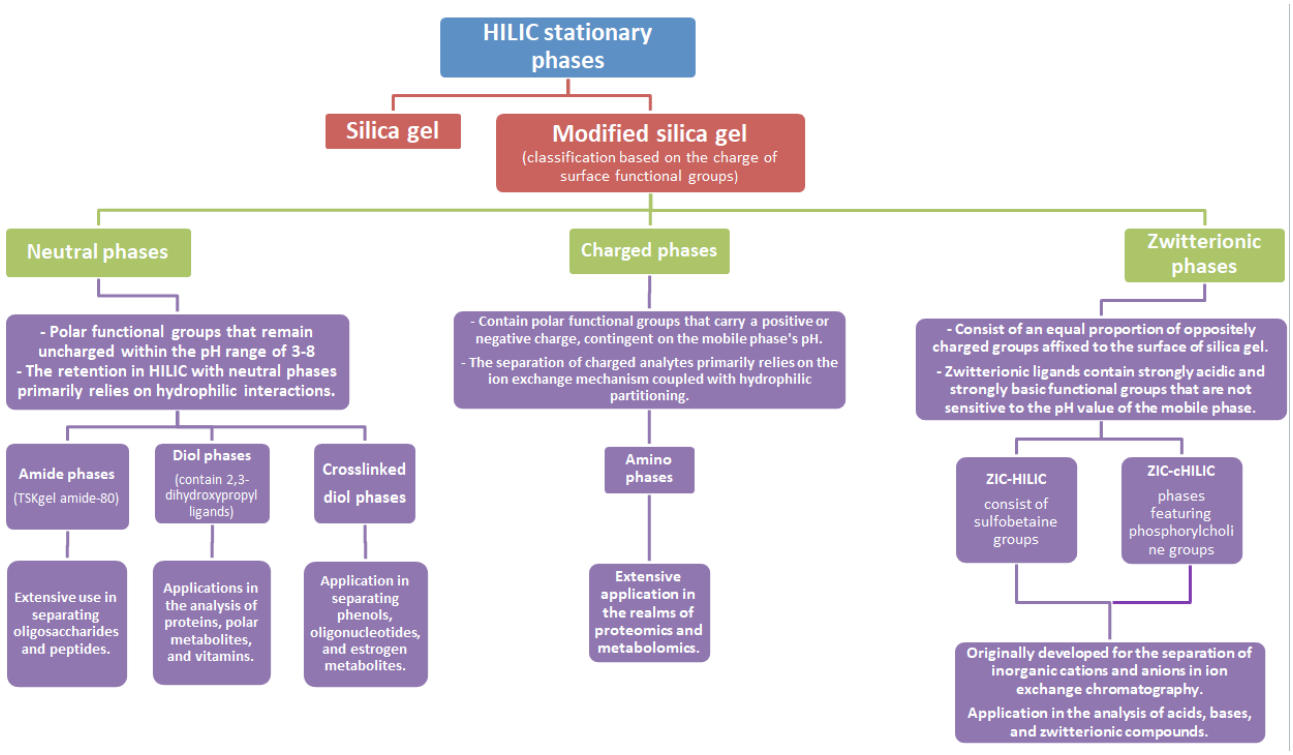


Fig. (1). Stationary phases used in HILIC. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Amino phases consist of a silica gel matrix bonded with an aminopropyl ligand and find extensive applications in the fields of proteomics and metabolomics [28].

Amide phases, including TSKgel amide-80, are achieved by surface functionalization of silica gel with carbamoyl or amide groups linked *via* alkyl chains. These phases have found extensive use in separating oligosaccharides and peptides. TSKgel amide-80 stands out as one of the highly favored amide phases [29].

Diol phases, which contain 2,3-dihydroxypropyl ligands, are used in the analysis of proteins, polar metabolites, and vitamins [26, 30]. Crosslinked diol phases with diol groups interconnected by ether bonds, such as the Luna HILIC column, are applied in the separation of phenols, oligonucleotides, and estrogen metabolites [28]. In general, the retention of polar analytes is stronger in the following sequence of different polar stationary phases: cyanopropyl- < diol- < aminopropyl- << silica gel, but the specific interactions of each individual stationary phase with the separated substances can strongly influence the selectivity of the separation.

In their study, Guo and Gaiki [29] compared the retention behavior of small polar compounds, such as carboxylic acids, nucleosides, and nucleotides. They examined both unmodified silica gel and various modified stationary phases, like amide, amino, aspartamide, and sulfobetaine, which are commonly employed stay HILIC. The results indicated that while silica gel exhibited the weakest retention, it demonstrated the highest selectivity among the polar stationary phases tested. Charged stationary phases contain polar functional groups that carry a positive or negative charge, typically contingent on the mobile phase's pH. In this scenario, the separation of charged analytes primarily relies on the ion exchange mechanism coupled with hydrophilic partitioning [28].

Zwitterionic stationary phases consist of an equal proportion of oppositely charged groups affixed to the surface of silica gel. Zwitterionic ligands usually contain strong acidic and basic functional groups that are not sensitive to the pH value of the mobile phase. Such stationary phases were originally developed for the separation of inorganic cations and anions in ion exchange chromatography but have found wide applications in HILIC [29]. Sulfobetaine and phosphorylcholine are widely recognized zwitterionic selectors utilized in HILIC stationary phases, where the permanently charged groups are positioned in opposite orientations. Additionally, various other zwitterionic compounds, such as 3-P,P-diphenylphosphoniumpropylsulfonate, imidazolium-based molecules, and quaternary imidazoline-based zwitterionic compounds, have been specifically designed and synthesized for the modification of supporting silica in HILIC stationary phases [30, 31].

Mixed-mode stationary phases have different functional groups, such as hydrophobic alkyl chain, exchange ionic moiety, polar groups, *etc*., which provide dual retention behavior and efficiency in the separation of compounds that differ in polarity [12-14, 32-35].

Introducing charged or ionizable groups into mixed-mode stationary phases for RPLC, ion exchange chromatography (IEX), and HILIC enables the development of a versatile stationary phase capable of accommodating all three chromatographic modes (RPLC/IEX and HILIC) [5].

Besides the commercial HILIC stationary phases, there is a trend of developing new ones, which are also categorized as neutral, anionic, cationic, and zwitterionic [36].

Several factors have been considered when selecting an appropriate column. Typically, neutral analytes are less hydrophilic than charged ones, necessitating the use of highly hydrophilic phases for their retention, such as charged, zwitterionic, and amide phases. Conversely, charged compounds experience excessive retention on columns with the same charge due to electrostatic attractions. As a result, better outcomes are achieved using neutral and zwitterionic phases [33].

2.2. Mobile Phases

In HILIC, aqueous mobile phases are used in combination with polar stationary phases, which usually contain 5-40%, or even less (2-3%), water or buffer in a mixture with organic solvents [37]. This quantity of water is essential to guarantee adequate hydration of the particles in the stationary phase and form a pseudo-stationary phase, enabling liquid-liquid partitioning between the rest of the mobile phase and the adsorbed aqueous layer. These HILIC systems are sometimes referred to as aqueous hydrophilic interaction chromatography (AQ-HILIC) [13]. However, Obradović *et al*. used non-aqueous versions of HILIC (NA-HILIC) with a mobile phase containing acetonitrile methanol with the addition of 20 mM ammonium formate and 20 mM ammonium formate with the addition of 0.1% (*v/v*) of formic acid [38]. Fig. (**2**) shows the mobile phases used in HILIC. It is advisable to commence initial experiments using a relatively elevated water content (*e.g*., 40%) in acetonitrile (ACN) to ensure the elution of all sample components. Subsequently, the elution strength can be adjusted by gradually increasing the concentration of ACN until satisfactory sample retention is attained.

Acetonitrile is most often used as the organic component of the mobile phase as it is well miscible with water, devoid of hydrogen-donor or acceptor functional groups that might compete with water for solvating the surface of the stationary phase. Other aprotic solvents, such as tetrahydrofuran dioxane and acetone, can also be used. Unlike other solvents, ACN contributes to satisfactory retention and peak symmetry [28]. Acetone has a similar polarity to ACN but leads to poorer retention under HILIC conditions [39]. Also, a high absorbance of acetone makes it impractical for applications in column high-performance liquid chromatography with UV detection, although it can be used with Mass Spectrometric (MS) detection [40]. On the other hand, protic solvents possess the capability to engage in hydrogen bonding with active sites on the stationary phase's surface, displacing water molecules. This results in the formation of a more hydrophobic stationary phase, leading to reduced retention of polar components, thereby causing a decrease in retention time [41].

In addition to the composition of the solvent, the chromatographic retention process is also influenced by the type and

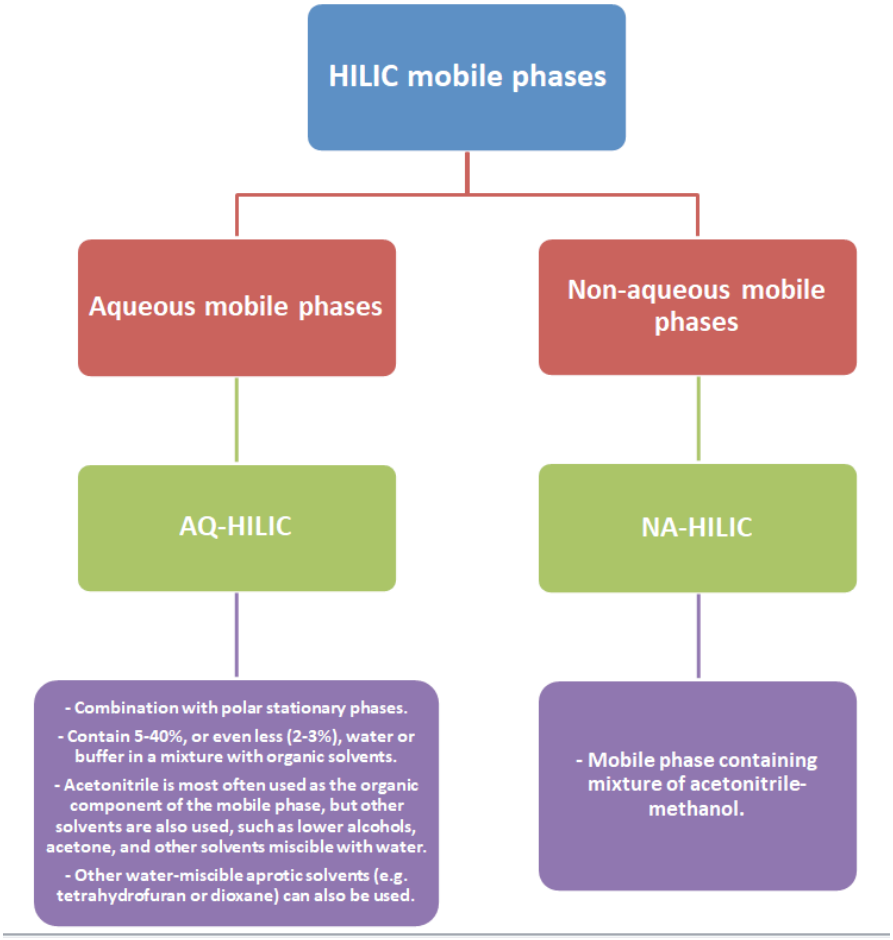


Fig. (2). Mobile phases used in HILIC. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

amount of salt, as well as the pH value and temperature [42, 43]. To enhance peak shape, various additives like acids, salts, and ion-pairing agents can be incorporated into mobile phases [44-46].

The novel proposal to utilize diethylamine as a modifier in the mobile phase to improve peak shape is a suggestion worth exploring in future investigations focused on amino acid analysis through HILIC [47].

Moreover, pH can affect the ionization state of analytes, especially for compounds with acidic or basic functional groups. The degree of ionization influences the interaction of analytes with the stationary phase. Also, the pH of the mobile phase can impact the surface charge of the stationary phase. This is particularly relevant for phases with ionizable functional groups. Changes in pH can affect the degree of ionization of these groups, influencing the electrostatic interactions with analytes [48].

Buffers are commonly employed in HILIC to maintain a stable pH in the mobile phase. This helps achieve reproducible and reliable separations. Common buffer systems used in HILIC include phosphate, acetate, and formate buffers [49].

2.3. Mechanisms of Retention Under HILIC Conditions

While the mechanism of chromatographic separation in RP mode is mostly known and explained, the mechanism and theoretical explanation of retention in HILIC is the subject of many research and review articles [24, 25, 28-30, 37, 50-52].

Retention behavior in HILIC is governed by hydrophilic partitioning, surface adsorption and electrostatic interaction between charged groups. Therefore, the mechanism can be viewed as multimodal, as it includes both hydrophilic and hydrophobic interactions [53, 54].

The dominant mechanism of separation in HILIC is not constant, and it is not always easy to predict because it depends on the nature of the analyzed components, the applied stationary, and the composition of the mobile phase, which can vary from adsorption to partition. Given the characteristics of the examined substances, it was noted that compounds with prominent hydrophilic properties exhibit a more pronounced emphasis on the HILIC mechanism, whereas those with pronounced lipophilic properties tend to favor the RP mechanism. Additionally, some compounds display nearly equal of both retention modes [55].

Using the same stationary phase, the interaction mechanism can shift from hydrophobic to hydrophilic by adjusting the water content in the mobile phase. The retention curve exhibits a distinctive U shape, which is indicative of the alteration (reversal) in the retention mechanism [41]. Many polar sorbents employed as the stationary phase in HILIC

demonstrate a dual (HILIC-RP) mechanism, acting as a reversed phase at elevated water content in the mobile phase and HILIC at a high concentration of the organic component [52].

A double retention mechanism, where normal-phase and reversed-phase effects simultaneously contribute to retention, is a common phenomenon in the chromatography of polar compounds on many polar-bound stationary phases. Moreover, the dual HILIC/RP mode presents an alternative biomimetic approach for efficiently and economically assessing the behavior of drugs within biological systems. In reversed-phase systems with binary mobile phases, retention decreases as the concentration of the less polar solvent increases, while in normal-phase chromatography, retention decreases as the concentration of the more polar solvent (water) increases. The composition of the mobile phase at the minimum of the U curve corresponds to the transition from reversed-phase to normal phase (NP) HILIC mechanism. For many compounds, retention on the so-called U-point is very weak. The composition of the mobile phase at this point mainly depends on the stationary phase, so with different stationary phases, one can expect a different composition of the mobile phase at the U-point [14]. It can be described by the multimodal equation:

$$F = \beta 0 + \beta p + \beta l \ln p, \quad (1)$$

Here, F represents the chromatographic function (such as logk for the HPLC system or R_M for the TLC system), and p denotes the physico-chemical property of the mobile phase (for instance, ϕ for the volume fraction of the mobile phase modifier, ptot for the total polarity of the mobile phase, or δtot for the total solubility of the mobile phase). The regression constants of the equation are denoted by β0, β, and βl.

Furthermore, the transition point (pmin) between the HILIC and RP modes can be mathematically represented by the following equation:

$$\text{pmin} = -\beta l/\beta, \quad (2)$$

Here, β and βl represent the regression constants in equation (1).

Typical normal-phase behavior was observed over the entire range of non-aqueous mobile phases, and typical reverse-phase dependence was observed with mobile phases containing less than 80% organic solvent in water. Therefore, employing less polar stationary phases, the U point shifts towards higher concentrations of water in the mobile phase. This adjustment facilitates the utilization of mobile phases with elevated water content in HILIC [26]. However, few works have been devoted to the mechanism transition from adsorption to partition in HILIC mode and the factors influencing these changes. Critical parameters for the retention mechanism in HILIC are the structure of the sample, the composition of the mobile phase, and the polar functional groups on the stationary phases [24, 51]. When the water content is greater than 50% (aqueous environment), the retention of polar compounds increases with increasing analyte hydrophobicity. Increasing the water content of the mobile phase leads to longer retention, which is similar to RP separation. Here, hydrophobic interactions are dominant. When the water content is less than 50% (organic environment), an increase in retention is observed for more hydrophilic analytes. Under these conditions, water becomes a stronger solvent, and increasing water content decreases analyte retention [40]. In such conditions, the retention sequence can be attributed to a combination of adsorption and partition mechanisms, and the transition between these two mechanisms is probably continuous and depends on the water content in the mobile phase, *i.e*., with increasing water content, the partition becomes dominant [26, 52].

3. PRACTICAL APPLICATIONS

3.1. HILIC Systems in the Analysis of Pharmaceuticals

Table **1** presents articles [6, 9, 12-17, 32-35, 48, 56-73] that focus on the applications of the HILIC method in the analysis of pharmaceutical compounds in different pharmaceutical formulations, body fluids, *etc*., and investigate their presence in the environment for the last five years. The most commonly used methods are High-Performance Liquid Chromatography (HPLC) and Ultra High-Performance Liquid Chromatography (UPLC). UPLC has significant advantages over traditional HPLC in terms of increased resolution, speed, and sensitivity, which simplify the characterization of complex samples [33]. LC can be coupled to different types of detectors. The most common are Ultraviolet-Visible (UV-Vis), Photodiode Array Detector (PAD or DAD), Mass Spectroscopic (MS), Charged Aerosol Detector (CAD), and Evaporative Light Scattering (ELSD). Refractive index detector (RI) is less in use because it is limited by the use of an isocratic mobile phase.

Methods of detection are various and depend on the nature of the target substance and the complexity of the tested mixture. UV spectroscopy, which is limited by solvent selection, and CAD are preferably used in non-biological applications. On the other hand, for biological applications, the detection is usually performed with a tandem mass spectrometer (MS) primarily used for targeted quantitative methodologies or high-resolution mass spectrometry (HRMS), such as quadrupole time-of-flight and Orbitrap MS technology to augment the methods with non-target and suspect screening capabilities.

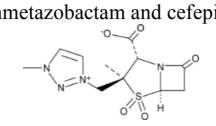
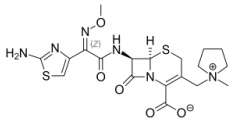
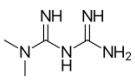
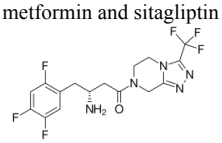
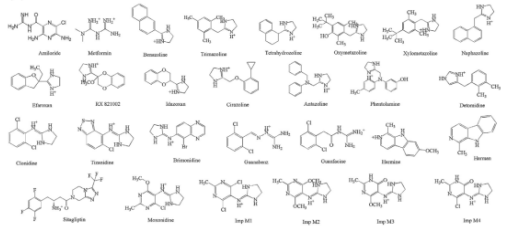
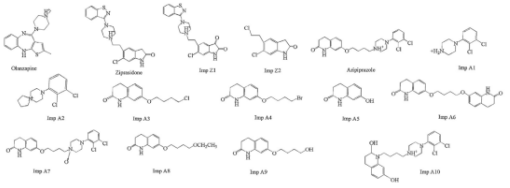
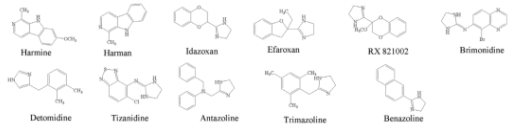
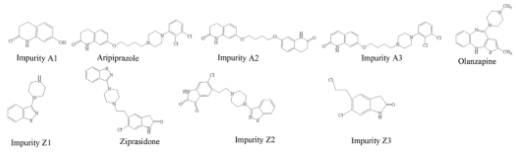
Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the most popular interfaces in HPLC–MS/MS techniques.

HPLC-MS/MS systems are very sensitive and quick and do not require complex sample preparation procedures [74]. Besides, MS detection is very suitable for HILIC systems due to the use of high concentrations of organic solvents in HILIC mobile phases.

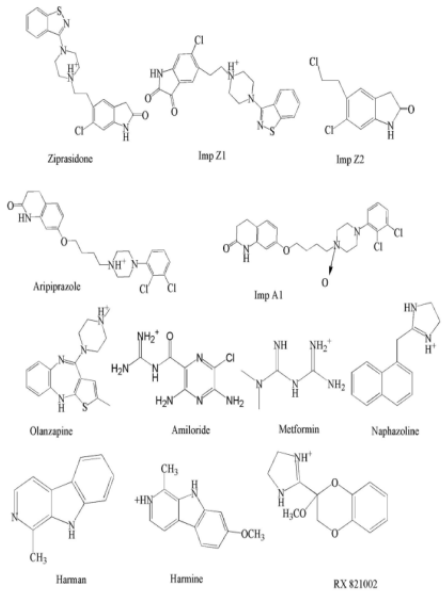
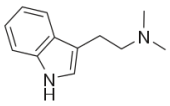
Evaporating Light-Scattering Detection (ELSD) is suitable for compounds that are poor UV absorbers and can detect any analyte less volatile than the mobile phase. Furthermore, it has a very low background noise with solvents commonly used in RP-HPLC lipid analysis (methanol, isopropanol, and acetonitrile).

Varachea *et al*. used this type of detection in the analysis of phospholipids, polyethylene glycol (PEG) compounds, and triglycerides, which are used as nanocarriers [33].

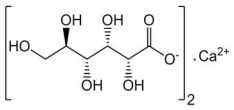
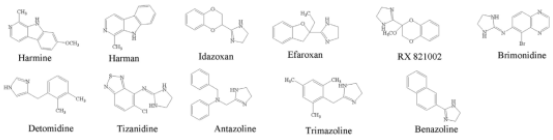
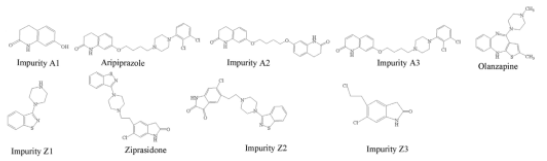
Table 1. The HILIC systems in analysis of pharmaceutical formulations.

Compound	Material	Type of Chromatography/ Detector	Stationary Phase	Components of Mobile Phase	References
Enmetazobactam and cefepime  Enmetazobactam  Cefepime	Human plasma	UPLC–MS/MS	Acquity BEH HILIC column (50 mm × 2.1 mm, 1.7 µm), Waters	20 mM ammonium formate in water) and ACN	[6]
Antidiabetic drugs metformin and sitagliptin  Metformin  Sitagliptin	Commercial standards, human plasma and urine	HPLC - MS/MS	Kinetex HILIC, 50 x 4.6 mm, 2.7 µm (Phenomenex, Aschaffenburg, Germany)	(A) 9 mM ammonium formate solution, formic acid and acetonitrile (450:0.25:50, *v/v/v*) (B) 80 mM ammonium formate solution, formic acid and acetonitrile (100:0.5:900, *v/v/v*)	[9]
Imidazoline and serotonin receptor ligands  Imidazoline receptor ligands  Serotonin receptor ligands	Standard solutions of tested compounds	HPLC/DAD	Acclaim Mixed-Mode HILIC-1 analytical column (150 mm × 4.6 mm, 5 µm) Thermo Fisher Scientific	ACN - 20 mM ammonium acetate adjusted with acetic acid to pH 6	[12]
Imidazoline and serotonin receptor ligands  Imidazoline receptor ligands  Serotonin receptor ligands	Standard solutions of tested compounds	HPLC/DAD	Acclaim Mixed-Mode HILIC-1 analytical column (150 mm × 4.6 mm, 5 µm) Thermo Fisher Scientific HILIC (Diol) column (150 mm × 4.6 mm, 2.6 µm), Phenomenex, USA	ACN and MeOH with 20 mM ammonium formate and 20 mM ammonium formate with addition of 0.1% (*v/v*) of formic acid to pH 6	[13]

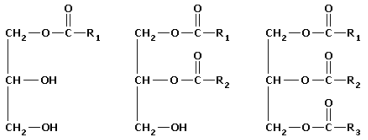
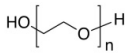
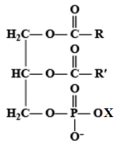
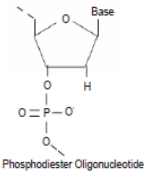
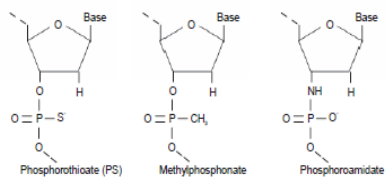
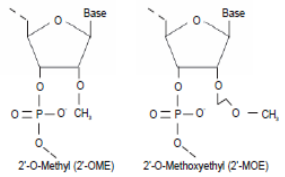
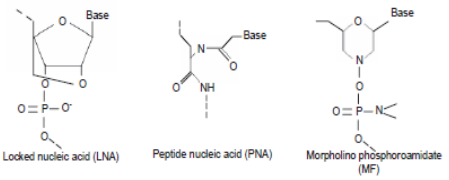
(Table 1) Contd….

Compound	Material	Type of Chromatography/ Detector	Stationary Phase	Components of Mobile Phase	References
Imidazoline and serotonin receptor ligands, and their related compounds 	Standard substances	TLC-UV	TLC RP-18 F254s (cat. no. HX389060); TLC plain silica gel 60 F254s (cat. no. HX44246354); HPTLC NPDIOL F254s (cat. no. HX85870368), all from Merck	(i) Mixture of acetonitrile and water acidified with 20 mM ammonium acetate and 0.1 volume% acetic acid, water ϕ values from 0.20 to 0.90 (ii) Mixture of acetonitrile and methanol (as a modifier) acidified with 20 mM ammonium acetate and 0.1 volume% acetic acid, water ϕ values from 0.05 to 0.90	[14]
N,N-dimethyltriptamine (DMT) 	Rat blood plasma and brain	2D-HPLC- UPLC/ HRMS/MS	Luna HILIC (150 ×3 mm, 3 µm, 200 Å, Phenomenex), Luna Omega Sugar (100 ×2.1 mm, 3 µm, 100 Å, Phenomenex); Luna NH2(150 × 2 mm, 3µm, 100 Å, Phenomenex); Kinetex C18 (50 × 2.1 mm, 2.6µm, 100 Å, Phenomenex); Luna Omega PS C18 (50 × 2.1 mm, 1.6 µm, 100 Å, Phenomenex); Acquity UPLC BEH C18 (50 × 2.1 mm, 1.7 µm, 130 Å,Waters); CORTECS UPLC C18+ (50 × 2.1 mm, 1.6 µm, 90 Å,Waters)	ACN - 50 mM ammonium formate	[15]

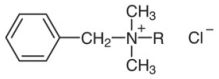
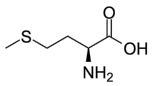
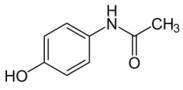
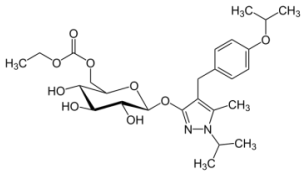
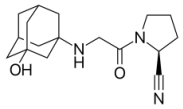
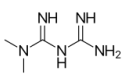
(Table 1) Contd….

Compound	Material	Type of Chromatography/ Detector	Stationary Phase	Components of Mobile Phase	References
Highly polar impurities in calcium gluconate injections 	Injection solution	2D-HPLC: (^{1}D) IPRP-LC-Q-TOF/MS; (^{2}D) HILIC/DAD	Xbridge C8 column (250 ×4.6 mm, 5 µm, Waters Corporation, Milford, MA, USA); Poroshell 120 HILIC-Z column (100 ×3.0 mm, 2.7 µm, Agilent Technologies Inc.)	Aqueous solution of 25 mmol/L potassium dihydrogen phosphate and 1 mmol/L tetrabutylammonium hydroxide (pH was adjusted to 5.8 with phosphoric acid); Ammonium acetate solution (pH 9.0; 10 mM), and ACN	[16]
Steroid hormons	Commercial internal standard MIX MassChrom ® Steroids in a Serum/Plasma (ISTD)	2D-HPLC/MS/MS	(1D)YMC—Triart Diol-HILIC column (100 × 2.1 mm, 3 µm; YMC, Japan); (2D) InfinityLab Poroshell 120 EC-C18 (3 × 100 mm; 2.7 µm; Agilent Technologies, Santa Clara, CA, USA)	(1D) Ammonium acetate (25 mM) and formic aciid (0.2%) in water, and (B): 100% ACN; (2D) Ammonium fluoride (0.5 mM) in water, and B: 100% MeOH	[17]
Imidazoline and serotonin receptor ligands  Imidazoline receptor ligands  Serotonin receptor ligands	Standard solutions of tested compounds	HPLC/DAD	Acclaim Mixed-Mode HILIC-1 (150 mm ×4.6 mm i.d., 5 µm) Thermo Fisher Scientific (Sunnyvale, CA, USA)	ACN and 20 mM ammonium acetate adjusted with a low quantity of acetic acid to pH 6	[32]

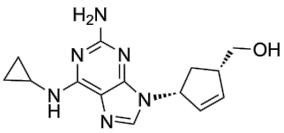
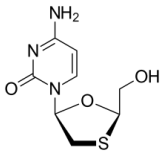
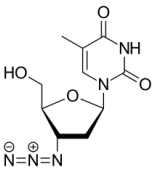
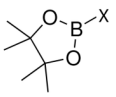
(Table 1) Contd….

Compound	Material	Type of Chromatography/ Detector	Stationary Phase	Components of Mobile Phase	References
Lipid nanocarriers -glycerides, polyethylene glycol (PEG)-stearates and phospholipids  Glycerides  Polyethylene glycol  Phospholipids	Suspension	UPLC/ELSD	CORTECS UPLC C18 column (1.6 µm, 150×2.1 mm, 90 A, Waters); CORTECS UPLC HILIC column (1.6 ×µm, 150×2.1 mm, 90 Å), Waters	Water (A), MeOH (B) and 25% *v/v* ACN in IPA (C) with gradient protocol; 10mM ammonium formate (pH=3) in ACN A) 5:95 (*v/v*) B) 50:50 (*v/v*) with gradient protocol	[33]
Inorganic ions in oligonucleotides*  Phosphodiester Oligonucleotide FIRST GENERATION ANTISENSE OLIGONUCLEOTIDES  Phosphorothioate (PS) Methylphosphonate Phosphoroamidate SECOND GENERATION ANTISENSE OLIGONUCLEOTIDES  2'-O-Methyl (2'-OME) 2'-O-Methoxyethyl (2'-MOE) THIRD GENERATION ANTISENSE OLIGONUCLEOTIDES  Locked nucleic acid (LNA) Peptide nucleic acid (PNA) Morpholino phosphoroamidate (MF)	Lyophilized antisense oligonucleotide (ASO material)	HPLC-CAD	Acclaim Trinity P2 column (3 × 50 mm, 3 µm, Thermo Scientific; Millipore SeQuant ZIC-cHILIC (2.1 × 150mm, 3 mµm) and Millipore SeQuant ZIC-pHILIC (2.1 × 150 mm, 3 µm and 2.1 × 50 mm, 3 µm)	Water - 100 mM ammonium formate, pH 3.50, with gradient elution; ammoniumacetate buffer and acetonitrile	[34]

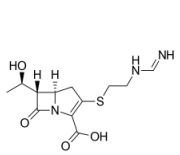
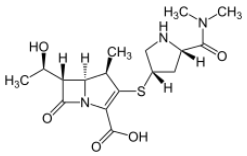
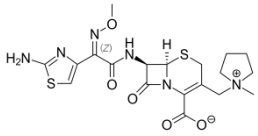
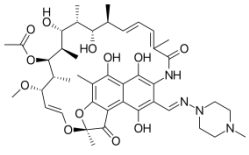
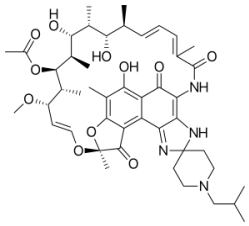
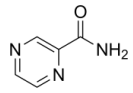
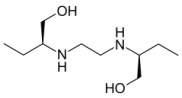
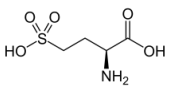
(Table 1) Contd….

Compound	Material	Type of Chromatography/ Detector	Stationary Phase	Components of Mobile Phase	References
Benzalkonium chloride and its homologues 	Commercial standards chemicals; commercial pharmaceutical formulations	HPLC-UV-Vis	Kinetex Biphenyl (150 ×3.0 mm, 5 µm) Phenomenex Nova Pak CN HP (150 ×3.9 mm, 4 µm) Waters	ACN: aqueous phase ranging from 60:40 to 95:5 (*v/v*) Aqueous phase: buffer (ammonium formate, ammonium acetate, or TEA formate, 10 - 100 mM)	[35]
Methionine and paracetamol  Methionine  Paracetamol	Reference standard	HPLC-DAD	Zorbax SB-CN column (150 × 4,6, 5 µm); Zorbax Rx-Sil column(150 × 4.6 mm, 5 µm, Agilent, Santa Clara, CA, USA); Alltech®ApolloTM Silica column (150 × 4.6 mm, 5 µm, Grace, Columbia,MD, USA); Supelco Ascentis®Si HPLC column (250 × 4.6 mm, 5 µm, Merck, Darmstadt, Germany); Hypersil Silica HPLC column (200 × 4.6 mm, 5 µm) (Thermo Fischer Scientific, Waltham, MA,USA)	ACN and aqueous formic acid - diethylamine solution at specific composition	[48]
Remogliflozin etabonate, vildagliptin, and metformin  Remogliflozin etabonate  Vildagliptin  Metformin	Commercial standard compounds, tablets	HPLC-DAD	Acclaim mixed-mode HILIC-1 column (150× 4.6 mm, 5µm, 120 Å) Thermo Fisher Scientific company, Göteborg, Sweden	ACN(65% *v/v*) and 20 mM phosphate buffer (35% *v/v*, pH 6)	[56]

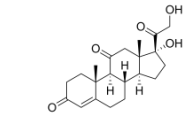
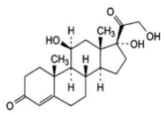
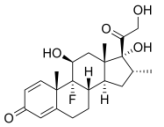
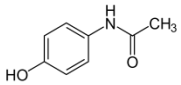
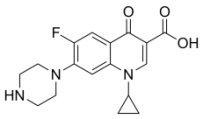
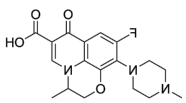
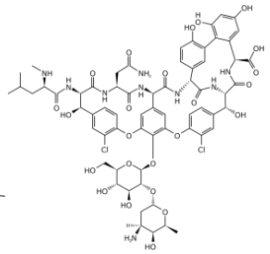
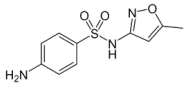
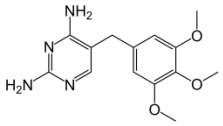
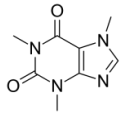
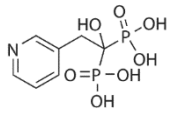
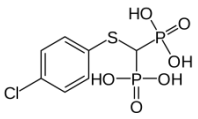
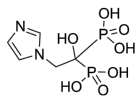
(Table 1) Contd….

Compound	Material	Type of Chromatography/ Detector	Stationary Phase	Components of Mobile Phase	References
16 nucleosides	Commercial standard compounds	HPLC	Thermo Fisher Scientific HILIC stationary phases: Accucore, 150×4.6 mm, 2.6 µm), amino (Syncronis amino, 150×4.6 mm, 3.0 µm), amide (Acclaim HILIC-10, 150×3.0 mm, 3.0 µm), and a zwitterionic stationary phase (Syncronis, 150×4.6 mm, 3.0 µm)	Formate buffer and acetonitrile	[57]
Abacavir, lamivudine, and zidovudine  Abacavir  Lamivudine  Zidovudine	Dosage form	HPLC-DAD	Columns Kinetex HILIC (150 mm ×4.6 mm, 2.6 µm), Kinetex HILIC (150 mm ×4.6 mm, 5 µm, Phenomenex, Torrance, CA, USA), XBridge HILIC (150 mm ×4.6 mm, 2.5 µm,Waters, Milford, MA, USA), and ZIC HILIC (250 mm ×4.6 mm, 5 µm, Merck KGaA, Darmstadt, Germany)	ACN – ammonium acetate or ACN - ammonium formate buffers	[58]
Pinacol boronate compounds 	Commercial obtained pinacol boronate ester compounds	HPLC-MS	Atlantis HILIC 4.6 x 150 mm, 5 µm, Poroshell HILIC-Z 4.6 x 150 mm, 5 µm, Acentis Express HILIC 2.1 x 75 mm, 2.7 µm, Xbridge BEH HILIC 4.6 x 150 mm, 5 µm, ACE HILIC-A 4.6 x 150 mm, 5 µm, ACE HILIC-N 4.6 x 150 mm, 5 µm, ACE HILIC-B 4.6 x 150 mm, 5 µm, Xbridge BEH Glycan Amide 3.0 x 150 mm, 2.5 µm, ZIC-pHILIC 4.6 x 150 mm, 5 µm, ZIC-cHILIC 4.6 x 150 mm, 3 µm	ACN with four different mobile phase additives were used, including TFA, formic acid, ammonium formate (pH 4.0) and ammonium acetate (pH 5.8)	[59]

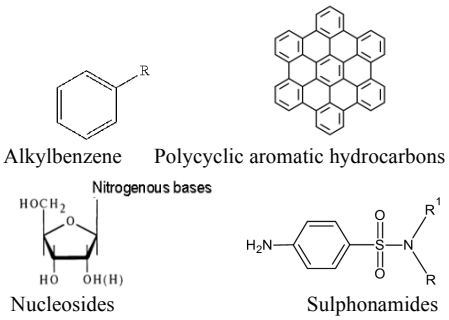
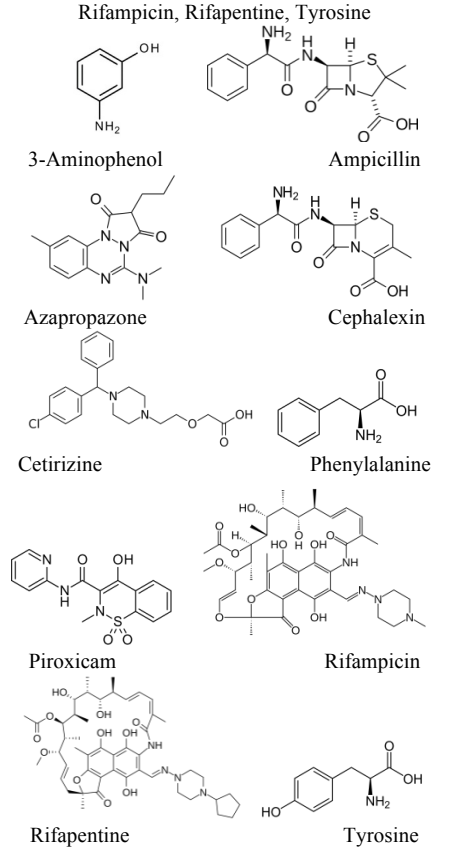
(Table 1) Contd….

Compound	Material	Type of Chromatography/ Detector	Stationary Phase	Components of Mobile Phase	References
β-lactam antibiotics: imipenem, meropenem and cefepime  Imipenem  Meropenem  Cefepime	Analytes stock solutions plasma and cerebrospinal fluid	HPLC-MS/MS	HypersilGOLD HILIC column (150 × 4.6 mm, 5 m, Thermo Fisher Scientific)	A: ammonium acetate (10 mM) and formic acid (0.1 %), B: acetonitrile-methanol(1:1, *v/v*) with ammonium acetate (10 mM) and formic acid (0.1 %)	[60]
Tuberculosis drugs: rifampicin, rifabutin, pyrazinamid, ethambutol  Rifampicin  Rifabutin  Pyrazinamid  Ethambutol	Commercial standards, human plasma	HPLC-ESI	Inertsil HILIC column (2.1×150 mm, 3 µm)	5 mM ammonium acetate contatining 0.05 % acetic acid (A) and 50 mM ammonium acetate solution-acetonitrile (1:9, *v/v*) (B)	[61]
Homocysteic acid 	Commercial standards, human plasma and urine, and cerebrospinal fluid	HPLC - MS/MS	Luna HILIC 200 Å 100 x 2 mm, 3 µm with a KrudKatcher in-line filter (both Phenomenex, Aschaffenburg, Germany)	(A) mixture of water, ACN and 0.1 M Ammonium acetate solution (88:10:2, *v/v/v*), (B) ACN containing 0.0025% formic acid	[62]

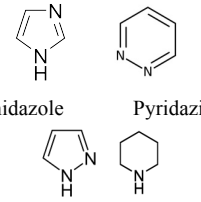
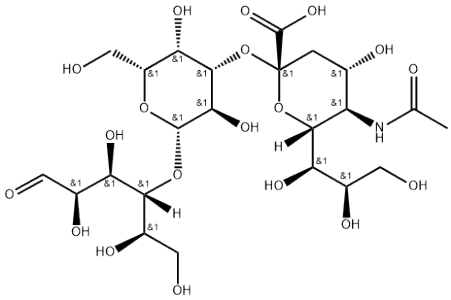
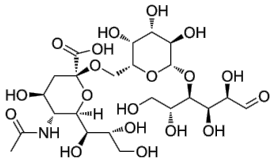
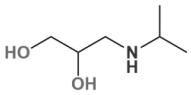
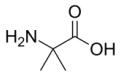
(Table 1) Contd….

Compound	Material	Type of Chromatography/ Detector	Stationary Phase	Components of Mobile Phase	References
β-lactam antibiotics: cortisone, hydrocortisone, dexamethasone, paracetamol, ciprofloxacin, ofloxacin, sulphamethoxazole, vancomycin, trimethoprim, caffeine  Cortisone  Hydrocortisone  Dexamethasone  Paracetamol  Ciprofloxacin  Ofloxacin   Sulphamethoxazole Vancomycin   Trimethoprim Caffeine	Analytes stock solutions; Human urine	HPLC-UV	Syncronis ™ HILIC silica- (250 ×4.6 mm, 5 µm, pore size 100 Å, carbon load 5%) coupled with the equivalent guard column (10 ×4 mm, 5 µm, Micro-column, Desio, Italy)	ACN and water 0.05% *v/v* formic acid	[63]
Bisphosphonates risedronate, tiludronate and zoledronate  Risedronate  Tiludronate  Zoledronate	Film-coated tablets	HPLC-DAD	Polymeric zwitterionic ZIC®-pHILIC analytical column (150.0 × 2.1, 200 Å, 3.5 µm), Merck Millipore, Darmstadt, Germany	Mixtures of ACN and ammonium formate or ammonium acetate aqueous solutions	[64]

(Table 1) Contd….

Compound	Material	Type of Chromatography/ Detector	Stationary Phase	Components of Mobile Phase	References
Underivatized neurotransmitters	Commercial standard substances, brain tissue, intestinal tissue, intestinal contents, and plasma of rats	UHPLC–QTRAP®/MS/MS, ESI+	Acquity UPLC BEH amide column (2.1 mm × 100 mm, 1.7 µm) equipped with an Acquity UPLC BEH Amide VanGuard Precolumn	Mobile phase was A (0.2% formic acid-5 mM ammonium formate and ammonium acetate water solution) and B (0.2% formic acid-1 mM ammonium formate and ammonium acetate water solution) with gradient elution	[66]
Alkylbenzene, polycyclic aromatic hydrocarbons (PHAs), nucleosides, bases and sulphonamides  Alkylbenzene, Polycyclic aromatic hydrocarbons, Nucleosides, Sulphonamides	Commercial standard chemicals	HPLC-UV	Sil-Ser-OCDs column; Welch Ultimate XB-C18 (4.6 × 250 mm); Spherisorb Amino (NH2) column (4.6 × 100 mm)	MeOH or ACN, and the aqueous phase was pure water or 10 mM ammonium acetate solution	[67]
3-Aminophenol, Ampicillin, Azapropazone, Cephalexin, Cetirizine, Phenylalanine, Piroxicam, Rifampicin, Rifapentine, Tyrosine 	Standard solutions of tested compounds	HPLC-PDA	(1) ZIC®-pHILIC column (100 mm ×4.6 mm, 5 µm SeQuant (Umeå, Sweden); (2) ZIC®-HILIC column (100 mm ×4.6 mm, 5 µm 200 Å, Umeå, Sweden) (3) ZIC®-cHILIC 100 mm ×4.6 mm, 3 µm, 100 Å, SeQuant,Umeå, Sweden)	ACN - ammonium formate or ACN - ammonium acetate buffers with different pH values	[68]

(Table 1) Contd….

Compound	Material	Type of Chromatography/ Detector	Stationary Phase	Components of Mobile Phase	References
Nitrogen-containing heterocycles: imidazole, pyrazole, pyridine, pyridazine, piperidine  Imidazole Pyridazine Pyrazole Piperidine	Standard solutions of tested compounds	HPLC/MS	Primesep 200 column (2.1 × 150 mm, 5 µm 100-A) and guard column (2.1 × 5 mm) from SIELC technology	ACN - water was acidified with formic acid	[69]
3-and 6-sialyllactose  3-sialyllactose  6-sialyllactose	Standar substances, mini-pig plasma	HPLC–MS/MS	Atlantis HILIC column (50 × 2.1 mm, 3 µm,Waters, Milford, MA, USA)	10 mM ammonium acetate buffer and ACN with gradient elution	[70]
3-isopropylamino-1,2-propanediol  2-Aminoisobutyric acid 	Commercial standard substances, injection, tablets. Commercial standard substances	HPLC-CAD HPLC - fluorescence detector	Halo Penta HILIC column, 150×4.6 mm, 5 µm, Advanced Materials Technology (Wilmington, DE, USA) COSMOSIL HILIC column (250 × 4.6 mm, 5 µm, Chromservis, Czech Republic)	ACNand ammonium formate buffer (pH 3.2 or 2.8; 100 mM). ACN and solution of 25 mM potassium acetate pH 5.5 in ratio 72:28 (*v/v*)	[71, 72]

*Structures are given in Ref [73].

Application of design of experiments (DoE) that involves the use of multivariate optimization of analysis conditions in mathematical models can be of great help in the development of optimal analytical methods. This technique is founded on pre-established objectives and the recognition of variable factors that exert a noteworthy interactive impact on the analysis, saving time, costs, and resources [28, 56].

There are several commonly used designs, such as the three-level Full Factorial Design, Box-Behnken Design (BBD), and Central Composite Design (CCD) [38].

Attimarad *et al*. employed a face-centered central composite design and a surface response design applied for the optimization of chromatographic conditions for the separation of metformin, vildagliptin, and remogliflozin, which were analyzed simultaneously for the first time [56].

Taraji *et al*. used the quality by design (QbD) concept to find optimal conditions of separation of new compounds. It features the application of quantitative structure-retention relationship (QSRR) methodology in conjunction with DoE principles. This QbD optimisation protocol made it possible

to find optimal chromatographic separation conditions for a mixture of nucleosides over four HILIC stationary phases based on the chemical structures of the analytes [57].

The influence of particle size on resolution and efficiency was performed by Erkmen *et al*. [58]. They investigated the simultaneous separation and quantification of abacavir, lamivudine, and zidovudine from dosage form on columns with different particle sizes, 2.6 µm, 2.5 µm, and 5 µm. A Kinetex HILIC column with 2.6-µm particles was selected as the most appropriate in the study of the simultaneous determination of these compounds, which belong to the class of medications called Nucleoside Reverse Transcriptase Inhibitors (NRTIs).

The analysis of some substances represents a real challenge because of many factors. Some compounds, such as boronate esters and boronic acids, cannot be effectively performed under RP, as well as under NP conditions. These substances are important building blocks for the synthesis of drugs and other materials and are used as boron carriers for controlled drug delivery devices, sensors, therapeutic drugs, and boron neutron capture therapy [59]. Under RP conditions, boronate esters tend to hydrolyze, and polar boronic acid is difficult to retain, so it is difficult to analyze them simultaneously. On the other hand, under NP conditions, they have limited solubility. Dai *et al*. developed a method for analyzing water-sensitive pinacol boronate compounds by hydrophilic interaction liquid chromatography on ZIC-cHILIC column with a high percentage of organic solvent mobile phase, which effectively prevents the on-column hydrolysis and provides excellent solubility for boronate ester samples [59].

Due to high polarity and low stability in water, beta-lactam antibiotics, imipenem, meropenem and cefepime, could not be efficiently determined in RP systems. For the first time, Rehm and Rentsch developed a HILIC MS/MS method on Hypersil GOLD HILIC column for simultaneous analysis of these compounds in plasma and cerebrospinal fluid with the presence of an effective stabilizer, which ensures their stability during work [60].

Wu *et al*. developed a method for the determination of antitubercular drugs, such as rifampicin, pyrazinamide, ethambutol, and isoniazid, in human plasma samples for detecting blood drug concentrations to improve therapeutic effects [61]. In order to compensate for matrix effects, the analysis was performed by using a co-eluting isotope labelled internal standard of each compound, which has almost identical chemical and physical properties to the target analyte. Stable isotope labelled internal standards for both antidiabetic drugs, metformin and sitagliptin, were also used in their determination in human plasma and urine by HILIC-MS/MS method [9].

The HPLC-MS/MS method for the determination of homocysteic acid (HCA), whose elevated concentrations are associated with the diagnosis of Alzheimer's disease, was the first method for the determination of HCA in human serum, urine, and cerebrospinal fluid in full accordance with the guidelines of European Medicines Agency (EMA) and Food and Drug Administration (FDA). Selectivity was achieved by using a combination of protein precipitation and solid phase extraction for sample preparation and analyte determination with a HILIC column [62].

Sometimes, a single separation HILIC mode can efficiently resolve the analytical mixture. Several HILIC methods have been developed for the separation of antibiotics enmetazobactam and cefepime [6], β-lactam antibiotics [63], and pinacol boronate esters [59]. The enmetazobactam and cefepime were analyzed simultaneously for the first time in human plasma. The developed HPLC-MS/MS method is useful over the calibration range of 0.05–50 g/mL and 0.5–500 g/mL, respectively. Moreover, it is very fast and suitable for routine analysis. The mixture of β-lactam antibiotics in human urine was successfully determined, and the authors stated the potential of using the developed method in application to real-life samples from both hospitalized and home-treated patients.

On the other hand, polar and ionic compounds, bisphosphonates, risedronate, tiludronate and zoledronate, which are used to treat various skeletal disorders, were investigated under zwitterionic hydrophilic interaction liquid chromatography conditions. Furthermore, the zwitterionic hydrophilic interaction liquid chromatography-photodiode array (ZIC-HILIC-DAD) method for the quantitation of risedronate in commercial film-coated tablets was optimized [64].

The ZIC-HILIC column was employed to assess the stability of two inorganic selenium forms (selenite Se(IV) and selenate Se(VI)), along with four organic selenium species (selenomethionine (SeMet), selenocystine (SeCys2), selenomethylselenocysteine (MeSeCys), and selenomethionine selenoxide (SeMetO)) in standard solutions and aqueous extracts of dietary supplements. This analysis was conducted using an isocratic mobile phase [65]. The results indicated that the sample matrix influenced the stability of investigated selenium compounds.

A highly sophisticated analytical method, hydrophilic interaction UPLC coupled with triple-quadrupole linear ion-trap tandem mass spectrometry (HILIC-UHPLC-QTRAP®/MS^2), was developed for the simultaneous analysis of fifteen neurotransmitters. The validated analytical method was employed to examine authentic samples (including brain, plasma, intestinal, and intestinal contents) from AD mice treated with a combination of Ginkgo ketoester tablet and donepezil. The findings led the authors to conclude that the combined use of Ginkgo ketoester tablet and donepezil at a single clinical equivalent dose exhibited a synergistic anti-dementia effect. This effect was attributed to the regulation of disordered neurotransmitter levels to normalcy across various biological matrices. Consequently, the analytical method proves to be a valuable tool for monitoring neurotransmitters in diverse clinical biological fluids [66].

Some mixtures cannot be completely resolved by one LC mode, so it is necessary to combine different LC modes [67] or use RP/HILIC mixed-mode columns [2, 12-14, 28, 32, 34, 56].

Two distinct chromatographic methods are used in the analysis, quantification, and identification of lipid nanoparticle components: the RP-UPLC-ELSD method for the determination of PEGylated surfactants and glycerides and a

HILIC-UPLC-ELSD method for the assessment of phospholipids. These methods are used for studying the manufacturing process and stability of the formulated lipid excipients during long-term storage and under accelerated conditions. Furthermore, these methods may be applied with minor modifications to other similar lipid nanoparticles and employed in quality control of commercial manufacture of lipid-based nanomedicines [67].

The study of 43 nitrogen-containing heterocyclic and guanidine derivatives, such as imidazoline and serotonin receptor ligands or their related compounds, was directed at the investigation of retention mechanisms and selectivity of their separation in different HILIC systems [12-14, 32]. First, their retention behavior in the mixed-mode stationary phase in the combined RP and HILIC modes was investigated [13]. The focus was to investigate the applicability of the linear retention modelling (adsorption, partition) within the HILIC and RP modes on the Acclaim Mixed-Mode HILIC-1 stationary phase based on the volume fraction of aqueous eluent, ϕ(aq), and total polarity index, P_{tot}, of the mobile phase. Besides, the turning point between the HILIC and the RP mode was established. The second direction was a comparison of their retention behavior in non-aqueous hydrophilic interaction chromatography (NA-HILIC), which implied the use of acetonitrile-methanol mobile phase and supercritical fluid chromatography (SF) on Acclaim Mixed Mode HILIC-1 and Kinetex HILIC (Diol) columns [12]. The authors concluded that adding formic acid to the mobile phase led to the change in retention on both columns, whereby the selectivity differences for serotonin receptor ligands increased more compared to imidazoline receptor ligands. It was found that the obtained results could help researchers solve problems of weak retention or poor resolution of novel pharmaceutical compounds related to imidazoline and piperazine structures. The third direction was the modelling of the bimodal HILIC/RP retention with a focus on different equations and different turning point estimation techniques [32].

The authors developed mathematical dependences that are useful in the characterization of the mixed-mode HILIC/RP retention data.

The same authors continued their investigation of dual retention mechanisms in thin-layer chromatography systems. They selected 12 compounds, imidazoline and serotonin receptor ligands, and their related compounds [69], which were investigated on three stationary phases of different polarity (RP-18, plain silica gel, and DIOL) and using binary mobile phases composed of acetonitrile or methanol as the modifiers and water. The presence of two retention mechanisms in TLC was effectively verified by examining the multimodal retention model in terms of the volume fraction of the mobile phase modifier, overall polarity, and total solubility of the mobile phase.

Simultaneous quantitation of inorganic ions in antisense oligonucleotide (ASO) drugs was performed using mixed-mode anion exchange/cation exchange-hydrophilic interaction liquid chromatography (AEX/CEX-HILIC) with a CAD [35]. The developed method demonstrated considerable potential for application in various analytical and quality control laboratories. It serves to support the development and characterization of ASO drugs, offering a valuable tool for the quantitative analysis of counter-ions. This allows for the confirmation of both the quantity and types of counter ions present in the final oligonucleotide drugs.

A core-shell column functionalized with biphenyl groups was used for the first time in mixed mode for the investigation of the retention of the four benzalkonium chloride (BAK) homologues possessing benzyl group, cationic group (ammonium), and alkyl chain [17]. The components of the mobile phase (salt concentration, pH, the type of anion and cation of the salt) were optimized, and a method of BAK analysis was proposed for a wide range of formulations.

Beside the commercially available mixed-mode stationary phases, with an aim to respond to the requirement of separation of complex mixtures, new stationary phases have been developed that can work under the combined LC modes and multiple retention mechanisms. Such a stationary phase was prepared and characterized by Zhao *et al*., which is known as Sil-Ser-OCDs. Ser-OCDs were synthesized using octadecylamine and serine as carbon sources, with hydrophilic and hydrophobic functional groups, such as hydroxyl, amino, and carboxyl groups, as well as alkyl chains, and bonded with silica gel. The prepared stationary phase with the dual retention mechanism of both HILIC and RPLC was tested on alkylbenzenes, polycyclic aromatic hydrocarbons (PHAs), nucleosides, bases, and sulphonamides [67].

The two new handmade columns, ZIC-S1 and ZIC-S4, which consist of sulfobetaine particles, 4-vinylbenzyldimethylammonio bmethanesulfonate and 4-vinylbenzyldimethylammonio butanesulfonate, respectively, were used for separation of angiotensin II receptor antagonists, telmisartan, valsartan, irbesartan, and losartan. The authors suggested that the validated methods could be used for therapeutic drug monitoring [67].

In the case of complex biological material, two-dimensional liquid chromatography (2D-LC) can be used, which implies the use of two different LC columns, each corresponding to a different separation, providing a number of possibilities to enhance selectivity and sensitivity for the determination of targeted compounds [15, 48, 70].

N,N-dimethyltriptamine (DMT) in rat blood plasma and brain [15] and a mixture of steroids in plasma [48] were determined using HILIC, followed by second-dimensional reversed-phase chromatography.

Besides the biologically active ingredients, final pharmaceutical products may contain residual impurities from different sources, such as reagents, starting materials, intermediates, and by-products, during the course of synthesis. To guarantee the safety and quality of drug substances and products, it is essential to effectively manage and control these impurities. A simple, sensitive, accurate, and precise HPLC-MS/MS method was developed for the trace-level quantification of 2,3-dichloroaniline, bis(2-chloroethyl) amine, and 2-chloroethan-1-amine in aripiprazole [16].

Polar impurities in calcium gluconate injection [16] were determined by combining ion-pair reversed-phase liquid chromatography (IPRP-LC) in the first dimension and HILIC in the second. A HILIC method with CAD detection was developed for the separation and quantitation of 3-

isopropylamino-1,2-propanediol, a degradation product of metoprolol in drug products, including metoprolol tartrate injection, metoprolol tartrate tablets, and metoprolol succinate extended-release tablets [71].

The HILIC method with fluorescence detection was developed for the quantification of 2-aminoisobutyric acid impurity in the enzalutamide bulk drug substance. Due to the good results in all requested parameters, it was suggested that the method could be applied in the analysis of 2-aminoisobutyric acid in routine quality control evaluation of commercial samples of enzalutamide bulk drug substance [72].

3.2. Chemometric Methods in Analyzing the Retention Data Obtained from HILIC Systems

Different chemometric methods represent a necessary tool for a complete understanding of the retention mechanism. They usually result in the derivation of an appropriate equation that relates the experimental parameters expressed through retention data and the structures of known analytes. Such relationships can be used to predict the retention characteristics of new analytes that have not been utilized in the modelling process. The final models are preceded by the optimization process of different variables of stationary and mobile phases and must include a large number of tested compounds. These mathematical models result in the evaluation of the most dominant properties (experimental and calculated) affecting the retention [28].

In the modelling process, different chemometric techniques, such as Multiple Linear Regression (MLR), Support Vector Machine (SVM), Partial Least Square Regression (PLSR) [54], PCA [12, 38, 68], *etc*., can be included.

A group of algorithm-based techniques includes multiple linear regression. It is an empirical model that requires information from previous projects in order to evaluate the present projects, according to Fedotova *et al*. [75]. MLR is one of the types of effort estimating approaches that are used to determine how the dependent variable (Y) (response) is related to the independent variables (Xi) (predictors) [76]. When one or more of the following criteria are present, the MLR technique is frequently employed:

(i) The number of cases is significantly greater than the number of parameters to be estimated.

(ii) The data behaves consistently.

(iii) There are few missing data.

(iv) A small number of independent variables are sufficient (after transformations if necessary) to linearly predict output variables (also transformed if necessary), allowing for an understandable representation.

When a straightforward model and analytic tool for effort estimation are required to support the initial attempts, regression may be utilized. Verification of the underlying assumptions is necessary before applying the MLR approach. Major theories that should be considered include linearity, in which each Xi and Y relationship is linear; hence, the model adequately represents the behavior of the data, and the error component has a mean value of zero, is independently distributed normally, and has a constant variance [75].

Choosing the independent variables to incorporate into the MLR model can be accomplished through various methods. One of these entails including all independent variables thought to be relevant, while others make use of stepwise techniques, including stepwise regression, backward regression, and forward regression. More people are familiar with the stepwise model than the others. The independent variables (Xi) are included in this procedure one at a time, starting with the one that has the strongest connection with Y. Each time the R^2 value is evaluated, it is checked to examine if any of the variables that were previously included contributed to the increase in R^2, and if not, they are eliminated [75].

In MLR, a mathematical function is used to express the connection between a number of independent variables (x_1, x_2, …, x_n) and a dependent variable, y. The relationship is frequently linear or can be linearized and has the following form:

$$y = b_0 + b_1x_1 + b_2x_2 + \cdots + b_nx_n \quad (3)$$

Where b_0 - b_n are the regression parameters. Both least squares regression and robust regression techniques can be used to estimate them when the error distribution is non-normal. Equations like 1 provide a model of the system under investigation that may be used to determine which variables affect its response and to what degrees, as well as to forecast the value of a single variable when the values of the other variables are known. In the absence of such data, cross-validation processes can be used to compare the predicted value of the variable of interest with the observed value. The model is often validated by applying it to independent experimental data (*i.e*., data not used to construct the model) [76].

In contrast to pattern recognition methods, MLR views experimental results from the perspective of connections between variables rather than similarities or differences between objects. The objective of MLR investigations is frequently to forecast one characteristic from the known values of other properties that may be tested more readily. For example, MLR is a good modelling approach that can be used to create a model for Quantitative Structure Retention Relationships (QSRRs). Chromatographic retention data are related to chemical descriptors and theoretical or experimental features of molecules *via* QSRRs models. Zisi, C. *et al*. analyzed metabolites by a HILIC system and in order to forecast retention duration and characterize them, they used MLR by means of the linear procedure in order to develop QSRRs models that can describe metabolites [57]. Moreover, Taraji and Haddad provided the first illustration of a QbD optimization protocol that predicts the retention of never-analyzed pharmaceutical compounds over a broad range of HILIC stationary phases and mobile phase conditions by combining dual-filtering-based QSRR calculations and DoE principles. The effectiveness of this methodology is illustrated by the prediction of potential separation conditions for a mixture of medicines analyzed in the HILIC mode [11].

Additionally, using Abraham's solvation parameter model, Xavier Subirats *et al*. characterized various reversed-phase and HILIC chromatographic systems with an emphasis on the effects of solute polarizability, dipolarity, hydrogen bonding, and molecular volume on chromatographic retention. The set of solutes was carefully chosen to have well-known and diverse molecular descriptors and to be large

enough to ensure the statistical significance of the system coefficients. In order to track how the solvent affected the chromatographic selectivity in RP and HILIC, the system constants and appropriate coefficients were determined by MLR analysis of the retention factors (dependent variable) and the molecular descriptors of the solutes [49]. According to a similar principle, applying the dual HILIC/RP mode (HILIC/RP), Obradović *et al*. made a drug design that includes modelling of pharmacokinetically favorable behavior and characterization of the physicochemical properties of newly synthesized molecules and chemical entities. In this case, HILIC/RP was used as an alternative biomimetic tool for the preliminary profiling of imidazoline and piperazine-related compounds. Their retention behavior on the mixed-mode HILIC/RP column was described as an equation of dependence of the logarithm of the retention factor (logk) in relation to the volume fraction of the water component in the mobile phase (φ), where the MLR analysis was used to calculate the coefficients in the mentioned equation [55]. Also, MLR and SVM can be used to select and evaluate the molecular properties of the tested compounds that affect retention behavior and turning point values. This is evidenced by the research of Obradović *et al*., who investigated the retention behavior of a wide range of analytes on a mixed-mode stationary phase in combined RP and HILIC modes. The obtained results combined with MLR and SVM were used to predict the retention characteristics and compare the used statistical approaches [12]. They also demonstrated that a variety of chemometric tools, such as MLR, can be employed to analyze retention patterns. Additionally, these tools can help identify the most crucial molecular mechanisms that significantly influence retention behavior in SFC and NA-HILIC, especially for pharmaceutically important compounds [38].

On the other hand, the categorization and prediction of biological data are also the main bioinformatics activities. It is crucial to employ computer programs to automate the classification procedure due to the biological databanks' rapid growth in size [77]. Support vector machines are the computer programs that now make the best predictions. They are one of the more well-known and effective methods, a family of supervised learning techniques that may be used for both classification and regressions [78]. The trained model generalizes well to new data because SVMs are made to maximize the margin between two classes. The majority of other computer programs create classifiers by minimizing errors that happen during training, which results in worse generalization [77]. Although SVMs are much more robust to data overfitting than other feature selection techniques [79], they are still being widely used in a variety of bioinformatics fields [77]. Compared to other classifiers, SVM classifiers have the best capacity to generalise to new data. The objective of a classification algorithm is to create a hyperplane that divides two classes and to identify a mapping function between input features (x) and class membership. SVMs discover a set of data points that are the most challenging training points to classify while searching for the best hyper-plane. The term "support vectors" is used to describe these data points. The support vectors nearest to the hyper-plane and on the margins separating two classes are used to build an SVM classifier. Utilizing SVMs has the advantage of searching the hyper-plane by maximizing this margin, so the SVM classifier is the most reliable and, as a result, has the finest generalization capability [77].

For the purpose of predicting protein retention time in linear-gradient HILIC systems, Jie Chen, Ting Yang, and Steven M. Cramer created a two-step technique. By combining relatively significant amounts of chromatographic data with well-known characteristics of protein structure and suitable modelling, they enabled the description of protein adsorption in HILIC systems. For a number of commercially available proteins with various characteristics, necessary isocratic retention parameters were found from ln(k)-salt concentration plots. In order to link the experimental results to certain physicochemical characteristics of the proteins, Quantitative Structure-Property Relationships models (QSPRs) were created utilizing cutting-edge non-linear SVM regression techniques. For estimating isocratic retention parameters for proteins not included in the model creation, models based on an SVM method were created. The results showed that this approach is well suited for forecasting experimental gradient retention data, which are subsequently calculated using the projected parameters. Investigating the effects of salt type on protein retention in ion exchange systems has been successfully done using a QSPR-based technique. To assess resin's impact on protein retention and selectivity, QSPR models have recently been used in HILIC systems [80].

3.3. Application of the HILIC Method in the Determination of Permeation Abilities of Pharmaceuticals

In the drug discovery process, due to the numerous advantages, chromatographic methods are widely used for the determination of lipophilicity. Over the traditional shake-flask procedure, this method is less sensitive to impurities, faster and more amenable to automation than the traditional biphasic method. Currently, reversed-phase chromatography has been considered a gold standard. The method of lipophilicity determination can be based on the use of extrapolated or isocratic retention values [81]. Voicu *et al*. used bimodal retention conditions (reversed-phase and hydrophilic interaction) for the determination of lipophilicity indices, which were better correlated to logP values than conventional indices resulting from extrapolation of the retention [82].

For the first time, Vallaro *et al*. investigated the application of HILIC systems in the characterization of the lipophilicity of zwitterions. Among six chromatographic systems, three different HILIC stationary phases (ZIC®-HILIC, ZIC®-pHILIC, and ZIC®-cHILIC) were combined with two different mobile phases (80% ACN/20% buffer and 90% ACN/10% buffer). The most effective system for evaluating the lipophilicity of the studied ampholytes involved employing a cHILIC column with a mobile phase consisting of 80% ACN (acetonitrile) and 20% buffer [68].

The HILIC method was used in the study of the permeation capabilities of pharmaceuticals through the blood-brain barrier (BBB). Two different columns were used: a butyl column to simulate the behavior of a drug regarding BBB permeability and a zwitterionic-HILIC to simulate blood. A new descriptor was introduced and symbolized as MT, which is equivalent to the logarithm of BBB permeability (logBB)

and represents the sum of each drug's retention time on the two columns, divided by its molar volume. The BBB parameter obtained in this way may indicate the ability of a new molecule to act as a candidate drug able to enter the BBB [83].

CONCLUSION

In recent decades, HILIC has become a very popular analytical technique in the analysis of small polar molecules, charged as well as uncharged, enabling improved selectivity and sensitivity compared to traditional RP chromatography. It is involved in all phases of pharmaceutics development, from the synthesis process through testing its stability and quality to the monitoring of the products. There is a great diversity of commercially available HILIC stationary phases and continuous development of new ones. The stationary phases are selected according to the nature of the analyte and combined with an optimized mobile phase to make chromatographic systems that can meet the demands of analyzing specific pharmaceutical formulations. Besides, the high compatibility of HILIC mobile phases with MS detectors enables the structural characterization of molecular components of samples, their masses, and elemental formulas, which makes HILIC suitable for analyzing complex biological materials.

Moreover, the HILIC method can be very useful in physicochemical characterization of hydrophilic pharmaceuticals. Through the quantitative structure-retention relationship, which correlates retention data and structural descriptors, employing chemometric approaches can be useful in predictive models, which can shorten the process of finding new drug candidates.

LIST OF ABBREVIATIONS

2D-LC	=	Two-Dimensional Liquid Chromatography
ACN	=	Acetonitrile
APCI	=	Atmospheric Pressure Chemical Ionization
AQ-HILIC	=	Aqueous Hydrophilic Interaction Chromatography
ASO	=	Antisense Oligonucleotide
BBB	=	Blood-Brain Barrier
BBD	=	Box-Behnken Design
CCD	=	Central Composite Design
ELSD	=	Evaporating Light-Scattering Detection
EMA	=	European Medicines Agency
ESI	=	Electrospray Ionization
FDA	=	Food and Drug Administration
HCA	=	Homocysteic Acid
HILIC	=	Hydrophilic Interaction Liquid Chromatography
HPLC	=	High-Performance Liquid Chromatography
IEX	=	Ion Exchange Chromatography
IPRP-LC	=	Ion-Pair Reversed-Phase Liquid Chromatography
LC	=	Liquid Chromatography
MLR	=	Multiple Linear Regression
MS	=	Mass Spectrometry
NP	=	Normal-Phase
NRTIs	=	Nucleoside Reverse Transcriptase Inhibitors
PAD	=	Photodiode Array Detector
PEG	=	Polyethylene Glycol
PLSR	=	Partial Least Square Regression
QbD	=	Quality by Design
QSRR	=	Quantitative Structure-Retention Relationship
RPLC	=	Reversed-Phase Liquid Chromatography
SF	=	Supercritical Fluid Chromatography
SVM	=	Support Vector Machine
UPLC	=	Ultra High-Performance Liquid Chromatography

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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