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Hydrophilic interaction chromatography in drug analysis

Review Article

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Abstract: Hydrophilic interaction chromatography (HILIC) is an increasingly popular alternative to conventional HPLC for drug analysis. It offers increased selectivity and sensitivity, and improved efficiency when quantifying drugs and related compounds in complex matrices such as biological and environmental samples, pharmaceutical formulations, food, and animal feed. In this review we summarize HILIC methods recently developed for drug analysis (2006-2011). In addition, a list of important applications is provided, including experimental conditions and a brief summary of results. The references provide a comprehensive overview of current HILIC applications in drug analysis.

Keywords: *HILIC • Stationary phases • Detectors • Drug Analysis*

1. Introduction

Hydrophilic interaction chromatography (HILIC), using a hydrophilic stationary phase and a hydrophobic eluent, was first described in 1990 [1], although it had been applied to the separation of sugars 15 years earlier [2,3]. Since then, HILIC has been successfully applied to all types of liquid chromatographic separations, including small molecules [4], pharmaceutical compounds [5], metabolites [6], drugs of abuse [7], toxins [8], carbohydrates [9], oligosaccharides [10], amino acids [11], peptides and proteins [1,12].

Several interesting reviews focusing on general aspects of HILIC have been published (fundamental principles, applications and design of the separation mechanism) [13-17]. For example, applications of HILIC to polar contaminants in food and environmental samples were described [13], as were examples of HILIC-based food analysis [14]. Several HILIC applications to modern bioanalytical chemistry were discussed [15]. Its use in quantitative bioanalysis was also reviewed [16], highlighting some practical considerations, including orthogonal and fast separations as well as techniques to overcome matrix effects. In addition, it was demonstrated that HILIC is not limited to polar drugs and metabolites, but relatively non-polar compounds, peptides and biomarkers can be determined as well.

Analysis is vital to ensure patient safety and minimize side effects. Fig. 1 highlights the increasing literature published on HILIC drug analysis over the last six years. Recently, approximately 60 HILIC applications to pharmaceutical analysis from 2005 to 2009 were reviewed [17], focusing primarily on hydrophilic stationary phases. In contrast, this review summarizes ~140 studies (2006-2011), focusing on the sample matrices. We have highlighted their most salient aspects including the stationary phases and detectors used, and we discuss the most significant applications.

The publications are grouped according to matrix type (Fig. 2), HILIC stationary phase (Fig. 3) and detection method (Fig. 4) to provide a more comprehensive overview of current trends. Readers interested in more specific instrumental or fundamental aspects of HILIC should refer to the above reviews.

2. HILIC drug analysis in different matrices

As summarized in Tables 1-3, this review is organized by the types of matrices analyzed, with a particular focus on HILIC stationary phases, the detection techniques employed and the compounds studied.

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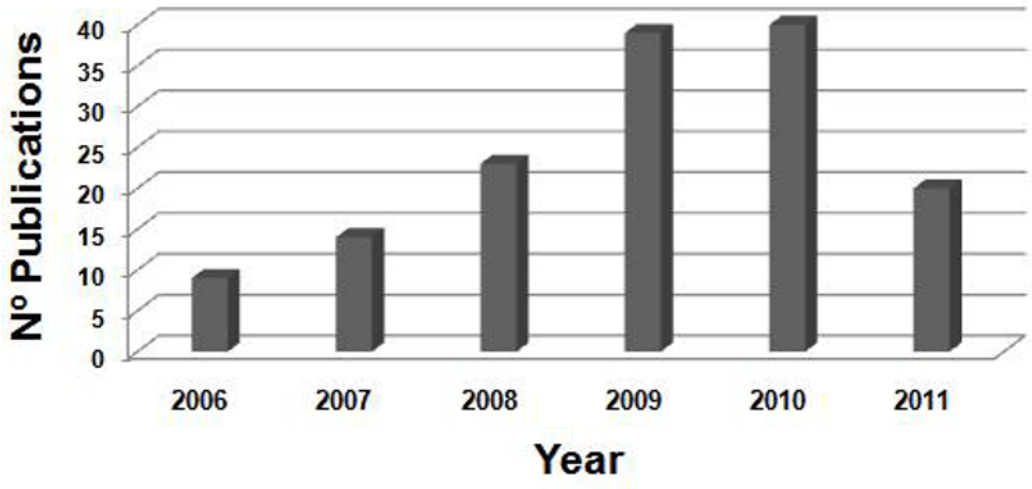

Figure 1. HILIC pharmaceutical analysis published research for the last six years (to end of May 2011) listed in ISI-Web of Knowledge, Scirus, and Scopus using keywords: [(Hydrophilic Interaction Chromatography) or (HILIC)] and [(Drugs) or (Pharmaceuticals) or (Drugs of abuse)]

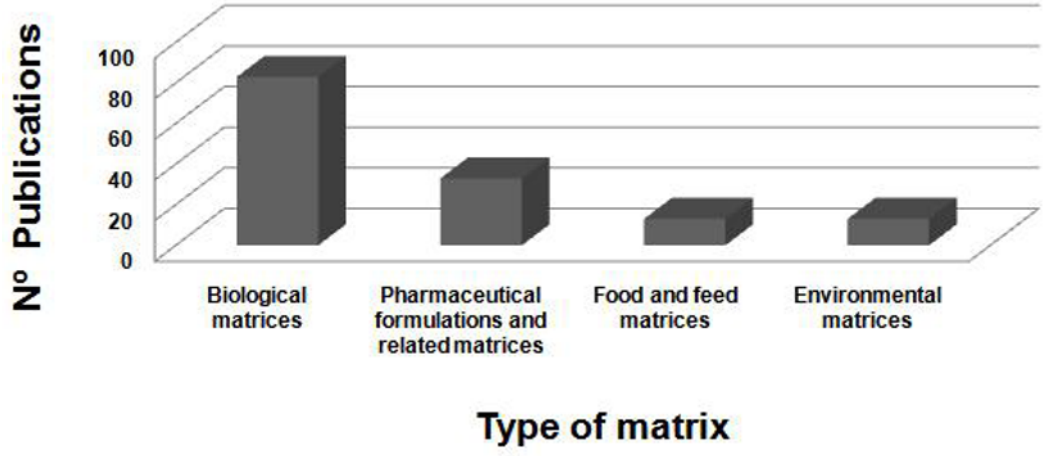

Figure 2. Sample matrices analyzed with HILIC between 2006 and 2011.

The matrices include biological samples, pharmaceutical formulations, food, animal feed, and environmental samples. 60% of the publications examined biological matrices (Fig. 2), most commonly plasma, urine and serum. A wide variety of compounds have been separated in these matrices including antibiotics, veterinary drugs, common pharmaceutical counter ions, drugs of abuse (DOAs), active pharmaceutical ingredients (APIs), and other compounds such as impurities.

HILIC stationary phases permit more organic solvent in the mobile phase, favoring mass spectrometric ionization, particularly electrospray (ESI). Acetonitrile (AcN) is the most commonly used organic solvent (Tables 1-3), while water or aqueous mixtures may be used to produce a stronger eluent. Aqueous eluents typically contain ammonium acetate or formate (Tables 1-3).

A variety of stationary phases have been described (Fig. 3). The most commonly used are Atlantis HILIC Silica (difunctionally bonded ODS), ZIC-HILIC (zwitterionic, sulfobetaine-functionalized silica) and Luna HILIC (aminopropyl phase) columns, which are classified as neutral (no electrostatic interactions), zwitterionic (weak electrostatic interactions) and charged (strong electrostatic interactions) columns, respectively.

To detect drugs tandem mass spectrometry (MS/MS, triple quadrupole) and ultraviolet-visible or diode array (UV-VIS, DAD) detectors are mainly employed (Fig. 4). Electrospray ionization was employed in the majority of studies (Fig. 4) as weak aqueous/strongly polar organic mobile phases are highly compatible with ESI-MS detection, and can augment sensitivity [18].

Table 1.- HILIC applications in the analysis of biological samples.

Matrix	Compounds of interest	Stationary phase	Mobile phase	Detection	Refs.
Plasma, urine and salive	Antiviral drugs (oseltamivir)	ZIC-HILIC (50 mm × 2.1 mm, 5 μm)	AcN / 10mM ammonium acetate (pH 3.5); gradient	ESI-MS/MS	[19]
Human plasma	Antiviral drugs (peramivir)	ZIC-HILIC for SPE	MeOH / AcN / AcN + 10mM ammonium acetate +1 % acetic acid; activate	ESI-MS/MS	[20]
Human plasma	Antiviral drugs (zanamivir)	ZIC-HILIC (50 mm × 2.1 mm, 5 μm)	AcN / aqueous ammonium acetate +1% acetic acid; (70:30, v/v); isocratic	APCI-MS/MS, ESI-MS/MS	[21]
Human plasma	Antiviral drugs (peramivir)	TSK-Gel Amide 80 (100 mm × 4.6 mm, 5 μm)	AcN / water containing 0.1% (v/v) formic acid (70:30, v/v); isocratic	ESI-MS/MS	[22]
Human and rat plasma	Antiviral drugs (ganciclovir and its prodrug)	Luna HILIC (50 mm × 2.0 mm, 3 μm)	Water-MeOH (15:85, v/v) and water-AcN (10:90, v/v), both containing 5mM ammonium formate and 0.2% formic acid; gradient	ESI-MS/MS	[23]
Rat and monkey plasma	Antiviral drugs (zanamivir)	Atlantis HILIC silica (50 mm × 2.1 mm, 3 μm)	10mM ammonium acetate + 1% MeOH / AcN; gradient	ESI-MS/MS	[24]
Human plasma	Antiviral drugs (adefovir)	Acquity UPLC BEH HILIC (50 mm × 2.1 mm, 1.7 μm)	MeOH–water–formic acid (85:15:0.2, v/v/v); isocratic	ESI-MS/MS	[25]
Human plasma	Adrenergic receptors (doxazosin)	Atlantis HILIC Silica (50 mm × 3 mm, 5 μm)	AcN / 100mM ammonium formate (pH 4.5) (93:7 v/v); isocratic	ESI-MS/MS	[26]
Human plasma	Adrenergic receptors (carvedilol)	Atlantis HILIC Silica (50 mm × 3 mm, 5 μm)	AcN / 50mM ammonium formate (pH 4.5) (90:10, v/v); isocratic	ESI-MS/MS	[27]
Serum and umbilical cord serum	Adrenergic receptors (ritrodine)	Unison UK-Silica (50 mm × 2.0 mm, 3 μm)	10mM ammonium acetate (pH 4.5) / AcN (10:90, v/v); isocratic	ESI-MS/MS	[28]
Human plasma	Angiotensin receptor (irbesartan)	Luna HILIC (100 mm × 2.0 mm, 3 μm)	AcN / 50mM ammonium formate (pH 6.5) (96:4, v/v); isocratic	ESI-MS/MS	[29]
Human plasma	Adrenergic receptors (gaboxadol)	Asahipak NH_2P-50 2D (150 mm × 2mm, 5 μm)	AcN / 20mM ammonium acetate (pH 4) (70:30, v/v); isocratic	ESI-MS/MS	[30]
Human plasma	Adrenergic receptors (phenylephrine)	Pentaflurophenylpropyl HILIC HS F5 (50 mm × 4 mm, 3 μm)	MeOH / 10mM ammonium acetate (90:10, v/v); isocratic	ESI-MS/MS	[31]
Rat plasma	Adrenergic receptors (fenoterol)	Atlantis HILIC Silica (150 mm × 2.1 mm, 5 μm)	AcN / 20mM ammonium acetate (pH 4.1) (85:15, v/v); isocratic	ESI-MS	[32]
Rat plasma	Adrenergic receptors (fenoterol)	Atlantis HILIC Silica (150 mm × 2.1 mm)	MeOH / 10 mM ammonium acetate (pH 6.8) (90:10, v/v); isocratic	ESI-MS	[33]
Rat plasma	Inhibitors and receptors (didanosine)	Acquity UPLC BEH HILIC (50 mm × 2.1 mm, 1.7 μm)	Water containing 0.1% formic acid / MeOH (85:15, v/v); isocratic	ESI-MS/MS	[34]
Rat cerebellar tissues	Inhibitors and receptors (mildronate)	Acquity HILIC BEH (100 mm × 2.1 mm, 1.7 μm)	10mM ammonium acetate (pH4) / AcN; gradient	ESI-MS/MS	[35]
Mouse plasma	Inhibitors and receptors (everolimus)	Acquity UHPLC BEH HILIC and UPHILIC (50 mm × 2.1 mm, 3 μm and 50 mm × 2.1mm, 1.8 μm)	water / acetonitrile containing both 0.1% formic acid or water and 95% acetonitrile containing both 0.1% formic acid and 4mM ammonium acetate; gradient	ESI-MS/MS	[36]
Monkey serum	Inhibitors and receptors (PF-00734200)	Atlantis HILIC Silica (50 mm × 2.1 mm, 3 μm)	Water / AcN / 200mM ammonium formate, (pH 3.0); gradient	ESI-MS/MS	[37]
Human plasma and cerebrospinal fluid	Inhibitors and receptors (miglustat)	Atlantis HILIC Silica (150 mm × 2.1 mm, 3 μm)	AcN / water / 100mM ammonium acetate buffer (pH 5) (75/10/15, v/v/v); isocratic	ESI-MS/MS	[38]

Continued **Table 1.**- HILIC applications in the analysis of biological samples.

Matrix	Compounds of interest	Stationary phase	Mobile phase	Detection	Refs.
Rat and monkey blood	Inhibitors and receptors (imipenem, cilastatin and an investigational β-lactamase inhibitor)	Atlantis HILIC Silica (50 mm × 2.1mm, 3 μm)	15mM ammonium formate (pH 3) / AcN (20:80, v/v); isocratic	ESI-MS/MS	[39]
Human plasma	Inhibitors and receptors (omeprazole)	Betasil Silica (50 mm × 3.0 mm, 5 μm)	AcN / water / formic acid; gradient	ESI-MS/MS	[40]
Human plasma	Inhibitors and receptors (donepezil, cetirizine and loratadine)	YMC Silica (50 mm × 4.0 mm)	AcN / 15% acetic acid + 0.35% TFA (93:17, v/v); isocratic	ESI-MS/MS	[41]
Human plasma	Inhibitors and receptors (donepezil)	Atlantis HILIC Silica	AcN / 50mM ammonium formate (pH 4.0) (85:15, v/v)	MS/MS	[42]
Mouse plasma	Inhibitors and receptors (dasatinib, imatinib and nilotinib)	Halo HILIC (50 mm × 2.1 mm, 3 μm)	Water / AcN containing both 0.1% formic acid and 4mM ammonium acetate; gradient	ESI-MS/MS	[43]
Murine plasma	Inhibitors and receptors (zebularine)	Zorba× NH_2 HILIC (250 mm × 4.6 mm, 5 μm)	Isopropanol:1M ammonium formate (pH 3.0):AcN (12:0.24:88, v/v/v) / isopropanol: 1M ammonium formate (pH 3.0):AcN (49:1:50, v/v/v); gradient	UV-VIS	[44]
Rat muscle tissue micro-dialysate	Inhibitors and receptors (bradykinin)	ZIC-HILIC (150 mm × 0.3 mm, 5 μm)	AcN-water (5/95, v/v) containing 9.5 mM ammonium acetate / CAN-water (95/5, v/v) containing 0.5 mM of ammonium acetate; gradient	ESI-TOF-MS	[45]
Human plasma	Inhibitors and receptors (chloro-substituted biarylmethoxyphenyl piperidine-4)	ZIC-pHILIC (100 mm × 4.6 mm, 5 μm)	AcN / (trifluoroacetic acid/ ammonium, pH 2) 95% (v/v); isocratic	UV-VIS	[46]
Human plasma	Anti-diabetic drugs (metformin)	Inertsil HILIC (150 mm × 2.1 mm, 5 μm)	Water / AcN + 0.1% formic acid (30:70, v/v); isocratic	ESI-MS/MS	[47]
Rat plasma	Anti-diabetic drugs (metformin and BMS-754807)	Acquity UPLC BEH HILIC (50 mm × 2 mm, 1.7 μm)	5mM ammonium bicarbonate / 5mM ammonium bicarbonate in 80:20 MeOH–water; gradient	ESI-MS/MS	[48]
Human and rat blood	Anti-diabetic drugs (metformin and its prodrugs)	Supelcosil LC-Si (250 mm × 4.6 mm, 5 μm)	0.01M ammonium acetate (pH5.0) / AcN (40:60, v/v); isocratic	UV-VIS	[49]
Human dried and mouse blood	Anti-diabetic drugs (metformin and sitagliptinin)	Kineti× HILIC UHPLC (100 mm × 2.1 mm, 2.6 μm)	5mM ammonium formate in can + 0.1% formic acid / 0.5mM ammonium formate in water + 0.1% formic acid; gradient	APCI-MS/MS	[50]
Human plasma	Anti-diabetic drugs (sitagliptinin)	Atlantis HILIC Silica (50 mm × 2.1 mm, 3 μm)	AcN / water (80/20, v/v) + 10mM ammonium acetate (pH 4.7); isocratic	ESI-MS/MS	[51]
Human and animal plasma	Anti-diabetic drugs (taspoglutide)	Atlantis HILIC Silica (50 mm × 2.1 mm, 3 μm) for online SPE	1% trifluoroacetic acid in water/ AcN / MeOH (40/30/30, v/v/v); isocratic	ESI-MS/MS	[52]
Human plasma	Anti-diabetic drugs (muraglitazar)	Hypersil silica (50 mm × 3 mm, 3 μm)	Methyl t-butyl ether / (90:10, v/v) AcN-water + 0.3% trifluoroacetic acid (85:15, v/v); isocratic	ESI-MS/MS	[53]
Plasma and urine samples	Opioid drugs (methadone)	TSK-Gel Amide 80 (250 mm × 4.1 mm, 3 μm)	AcN + 0.01% formic acid / 3mM ammonium formate in water + 0.01% formic acid (28:72 v/v); isocratic	ESI-MS/MS	[54]
Human plasma	Opioid drugs (sufentanil)	Alltima HP HILIC (50 mm × 2.1 mm, 3 μm)	Water-formic acid-1M ammonium acetate (100:0.25:0.5, v/v/v, pH 2.8) / AcN-formic acid/1M ammonium acetate (100:0.25:0.5, v/v/v); gradient	ESI-MS/MS	[55]
Dog plasma	Opioid drugs (fentanyl)	Atlantis HILIC Silica (150 mm × 4.6 mm, 5 μm)	AcN / 10mM ammonium acetate + 0.1% formic acid; gradient	ESI-MS/MS	[56]

Continued **Table 1.** - HILIC applications in the analysis of biological samples.

Matrix	Compounds of interest	Stationary phase	Mobile phase	Detection	Refs.
Microdialysate samples	Opioid drugs (ketobemidone)	ZIC-HILIC (100 mm × 0.075 mm, 3.5 μm)	Ammonium formate, acetate, MeOH or AcN were tested	ESI-MS/MS	[57]
Micro-whole blood samples	Opioid, benzodiazepine drugs and others	ZIC-HILIC (100 mm × 2.1 mm, 3.5 μm)	AcN / 15mM ammonium acetate (97:3, v/v); isocratic	ESI-MS/MS	[58]
Human serum	Anticonvulsant drugs (gabapentin, pregabalin and vigabatrin)	TSK-Gel Amide 80 (150 mm × 2 mm, 3 μm)	AcN / 2 mM ammonium acetate and formic acid (5:95:0.2, v/v) / (95:5:0.2, v/v); gradient	ESI-MS/MS	[59]
Rat brain and serum	Psychostimulant drug (methylphenidate)	TSK-Gel Amide 80 (150 mm × 2.1 mm)	AcN / 10mM ammonium formate (pH 3.0) (85:15, v/v); isocratic	ESI-MS/MS	[60]
Serum samples	C10 to C18 acylcarnitines	Atlantis HILIC Silica (150 mm × 2.1 mm)	10 mM ammonium formate / AcN (40:60, v/v); isocratic	ESI-MS/MS	[61]
Rat plasma, urine, feces and tissue samples	Anti-tumor drug candidate (3-Deazaneplanocin A)	Zorbax HILIC plus (50 mm × 2.1 mm)	0.1% formic acid in water / AcN + 0.1% formic acid; gradient	ESI-MS/MS	[62]
Human plasma	Anti-cancer agent (5-Fluorouracil)	Asahipak NH_2P-50 2D (150 mm × 2 mm, 5 μm)	0.1% formic acid in AcN / 0.1% formic acid in water; isocratic	ESI-MS/MS	[63]
Human plasma	Chemotherapy drugs (melphalan)	Atlantis HILIC Silica (150 mm × 2.1 mm, 3 μm)	Water / AcN; gradient	ESI-MS/MS	[64]
Human plasma	Chemotherapy drugs (dacarbazine)	TSK-Gel Amide 80 (100 mm × 4.6 mm, 5 μm)	AcN / 0.1% formic acid in water; gradient	ESI-MS/MS	[65]
Human plasma	Antiemetic drugs (metoclopramide)	Atlantis HILIC Silica (50 mm × 3.0 mm, 3 μm)	AcN / 100mM ammonium formate (pH 6.5) (85:15, v/v); isocratic	ESI-MS/MS	[66]
Human plasma	Antibacterial drug (levofloxacin)	Atlantis HILIC Silica (50 mm × 3 mm, 5 μm)	AcN / 100mM ammonium formate (pH 6.5) (82:18, v/v) isocratic	ESI-MS/MS	[67]
Human plasma	Anaesthetic drugs (propofol)	Atlantis HILIC Silica (150 mm × 2.1 mm, 3 μm)	AcN / water / 100mM ammonium acetate buffer (pH 5) (87/1/12, v/v/v); isocratic	ESI-MS/MS	[68]
Human plasma	Antimuscarinic drug (tolterodine)	Hypersil Silica (30 mm × 4 mm, 3 μm)	AcN / 20mM ammonium acetate (70:30, v/v); isocratic	ESI-MS/MS	[69]
Human plasma	Anti-tuberculosis drug (isoniazid)	Hypersil Silica (50 mm × 2.1 mm, 3 μm)	Water + 0.1% acetic acid + 2.5mM ammonium acetate / AcN + 0.1% acetic acid;	ESI-MS/MS	[70]
Human serum	Antidepressant drug (sertraline)	Restek Silica (100 mm × 3.2 mm, 3 μm)	AcN / 0.25% ammonium hydroxide + 0.25% glacial acetic acid in methanol (80:20, v/v); isocratic	APCI-MS	[71]
Human plasma breast milk samples	GTIs (ethyl methanesulfonate)	Atlantis HILIC Silica (100 mm × 4.6 mm, 3.0 μm)	AcN / 100mM ammonium acetate (93:7, v/v); isocratic	APCI-MS/MS	[72]
Human plasma and urine plasma	Endogenous metabolites	Luna HILIC (100 mm × 2 mm, 3μm), Acquity UPLC BEH HILIC (100 mm × 2.1 mm, 1.7 μm), Sielc Obelisc N (150 mm × 2.1 mm, 5 μm)	10mM ammonium acetate and 10mM ammonium acetate in 98:2 ACN/water; gradient	ESI-MS/MS	[73]
Rat plasma	Daumone and glucoraphanin	ZIC-HILIC (100 mm × 2.1 mm, 3 μm)	10 mM ammonium acetate / AcN (20:80, v/v); isocratic	ESI-MS/MS	[74]
Dog and rat plasma	Glucoraphanin	Luna HILIC (50 mm × 2.0 mm, 5 μm)	200mM ammonium acetate and formic acid (99:1, v/v) / AcN; gradient	ESI-MS/MS	[75]
Rabbit plasma	Drug transporter (tetraethylammonium)	ZIC-HILIC (50 mm × 4.6 mm, 3.5 μm)	AcN / 5mM ammonium acetate + 0.1% formic acid (80:20, v/v); isocratic	ESI-MS/MS	[76]

Continued **Table 1.**- HILIC applications in the analysis of biological samples.

Matrix	Compounds of interest	Stationary phase	Mobile phase	Detection	Refs.
Dog plasma	Osteoarthritis drug (glucosamine)	Zorba× SBCN HILIC (250 mm × 4.6 mm, 5 μm)	MeOH / 5mM ammonium hydrogen carbonate buffer (pH 7.5, 95/5, v/v); isocratic	ESI-MS	[77]
Rat plasma	Antimalarial agent (1,2,4-trioxolane)	Atlantis HILIC Silica (100 mm × 2.1 mm, 5 μm)	0.1M ammonium formate (pH 3.0)/AcN (5:95, v/v) / 0.1M ammonium formate (pH 3.0)/ IPA (5:95, v/v); gradient	ESI-MS	[78]
Human plasma	DOAs (nicotine)	Atlantis HILIC Silica (100 mm × 300 μm, 5 μm)	7.5mM ammonium acetate/ AcN (23:77, v/v) + 0.4% acetic acid or water/AcN/formic acid (15:85:0.2, v/v/v); isocratic	ESI-MS/MS	[79]
Human urine	Antineoplastic drug (5-fluorouracil)	ZIC-HILIC (100 mm × 2.1 mm, 5 μm)	25mM ammonium formate / AcN; gradient	ESI-MS/MS	[80]
Human urine	Hyperuricaemia agent (oxypurinol)	Atlantis HILIC Silica (100 mm × 2.1 mm, 3 μm)	AcN / MeOH / 50mMammonium acetate in 0.2% formic acid (95/2/3, v/v); isocratic	ESI-MS/MS	[81]
Urine samples	Drugs (tolterodine, amperozide, BVT 2938)	HILIC-PFPP (HSF 5, 100 mm × 2.1 mm, 3 μm)	AcN in 15mM ammonium formate (pH 3.6, 20:80, v/v) / 3mM ammonium formate in AcN + 2% water; gradient	ESI-MS/MS	[82]
Urine	β_2-agonists (clenbuterol, hydrochloride, salbutamol hemisulfate, and terbutaline)	Luna HILIC (250 mm × 2.0 mm, 5 μm)	AcN / water (85:15, v/v), containing 0.3% formic acid; isocratic	ESI-MS	[83]
Human urine	DOAs (morphine, codeine and their glucuronide conjugates)	Zorba× HILIC Plus (100 mm × 2.1 mm, 3.5 μm)	10mM ammonium formate (pH 6.4) / 10mM ammonium formate (pH 6.4) in 90% AcN; gradient	ESI-TOF-MS	[84]
Human urine	DOAs (nicotine and metabolites)	Luna HILIC (150 mm × 3.0 mm, 5 μm)	AcN / 10mM ammonium formate (pH 3.0); gradient	ESI-MS/MS	[85]
Urine samples	Alkaloid (mitragynine)	Atlantis HILIC Silica (50 mm × 3.0 mm, 3 μm)	5mM ammonium acetate / MeOH; gradient	ESI-MS/MS	[86]
Human urine	Cimetidine, creatinine, uric acid	Spherisoro NH_2 (250 mm × 4.6 mm, 5 μm)	AcN / 10mM sodium phosphate buffer (pH 4.75) (50:50, v/v); isocratic	UV-VIS	[87]
Hair samples	DOAs (cocaine and its metabolites)	Atlantis HILIC Silica (100 mm × 2.1 mm, 3 μm)	0.1 M ammonium acetate buffer, pH 4.5 / AcN	ESI-MS/MS	[88]
Meconium and hair	Alcohol marker (ethyl glucuronide)	Luna HILIC (150 mm × 3 mm, 5 μm)	2 mM ammonium acetate / AcN; gradient	ESI-MS/MS	[89]
Head and pubic hair	Alcohol marker (ethyl glucuronide)	Acquity BEH HILIC column (100 mm × 2.1 mm, 1.7 mm)	AcN–formate buffer (pH 3.0) (95:5, v/v); gradient	ESI-MS/MS	[90]
Rat hair	Metabolic profiling	ACQUITY UPLC BEH HILIC (100 mm × 2.1mm, 1.7 μm)	0.1% formic acid in water / 0.1% formic acid in AcN; gradient	ESI-TOF-MS	[91]
Oral fluid	DOAs (opiates, amphetamines, flunitrazepam, cocaine and metabolites)	ACQUITY UPLC BEH HILIC (100 mm × 2.1 mm, 1.7 μm)	AcN / 10mM ammonium formate with 10mM formic acid in water (pH 3.4); gradient	ESI, APCI, APPI-MS/MS	[92]
Fingertips	Benzodiazepine drugs (lorazepam and its glucuronide)	TSK-Gel Amide 80 (250 mm × 4.1 mm, 3 μm)	AcN + 0.01% formic acid / 3 mM ammonium formate in water + 0.01% formic acid (70:30 v/v); isocratic	ESI-MS/MS	[93]
Pooled liver cytosol	DOAs (3,4-ethylenedioxymethamphetamine)	ZIC-HILIC (150 mm × 4.6 mm, 5 μm)	10mM aqueous ammonium formate buffer + 0.1% formic acid / AcN + 0.1% formic acid; gradient	UV-VIS	[94]
Monkey and human liver subcellular	Anticancer agent (trabectedin)	Atlantis HILIC Silica (30 mm × 2.1 mm, 3 μm)	Water / MeOH (95/5; v/v); isocratic	ESI-MS/MS	[95]
Human liver microsomes	Antimarial drug (amodiaquine)	BETASIL Silica-100 (50 mm × 2.1 mm, 5 μm)	AcN + 5mMammonium acetate / 0.1% formic acid (85:15, v/v); isocratic	ESI-MS/MS	[96]

Continued **Table 1.**- HILIC applications in the analysis of biological samples.

Matrix	Compounds of interest	Stationary phase	Mobile phase	Detection	Refs.
Cells	Drug candidate	ZIC-HILIC	Water / AcN; gradient	ESI-MS/MS, DAD	[97]
Cells	Teratogens and not-teratogens compounds	Luna HILIC (100 mm × 3 mm, 3 μm)	0.1% formic acid in water / 0.1% formic acid in AcN;	ESI-MS/MS	[98]
Manure	Aminoglycoside and lincosamide antibiotics (spectinomycin and lincomycin)	Alltima HP HILIC (150 mm × 2.1 mm, 3 μm)	AcN / 0.1% formic acid (90:10, v/v); isocratic	APCI-MS/MS	[99]
Zebra finch feces	DOAs (nicotine and cotinine)	Polaris Si-A (50 mm × 3.0 mm, 5 μm)	1:1 AcN:MeOH with 0.05% formic acid / 10mM ammonium formate; gradient	ESI-MS/MS	[100]

AcN: acetonitrile; MeOH: methanol; GTIs: genotoxic impurities; IPA: Isopropyl alcohol; NaH_2PO_4: sodium dihydrogen phosphate

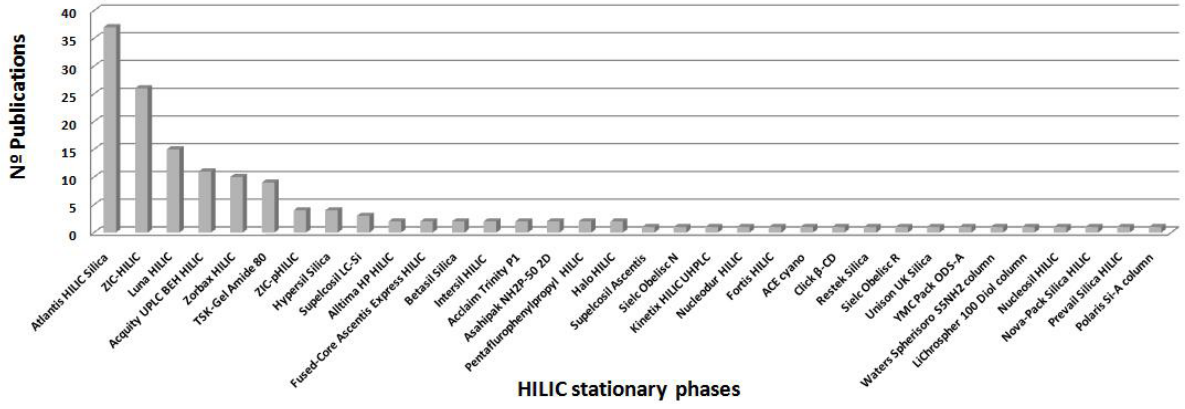

Figure 3. HILIC stationary phases used in pharmaceutical analysis

2.1. Drug analysis in biological matrices

Pharmacokinetic and metabolic analyses in animal models form a critical part of drug discovery. Efficient analytical methods for both *in vitro* and *in vivo* samples are essential. Many HILIC methods have been developed for these analyses (Table 1). Human and animal plasma, serum, and urine are the most common body fluids analyzed (more than 50% of publications).

2.1.1. Drug analysis in plasma and serum samples

Anti-viral, antibiotic, antifungal and antiparasitic drugs have been analyzed using HILIC [19-25], in all cases coupled to ESI-MS/MS detection, although separation was achieved using a variety of stationary phases. For example, a zwitterionic ZIC-HILIC column was used to determine oseltamivir and its metabolite oseltamivir carboxylate (influenza virus neuraminidase inhibitor) in human plasma [19]. Similarly, the anti-influenza drugs peramivir [20] and zanamivir [21] were quantified in human plasma using ZIC-HILIC solid phase extraction (SPE) in a 96-well plate format. The sample preparation step in ZIC-HILIC SPE allows peramivir to be selectively extracted, minimizing co-extraction of lipophilic phospholipids. Permavir was also determined in human plasma using an isocratic mobile phase on a TSK-Gel Amide column [22]. Other column types have also been employed to analyze similar compounds such as: ganciclovir and its prodrug valganciclovir in human and rat plasma using a Luna Amine HILIC column [23]; zanamivir (neuraminidase inhibitor) in rat and donkey plasma using an Atlantis HILIC stationary phase [24]; and adefovir, identified for the first time in human plasma, using an Acquity ultra performance liquid chromatography (UPLC) BEH HILIC column [25].

Adrenergic receptors are activated by catecholamines. There are several rapid, selective and sensitive ESI-MS/MS methods to analyze adrenergic receptor ligands using silica-derived [26-28] and amine-derived [29,30] stationary phases. The anti-hypertensive drugs doxasozin [26] and carvedilol [27] were measured in human plasma using an Atlantis HILIC Silica column. Similarly, Ritrodine, which acts on uterine smooth muscles, was also analyzed in serum and umbilical cord serum using a Unison UK-Silica column [28]. In the analysis of human plasma, a Lune Amine HILIC stationary phase was selected to quantify the anti-hypertensive ibersatan [29], while gabaxodolol (a GABA agonist) was quantified using an Asahipak NH_2P-50 column [30]. Phenylephrine (decongestant) in human plasma has been separated on a pentaflurophenylpropyl

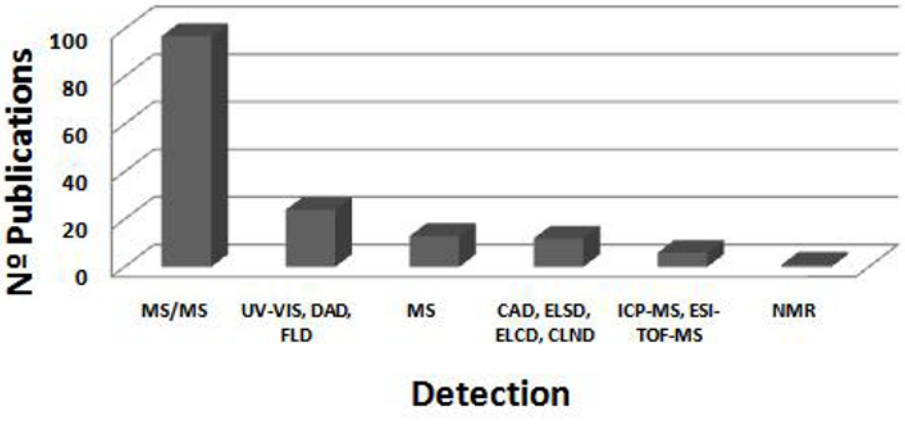

Figure 4. Detection techniques employed in HILIC pharmaceutical analysis.

(PFPP-HILIC) column [31]. In rat plasma, fenoterol (asthma treatment) was successfully identified using an Atlantis HILIC column by isocratic separation with AcN [32] or MeOH [33] in the mobile phase and ESI-MS detection.

Many other receptor ligands have been analyzed using HILIC (Table 1) most often employing MS/MS detection [34-43]. Didanosine and its amino acid prodrug were simultaneously detected in rat plasma [34] using an Acquity UPLC BEH HILIC stationary phase, which was also successfully used to detect two other inhibitors, mildronate [35] and everolimus [36], in rat and mouse plasma respectively. PF-00734200 (dipeptidyl peptidase IV inhibitor) was quantified in monkey serum on an Atlantis HILIC column [37], as was the glucosylceramide synthase inhibitor miglusat in human cerebrospinal fluid [38]. This column was also selected to simultaneously determine β-lactam inhibitors in rat and monkey blood [39]. Silica-derived HILIC stationary phases have also been used: Betasil Silica for omeprazole analysis [40], YMC Silica to determine histamine antagonists (donepezil, cetirizine and loratadine) [41] and an Atlantis HILIC Silica column for the detection of donepezil in human plasma [42]. In addition, three tyrosine kinase inhibitors used in leukemia treatment were successfully identified in mouse plasma using a Halo HILIC column [43]. A novel analytical method has also been developed to identify and quantify the DNA inhibitor zebularine and its metabolites in mouse plasma, using a Zorbax NH_2 HILIC column and UV-VIS detection [44].

In a study that separated bradykinin in rat muscle distinct technologies were successfully coupled [45]: a 2D SPE-HILIC x SPE-RPLC method using a zwitterionic ZIC-HILIC stationary phase in the first dimension was coupled to ESI-TOF-MS detection. ZIC-p-HILIC with UV-VIS detection was also described in a study of biarylmethoxy isonipecotanilides as potential inhibitors of the factor Xa blood coagulation enzyme [46].

Anti-diabetic drugs (oral hypoglycemic and antihyperglycemic agents) have received attention. Several methods have been used to detect metformin and related compounds, including ESI-MS/MS [47,48], UV-VIS [49] and atmospheric pressure chemical ionization (APCI-MS/MS) [50]. An Inertsil HILIC column separated metformin in human plasma [47] and an Acquity UPLC BEH HILIC separated BMS-754807 (insulin-like growth factor inhibitor) and metformin in rat plasma [48]. With a Supelcosil LC-Si column HILIC separation was faster and better than RPLC when analyzing human and rat blood samples [49]. A high-throughput assay to simultaneously analyze metformin and sitagliptin in dried mouse and human blood spots used a Kinetix HILIC UHPLC column with laser diode thermal desorption [50]. Sitagliptin was also determined in human plasma using ESI-MS/MS and an Atlantis HILIC Silica column [51], and a similar set up was used to separate taspoglutide in human and animal plasma [52]. A Hypersil Silica column and ESI-MS/MS detection has been used to analyze muraglitazar in human plasma [53].

Synthetic opioid analgesics have been successfully measured by ESI-MS/MS in human plasma [54,55], dog [56], and in microdialysate samples [57]. Methadone [54], fentanyl [56] and sufentanil [55], were quantified using silica stationary phases such as carbamoyl-functionalized silica (TSK-Gel amide), difunctionally bonded ODS (Atlantis HILIC Silica) and Alltima HP HILIC, respectively. A ZIC-HILIC stationary phase was tested to identify ketobemidone and its metabolites, but failed to separate one metabolite [57]. A HILIC (ZIC-HILIC) x RPLC-ESI-MS/MS method was developed to simultaneously determine opioid and benzodiazepine derivatives and metabolites in micro-whole blood samples [58].

ESI-MS/MS has been the method of choice to detect a variety of drug classes in both serum [59,60] and

Table 2. - HILIC applications in drug analysis in pharmaceutical formulations and related matrices.

Matrix	Compounds of interest	Stationary phase	Mobile phase	Detection	Refs.
API starting material	API (4-(aminomethyl)pyridine) and related compounds	Atlantis HILIC Silica (150 mm × 4.6 mm, 3 μm)	AcN / 50 mM ammonium formate (pH 3.0); gradient	DAD	[101]
Drug substances	GTIs (alkyl sulfonates and dialkyl sulfates)	Atlantis HILIC Silica (50 mm × 2.1mm, 3 μm)	AcN / 50mM ammonium formate and 0.1% (v/v) formic acid (85:15, v/v); isocratic	ESI-MS	[104]
Single drug	GTIs	Atlantis HILIC Silica (50 mm × 2.1mm, 3 μm)	0.1% formic acid + 50mM ammonium formate in water, acetonitrile	ESI-MS	[105]
Pharmaceutical genotoxic impurities	GTIs	Atlantis HILIC Silica (50 mm × 2.1 mm, 3 μm)	Formic acid (0.1%) and 50mM ammonium formate (70:30, v/v) / AcN; isocratic	ESI-MS	[106]
API starting material	Impurities of API (phenyl methyl amino propane)	Zorba× NH_2 HILIC (250 mm × 4.6 mm, 5 μm)	10mM phosphoric acid (pH 6.1) / AcN (15:85, v/v); isocratic	UV-VIS	[107]
Mildronate	Mildronate and impurities	ZIC-HILIC (100 mm × 2.1 mm, 5 μm)	AcN / 5mM ammonium formate pH 5.0 (85:15, v/v); isocratic	ESI-MS/MS	[108]
Veterinary drugs (streptomycin and dihydrostreptomycin)	Impurities	Halo HILIC (100 mm × 2.1mm, 2.7 μm)	200mM ammonium formate buffer (pH 4.5) / AcN; isocratic	ESI-MS/MS	[109]
Basic drugs (anisole and 1,2-dinitrobenzene)	Impurities	ACE Cyano (150 mm × 3 mm, 3μm)	AcN / 3.25mM ammonium acetate in water (95:5); isocratic	ESI-MS/MS	[110]
Pharmaceutical formulations	Polar compounds	Ascentis Supelcosil LC-Si, (50 mm × 4.6 mm, 3 μm), Prevail Silica (150 mm × 4.6 mm, 3 μm)	10mM ammonium formate (pH 3) / AcN; gradient	ESI-TOF-MS, CAD, DAD	[111]
Pharmaceutical formulations	Anti-epileptic drug (gabapentin)	Luna HILIC and Atlantis HILIC Silica (100 mm × 2.0 mm, 3 μm), ZIC-HILIC (100 mm × 2.1 mm, 3.5 μm) and ZIC pHILIC (150 mm × 2.1 mm, 5 μm)	Composition, AcN content, and mobile phase pH were evaluated.	UV-VIS, ELSD, CAD	[112]
Cough-cold formulations	Hydrochloride, diphenhydramine hydrochloride,dextromethorph, hydrobromide	Supelcosil LC-Si (250 mm × 4.6 mm, 5 μm)	MeOH / (ammonium acetate and triethylamine, pH 5.2) (95:5, v/v); isocratic	UV-VIS	[113]
Tablet formulations	Metformin hydrochloride and its related compound	Nova-Pak Silica (150 mm × 3.9 mm, 5 μm)	Ammonium dihydrogen phosphate buffer / MeOH (21:79, v/v); isocratic	UV-VIS	[114]
Tablets	Metformin hydrochloride, cyanoguanidine, melamine	Atlantis HILIC Silica (250 mm × 4.6 mm, 5 μm)	AcN / 25mM ammonium dihydrogen phosphate buffer, (pH 3.0) (84:16, v/v); isocratic	UV-VIS	[115]
Pharmaceutical intermediates	Hydrazine and 1,1-dimethylhydrazine	ZIC-HILIC (250 mm × 4.6 mm, 5 μm)	Ethylalcohol / water / TFA (70:30:0.1); isocratic	CLND	[116]
Pharmaceutical gel	Dimethindene maleate	ZIC-HILIC (50 mm × 2.1 mm, 5 μm)	AcN / 25mM acetic acid + 2.5mM ammonium acetate (87.5:12.5, v/v); isocratic	UV-VIS	[117]
Drug substances (naproxen sodium, adenine hydrochloride and compound X)	Inorganic common pharmaceutical counterions	Acclaim Trinity P1 (50 mm × 3.0 mm, 3 μm)	200mM ammonium formate (pH 4.0) / Water / AcN; gradient	CAD	[118]
Drug substances	Inorganic common pharmaceutical counterions	ZIC-pHILIC (150 or 50 mm × 4.6 mm, 5 μm)	0.1M ammonium formate o acetate / AcN (25:75, v/v); isocratic	CAD	[119]

Continued **Table 2.** - HILIC applications in drug analysis in pharmaceutical formulations and related matrices.

Matrix	Compounds of interest	Stationary phase	Mobile phase	Detection	Refs.
Sympathomimetic drugs	Polar compounds	Luna HILIC (100 μm × 25 cm, 3 μm)	10mM ammonium formate (pH 3) in water / AcN (92:8, v/v); isocratic	UV-VIS	[120]
Cardioprotectant dexrazoxane	Dexrazoxane and its three polar metabolites	ZIC-HILIC (150 mm × 2.1 mm, 3.5 μm)	0.5mM ammonium formate / AcN (pH 6) (15:75, v/v); isocratic	ESI-MS/MS	[121]
Antibiotics fermentation extracts: Penicillin and cephalosphorin	Metabolites in penicillin and cephalosporin	ZIC-HILIC (150 mm × 4.6mm, 5 μm)	20mM ammonium formate (pH 3.5) in water / 20mM ammonium acetate (pH 7.5) in AcN; gradient	ESI-MS/MS	[122]
Cephalosporins	Cephalosporin	Click β-CD column (150 mm × 2.1 mm, 5 μm), Atlantis HILIC Silica (100 mm × 2.1 mm, 5 μm)	10mM ammonium formate (pH 6.8) / AcN /100mM ammonium formate (90:10, v/v, pH 6.8); Different gradients	ESI-MS/MS	[123]
Drugs molecules	Penicillin potassium salt	Acclaim Trinity P1 (50 mm × 3 mm, 3 μm)	AcN / 10mM ammonium acetate (pH 5.2); isocratic	ELSD	[124]
Tablet samples	Vitamins (ascorbic and dehydroascorbic acids)	Sielc Obelisc R (100 mm × 3.2 mm, 5 μm)	AcN / 75mM ammonium acetate pH 4.2 (15:85, v/v); isocratic	CAD	[125]
Pharmaceutical formulations	Vitamins (B and C)	HILIC Inertsil diol (150 mm × 64.6 mm, 5 μm)	AcN–water (90:10, v/v) + containing 10 mM ammonium acetate and 20 mM triethylamine (pH 5.0); gradient	DAD	[126]
Ophthalmic solution test samples	Brimonidine tartrate	Supelcosil LC–Si (250 mm × 4.6 mm, 5 μm)	AcN / aqueous ammonium acetate buffer (pH 7.1) (92:8, v/v); isocratic	UV-VIS	[127]
Ophthalmic solution	Sodium Cromoglicate	Atlantis HILIC Silica (250 mm × 64.6 mm, 5 μm)	AcN / 30mM ammonium acetate (pH 3.0) (86:14, v/v); isocratic	UV-VIS	[128]
Standard solution mixtures	Adrenoceptors	LiChrospher 100 Diol (250 mm × 2 mm, 5 μm)	AcN / 10mM formate ammonium; isocratic a different compositions	UV-VIS	[129]
Pharmaceutical dry inhalation products	Lactose	APS-2 Hypersil (100 mm × 3mm, 5 μm)	AcN / water (80:20, v/v); isocratic	ELSD	[130]
Gemzar® pharmaceutical formulations	Mannitol	TSK-Gel Amide 80 (250 mm × 64.6 mm, 5 μm)	AcN / 0.1% TFA in water, (75:25, v:v); isocratic	ELSD	[131]
Antiepileptic drugs	C10 to C18 acylcarnitines	Atlantis HILIC Silica (150 mm × 2.1 mm)	10mM ammonium formate / AcN (40:60, v/v); isocratic	ESI-MS/MS	[61]
Pharmaceutical formulations	Lysine	Atlantis HILIC Silica (150 mm × 4.6 mm, 3 μm), Luna HILIC (150 mm × 4.6 mm, 5 μm), Nucleosil 100-5 $N(CH_3)_2$ (250 mm × 4.6 mm, 5μ m)	AcN / 10mM potassium salt; isocratic	FLD	[132]
Standard solution mixtures	76 model compounds relevant to biomedical and pharmaceutical studies	ZIC-HILIC (150 mm × 4.6 mm, 5 μm), Nucleodur HILIC (125 mm × 3 mm, 3 μm)	Different compositions of AcN / ammonium acetate buffer were evaluated	ESI-MS/MS	[133]
Standard solution mixtures	Oxime-type acetylcholinesterase reactivators	ZIC-HILIC (150 mm × 4.6 mm, 3.5 μm), Luna HILIC (100 mm × 2 mm, 3 μm)	AcN / 0.9% NaCl in water. Different compositions for each column.	DAD	[134]

AcN: acetonitrile; MeOH: methanol; TFA: trifluoroacetic acid API: active pharmaceutical ingredient; GTIs: genotoxic impurities

plasma [62-70]. When four different HILIC stationary phases were tested to simultaneously analyze three anticonvulsants (gabapentin, pregabalin and vigabatrin) in human serum, the TSK-Gel Amide column provided the best peak shape [59]. This column has also been used to determine methylphenidate (psychostimulant) in rat serum and brain [60]. C10 to C18 acylcarnitines were analyzed in serum from epileptic children using HILIC–MS/MS with an Atlantis HILIC Silica column [61]. The chemotherapy drugs 3-deazaneplanocin A, 5-fluorouracil, melphalan and dacarbazine were quantified using Zorbax HILIC [62], amine (NH_2P-50 2D) [63], Atlantis HILIC Silica [64] and TSK-Gel Amine [65] stationary phases, respectively. Atlantis HILIC Silica was also used to separate antiemetic (metoclopramide) [66], antibacterial (levofloxacin) [67] and anesthetic (propofol) [68] drugs, while analysis of antimuscarinic (tolterodine) [69] and anti-tuberculosis (isoniazid) [70] drugs was described using Hypersil Silica.

Two studies focused on non-ESI-MS/MS applications, employing an APCI interface to determine serum sertraline levels using a silica column under non-aqueous-HILIC conditions [71], and measuring ethyl methanesulfonate in human plasma using an Atlantis HILIC Silica column [72].

HILIC coupled to MS/MS has emerged as an important complementary technique to RPLC to obtain metabolite profiles; endogenous metabolites in human urine and plasma were analyzed with a range of different HILIC columns (Luna HILIC, Acquity UPLC BEH HILIC and Sielc Obelisc N) [73]. Finally, other compounds including daumone, glucoraphanin, nicotine, and glucosamine have been analyzed in animal plasma by ESI-MS/MS [74-76] and ESI-MS [77,78] detection using ZIC-HILIC [74,75], Luna HILIC [75], Zorbax SBCN [77] and capillary Atlantis HILIC Silica columns [78,79] (Table 1).

2.1.2. Drug analysis in urine samples

HILIC has been widely used to analyze a wide variety of drugs and metabolites in urine [19,73,80-87], and many metabolites have been identified using ESI-MS/MS [19,80-82]. The first such study [80] described a sensitive and specific method to quantify the main metabolite of the antineoplastic drug 5-FU using a ZIC-HILIC column, as part of an evaluation of health care workers exposed to this compound. The same stationary phase was also chosen to identify peramivir in urine and saliva [19]. Renal elimination of oxypurinol was determined in gout patients using an Atlantis HILIC Silica column [81], and a 2D-RPLC-HILIC method was developed to identify three drugs and their metabolites with a pentaflurophenylpropyl (PFPP-HILIC) second dimension column [82]. The strong separating power of this approach may be useful when using detectors less selective than MS. A further example of HILIC columns in drug analysis is the use of a Luna HILIC stationary phase as an effective interface between polymer monolith microextraction and ESI-MS in the analysis of β_2 agonists [83].

HILIC has been used to determine drugs of abuse in human urine [84,85]. Nicotine and its metabolites have been separated *via* Luna Amine HILIC with MS/MS detection [84], and morphine, codeine, and their glucuronide conjugates were effectively detected using a Zorbax HILIC Plus column and ESI-TOF-MS [85]. In a study of the potential abuse of mitragynine, the primary active alkaloid in *Mitragyna speciose*, it was effectively quantified using an Atlantis HILIC Silica column and MS/MS [86]. Cimetidine, used to treat heartburn, was selected as the internal standard when simultaneously determining uric acid and creatinine using UV-VIS and a Spherisorb amine column [87].

2.1.3. Drug analysis in other biological samples

Drug analysis using HILIC has been described for a number of other biological matrices, including hair [88-91], saliva [92], fingerprints [93], liver [94-96], cells [97,98], manure [99] and feces [100]. Cocaine and its metabolites benzoylecgonine, ecgonine methyl ester, and cocaethylene were quantified by ESI-MS/MS in hair using an Atlantis HILIC Silica column [88]. Similarly, ethyl glucuronide, a marker of recent alcohol consumption, was also determined in meconium and hair using Luna Amine HILIC and ESI-MS/MS [89]. An Acquity BEH HILIC column was used for a similar analysis of head and pubic hair [90] and this column also successfully identified seventeen illicit drugs in saliva (opiates, amphetamines, flunitrazepam, cocaine, and their metabolites), using three different atmospheric pressure ionization (API) sources [92]. Successful detection of DOAs in rat hair has also been reported using the UPHLC-ESI-TOF-MS method (Acquity BEH HILIC: [91]).

The use of fingerprints as matrix to test for drugs and metabolites is a novel area of analytical toxicology research. Benzodiazepines (lorazepam and its 3-O-glucuronide conjugate) were successfully detected in fingerprints using a TSK-Gel Amide stationary phase with ESI-MS/MS detection [93].

HILIC has also been employed for drug analysis of liver samples. In a recent study [94], a ZIC-HILIC column was chosen to investigate the sulfation of two metabolites of 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) in human liver cytosol. Other *in vitro* applications involving liver samples have also been described, such as analyzing the anticancer agent

trabectedin in monkey liver, and an antimalarial drug metabolite in human liver tissue [95] and in human liver microsomes [96], using a Silica HILIC column with ESI-MS/MS detection.

Several techniques to analyze drugs in cells have been reported using HILIC, including a HILIC-fluorescence-based assay for measuring trypanosome uptake of CPD0801, a candidate for trypanosomiasis treatment [97]. Sample fluorescence was measured in a 96-well format using a benchtop fluorimeter and a ZIC-HILIC column. In a study designed to predict the toxic effects of several drugs on human embryonic stem cells [98] a Luna Amine HILIC stationary phase coupled with ESI-MS/MS [98] was used.

Liquid manure from intensive livestock operations is often used as fertilizer and is a potential source of environmental antibiotic contamination. A robust, specific and sensitive HILIC-APCI-MS/MS method was developed to determine aminoglycoside and lincosamide antibiotics (spectinomycin and lincomycin) in liquid hog manure and runoff from manure-treated cropland [99]. This technique used an Alltima HP HILIC analytical column. Finally, a HILIC (Polaris Si-A column)-ESI-MS/MS method was developed to quantify nicotine and cotinine levels in zebra finch feces [100].

2.2. Drug analysis in pharmaceutical formulations and related matrices

Polar drugs are poorly retained in most common RPLC approaches [101]. HILIC has been widely adopted in recent decades to overcome this problem; it is currently the predominant technique used to separate polar drugs (Table 2).

Bioanalytical support is a fundamental requirement for lead optimization in drug discovery and development. HILIC-MS/MS studies up to 2006 quantitatively tracing drugs *in vitro* and *in vivo* have been reviewed by Hsieh [102] and HILIC methods employed in pharmaceutical analysis from 2005 to 2009 were reviewed [17].

Genotoxic impurities (GTIs) can induce genetic mutations, chromosomal breaks, or chromosomal rearrangements. Newer methods (including HILIC) used to monitor trace levels have been evaluated [103]. They have also been studied using an Atlantis HILIC Silica stationary phase [104-106]. In all cases, ESI-MS was employed to distinguish alkyl sulfonate and dialkyl sulfate GTIs from the main API.

The use of several bonded silica stationary phases to analyze 4-(aminomethyl) pyridine (a common basic and highly polar starting material in API synthesis) has been investigated [100]. Of these, the Atlantis HILIC Silica column provided the most satisfactory DAD separation and offered an attractive alternative to RP-HPLC. Impurity retention from the starting material (phenyl methyl amino propane) was studied using multiple analytical methods (ion-pairing, ion interaction, and hydrophilic interaction chromatography modes), with a Zorbax Amine HILIC column coupled to UV-VIS giving best results [107].

Other HILIC columns have been used to analyze pharmaceutical formulations in combination with ESI-MS/MS [108-110]. For example, ZIC-HILIC and Halo HILIC columns were chosen to separate impurities in mildronate (anti-ischemic) [108], demonstrating HILIC to be a valid alternative to reverse phase and ion-pair chromatography. The Halo HILIC stationary phase has also been used in the analysis of impurities in streptomycin and dihydrostreptomycin. The retention mechanism of basic drugs has been investigated using an ACE cyano-column in HILIC mode [110]. Multidimensional chromatography has also been successfully applied to drug analysis [111], employing a RPLC x HILIC-ESI-MS/MS method using an Ascentis Si HILIC column to analyze a mixture of sugars and sulfonamides, and a Prevail Silica analytical column to examine a mixture of arylamines and aminopyridines (model pharmaceuticals).

HILIC determination of APIs in pharmaceutical formulations has been reported previously [112-117]. Recently four HILIC columns (ZIC HILIC, ZIC *p*-HILIC, Luna Amine HILIC and Atlantis HILIC Silica) and two detection methods (evaporative light scattering detector [ELSD] and charged aerosol detector [CAD]) were tested to analyze gabapentin in pharmaceutical formulations [112]. Similarly, the main APIs, including the sympathomimetic pseudoephedrine, the antihistamine diphenhydramine, and the antitussive dextromethorphan were simultaneously examined in cough syrup using a Supelcosil LC-Si stationary phase and UV-VIS [113]. This same detector was also used in combination with a Nova Pak Silica HILIC column to analyze metformin and the related 1-cyanoguanidine in tablets [114]. Metformin and its impurities were simultaneously evaluated on an Atlantis HILIC Silica stationary phase under isocratic conditions [115].

Hydrazine and related compounds have been analyzed using a zwitterionic column (ZIC-HILIC) in combination with chemiluminescent nitrogen detection [116]. A similar column coupled to a UV-VIS detector was also used to determine dimethindene maleate in a topical gel [117].

Identifying and quantifying counter ions and residual salts is critical when determining drug purity and mass balance, and in salt formation. Mixed-mode chromatography with CAD detection was developed to screen for and quantify counter ions, unknown ionic

Table 3. - HILIC applications in the analysis of pharmaceuticals in food, feed and environmental matrices.

Matrix	Compounds of interest	Stationary phase	Mobile phase	Detection	Refs.
Food and feed matrices					
Poultry tissue	Anti-influenza drugs (amantadine, rimantadine, zanamivir and oseltamivir and its carboxylate metabolite), anti-herpes drugs (acyclovir and ganciclovir) and an immunomodulator (imiquimod)	ZIC-HILIC (150 mm × 2.1 mm, 5 μm)	20mM ammonium acetate (pH5.5) / AcN; gradient	ESI-MS/MS	[136]
Veal muscle	Veterinary drugs (aminoglycosides)	ZIC-HILIC (50 mm × 2.1 mm, 5 μm)	0.4% formic acid in water / AcN; gradient	ESI-MS/MS	[137]
Swine/bovine meat and kidney	Veterinary drugs (spectinomycin, dihydrostreptomycin, streptomycin, kanamycin, gentamicin, apramycin, neomycin)	ZIC-HILIC (100 mm × 2.1mm, 5 μm)	AcN / 150mM ammonium acetate + 1% formic acid in water; gradient	ESI-MS/MS	[138]
Chicken muscle	Veterinary drugs (aminoglycosides, β-lactams, lincosamides, macrolides, quinolones, sulfonamides, tetracyclines, and amprolium)	ZIC-HILIC (100 mm × 2.1 mm, 3.5 μm)	50mM ammonium formate in water (pH 2.5) / AcN; gradient	ESI-MS/MS	[139]
Chicken muscle, eggs and animal feed	Veterinary drug (amprolium)	Fused-Core Ascentis E×press HILIC (100 mm × 2.1 mm, 2.7 μm)	AcN / 50mM formic acid–ammonium formate buffer (pH 4) (60:40, v/v); isocratic	ESI-MS/MS	[140]
Milk	Veterinary drugs (streptomycin and dihydrostreptomycin)	HILIC Fortis (100 mm × 3.0 mm, 3 μm)	150mM ammonium formate in water (pH 4.5) / AcN (35:65, v/v); isocratic	ESI-MS	[141]
Milk and eggs	Sulfonamides antibacterial residues	Luna HILIC (150 mm × 2.0 mm, 3 μm)	AcN + 0.05% formic acid / water; gradient	ESI-MS	[142]
Milk	Pharmaceutical (bicozamycin)	TSK-Gel Amide 80 (150 mm × 2.0 mm, 3 μm)	50mM ammonium acetate in water (pH 4.0) /AcN; gradient	ESI-MS/MS	[143]
Milk	Fluoroquinolone residues	Atlantis HILIC Silica (250 mm × 4.6 mm, 5 μm)	Acetate buffer (pH 5.4) / AcN (75:25, v/v); isocratic	ELCD	[144]
Apples	Antibiotic residues (streptomycin)	Atlantis HILIC Silica (150 mm × 2.1 mm, 3 μm)	0.05% formic acid in water / in AcN; gradient	ESI-MS/MS	[145]
Distillers grains	Antibiotic residues (streptomycin)	Atlantis HILIC Silica (100 mm × 2.1 mm, 5 μm)	0.1% formic acid in water / AcN; gradient	APCI-MS/MS	[146]
Swine feed	Antibacterial drugs (carbadox and olaquindox)	Acquity UPLC BEH HILIC column (100 mm × 2.1 mm, 1.7 μm)	10mM ammonium acetate in AcN / water (95:5, v/v); isocratic	DAD	[147]
Baby food (fruit, vegetable, chicken)	Antibiotics (nitrofurans)	TSK-Gel Amide 80 (250 mm × 2.0 mm, 5 μm)	AcN / water / 0.2% formic acid; gradient	ESI-MS/MS	[148]
Environmental matrices					
River water	Estrogen conjugates (estrone, estradiol, and estriol)	ZIC–pHILIC (100 mm × 2.1 mm, 5 μm)	AcN / 5mM ammonium acetate; gradient	ESI-MS/MS	[150]
Waste water	Cytostatics (5-fluorouracil, cytarabine, and gemcitabine) and human metabolites	ZIC-HILIC (150 mm × 2.1 mm, 3.5 μm)	AcN / 30mM ammonium acetate in water; gradient	UV-VIS, ESI-MS/MS	[151]
Waste and surface water	DOAs (cocaine and metabolites)	Zorba× R×-SIL HILIC (150 mm × 2.1 mm, 5 μm)	15mM ammonium acetate (pH 4.5) / acetonitrile; gradient	ESI-MS/MS	[152-155]

Continued **Table 3.** - HILIC applications in the analysis of pharmaceuticals in food, feed and environmental matrices.

Matrix	Compounds of interest	Stationary phase	Mobile phase	Detection	Refs.
Composite waste water	DOAs (amphetamine, methamphetamine, methylenedioxymethamphetamine, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, cocaine, benzoylecgonine, ecgonine methyl ester and 6-monoacetylmorphine and their metabolites)	Luna HILIC (150 mm × 3 mm, 5 μm)	5mM ammonium acetate in water / AcN; gradient	ESI-MS/MS	[156]
River water and effluent waste water	Pharmaceuticals (trimethoprim and atenolol) and DOAs and their metabolites (cocaine, morphine, codeine)	Fused-Core Ascentis E×press HILIC (50 mm × 2.1 mm, 2.7 μm)	15mM ammonium acetate (pH 4.5) / AcN; gradient	ESI-MS	[157]
Waste water	Pharmaceuticals (omeprazole, pantoprazole, ranitidine, citalopram, fluoxetine, paroxetine, venlafaxine, tramadol, nebivolol, metoprolol, atenolol, bisoprolol and metformin)	Luna HILIC (150 mm × 3 mm, 5 μm)	5mM ammonium acetate in water / AcN/ MeOH (87.5:12.5, v/v); gradient	ESI-MS/MS	[158]
Sewage and surface waters	Antidiabetic drug (metformin)	ZIC-HILIC (150 mm × 2.1 mm, 3.5 μm)	AcN / 10mM ammonium formate in water (pH3); gradient	ESI-MS/MS	[159]
Waste water	Gadolinium Chelates	ZIC-HILIC (150 mm × 2.1 mm, 3.5 μm)	12.5mM ammonium formate and 12.5mM formic acid in AcN / water (pH 3.75); isocratic	ICP-MS	[160]
Plant samples	Acetylcholinesterase	Atlantis HILIC Silica (150 mm × 2.1 mm, 3 μm)	90% AcN in water + 10mM ammonium formate (pH 3.5) / 50% AcN in water + 10mM ammonium formate, (pH 3.5); gradient	ESI-TOF-MS	[161]

AcN: acetonitrile; MeOH: methanol; DOAs: drugs of abuse

impurities, and salts in APIs. It also processed control samples with excellent accuracy, precision and sensitivity [118]. Of the four different HILIC columns tested, an Acclaim Trinity P1 HILIC column provided the best results, although another study reported comparable findings using a ZIC p-HILIC column coupled to CAD [119].

In analyzing the retention of six sympathomimetic drugs by nano-LC with ESI-MS/MS detection [120], complete separation was only achieved using a Luna Amine HILIC stationary phase. In another study, the retention and selectivity of two bare silica (Ascentis Express HILIC, Atlantis HILIC) and two zwitterionic (Obelisc N and ZIC HILIC) HILIC columns were compared analyzing dexrazoxane (a bisdioxopiperazine) and its three polar metabolites, demonstrating that only the ZIC HILIC column achieved complete separation [121].

Metabolic profiling of intracellular metabolites in β-lactam antibiotic fermentation broths was investigated by HILIC-ESI-MS/MS (zwitterionic phase) [122]. This method enabled 87 metabolites in penicillin fermentation broths to be defined, and 94 compounds in cephalosporin extracts. The same detection method was used in conjunction with β-CD and Atlantis HILIC Silica columns to determine cephalosporins [123]. Penicillin has also been analyzed using an Acclaim Trinity P1 stationary phase and ELSD [124].

Tablets of ascorbic acid (vitamin C) and related compounds have been successfully separated using a Sielc Obelisk R column and CAD detection [125]. Several water soluble vitamins (B complex and vitamin C) were quantified in pharmaceutical formulations using a dihydroxypropyl bonded phase (Inertsil Diol) and DAD [126]. Commercial drugs have been detected in several studies by UV-VIS, as in ophthalmic solutions [127-129]. Brimonidine tartrate (α_2-adrenoreceptor agonist) was quantified on a Supelcosil LC-Si HILIC column [127]; sodium cromoglicate (anti-allergy) was analyzed on an Atlantis HILIC Silica column [128]; and adrenoreceptor agonists and antagonists were separated on a Diol HILIC column [129].

Dry inhalation pharmaceuticals are often lactose-based mixtures containing micronized drug particles. Analyzing the drug distribution in these mixtures shows the effect of the carrier on drug deposition. For this purpose, a HILIC-ELSD method was developed to quantify the fine particle dose of lactose using an aminopropyl column (APS-2 Hypersil) [130]. Another HILIC method (TSK-Gel Amide) was also described to measure mannitol, which contributes to the lyophilized

plug and maintains an amorphous form in Gemzar® formulations [131].

Valproate (VPA) is widely used to treat epilepsy. The effects of long-term VPA treatment on carnitine and isomer-differentiated acylcarnitine disposition have been evaluated in epileptic children using a HILIC (Atlantis HILIC Silica)-MS/MS method [61].

A rapid procedure was developed to separate lysine and arginine using HILIC with fluorescence detection [132], in which five different HILIC stationary phases were tested: Atlantis HILIC, Luna HILIC, TSKgel Amide-80, Kromasil 60-5DIOL Nucleosil100-5 $N(CH_3)_2$ and ZIC HILIC. Luna HILIC, and Nucleosil100-5 $N(CH_3)_2$. Of these, the Atlantis HILIC Silica column best separated lysine and arginine.

The separation of 12 model compounds that possess different ionization states, including neurotransmitters, was compared using two stationary phases coupled to ESI-MS/MS: ZIC-HILIC and Nucleodur HILIC [133]. The columns displayed identical retention behavior, although better peaks were generally obtained for the Nucleodur HILIC column. The ZIC-HILIC column coupled to a DAD detector has also been used to analyze several oxime-type acetylcholinesterase reactivators [134].

2.3. Other matrices

2.3.1. Drug analysis in food and animal feed matrices

The European Union has established maximum residue limits (MRLs) for antibiotics in meat, fish, milk, and eggs [135]. HILIC methods have been applied to measure pharmaceuticals in food and animal feed (Table 3).

Veterinary antibiotics (aminoglycosides, β-lactams, coccidiostats, lincosamides, macrolides, quinolones, sulfonamides and tetracyclines) are commonly used for therapeutic and prophylactic purposes, and as feed additives to promote livestock growth. Thus, monitoring their residues is important to ensure food safety. Pork, beef and poultry are frequently analyzed, commonly using ESI-MS/MS detection [136-140]. Several of these studies use the ZIC-HILIC column to measure anti-influenza and anti-herpes drugs and immunomodulators in poultry tissues [136], or aminoglycoside residues in veal muscle [137]. It has also been used to identify aminoglycoside antibiotics in kidneys and meat [138], and to analyze a range of veterinary drugs in chicken meat [139]. A Fused-Core Ascentis Express HILIC column has also been used to analyze *Coccidiostast amprolium* in chicken eggs and meat [140].

Antibiotic residues in dairy products have been analyzed by HILIC, usually with MS detection. Two of the most commonly used veterinary aminoglycoside antibiotics, streptomycin and dihydrostreptomycin, have been examined in milk using a HILIC Fortis column [141], while sulfonamide residues have been monitored in milk and eggs on a Luna Amine HILIC phase [142]. Both cases used ESI-MS detection. Bicozamycin was also studied in milk using a TSK-Gel Amide HILIC column and ESI-MS/MS detection [143]. Finally, a simple, stable, and sensitive electrogenerated chemiluminescence detector was recently described to assess multiple fluoroquinolone residues in milk using an Atlantis HILIC column [144].

Fruits and vegetables are essential elements of a balanced human diet. Significantly, streptomycin has been successfully separated and quantified in apples using an Atlantis HILIC Silica column and ESI-MS/MS detection [145].

Distillers grain, a major byproduct of dry-grind ethanol processing, is a livestock feed supplement. However, antibiotics are commonly used in the dry-grind process to increase fermentation efficiency by preventing bacterial growth. This can result in antibiotics in the animal tissues, a human health risk. Accordingly, the Food and Drug Administration Center for Veterinary Medicine has established limits for feed contaminants and has approved a method to determine antibiotic residues in distillers grain [146]. To analyze streptomycin with sufficient accuracy and precision, this method uses an Atlantis HILIC Silica column and APCI-MS/MS detection. Antibacterial drugs like carbadox and olaquindox, used to prevent a number of diseases and as growth promoters, have been quantified in swine feed using a HILIC-DAD method (Acquity UPLC BEH HILIC column) [147]. Similarly, amprolium was assessed in animal feed using a Fused-Core Ascentis Express HILIC stationary phase and ESI-MS/MS detection [140].

Nitrofurans are cheap and highly effective drugs used to treat bacterial diseases. Azodicarbonamide (ADC) is thought to be a marker of illegal nitrofurazone use in aquaculture and poultry production. A novel HILIC-ESI-MS/MS method discriminating nitrofurazone from azodicarbonamide in food was developed using a TSK-Gel Amide phase to determine nitrofurans [148].

2.3.2. Drug analysis in environmental matrices

Pharmaceuticals or DOAs can contaminate the environment if not eliminated by wastewater treatment. Table 3 contains a brief list of compounds in environmental samples analyzed using HILIC, and the methods used.

Estrogens influence plant growth and human health, and act as endocrine obstructors, provoking feminization in fish [149]. A method to detect estrogen derivatives in river water has been described using a column-switching-HILIC-ESI-MS/MS system [150] with a ZIC-pHILIC stationary phase. Cytostatic drugs

in wastewater have been separated on a ZIC-HILIC column and identified by ESI-MS/MS [151].

HILIC with ESI-MS/MS detection is the technique of choice for determining DOAs. A Zorbax Rx-SIL HILIC column was employed to analyze cocaine and its metabolites in waste and surface water [152-155], while simultaneous analysis of nine DOAs and their metabolites in composite wastewater was achieved using a Luna Amine HILIC [156]. ESI-MS detection was only reported in one study of environmental matrices, where it was used with an optimized HILIC (Fused-Core Ascentis Express) method to separate and detect two pharmaceuticals (trimethoprim and atenolol), and three illicit drugs and their metabolites (cocaine, morphine, codeine) in river and wastewater [157].

A ESI-MS/MS detector was used to quantify 13 pharmaceutical compounds in waste water using a Luna Amine HILIC column [158], and to determine metformin in waste and surface water when coupled to a ZIC-HILIC column [159].

Gadolinium (Gd) chelates are are widely used in magnetic resonance imaging, which can result in toxic Gd species in the environment. A novel approach has been described to analyze the most important Gd chelates in hospital effluent, municipal sewage, and treated wastewater based on HILIC-ICP-MS analysis using a ZIC-HILIC column [160].

Acetylcholinesterase inhibitors, important in Alzheimer´s therapy, have been analyzed in plants using a very efficient, selective and sensitive molecular screening technique consisting of an Atlantis HILIC Silica column combined with ESI-TOF-MS detection [161].

3. Conclusions

HILIC is a powerful tool to analyze drugs and their metabolites or impurities in a broad range of matrices, including pharmaceutical formulations, biological samples, food, animal feed, and environmental samples. Especially for polar compounds, it offers increased selectivity compared to traditional RP chromatography.

A typical pharmaceutical approach is to analyze samples separately by reversed-phase liquid chromatography, followed by normal-phase liquid chromatography or supercritical fluid chromatography. These techniques have been gradually supplanted by HILIC, as demonstrated by the large number of HILIC studies published in recent years.

While a wide variety of HILIC stationary phases have been used, difunctionally bonded ODS, zwitterionic, and aminopropyl based stationary phases are the most common. MS/MS is the most popular method of detection, followed by DAD and UV.

In the future, the development of new HILIC stationary phases and more powerful MS detectors compatible with HILIC will probably increase its use in drug analysis.

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