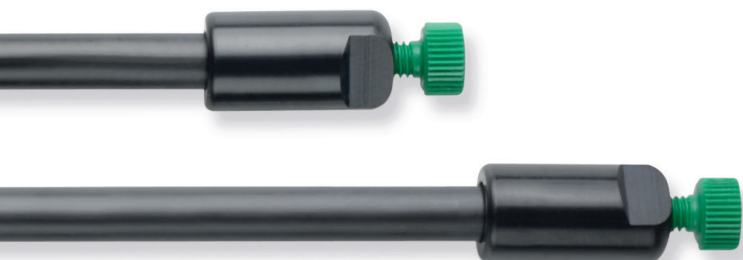


Polar Hydrophilic Compounds in Pharmaceutical Analysis

SeQuant[®] ZIC[®]-HILIC HPLC for control
of active ingredients, impurities,
counterions and excipients.



Introduction

For hydrophobic substances, reliable and robust methods have successfully been developed on reversed phase columns over the past 30 years, but analyzing hydrophilic compounds in clean or complex samples have been a massive undertaking, involving derivatization and tedious sample preparation procedures. To retain polar and hydrophilic molecules on reversed phase columns ion-pairing reagents or highly or completely aqueous mobile phases are needed. Ionizable polar compounds could be retained on ion-exchange columns, but then only negative or positive analytes in each method, since ion exchange columns are either for cations or anions.

Technical developments have provided us with new instrumentation and column chemistries, and thereby the possibility to develop comprehensive and sensitive methods. The challenge is to make them reliable and robust to work in diverse matrices and to combine the best detection technique with the most favorable separation mode for analytes of interest. Developing methods that are fast and accurate in a single matrix is straightforward, but in many cases a lot of information can be lost if the samples are analyzed with only one chromatographic selectivity, especially for impurity profiling or when sample composition is unknown. Screening methods, based on complementary chromatographic selectivities together with sensitive and specific detection techniques can therefore, all together, provide us with much more complete information.

The globalization process changes the demands and requirements literally as we speak. Currently official controlling organizations such as the United States Pharmacopeia (USP) are looking for new analytical alternatives to replace and modernize old regulated pharmaceutical control methods. EMD Millipore contributes in this context by offering analytical solutions and prompt technical support.

This compilation exemplifies how hydrophilic interaction liquid chromatography (HILIC) in general, and the bonded zwitterionic SeQuant® ZIC®-HILIC stationary phase in particular, is the ideal tool for analysis of polar compounds. The SeQuant® ZIC®-HILIC and ZIC®-cHILIC columns are ideal for replacing RP methods based on ion-pairing reagents and methods with excessively aqueous conditions in the mobile phase. With SeQuant® HILIC columns simultaneous separation of both cationic and anionic species in one run is easily accomplished due to the permanent zwitterionic bonded stationary phase.

Application examples are presented herein, with full experimental details, to demonstrate the usefulness of HILIC for pharmaceutical quality control, and as you will experience, HILIC is useful for regulated pharmaceutical assay and impurity profiling methods.

EMD Millipore offers virtually everything but the instrument to successfully implement these methods in your laboratory. Besides analytical chromatography columns we provide solvents, reagents, filters, water and water purification systems.

Active Pharmaceutical Ingredients (API's)

Chemotherapeutic Pharmaceuticals (anti-cancer drugs)

Anti-cancer drugs target rapidly dividing cells either by halting cell division or by introducing errors in and halting DNA replication. Drugs that target DNA synthesis such as anti-metabolites and alkylating agents are often highly hydrophilic. Anti-metabolites masquerading as purines (azathioprine, mercaptopurine) or pyrimidines become incorporated into the cell DNA stopping normal development and division. They also affect RNA synthesis. Due to their efficiency, these drugs are the most widely used cytostatics.

Purines and pyrimidines are hydrophilic molecules but the compounds actually introduced into the growing DNA chain are nucleoside monophosphates. These are very difficult to retain in reversed phase due to their high hydrophilicity. (**Azacitidine, Decitabine, 5-fluorouracil, Temozolomide**). The USP method for Temozolomide is an ion-pairing method, no methods are available for Azacitidine or Decitabine. The Indian Pharmacopoeia (IP) will, however, introduce in Q1, 2014, a monograph for Azacitidine using the SeQuant® ZIC®-HILIC column. Alkylating agents are highly reactive molecules often coupled to some molecule actively transported into cells like uracil or phenylalanine. USP methods are often wet chemistry methods like the one for **Busulfan**. The US EPA method for degradation (hydrolysis) products of nitrogen mustards (a common alkylating anti cancer drug family) is a HILIC method. Included here is also **Cisplatin** a platinum containing anti cancer drug that crosslinks DNA similar to alkylating agents. The USP methods for Limit of trichloroamineplatin, Limit of transplatin and assay use columns L14, L9 and L8 respectively. **Methotrexate** competitively inhibits dihydrofolate reductase (DHFR), an enzyme that participates in the tetrahydrofolate synthesis. DHFR catalyses the conversion of dihydrofolate to the active tetrahydrofolate. Folic acid is needed for the de novo synthesis of the nucleoside thymidine, required for DNA synthesis. Also, folate is needed for purine base synthesis, so all purine synthesis will be inhibited. Methotrexate, therefore, inhibits the synthesis of DNA, RNA, thymidylates, and proteins.

Other Therapeutic Areas

Hydrophilic API's are not limited to chemotherapeutic pharmaceuticals, included in this compilation is also a selection of API's from other segments that have been analyzed in HILIC mode; including:

Acetylcysteine - Mucolytic agent

Adrenaline (Epinephrine) - Cardiac arrest and other cardiac dysrhythmias, Anaphylaxis, Asthma

Allantoin - Moisturizer with keratolytic effect

Citicoline - Psycho stimulant / Nootropic drug

Eflornithine (DFMO) - Hirsutism and sleeping sickness

Metformin - Diabetes

Pyrimethamine - Antimalarial drug

Risedronate - Osteoporosis

Urea - Dermatology

Excipients

In many cases, once an active ingredient has been purified, it will denature, fall out of solution, or stick to the sides of the container. To stabilize the API, excipients are added, ensuring that the API stays "active", and, just as importantly, stable for a sufficiently long period of time that the shelf-life of the product makes it competitive with other products.

An excipient is most commonly a pharmacologically inactive substance used as a carrier for the API of a medication. The "active" substance(s) may not be easily administered and absorbed by the human body but the substance in question may be dissolved into or mixed with an excipient.

Excipients are also used to bulk up formulations that contain very potent active ingredients, to allow for convenient and accurate dosage. Excipients can also be used in the manufacturing process to aid in the handling of the API. Pharmacopoeia require that all ingredients in drugs, as well as their chemical decomposition products, be identified and guaranteed to be safe. Therefore, excipients are only used when absolutely necessary and in the smallest amounts possible. Excipients can be classified as:

Antiadherents	Binders	Coatings
Disintegrants	Fillers	Flavours
Colours	Lubricants	Glidants
Sorbents	Preservatives	Sweeteners

Reactive excipient impurities must also be controlled due to potential reactions with API's. Reducing sugars, formaldehyde (and other low molecular aldehydes), hydrogen peroxide, Nitrite/nitrate, heavy metals and organic acids are the most common such reactive impurities. Heavy metals are easily analyzed by ICP-AES (using Merck's ultra pure acids), all other are hydrophilic compounds.

Cosmetics

From a regulatory point of view, at least in the EU, there do not exist any requirements with respect to the analytics of cosmetic ingredients or even more for cosmetic formulations, creams, lip stick etc. The regulatory basis for the cosmetic industry in the EU is the Cosmetics Regulation (EC) No. 1223/2009. In general any adequate and suitable method is acceptable.

A few countries have cosmetic monographs in place, including analytical methods for cosmetic ingredients, e.g. Japan. In a few cases you can find limits for substances, e.g. heavy metals, but not linked to any method. Anything else depends on the manufacturer, the chemical substance and the equipment which is available.

In the US cosmetics are controlled by the FDA's Center for Food Safety and Applied Nutrition. Manufacturers are not required to register their cosmetic establishments, file data on ingredients, or report cosmetic-related injuries to FDA. The FD&C Act does not subject cosmetics to FDA premarket approval in order to be marketed legally.

In 2011 the Swedish Medical Products Agency (www.lakemedelsverket.se) published a [screening method for preservatives in cosmetics products](#)* where the ZIC-HILIC column was used. Two methods were developed; one for the determination of diazolidinyl urea, imidazolidinyl urea, and Quaternium-15 and the other for formaldehyde.

Extraction procedures from topical gels are typically very high in organic solvent. 50 to 100% methanol, ethanol or acetonitrile. This is perfect for direct injection in HILIC. In this context **Allantoin** and **Urea**, common ingredients in cosmetic creams and pharmaceutical formulations can be seen as examples.

* <http://www.lakemedelsverket.se/upload/foretag/kosmetika/Screening%20procedure%20for%20determination%20K%20o%20H.pdf>

Impurities and Impurity Profiling

The definition of an impurity is any component of the new drug substance that is not the chemical entity defined as the new drug substance. Impurities in pharmaceuticals are the unwanted chemicals that remain with the API's, or develop during formulation, or upon aging of both API and formulated API's to medicines. The definition of an impurity profile is a description of the identified and unidentified impurities present in a new drug substance.

Drug Manufacturing follow Pharmacopoeia and thereby the ICH Quality Guidelines. (<http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html>).

Stability Q1A - Q1F

Q1A – Stability Testing of new Drug Substances and Products

Analytical Validation Q2

Impurities Q3A - Q3D

Q3A – Impurities in New Drug Substances

The guideline addresses the chemistry and safety aspects of impurities, including the listing of impurities in specifications and defines the thresholds for reporting, identification and qualification.

Q3B - Impurities in New Drug Products

Complements Q3A and provides advice in regard to impurities in products containing new, chemically synthesized drug substances. Specifically deals with those impurities which might arise as degradation products of the drug substance or arising from interactions between drug substance and excipients or components of primary packaging materials. The Guideline sets out a rationale for the reporting, identification and qualification of such impurities based on a scientific appraisal of likely and actual impurities observed, and of the safety implications, following the principles elaborated in the parent guideline. Threshold values for reporting and control of impurities are proposed, based on the maximum daily dose of the drug substance administered in the product.

Pharmacopoeias Q4

Quality of Biotechnological Products Q5A - Q5EC

Specifications Q6A- Q6B

Q6A- Specifications

Test procedures and acceptance criteria for new drug substances and new drug products: chemical substances.

Good Manufacturing Practice Q7

Pharmaceutical Development Q8

Quality Risk Management Q9

Pharmaceutical Quality System Q10

Development and Manufacture of Drug Substances Q11

Impurities and Impurity Profiling

In pharmaceutical products, impurities are not wanted. Detecting impurities early in the product development cycle could save a lot of both development time and money. A lot of effort has been spent on finding the set of columns having the most dissimilar selectivity to find possible impurities co-eluting with the main component. If the API has low retention or if ion pairing (IP) agents are needed for retention impurity profiling is even more difficult, in IP-chromatography retention is dominated by the IP agent making resolution of closely related compounds difficult.

Ionizable polar compounds can be retained on ion-exchange columns, but then only negative or positive analytes in each method, since ion exchange columns are either for cations or anions. Chromatography is all about the molecules, their physical and chemical properties, and how they interact with the mobile and stationary phase. Knowing the LogP/ LogD values, pKa values are important – Is it possible to ionize or de-ionize functional groups within the molecules? Solubility can sometimes be an issue and should be checked in a mobile phase prior injection on a HPLC column. Right selection of mobile phase components are crucial for optimum detection sensitivity, and with HILIC one mostly uses high percentage organic solvent and smaller amounts of buffer. The SeQuant® ZIC®-HILIC and ZIC®-cHILIC columns provide advantages for impurity profiling requirements for hydrophilic and polar molecules; either as raw materials, API's, excipients, or as counter-ions. With these columns the chromatographer can develop more straightforward robust and reliable methods; in many cases with much better sensitivity than a former RP based method.

HILIC can replace methods with RP columns and ion-pairing reagents in the mobile phase
HILIC can replace methods with RP columns and highly or completely aqueous mobile phases
HILIC can replace ion-exchange methods

New opportunities are, in addition, opened with the United States Pharmacopoeia Monograph Modernization initiative. <http://www.usp.org/usp-nf/development-process/monograph-modernization>

HILIC can be used with any liquid chromatography detection principle, and not only with mass spectrometry. A HILIC column can be connected with UV (DAD), refractive index (RI), fluorescence (FL), electrochemical (EC), evaporative light scattering (ELSD), corona discharge (CAD), and also with more recent aerosol-based detectors like the Quant Nano Quantity Analyte detector (NQAD).

In the context of impurity profiling a number of relevant applications, such as **Acetylcysteine, Azacitidine, Chondroitin sulfate, Citicoline, Decitabine, Metformin, Methotrexate, Pyroglutamic acid, Temozolomide** are included in this compilation. A detailed example, Metformin and related substance is presented where a SeQuant® ZIC®-HILIC are used. The new method comply with all required system suitability criteria. In addition, overall retentivity and the resolution among the two impurities are better than current monograph method (USP35-NF30 S2) where a column of L9 classification (strong cation exchanger) is used.

Pharmaceutical Counterion Analysis

The majority of drugs are produced in a salt form, where counterions can be of inorganic and organic, acidic or basic character. A drug's salt form has a major effect on quality, safety and performance. Salt formation can be used to increase or decrease solubility, alter dissolution rate, improve stability, improve (decrease) toxicity, reduce hygroscopicity, improve permeability, improve drug efficacy and extend patent protection. The ten most common counterions in pharmaceutical salts are; chloride, bromide, nitrate, ammonium, sulfate, tosylate (**Pyrimethamine**), phosphate, tartrate, maleate and ethylenediamine. In this compilation a HILIC separation of five common organic anions are presented (**acetate, succinate, maleate, tartrate, and citrate**). Depending on the property of the drug molecules, the stoichiometry can differ also. If an acidic drug is balanced with 3.5 % magnesium (Mg^{2+}), it needs double the amount or 7% of potassium (K^+) for counterion exchange. Accurate quantitation of drug salts is a needed step in pharmaceutical method development. The analysis of the drug and its counterion often, however, requires two separate methods. One for the drug and one for the counter-ion(s).

Counterion Analysis Requirements

Methods need to be precise, accurate, robust and easily transferred from one lab to another, from one site to another etc. Ultimately the API will be monitored for safety, identity, strength, purity and quality. Traditionally, counterion analysis have been performed with capillary electrophoresis, HPLC, ion chromatography (both suppressed and non-suppressed), wet chemistry (titration).

In 2006, Don Risley and Brian Pack at Eli Lilly published a groundbreaking paper in LCGC North America.¹⁾ The study illustrated how a SeQuant® ZIC®-HILIC column in conjunction with evaporative light scattering detection is capable of simultaneously retaining and separating both cations and anions within a single chromatographic analysis for the purpose of quantification in pharmaceutical products. In total 33 commonly used pharmaceutical counter ions, 12 cations, and 21 anions were investigated. A single gradient chromatographic HILIC method was finally developed, for analysis of both anions and cations, well separated from each other as well as from the parent API's. The benefit with ELSD is that it has no reliance on conductance (mobile phase Vs analyte); UV activity (chromophore) is not required; it is gradient compatible; and it is a universal detector useful for both ions and organic molecules. Hence, HILIC-ELSD is a truly platform independent technique. In this compilation, three applications with HILIC and ELSD is introduced:

Bis(2-chloroethyl)amine; Carnitine, "Caffeine, Choline, Taurine, Inositol and Carnitine", and Chondroitin sulfate.

Beside counterion analysis, there are requirements to monitor potential hazardous or harmful agents that are being used in the synthetic pathway to obtaining the final API, or alternatively monitor such degradation products from a formulation. Herein, this is represented by the applications on **Bis(2-chloroethyl)amine** (material for Cyclophosphamide synthesis), **2-chloroethanol** (Hydroxyzine HCl), **methanesulfonic acid** in Busulfan solution.

1) Simultaneous Determination of Positive and Negative Counterions Using a Hydrophilic Interaction Chromatography Method
D. S. Risley, B. W. Pack; LCGC North America, 24 (2006) 776-785

Dietary Supplements

The European Union's Food Supplements Directive of 2002 requires that supplements be demonstrated to be safe, both in dosages and in purity. The Food and Drug Administration (FDA) regulates dietary supplements as a category of foods, and not as drugs. In the US there is no such pre-marketing approval requirement but it is stipulated under requirements for cGMP "that the dietary supplement consistently meets the established specifications for identity, purity, strength, and composition and has been manufactured, packaged, labeled, and held under conditions to prevent adulteration".

Acetylcysteine (also known as N-acetylcysteine or N-acetyl-L-cysteine) is a derivative of cysteine. It is a pharmaceutical drug and nutritional supplement used primarily as a mucolytic agent and in the management of paracetamol (acetaminophen) overdose. As a dietary supplement acetylcysteine is commonly claimed to provide antioxidant and liver protecting effects.

4-aminobutyric acid (γ -Aminobutyric acid or GABA) is the chief inhibitory neurotransmitter in the mammalian central nervous system. Although chemically it is an amino acid, GABA is rarely referred to as such in the scientific or medical communities, because the term "amino acid," used without a qualifier, conventionally refers to the alpha amino acids, which GABA is not, nor is it ever incorporated into a protein. A number of commercial sources sell formulations of GABA for use as a dietary supplement

Chondroitin sulfate is a sulfated glycosaminoglycan (GAG) composed of a chain of alternating sugars (N-acetylgalactosamine and glucuronic acid). Chondroitin is in dietary supplements used as an alternative medicine to treat osteoarthritis and also approved and regulated as a symptomatic slow-acting drug for this disease. It is commonly sold together with glucosamine. Chondroitin and glucosamine are also used in veterinary medicine. A chondroitin chain can have over 100 individual sugars, each of which can be sulfated in variable positions and quantities. Since chondroitin is not a uniform substance, and is naturally present in a wide variety of forms, the precise composition of each supplement may vary. Although many food supplement companies produce their products in compliance with human food processing Good Manufacturing Practice (GMP), most of them do not produce their products in compliance with the GMP regulations for pharmaceuticals, resulting in products without pharmaceutical requirements. Along with glucosamine, chondroitin sulfate has become a widely used dietary supplement for treatment of osteoarthritis.

Sports nutrition supplements are especially rich in hydrophilic components such as amino acids, Creatine, Carnitine and Choline. Compounds that are very difficult to analyze with traditional C-18 reversed phase columns are easily done using the ZIC-HILIC analytical columns. **Pyroglutamic acid** (also known as 5-oxoproline, pidolic acid, or pyroglutamate for its basic form) is an uncommon amino acid derivative in which the free amino group of glutamic acid or glutamine cyclizes to form a lactam. Pyroglutamic acid is sold as a dietary supplement in the United States.

What is HILIC and why ZIC[®]-HILIC?

Analysis of polar molecules in complex mixtures is problematic since the separation is difficult due to their inherently poor retention in traditional reversed-phase liquid chromatography (RP-LC). As a solution, EMD Millipore have developed the high-quality SeQuant[®] ZIC[®]-HILIC range of HPLC columns, designed to retain and separate all types of polar and hydrophilic compounds and for robust chromatography with high selectivity and reproducibility. These columns are used in Hydrophilic Interaction Liquid Chromatography (HILIC) mode, which means buffered aqueous eluents rich in organic solvents such as acetonitrile. With this mode of operation follows also a couple of characteristic advantages such as low column back-pressure allowing high-speed separations, enhanced sensitivity when interfaced with mass spectrometry (MS), and simplified sample preparation schemes. By employing ZIC[®]-HILIC columns, laboratories can be more efficient and deliver more secure analysis results for polar and hydrophilic analytes. And this is regardless if it is well-equipped with sophisticated instrumentation such as LC-MS/MS, or rely on more traditional HPLC with detection by UV light absorption, ELSD (evaporative light scattering) or refractive index (RI) detection.

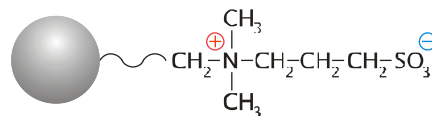
Separations are equally easy to develop on ZIC[®]-HILIC columns as on traditional RP HPLC columns since the eluents are similar, however, the difference is the effect of the water in the separation. In HILIC mode, water is the strongest solvent. To increase analyte retention, the organic portion of the mobile phase needs to be increased, and the water or buffer portions decreased. This will increase the hydrophilic partitioning into the water-enriched stationary phase, and thus increase the retention of the analyte.

The zwitterionic character of ZIC[®]-HILIC with a 1:1 balanced charge, gives further possibilities for selectivity by weak electrostatic interactions between the stationary phase and the molecules that are separated. This interaction can be tuned by changing buffer type and concentration, typically in the interval 5–50 mM. Buffer pH is also an important parameter to control retention, but also here the thinking is opposite to that of RP mode; more ionized compounds will be more hydrophilic and thus have more retention on ZIC[®]-HILIC.

More technical details on how to develop methods with ZIC[®]-HILIC can be found in the booklet 'A Practical Guide to HILIC', which is available free of charge from EMD Millipore printed or in online format (www.sequant.com/hilicguide).

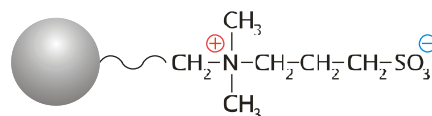
SeQuant® ZIC®-HILIC

Sorbent characteristics:	high-density zwitterionic sulfobetaine modification
Charge balance:	1:1
Particle material:	high-purity type B silica
Particle type:	spherical, fully porous
Particle size:	3.5 μm, 5 μm
Pore size:	100 Å, 200 Å
pH range:	pH 3 - 8
Max temperature:	70 °C
Max pressure:	350 bar (PEEK columns) or 400 bar
Column inner diameters:	0.075, 0.1, 0.3, 1.0, 2.1, 4.6, 7.5, 10, 21.2 mm
Column lengths:	20, 30, 50, 100, 150, 250 mm



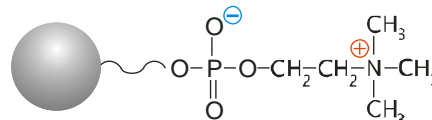
SeQuant® ZIC®-pHILIC

Sorbent characteristics:	high-density zwitterionic sulfobetaine modification
Charge balance:	1:1
Particle material:	high-purity polymer
Particle type:	spherical, fully porous
Particle size:	5 μm
pH range:	pH 2 - 12
Max temperature:	50 °C
Max pressure:	200 bar
Column inner diameters:	2.1, 4.6 mm
Column lengths:	50, 100, 150 mm

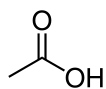


SeQuant® ZIC®-cHILIC

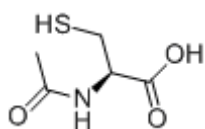
Sorbent characteristics:	high-density zwitterionic phosphorylcholine modification
Charge balance:	1:1
Particle material:	high-purity type B silica
Particle type:	spherical, fully porous
Particle size:	3 μm
Pore size:	100 Å
pH range:	pH 3 - 8
Max temperature:	70 °C
Max pressure:	350 bar (PEEK columns) or 400 bar
Column inner diameters:	0.3, 1.0, 2.1, 4.6 mm
Column lengths:	50, 100, 150, 250 mm



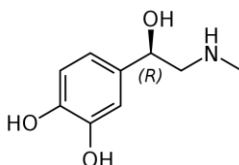
Molecular Structure Index



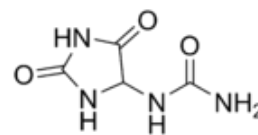
Acetic acid



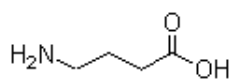
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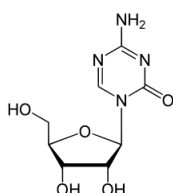
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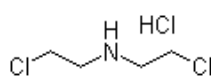
Allantoin



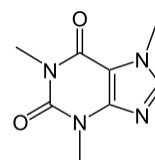
4-Aminobutyric acid



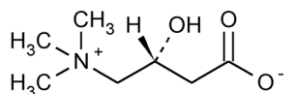
Azacitidine



Bis(2-chloroethyl)amine



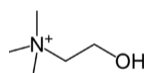
Caffeine



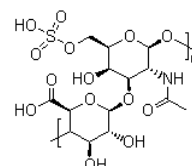
Carnitine



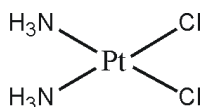
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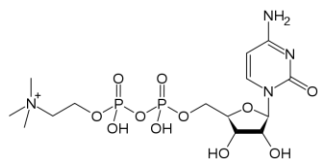
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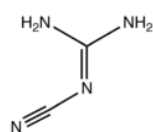
Chondroitin sulfate



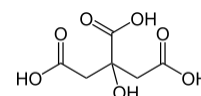
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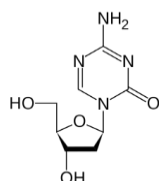
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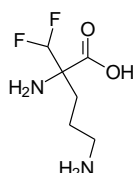
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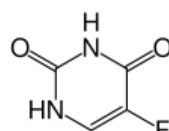
Citric acid



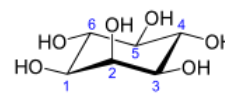
Decitabine



Eflornithine

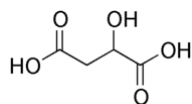


5-Fluorouracil

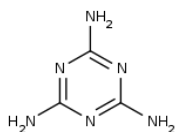


Inositol

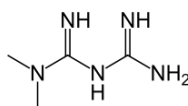
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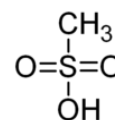
Malic acid



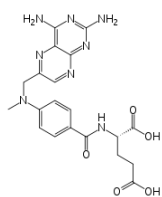
Melamine



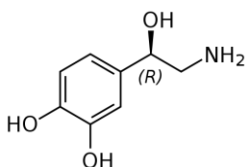
Metformin



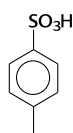
Methanesulfonic acid



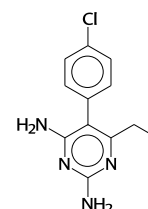
Methotrexate



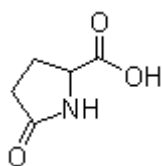
Noradrenaline



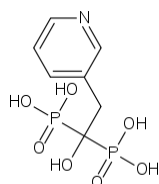
Para-toluenesulfonic acid



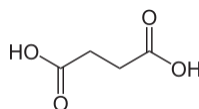
Pyrimethamine



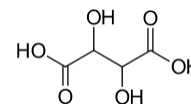
Pyroglutamic acid



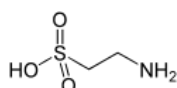
Risedronate



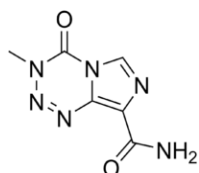
Succinic acid



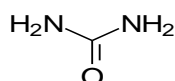
Tartaric acid



Taurine



Temozolomide



Urea

Tips and Tricks

Injection Solvent Effect in HILIC

Injection solvents with higher elution strength (i.e. more H₂O) than the mobile phase might cause problems in HILIC.

The effect of the injection solvent is a combination of solvent volume and elution strength. Therefore try to have the sample in mobile phase not water. Any organic solvent compatible with PEEK (no DMSO) will be better than water. If high water content samples are injected – reduce injection volume.

Be aware of the risk of sample precipitation with high concentration sample solutions dissolved in other solvents than the mobile phase.

Injection solvents with higher elution strength than the mobile phase might decrease the loading capacity.

Do's and Don'ts

Wash the system before and after use with water, to ensure a clean system without buffers that risk precipitation or contamination.

Change auto sampler needle-wash solvent to something HILIC-compatible, for example initial mobile phase composition without buffer salt or 90% acetonitrile in water.

Run slower gradients than in reversed phase, typically do not change more than 3% B/minute to achieve reproducible separations.

Try to use a mobile phase pH where the analytes of interest are ionized, this will enhance retention. A buffer or acid will be needed for most.

TFA and other ion pairing agents should be avoided since it will reduce retention dramatically.

Tips and Tricks

Guidelines for working with UV transparent buffers (i.e. phosphate) in HILIC

HILIC has up till now mainly been used as an MS friendly technique with volatile acetate or formate buffers. For applications with UV detectors RP columns and phosphate buffer has been predominant.

Using HILIC with low UV cut of buffers like phosphate is possible despite the limited solubility of potassium phosphate in high acetonitrile eluents. There are some guidelines to using phosphate buffer in HILIC, the same rules apply to RP when using a high proportion of acetonitrile in the eluent.

1. Always use premixed eluents. Never use pure Acetonitrile as one mobile phase constituent as precipitation can occur at the point of mixing in the HPLC system.
2. Try not to use over 80% Acetonitrile, at low buffer strengths 85% is the absolute maximum.
3. If using gradients make the difference between mobile phase A and B as small as possible to limit the risk of phosphate precipitation.
4. HILIC gradients should be shallower than in RP since changes in mobile phase has a larger effect in HILIC than in RP. And start the gradient with as strong eluent as possible.
5. In HILIC start the gradient with as strong eluent as possible, i.e. with as high aqueous portion as possible. This reduces column equilibration time and reduces the risk of precipitation.

HILIC is not just HILIC

Don't give up if the first HILIC column you try has retention but not enough selectivity. There are plenty of HILIC columns commercially available, but it is not as with C18 columns where the selectivity is similar. Different types of HILIC columns do not behave in same manner, but rather VERY differently.

The bonded zwitterionic SeQuant® HILIC columns from EMD Millipore can be used for all type of HILIC separations (differently to diol, plain silica and amino phases which are of more limited use). The weak electrostatic interactions provided by the stationary phase zwitterionic functional groups, overlaid with the hydrophilic partitioning, provide a very powerful tool for successful operation in your laboratory. Separation of polar and hydrophilic compounds are not difficult to separate any more, but you need to pay more attention to the chemistry of the sample applications

Application Index

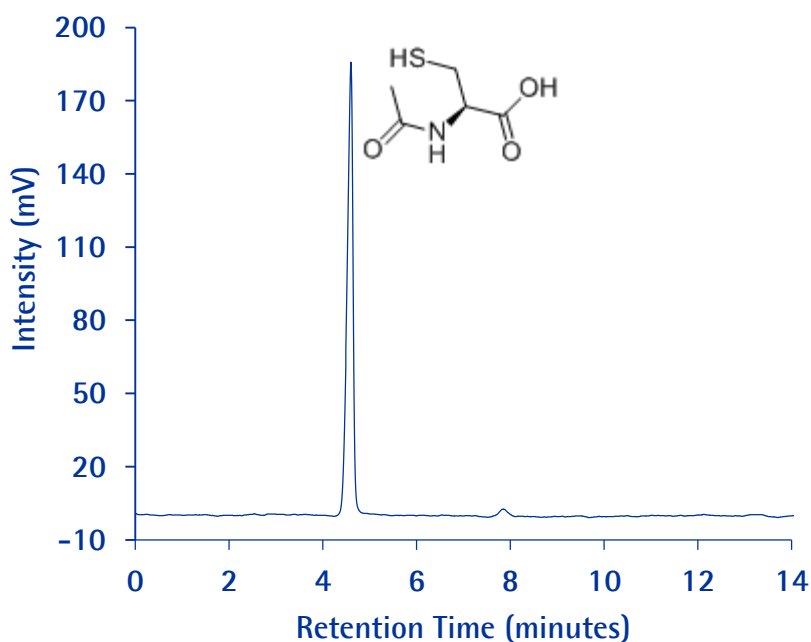
Molecule Name	Column Used	Page
Acetylcysteine Granules	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 250x4.6 mm	
Adrenaline and Noradrenaline	SeQuant® ZIC®-HILIC (3.5 µm, 100 Å) 100x2.1 mm	
Allantoin	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 150x4.6 mm	
4-Aminobutyric Acid	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 250x4.6 mm	
Azacitidine and Related Compound	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 150x4.6 mm	
Bis(2-chloroethyl)amine	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 150x4.6 mm	
Carnitine and Impurity A	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 150x4.6 mm	
Caffeine, Choline, Taurine, Inositol and Carnitine	SeQuant® ZIC®-HILIC (3.5 µm, 100 Å) 150x2.1 mm	
2-Chloroethanol	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 150x4.6 mm	
Chondroitin sulfate and Impurity A	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 250x4.6 mm	
Cisplatin and Mono-hydrated Cisplatin	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 150x2.1 mm	
Citicoline	SeQuant® ZIC®-cHILIC (3 µm, 100 Å) 150x4.6 mm	
Decitabine	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 150x4.6 mm	
Eflornithine	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 50x4.6 mm	
5-Fluorouracil and 5-Fluorouracil Dimer	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 150x4.6 mm	
Methanesulfonic Acid in Busulfan Injection	SeQuant® ZIC®-HILIC (3.5 µm, 100 Å) 150x4.6 mm	
Metformin and Related Impurities	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 250x4.6 mm	
Methotrexate	SeQuant® ZIC®-pHILIC (5 µm) 100x2.1 mm	
Organic Acids	SeQuant® ZIC®-cHILIC (3 µm, 100 Å) 150x2.1 mm	
Pyrimethamine and Para-toluenesulfonic acid	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 150x4.6 mm	
Pyroglutamic Acid and Impurity	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 250x4.6 mm	
Risedronate sodium and Related Impurities	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 100x4.6 mm	
Temozolomide and Related Impurities	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 250x4.6 mm	
Urea	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 50x4.6 mm	

Acetylcysteine Granules

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 250x4.6 mm	1.50458.0001
Injection:	1 µL	
Detection:	Agilent 1200, UV 205 nm	
Cell:	1 µl/10 mm	
Flow Rate:	1.0 mL/min	
Mobile Phase (v/v):	Buffer: 10mM of KH ₂ PO ₄ ,pH=5.6, Mix Acetonitrile and Buffer 15:85 (v/v) Total ionic strength: 1.5 mM	
Temperature:	30 °C	
Diluent:	Acetonitrile/water solution (70/30 v/v)	
Sample:	1mg/ml of Acetylcysteine in diluent	



Chromatographic Data

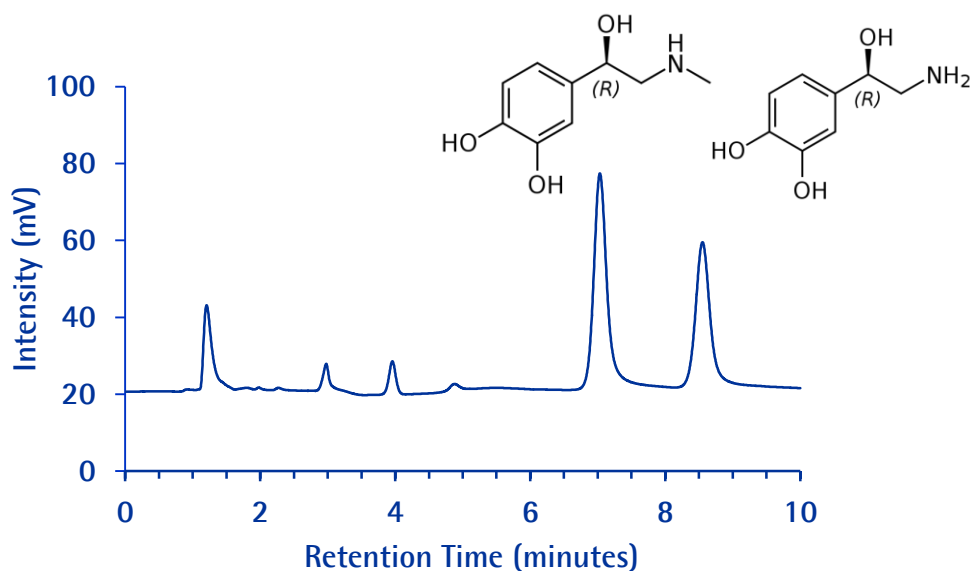
No.	Compound	Time	Tailing Factor	Resolution*
1	Acetylcysteine	4.6	0.8	0.0
2	Impurity A	7.8	1.1	2.4

Adrenaline and Noradrenaline

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-HILIC (3.5 µm, 100 Å) 100x2.1 mm	1.50441.0001
Injection:	2 µL	
Detection:	UV @ 210 nm	
Cell:	Micro-flow cell (2.5 µl)	
Flow Rate:	0.20 mL/min	
Mobile Phase (v/v):	Buffer: 40 mM ammonium acetate in water, pH 6.8 Mix acetonitrile and buffer by volume 80:20. Total ionic strength: 8 mM	
Temperature:	Ambient	
Diluent:	Mobile phase	
Sample:	25 µg/mL (25 ppm) Noradrenaline and 25 µg/mL (25 ppm) Adrenaline	
Pressure Drop:	56 Bar (806 psi)	



Chromatographic Data

No.	Compound	Time	Tailing Factor	Retention Factor (k')	Resolution
1	void volume	1.0	-	-	-
2	Adrenaline (A)	7.0	1.2	6.0	-
3	Noradrenaline (NA)	8.6	1.2	7.6	4.2

Allantoin

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column: SeQuant® ZIC®-HILIC (5 µm, 200 Å) 150x4.6 mm 1.50455.0001
 Injection: 20 µL
 Detection: UV at 210 nm. Shimadzu Prominence
 Cell: 10 µL
 Flow Rate: 0.5 mL/min.

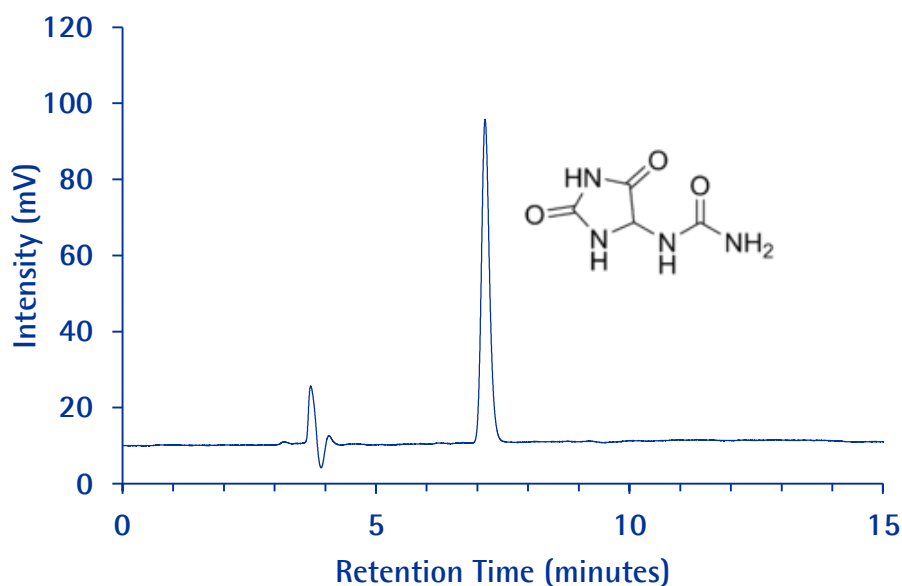
Mobile Phase (v/v): Add 1.0 ml of formic acid in 900 ml water. Mix well and dilute to 1L with water.
 Mix buffer and acetonitrile 25:75 (v/v)

Temperature: 30 °C

Diluent Mobile phase

Sample: Weigh 30 mg of Allantoin in 100 ml volumetric flask. Dilute up to the mark with mobile phase.
 Pipette out 5 ml of the above solution and dilute to 50 ml with mobile phase.

Pressure Drop: 16 bar (232 psi)



Chromatographic Data

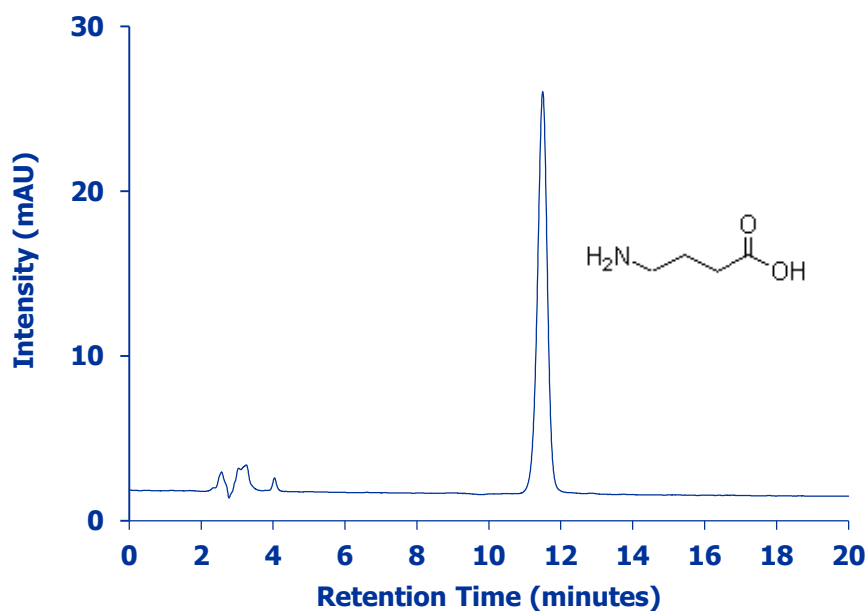
No.	Compound	Time (min)	Tailing Factor	Theoretical Plates
	Void volume (t ₀)	4.0	-	-
1	Allantoin	7.2	1.2	9907

4-Aminobutyric acid (GABA)

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 250 x 4.6 mm	1.50458.0001
Injection:	20 µl	
Detection:	Agilent 1200 system with UV detection@205 nm	
Cell:	10 µl	
Flow Rate:	1.0 mL/min	
Mobile Phase (v/v):	Buffer: Potassium dihydrogenphosphate (KH ₂ PO ₄) 20mM pH 4.5. Mix acetonitrile and buffer 70:30 (v/v). Total ionic strength: 6mM.	
Temperature:	Ambient	
Diluent:	Mobile phase	
Sample:	0.5mg/ml of 4-Aminobutyric acid in diluent	
Pressure Drop:	48 Bar (696 psi)	



Chromatographic Data

No.	Compound	Time	Retention Factor	Theoretical Plates	(TUSP)
1	Void volume	2.5	-	-	-
2	4-Aminobutyric acid	11.5	3.6	8919	0.92

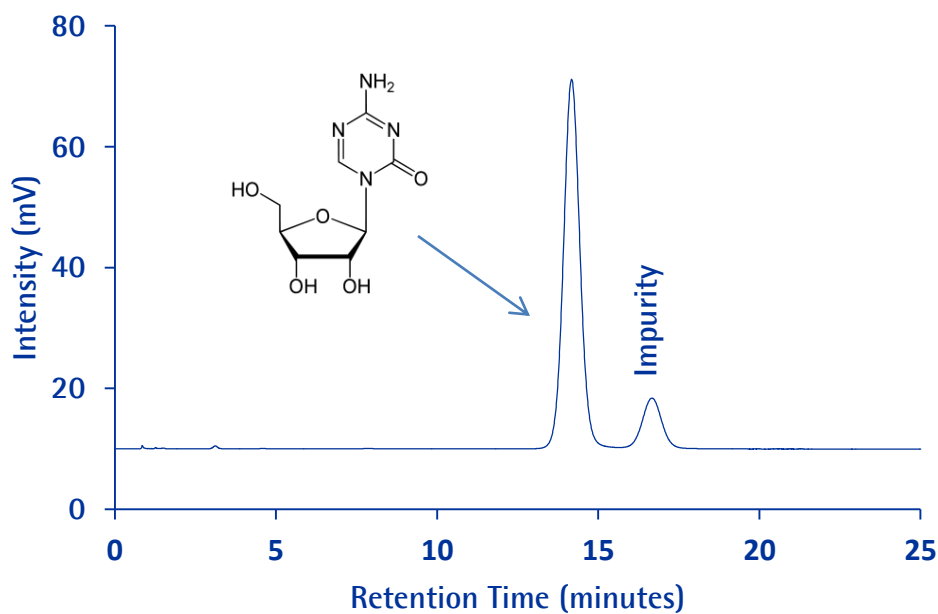
Azacitidine and Related Impurities

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column: SeQuant® ZIC®-HILIC (5 µm, 200 Å) 150x4.6 mm 1.50455.0001
 Injection: 10 µL
 Detection: UV at 242 nm. Shimadzu Prominence
 Cell: 10 µL
 Flow Rate: 2.0 mL/min.

Mobile Phase (v/v): Dissolve 0.77 g of ammonium acetate in 1L water (10 mM).
 Mix buffer and acetonitrile 10:90 (v/v). Total ionic strength: 1 mM
 Temperature: 25 °C
 Diluent: Mobile phase
 Sample: Weigh 50 mg of sample in 100 ml volumetric flask. Dilute up to the mark with mobile phase.
 Pressure Drop: 57 bar (826 psi)



Chromatographic Data

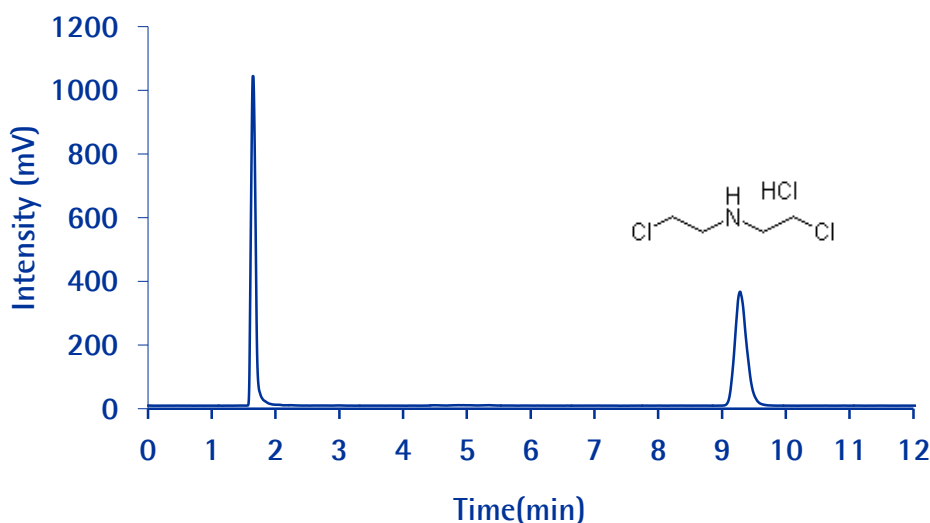
No.	Compound	Time (min)	Tailing Factor	Resolution
1	Azacitidine	14.2	1.1	-
2	Impurity	16.7	1.0	2.5

Bis(2-chloroethyl)amine

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 150x4.6 mm	1.50455.0001
Injection:	10 µL	
Detection:	Agilent 1260, ELSD	
Flow Rate:	1.0 mL/min.	
Mobile Phase (v/v):	Acetonitrile and 50 mM ammonium acetate, pH 5.9) 85:15 (v/v). Total ionic strength: 7.5 mM	
Temperature:	30 °C	
Diluent	Mobile phase	
Sample:	0.5 mg/ml of Cyclophosphamide and Bis(2-chloroethyl)amine in diluent.	
Pressure Drop:	80 bar (1200 psi)	



Chromatographic Data

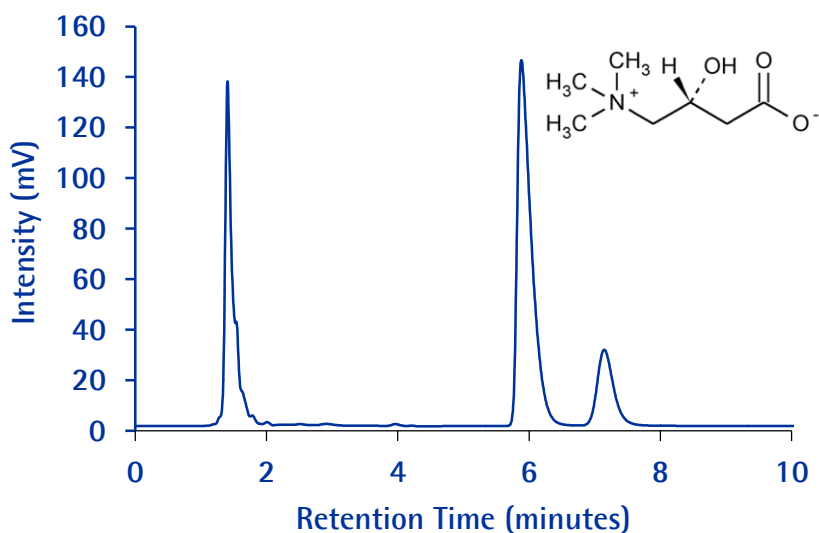
No.	Compound	Time (min)	Retention Factor	Asymmetry
1	Cyclophosphamide (CTX)	1.6	-	-
2	Bis(2-chloroethyl)amine	9.3	11.5	1.10

Separation of Carnitine and Impurity A

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 150x4.6 mm	1.50455.0001
Injection:	20 µL	
Detection:	UV 205 nm	
Flow Rate:	1.0 mL/min	
Mobile Phase (v/v):	Acetonitrile and Potassium Dihydrogen phosphate (KH ₂ PO ₄) 20 mM, pH 4.2. Mix Acetonitrile and buffer 70:30.	
Temperature:	30°C	
Diluent:	Mobile phase	
Sample:	1 mg/mL of L-Carnitine and 0.3mg/ml of impurity A in mobile phase	



Chromatographic Data

No.	Compound	Time (min)	Retention Factor (k')	Resolution
1	L-Carnitine	5.9	2.9	-
2	Impurity A	7.1	3.7	3.1

Caffeine, Choline, Taurine, Inositol and Carnitine

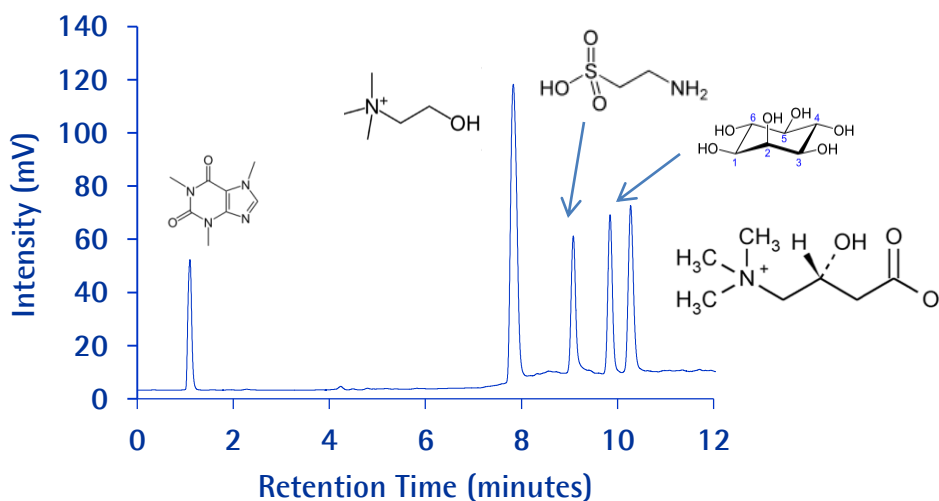
SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column: SeQuant® ZIC®-HILIC (3.5 µm, 100 Å) 150x2.1 mm 1.50442.0001
 Injection: 5 µl
 Detection: ELSD (Sedere Sedex 85LT), 40°C, 3,5 bar pressurized air, Gain 6
 Flow Rate: 0.4 mL/min
 Mobile Phase (v/v): A: Acetonitrile and ammonium acetate 100 mM, pH 4.5. (90:10 v/v)
 B: Ammonium acetate 100 mM, pH 4.5
 Gradient: See Table

Time (min)	Solution A	Solution B	Elution
0.0-2.0	100	0	isocratic
2.01-12.0	100→60	0→40	gradient
12.01-17.0	100	0	equilibration

Temperature: 30°C
 Diluent: Initial mobile phase
 Sample: 0.5 mg/mL of caffeine, taurine, inositol carnitine, and 0.75 mg/mL of Choline in diluent



Chromatographic Data

No.	Compound	Retention Time (min)	Resolution	Tailing Factor
1	Caffeine	1.1	-	1.1
2	Choline	7.8	35.6	1.3
3	Taurine	9.1	6.2	1.3
4	Inositol	9.8	4.6	1.1
5	Carnitine	10.3	2.3	1.2

2-Chloroethanol

SeQuant® ZIC®-HILIC

Chromatographic Conditions

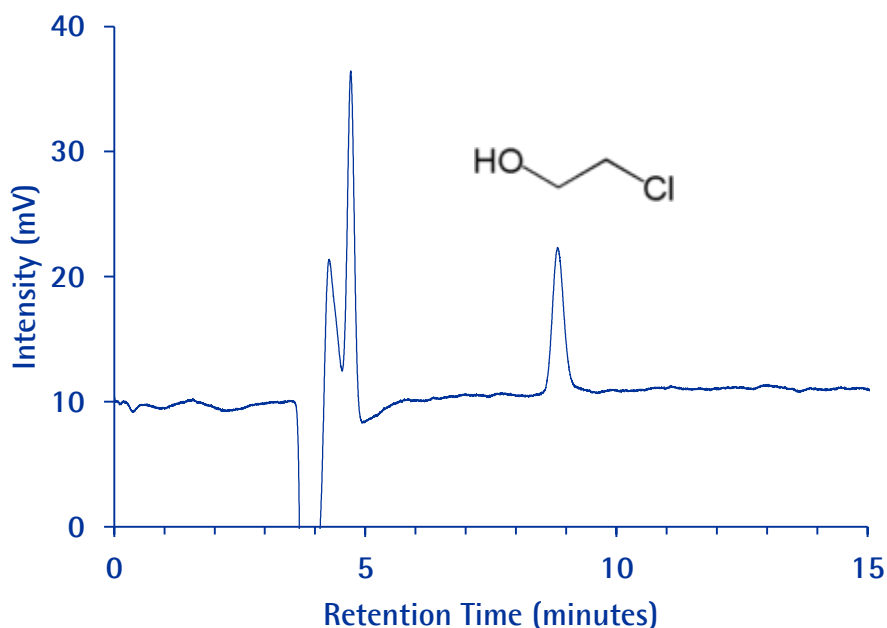
Column: SeQuant® ZIC®-HILIC (5 µm, 200 Å) 150x4.6 mm 1.50455.0001
 Injection: 25 µL
 Detection: RI (Detection Range: 1, Cell temperature: 40°C) Shimadzu Prominence
 Cell: 9 µL
 Flow Rate: 0.5 mL/min.

Mobile Phase (v/v): Dissolve 3.08 g of ammonium acetate in 1L water (40 mM).
 Mix buffer and acetonitrile 25:75 (v/v). Total ionic strength: 10 mM

Temperature: 40 °C
 Diluent: Mobile phase

Sample: Weigh 100 mg of sample in 100 ml volumetric flask. Dilute up to the mark with mobile phase.
 Pipette out 10 ml of the above solution & dilute to 100 ml with mobile phase.

Pressure Drop: 83 bar (1203 psi)



Chromatographic Data

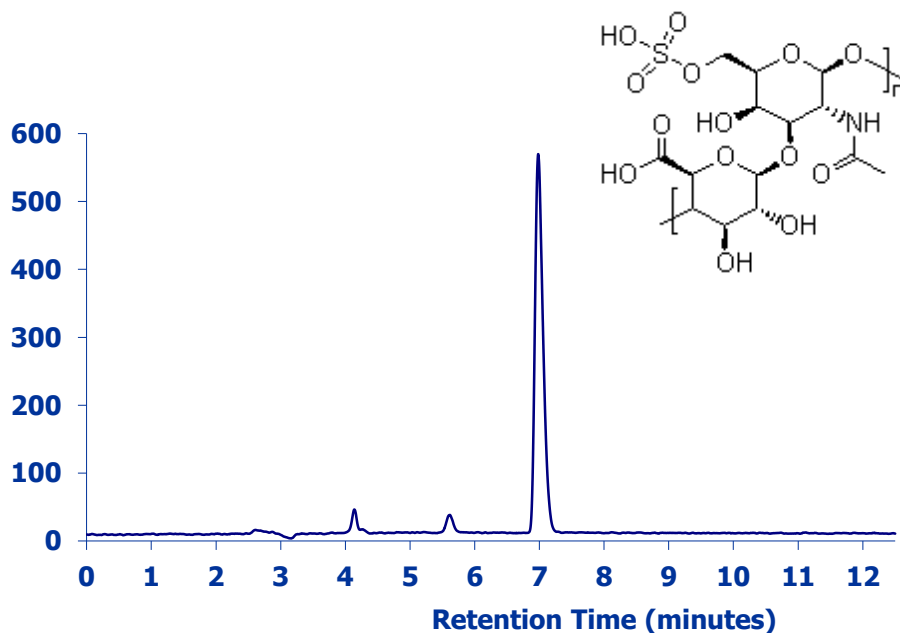
No.	Compound	Retention Time (min)	Tailing Factor	Theoretical Plate
1	2-Chloroethanol	8.8	1.2	7149

Chondroitin Sulfate

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 250×4.6 mm	1.50458.0001
Injection:	10 µL	
Detection:	Agilent 1200, ELSD	
Flow Rate:	1.0 mL/min	
Mobile Phase (v/v):	Buffer: Ammonium acetate 50mM pH 5.0. Mix Acetonitrile and Buffer 60:40 (v/v) (Total ionic strength: 20 mM)	
Temperature:	30 °C	
Diluent:	Mobile phase	
Sample:	1mg/ml of Chondroitin sulfate in diluent	
Pressure:	80 Bar (1200 psi)	



Chromatographic Data

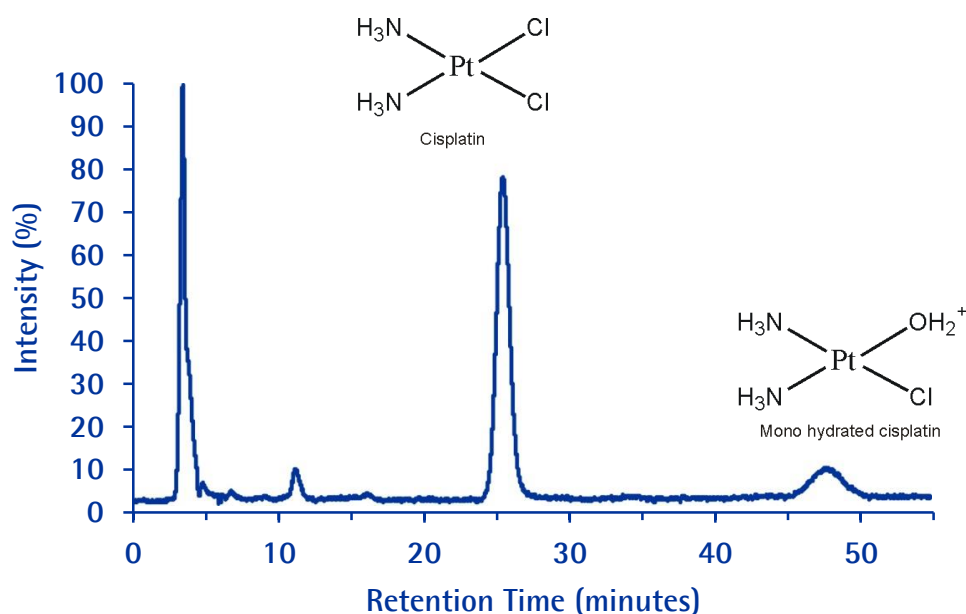
No.	Compound	Time	Retention Factor	Resolution*
1	Impurity A	5.6	1.3	-
2	Chondroitin sulfate	7.0	1.8	6.4

Cisplatin

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column: SeQuant® ZIC®-HILIC (5 µm, 200 Å) 150x2.1 mm 1.50454.0001
 Injection: 1 µl
 Detection: UV @ 305nm
 Flow Rate: 0.1 mL/min
 Mobile Phase : Buffer: Ammonium formate 25mM pH 6.5. Mix 1,4-dioxane and Buffer 80:20 (v/v)
 (Total ionic strength: 5mM).
 Temperature: Ambient
 Diluent: Mobile phase without buffer
 Sample: Cisplatin



Chromatographic Data

No.	Compound	Retention Time (min)	Resolution	Asymmetry
1	Cisplatin	25.6	-	-
2	Monohydrated cisplatin	47.8	-	-

Citicoline

SeQuant® ZIC®-cHILIC

Chromatographic Conditions

Column: SeQuant® ZIC®-cHILIC (3 µm, 100 Å) 150×4.6 mm 1.50661.0001
 Injection: 20 µL
 Detection: Shimadzu Prominence, UV 276 nm
 Flow Rate: 0.75 mL/min

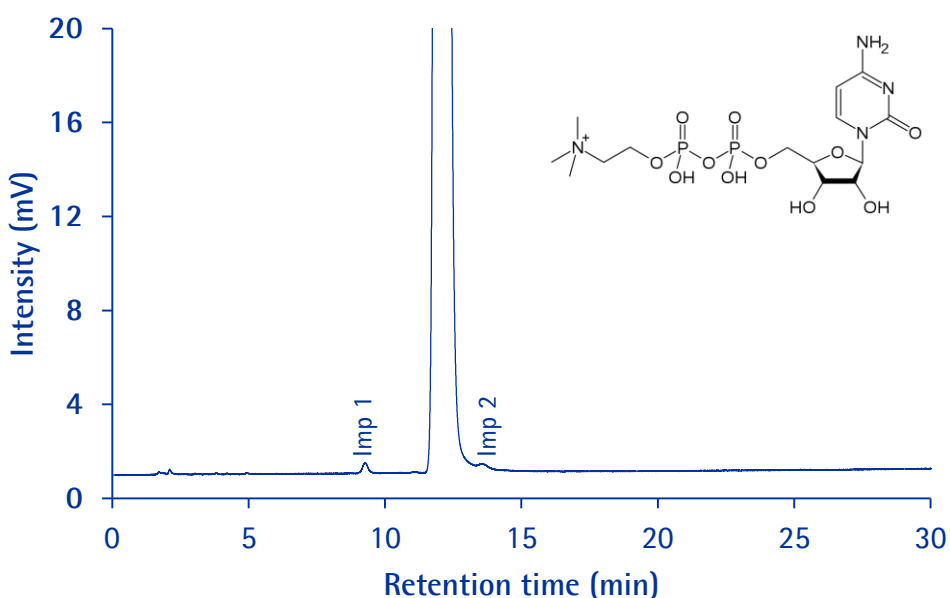
Mobile Phase (v/v): Buffer: Weigh 7.7 g ammonium acetate and dissolve in 1L MilliQ water.
 Mix acetonitrile and buffer 70:30 (v/v). Total ionic strength: 30 mM

Temperature: 30 °C

Diluent: Mobile phase

Sample: Weigh 25 mg substance in 50 ml volumetric flask. Dissolve in diluent.

Pressure: 59 Bar (856 psi)



Chromatographic Data

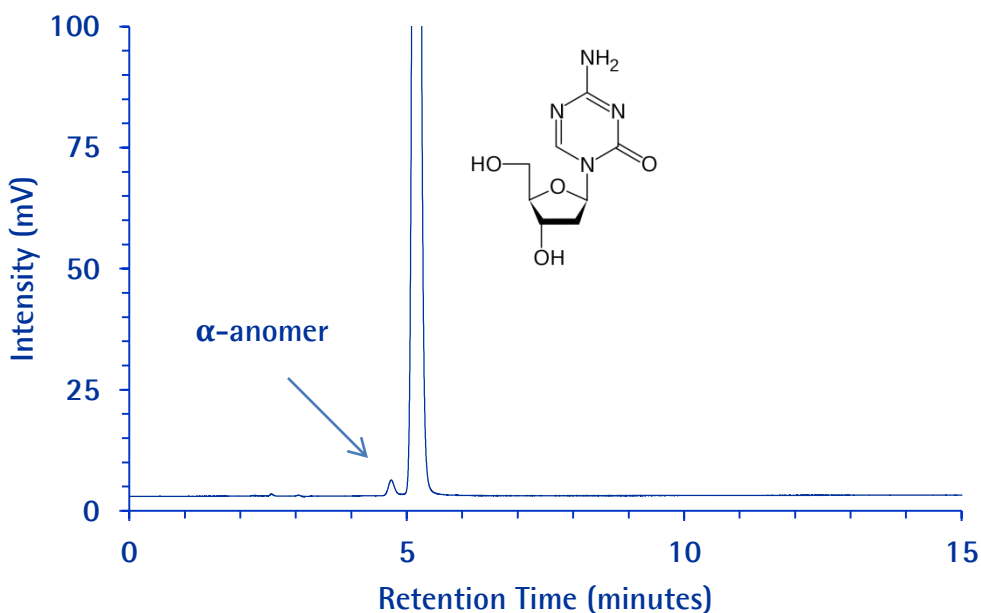
No.	Compound	Time	T _{USP}	Resolution*
1	Impurity 1	9.3	1.1	-
2	Citicoline	12.0	1.1	7.1
3	Impurity 2	13.6	1.1	3.9

Decitabine

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 150x4.6 mm	1.50455.0001
Injection:	10 µL	
Detection:	Shimadzu Prominence, UV@254 nm	
Cell:	10 µl	
Flow Rate:	0.75 mL/min	
Mobile Phase (v/v):	Acetonitrile and 50 mM ammonium acetate in water, pH 6.8 (85:15 v/v) Total ionic strength: 7.5 mM.	
Temperature:	Ambient	
Diluent	Mobile phase	
Sample:	200 ppm of Decitabine in mobile phase	
Pressure Drop:	30 Bar (435 psi)	



Chromatographic Data

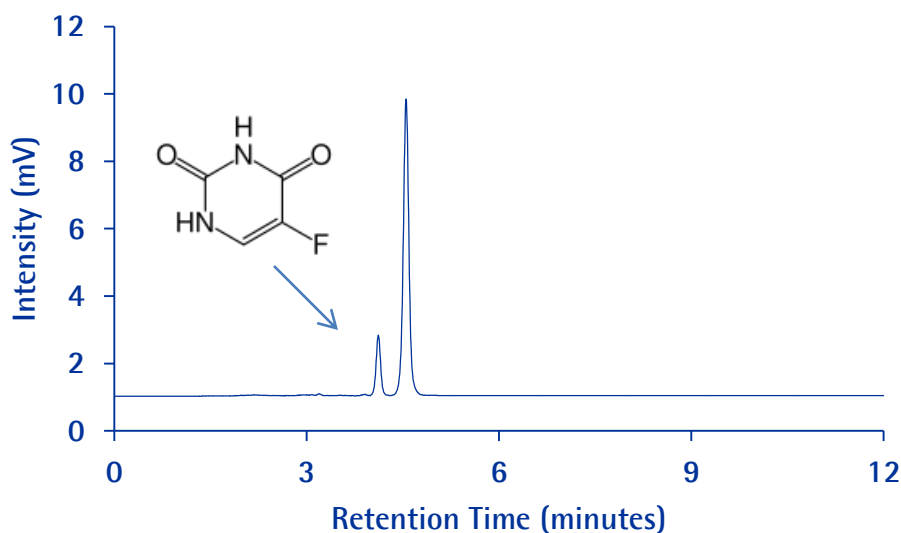
No.	Compound	Time	Tailing Factor	Retention Factor (k')	Resolution
1	α- Anomer	4.7	1.1	1.4	-
	Decitabine	5.2	1.1	1.6	2.2

5-Fluorouracil and 5-Fluorouracil Dimer

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 150x4.6 mm	1.50455.0001
Injection:	10 µL	
Detection:	Shimadzu Prominence, UV@254 nm	
Cell:	10 µl	
Flow Rate:	0.5 mL/min	
Mobile Phase (v/v):	Buffer: Dissolve 1.54 g of ammonium acetate in 1L water (20 mM). Mix acetonitrile and buffer by volume 80:20. Total ionic strength: 4 mM	
Temperature:	25°C	
Diluent	Mobile phase	
Sample:	Weigh 50 mg of each substance in 100 ml volumetric flask. Dilute up to mark with mobile phase. Pipette out 10 ml of the above solution & dilute to 100 ml with mobile phase.	
Pressure Drop:	29 Bar (418 psi)	



Chromatographic Data

No.	Compound	Time	Tailing Factor	Resolution
1	5-Fluorouracil	4.1	1.1	
2	5-Fluorouracil Dimer	4.6	1.1	2.6

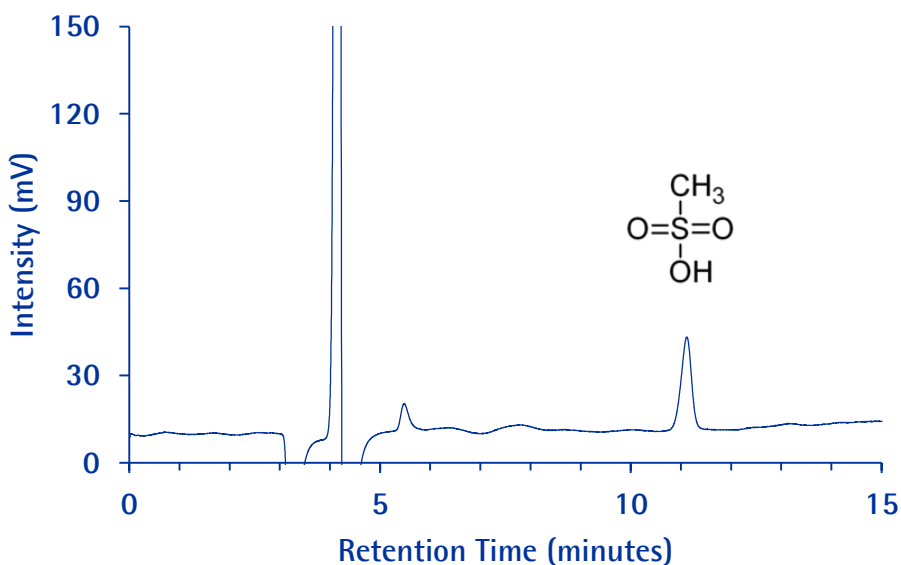
Methanesulfonic Acid in Busulfan Injection

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column: SeQuant® ZIC®-HILIC (3.5 µm, 200 Å) 150×4.6 mm 1.50444.0001
 Injection: 20 µL
 Detection: Shimadzu Prominence, RI (cell temperature 40 °C, Range 1.0)
 Cell: 10 µL
 Flow Rate: 0.5 mL/min

Mobile Phase: Buffer: Dissolve 7.7 g of ammonium acetate in 1000 ml water (100 mM).
 Adjust pH to 4.5 with glacial acetic acid. Mix Acetonitrile and Buffer 80:20 (v/v)
 Total ionic strength: 20 mM
 Temperature: 40 °C
 Diluent: Mobile phase
 Sample: Weigh 20 mg of sample in 100 ml volumetric flask. Dilute up to the mark with mobile phase.
 Pressure Drop: 57 bar (826 psi)



Chromatographic Data

No.	Compound	Time	Theoretical Plates	Tailing Factor
1	Methanesulfonic Acid	11.1	12917	1.0

USP Method – Metformin RS (USP35–NF30 S2)

Column: L9 classification – Partisil 10 SCX Related compounds 4.6 mm x 25 cm, 10 µm.
(Not official USP–NF text; and thereby not bound to specific brand)

Mobile phase:

Prepare a solution in water, containing 17 g of monobasic ammonium phosphate per L, adjust with phosphoric acid to a pH of 3.0, and mix.

Standard solution:

Prepare a solution of USP Metformin Related Compound A RS in water having a known concentration of about 0.2 mg per mL. Transfer 1.0 mL of this solution to a 200–mL volumetric flask, dilute with Mobile phase to volume, and mix. (note—Metformin related compound A is 1–cyanoguanidine)

Test solution:

Transfer about 500 mg of Metformin Hydrochloride, accurately weighed, to a 100–mL volumetric flask, dissolve in and dilute with Mobile phase to volume, and mix.

Diluted test solution:

Transfer 1.0 mL of the Test solution to a 10–mL volumetric flask, dilute with Mobile phase to volume, and mix. Transfer 1.0 mL of this solution to a 100–mL volumetric flask, dilute with Mobile phase to volume, and mix.

Resolution solution:

Prepare a solution in water containing about 0.25 mg of metformin hydrochloride and about 0.1 mg of melamine per mL. Transfer 1.0 mL of this solution to a 50–mL volumetric flask, dilute with Mobile phase to volume, and mix.

Chromatographic system:

The liquid chromatograph is equipped with a 218–nm detector and a 250x4.6 mm column containing packing L9. The flow rate is about 1.0 to 1.7 mL per minute. Chromatograph the Resolution solution, and record the peak responses as directed for Procedure: **the resolution, R, between melamine and metformin is not less than 10.**

Procedure:

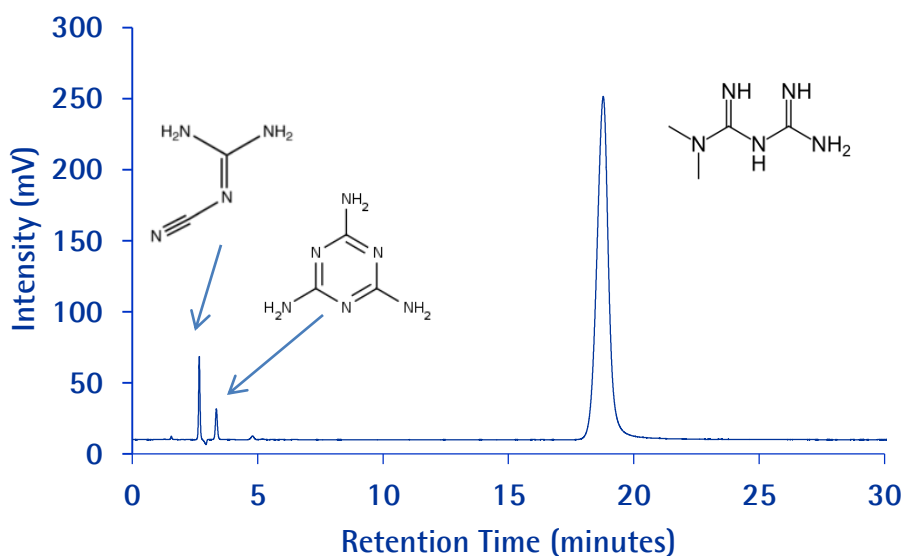
Separately inject equal volumes (about 20 µL) of the Test solution, the Standard solution, and the Diluted test solution into the chromatograph, **record the chromatograms for not less than twice the retention time of metformin**, and measure the peak areas. Calculate the percentage of metformin related compound A in the portion of Metformin Hydrochloride

Metformin and Related Impurities

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 250×4.6 mm	1.50458.0001
Injection:	10 µL	
Detection:	Shimadzu LC-10, UV 218 nm	
Cell:	10 µl	
Flow Rate:	1.5 mL/min	
Mobile Phase:	Buffer: Dissolve 0.77 g of ammonium acetate in 1000 ml water (10 mM). Mix Acetonitrile and Buffer 85:15 (v/v). Total ionic strength: 1.5 mM	
Temperature:	30 °C	
Diluent:	Mobile phase	
Sample:	500 ppm metformin and 4 ppm of each impurity in mobile phase	
Pressure Drop:	63 bar (913 psi)	



Chromatographic Data

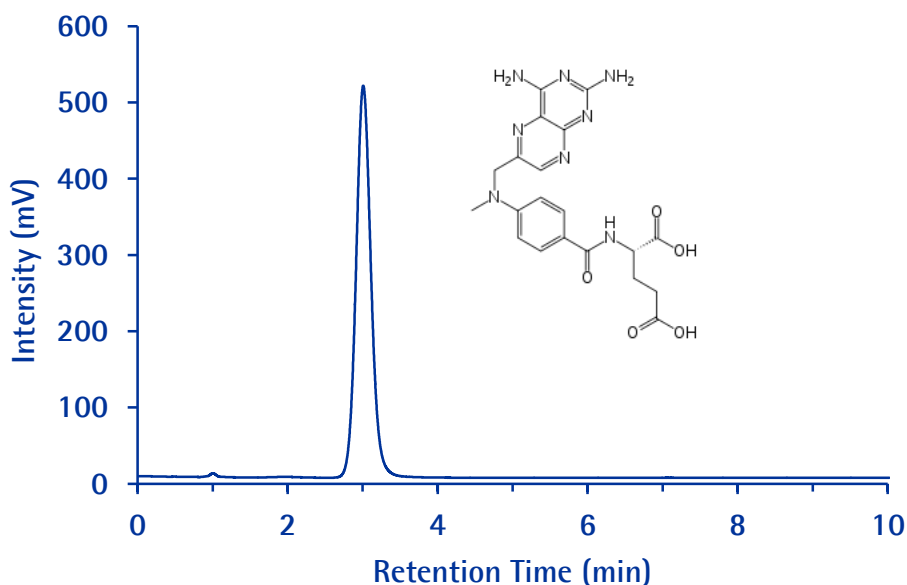
No.	Compound	Time	Theoretical Plates	Tailing Factor
1	Cyanoguanidine	2.7	11209	1.1
2	Melamine	3.3	8937	1.1
3	Metformin	18.8	8042	1.0

Methotrexate

SeQuant® ZIC®-pHILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-pHILIC (5 µm) 100x2.1 mm	1.50462.0001
Injection:	3 µL	
Detection:	Shimadzu UFLC XR, PDA 302 nm with 10 µL flow-cell	
Flow Rate:	0.5 mL/min.	
Mobile Phase:	Acetonitrile and 20mM ammonium acetate buffer pH 6.8 (80:20 v/v) Total ionic strength: 4 mM	
Temperature:	40 °C	
Diluent	Mobile phase	
Sample:	500 ppm of Methotrexate in mobile phase	
Pressure Drop:	103 Bar (1494 psi)	



Chromatographic Data

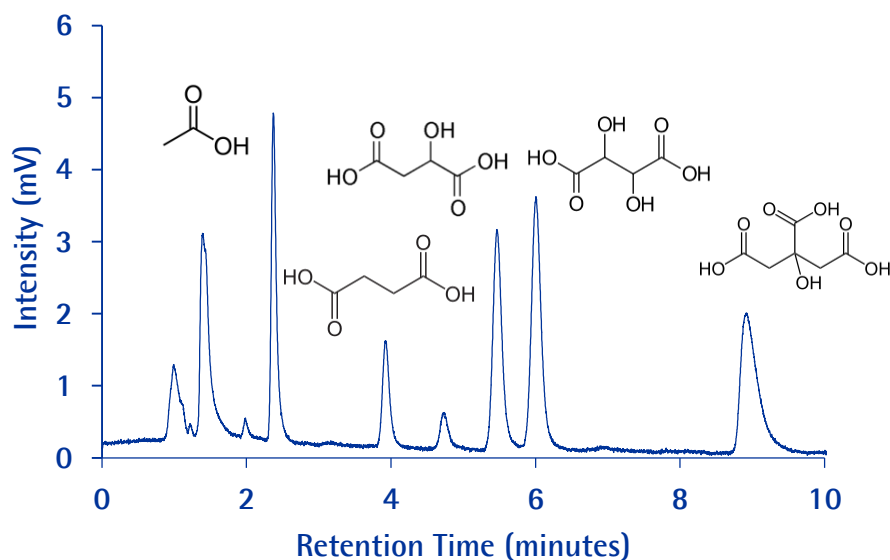
No	Compound	Time (min)	Resolution	Asymmetry
1	Impurity 1	2.4	-	1.0
2	Methotrexate	3.0	2.2	1.1
3	Impurity 2	5.5	4.5	0.9
4	Impurity 3	8.9	2.2	1.2

Organic Acids

SeQuant® ZIC®-cHILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-cHILIC (3 µm, 100 Å) 150x2.1 mm	1.50658.0001
Injection:	5 µL	
Detection:	UV at 200 nm. Shimadzu LC-10Vp equipped with 2.5µL semi-micro flow-cell	
Flow Rate:	0.3 mL/min.	
Mobile Phase:	Acetonitrile and 25mM Potassium Phosphate buffer pH 6.0 (75:25 v/v) Total ionic strength: 6.25 mM	
Temperature:	30 °C	
Diluent	Mobile phase	
Sample:	10 ppm of each analyte diluted in mobile phase	



Chromatographic Data

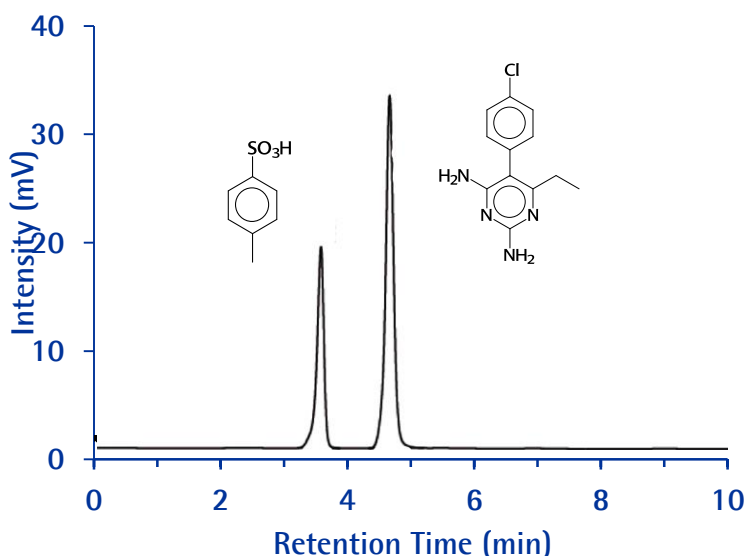
No.	Compound	Time (min)	Retention Factor
	Void volume (t ₀)	1	-
1	Acetic acid	2.4	1.4
2	Succinic acid	3.9	2.9
3	Malic acid	5.5	4.5
4	Tartaric acid	6.0	5.0
5	Citric acid	8.9	7.9

Pyrimethamine and Para-Toluenesulfonic Acid

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 150×4.6 mm	1.50455.0001
Injection:	1 µL	
Detection:	Shimadzu Prominence, UV 254	
Cell:	10 µl	
Flow Rate:	0.5 mL/min	
Mobile Phase:	Acetonitrile and Ammonium Acetate 10 mM, pH 6.8 mixed in 90:10 (v/v) Total ionic strength: 1 mM	
Temperature:	40 °C	
Diluent:	Mobile phase	
Sample:	100 ppm solution of each diluted in mobile phase.	
Pressure Drop:	14 bar (202 psi)	



Chromatographic Data

No.	Compound	Time (min)	Retention Factor
	Void volume (t ₀)	3.0	-
1	p-toluenesulfonic acid (LogP: 1.7)	3.5	0.2
5	Pyrimethamine (LogP: 2.7)	4.6	0.5

Pyroglutamic Acid and Related Impurity

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column: SeQuant® ZIC®-HILIC (5 µm, 200) 250×4.6 mm

1.50458.0001

Injection: 5 µL

Detection: Agilent 1200, UV 210 nm

Flow Rate: 1.0 mL/min

Mobile Phase (v/v): A: 10 mM of potassium dihydrogenphosphate (KH₂PO₄) pH 3.
B: 100% Acetonitrile

Gradient: See Table:

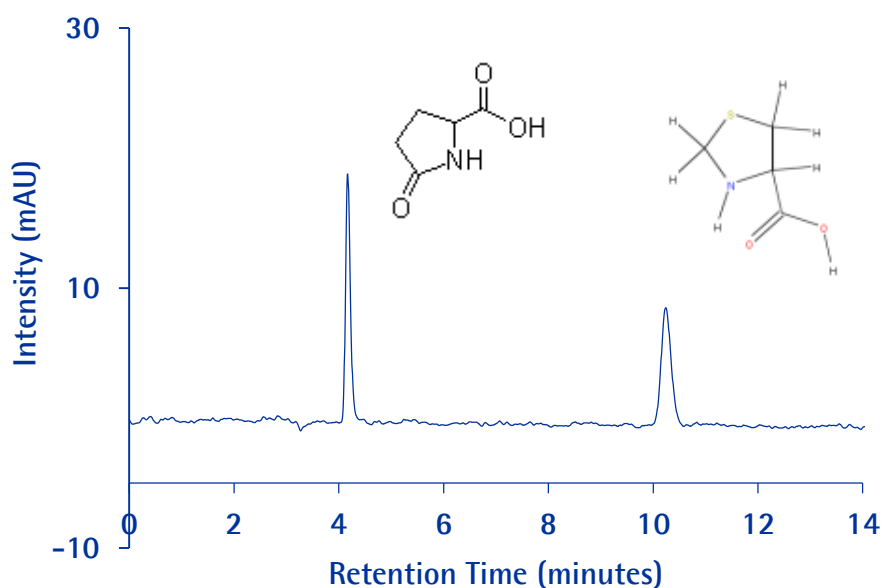
Temperature: Ambient

Diluent: Mobile phase

Sample: 1000 ppm (1 mg/mL) of Pyroglutamic acid in diluent

Pressure: 48 Bar (696 psi)

Time (min)	A (%)	B (%)
0.0	18	82
10-20	18-40	82-60
20-25	40	60



Chromatographic Data

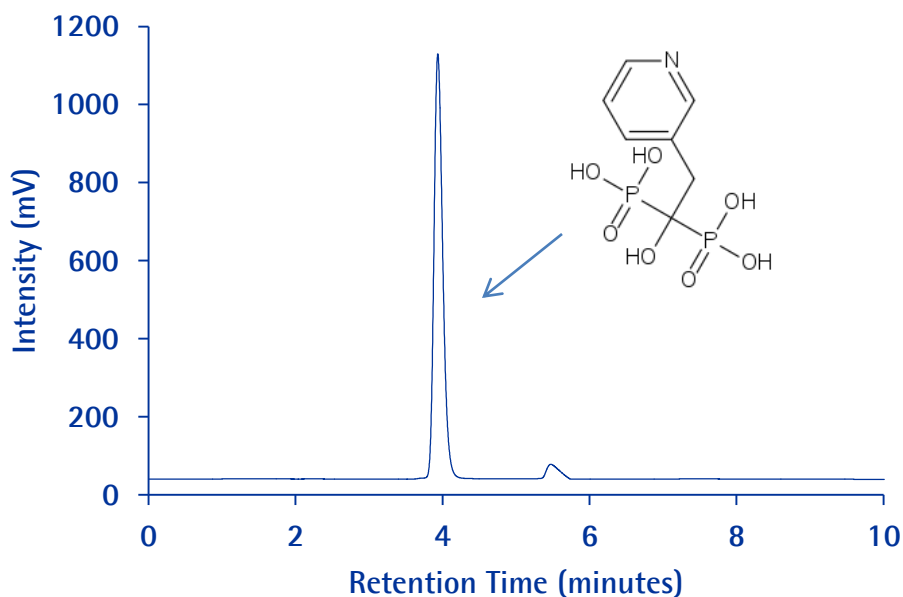
No.	Compound	Time	Tailing Factor	Theoretical Plate
1	Pyroglutamic acid	4.2	1.2	10303
2	Impurity A	10.2	1.1	13895

Risedronate sodium and Related impurities

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 100×4.6 mm	1.50453.0001
Injection:	5 µL	
Detection:	Shimadzu LC 2010, UV 226 nm	
Cell:	10 µl	
Flow Rate:	0.5 mL/min	
Mobile Phase:	Dissolve 1.54 g of ammonium acetate in 1L water (20 mM). Mix Acetonitrile and Buffer 80:20 (v/v). Total ionic strength: 4 mM	
Temperature:	25 °C	
Diluent	Mobile phase	
Sample:	Weigh 100 mg of sample in 100 ml volumetric flask. Dilute up to the mark with mobile phase. Pipette out 10 ml of the above solution & dilute to 100 ml with mobile phase.	
Pressure Drop:	46 bar (667 psi)	



Chromatographic Data

No.	Compound	Time	Tailing Factor	Theoretical Plate
1	Risedronate Na	3.9	1.4	5212

Temozolomide and Related Impurities

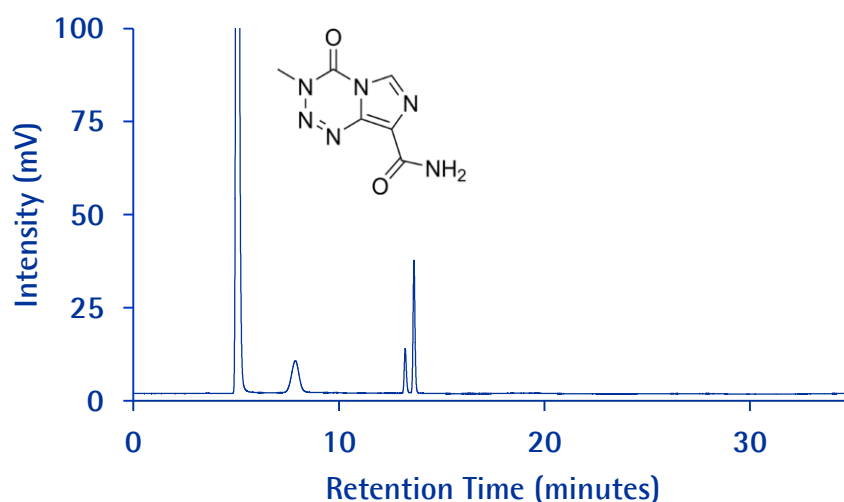
SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 250×4.6 mm	1.50458.0001
Injection:	10 µL	
Detection:	Shimadzu Prominence, UV 254 nm	
Cell:	10 µl	
Flow Rate:	0.8 mL/min	
Mobile Phase (v/v):	A: Dissolve 3.08 g of ammonium acetate in 1000 ml water (40 mM). B: 100% Acetonitrile	
Gradient:	See Table:	
Temperature:	25 °C over column and autosampler cooler set at 15 °C	
Diluent	Mobile phase	

Time (min)	A (%)	B (%)
0.0	3	97
2.0	3	97
25.0	50	50
30.0	3	97
35.0	3	97

Sample: 400 ppm of Temozolomide and 1 ppm of each impurity A, B and C in acetonitrile. Keep the solution for 4 hrs in amber glassware before analysis for stabilization. Use amber colored vial for analysis.



Chromatographic Data

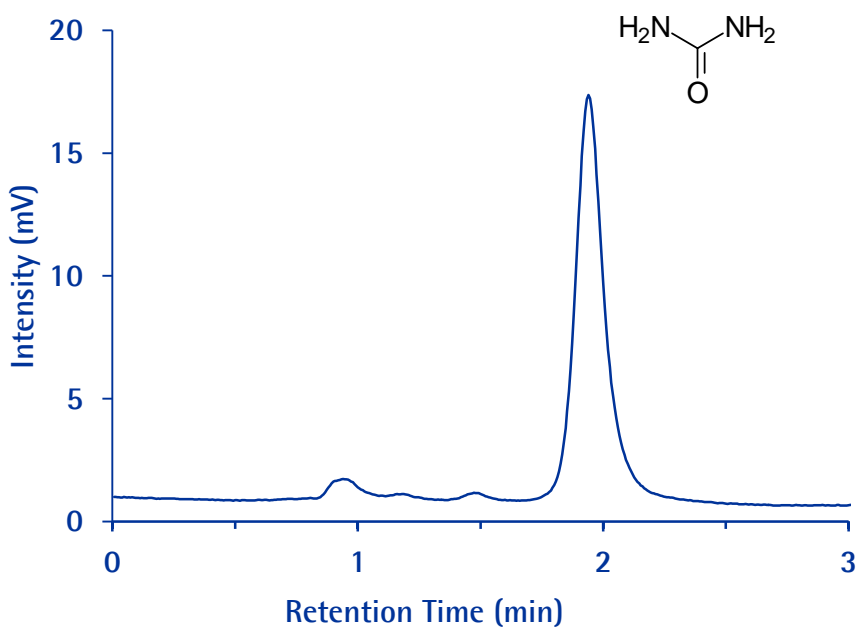
No.	Compound	Time	Tailing Factor	Resolution*
1	Temozolomide	5.1	1.3	0.0
2	Impurity C	7.9	1.0	6.3
3	Impurity B	13.2	1.1	12.5
4	Impurity A	13.7	1.1	2.4

Urea

SeQuant® ZIC®-HILIC

Chromatographic Conditions

Column:	SeQuant® ZIC®-HILIC (5 µm, 200 Å) 50x4.6 mm	1.50451.0001
Injection:	5 µL	
Detection:	Shimadzu LC-10, UV@204 nm	
Cell:	2.5 µl micro-cell	
Flow Rate:	0.5 mL/min	
Mobile Phase (v/v):	Acetonitrile and 5 mM ammonium acetate in water, pH 6.8. 90:10 (v/v)	
	Total ionic strength: 0.5 mM	
Temperature:	Ambient	
Diluent:	Mobile phase	
Sample:	1000 ppm of Urea in mobile phase	
Pressure Drop:	5 Bar (72 psi)	



Chromatographic Data

No.	Compound	Time	Tailing Factor	Retention Factor (k')
1	Urea	1.9	1.3	0.9

Recommended Reagents and Chemicals

Product	Ordering Number
Acetic acid for Chromatography	1.00063
Acetonitrile for Chromatography	1.14291
Acetonitrile Gradient Grade for Chromatography	1.00030
Ammonium acetate	1.01116
1,4-Dioxane for liquid chromatography	1.03132
Formic acid	1.00264
Potassium dihydrogen phosphate	1.05108
Potassium hydroxide	1.05002
Water for chromatography*	1.15333
* Or use a Milli-Q Integral Water Purification System	

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