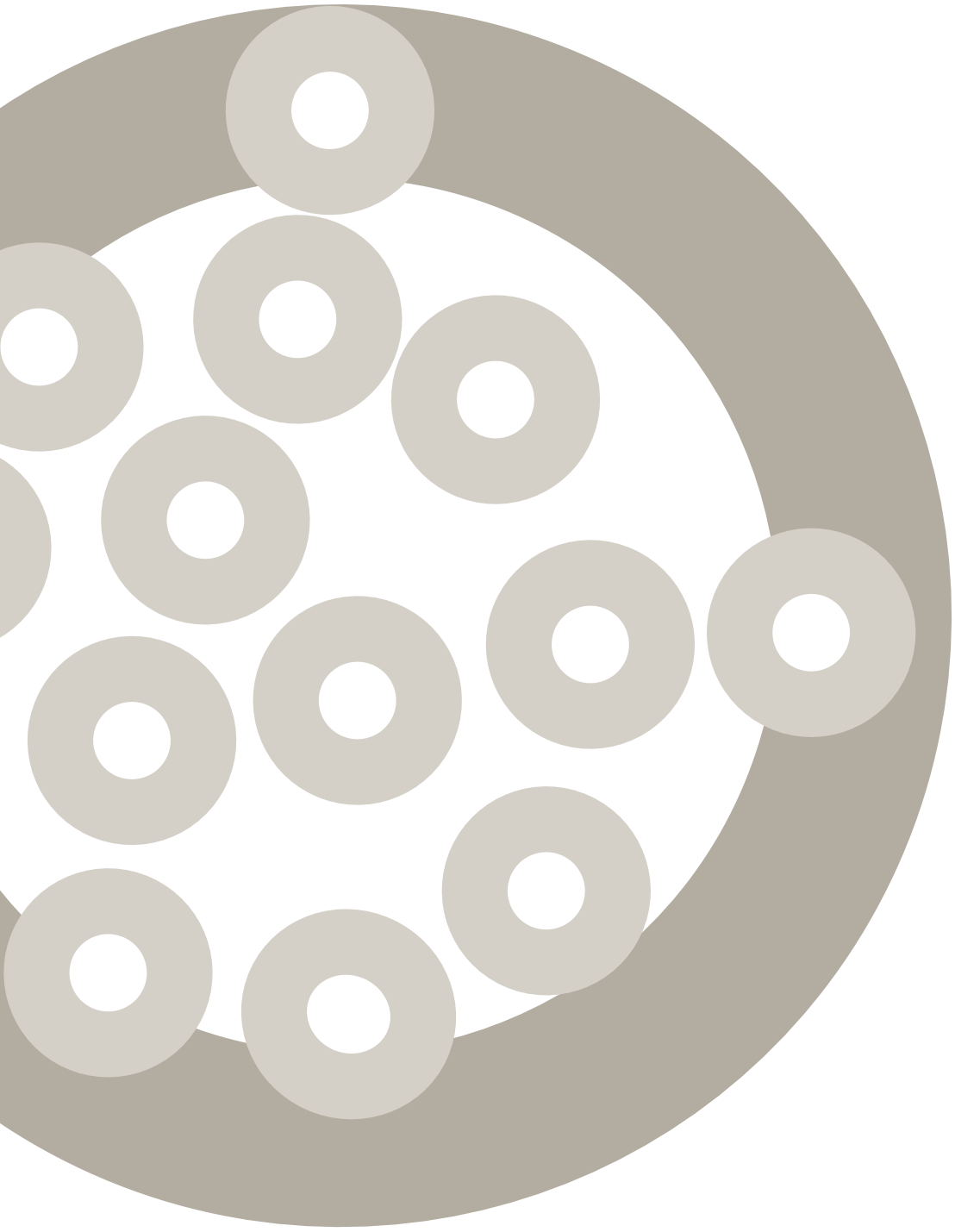


HILIC
HYDROPHILIC INTERACTION CHROMATOGRAPHY

HILIC PRODUCTS

SILICA BASED HILIC COLUMNS

TSKgel Amide-80
TSKgel NH_2-100

TOSOH FACT

The first columns used in chromatography were glass, both for liquid-solid chromatography by Tswett in his separation of plant pigments and by James and Martin in their first gas chromatograph. However, as the technique developed and particle size was reduced, the length of the columns in liquid chromatography was decreased. This resulted in the columns having to be operated at higher pressures. To accommodate these higher pressures, stainless steel columns were introduced. Tosoh introduced its first HPLC (GPC) columns in 1971, which were composed of stainless steel. Recently, columns packed in PEEK, a biocompatable fluorocarbon polymer, became available. PEEK can withstand the pressures commonly encountered in HPLC.

INTRODUCTION TO TSKgel HILIC COLUMNS

HIGHLIGHTS

- Stable bonding chemistries
- Unique polar phases
- Handle a wide spectrum of sample polarities
- Stable in 100% organic
- Separate many different types of polar molecules
- 3 µm particle size for LC/MS analysis

Hydrophilic interaction chromatography (HILIC) is used primarily for the separation of polar and hydrophilic compounds. HILIC has similarities with traditional normal phase chromatography, but the mobile phases for HILIC are similar to those known from reversed phase chromatography (RPC). They include polar organic solvents like acetonitrile. Based on hydrogen bonds the aqueous content of the mobile phase creates a water-rich layer on the particle surface. This allows for partitioning of polar compounds between the more organic mobile phase and the aqueous layer (FIGURE 1). The number of polar groups, as well as the conformation and solubility of the sample in the mobile phase determines the elution order.

Typical mobile phases consist of acetonitrile buffer mixtures. Samples are eluted from the column by increasing the percentage of the aqueous component. Compared to RPC the elution order in HILIC mode is inversed for most substances.

HILIC is often used to separate hydrophilic compounds such as peptides, carbohydrates and small polar drug candidates or metabolites. Hydrophilic compounds are retained on the polar bonded phase column while non-polar sample impurities elute unretained in the void volume. In addition it is ideally suited for sensitive LC-MS analysis of water soluble polar compounds because the high organic content in the mobile phase provides rapid evaporation of solvent during electrospray ionization.

TSKgel HILIC columns are available in various dimensions and particle sizes, functionalized with carbamoyl-groups (TSKgel Amide-80) or amino-groups (TSKgel NH_2-100).This enables the user to perfectly match HILIC selectivity to specific application needs.

For more detailed information, please refer to our ***TSKgel HILIC brochure*** on www.tosohbioscience.de, or request a printed copy at sales-marketing.tbg@tosoh.com.

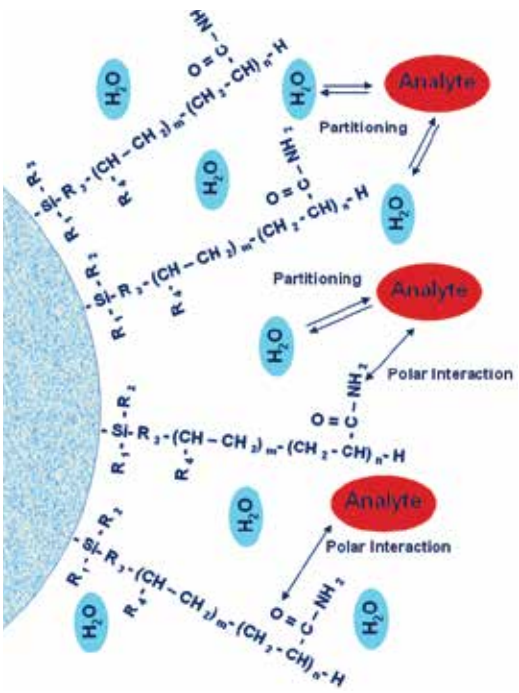
The TSKgel Amide-80 column offers an excellent alternative to amino-bonded stationary phases and consists of 3, 5 or 10 µm silica particles in a stainless steel format. Spherical silica particles are covalently bonded with carbamoyl groups. For years TSKgel Amide-80 columns have been the standard for the analysis of glycans. TSKgel Amide-80 columns packed with 3 µm particles are the newest addition to the TSKgel Amide-80 series. The 3 µm HILIC columns reduce analysis time and improve peak capacity and sensitivity for HPLC and LC-MS analysis.

TSKgel NH_2-100 3 µm columns are the latest addition to the TSKgel HILIC family. They expand the selectivity range of TSKgel HILIC solutions by a new, robust amino-phase. In contrast to conventional silica-based amino phases the new column offers expanded stability under HILIC conditions. It is well suited for the analysis of all types of hydrophilic compounds like carbohydrates, peptides, vitamins, polar drugs or metabolites.

The NH_2-100 phase is based on a silica particle with 3 µm particle and 10 nm pore size, treated with a special endcapping procedure. Amino groups are introduced step wisely after endcapping. These columns are unique in that the bonded phase ligand not only, as expected, has a terminal primary amino group, but that the spacer also incorporates secondary as well as tertiary amino groups. The amino groups act as HILIC functional groups without any peak splits. Due to their high ligand density and large surface area TSKgel NH_2-100 3 µm columns show high retention for very polar compounds. Anionic compounds are retained on the column by ionic interaction. This allows for the use of salt gradients, in addition to gradient elutions with acetonitrile. Since the TSKgel NH_2-100 has cationic sites, it can be used as mixed mode phase under some conditions.

FIGURE 1

HILIC principles



HILIC

COLUMN OPERATION AND SPECIFICATIONS

TSKgel HILIC columns can be operated over a broad range of mobile phase conditions. Factors to consider when employing these columns include:

Sample loading capacity is dependent upon the polarity of the mobile phase. It increases with decreasing mobile phase polarity. For example, on a TSKgel Amide-80 column the highest loading capacity for mannitol (200 µg) occurs with a mobile phase of 75:25 acetonitrile/water. However, <100 µg of mannitol can be loaded in a mobile phase of 65:35 acetonitrile/water. The maximum sample volume for a 4.6 mm ID x 25 cm L Amide-80 analytical column is 50 µL.

Temperature range: TSKgel Amide-80 columns can be operated over a temperature range of 10-80°C (10-50°C for Amide-80 3 µm), TSKgel NH_2-100 columns in the range of 10-50 °C. In general, retention times for carbohydrates decrease with increasing temperature, thereby shortening analysis time. Below certain temperatures some carbohydrates may elute as split peaks. In this case, column heating or addition of triethylamine to the mobile phase is required.

Choice of mobile phase: the pH range of TSKgel Amide-80 and NH_2-100 columns is 2.0-7.5 with a maximum salt concentration of 100 mmol/L. The columns are stable in 100% organic for normal phase separations; however, in HILIC mode a combination of aqueous and organic solvents is necessary in order to create the water-rich surface layer. As the mobile phase polarity decreases (higher organic content) the sample is retained longer on the column.

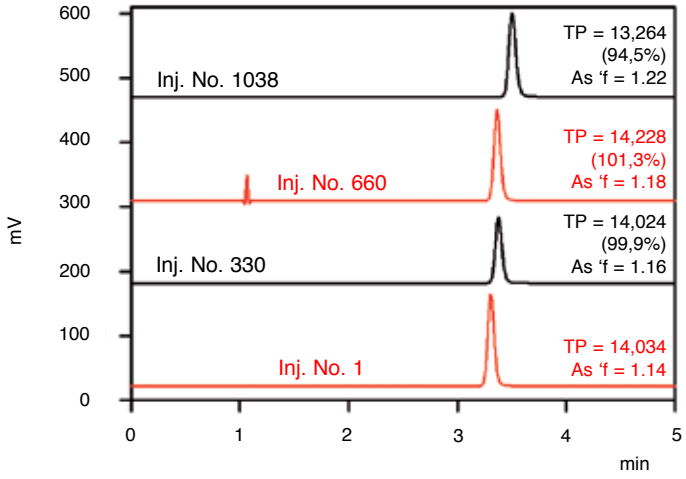
LONG TERM STABILITY

The high stability of TSKgel Amide-80 columns is demonstrated in FIGURE 2 showing the same analysis after 330, 660 and more than 1000 runs compared to the first injection. Only 5% reduction of column performance (theoretical plates) is observed after more than 1000 injections.

FIGURE 3 shows the high stability of TSKgel NH_2-100 columns. Compared to the first injection only a slight reduction of retention time of inositol is observed with the TSKgel NH_2-100 column after more than 400 hours of flushing with mobile phase.

FIGURE 2

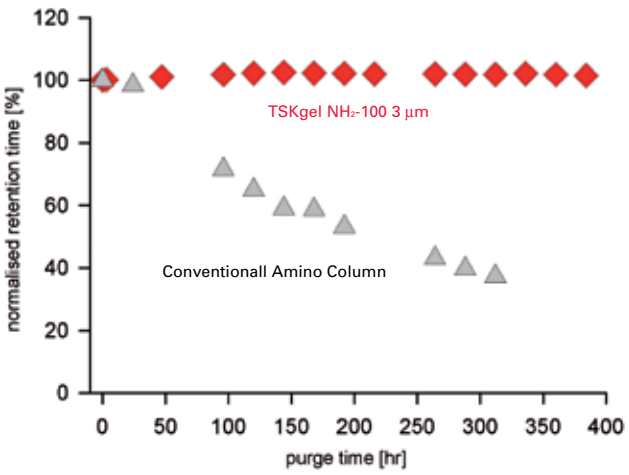
Durability of TSKgel Amide-80 3 µm



Column: TSKgel Amide-80 3 µm (2.0 mm ID x 15 cm L)
Eluent : H_2O/ACN = 15/85; Flow rate: 0.2 mL/min; Inj. volume: 2 µL
Detection : UV @ 254 nm; Temp. : 25 °C; Samples: Uracil (37 mg/L)

FIGURE 3

Long term stability of TSKgel NH_2-100 columns



Column: TSKgel NH_2-100 3 µm, 4.6 mm ID x 15 cm L
Conventional Amino Column, 4.6 mm ID x 25 cm L;
Eluent: H_2O/ACN (25/75); Flow rate: 1.0 mL/min; Detect: RI;
Temp.: 40 °C; Injection: 10 µL; Sample: Inositol

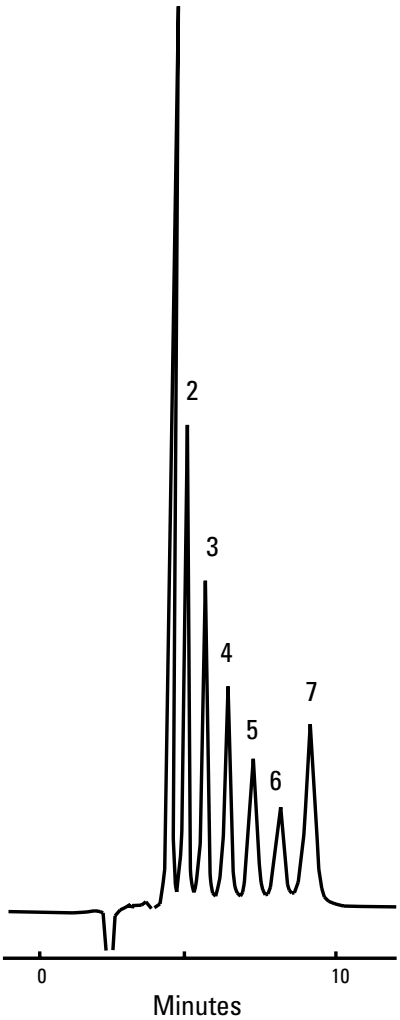
APPLICATIONS OF TSKgel AMIDE-80 COLUMNS

OLIGOSACCHARIDES

The TSKgel Amide-80 can separate oligosaccharides very rapidly and efficiently. FIGURE 4 shows a separation of a ß-cyclodextrin hydrolysate in less than 10 minutes. The labels indicate the number of base sugars such as glucose in each oligomer.

FIGURE 4

Separation of ß-cyclodextrin hydrolysate on TSKgel Amide-80 column



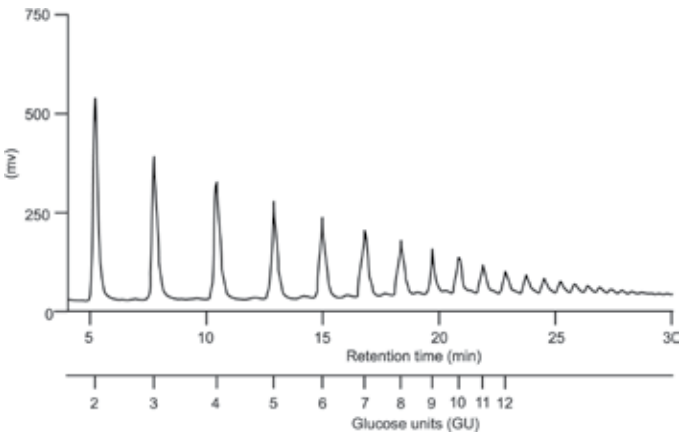
Column: TSKgel Amide-80 (4.6 mm ID x 25 cm L); Sample: 2 µL, β-cyclodextrin hydrolysate, 1 - 7 degrees of polymerization (4.6 mg/mL); Elution: ACN/water (55/45); Flow rate: 1.0 mL/min; Detection: RI; Temperature: 25 ºC

GLYCANS

Glycosylation is one of the most common post-translational modifications in eukaryotic cells. Complex N- and O-linked structures composed of repeating sugar moieties form the so called glycans. HILIC with fluorescence detection is the method of choice to effectively separate, identify and quantify glycans after exoglycosidase cleavage and fluorescent labeling. In order to normalize retention times of complex glycan structures a dextran ladder consisting of glucose oligomers is used as calibration reference. The calculated numbers of glucose units (GU) can be used in subsequent database queries (Glycobase, autoGU) to predict the glycan structure. For years TSKgel Amide-80 columns have been used successfully in glycan analysis. Amide-80 chemistry is ideally suited for the separation of carbohydrate structures. FIGURE 5 shows the high-resolution separation of a 2-aminobenzamide (2AB) labeled dextran ladder within 30 minutes on a TSKgel Amide-80 3 µm column. This ladder can be used as a calibration standard for HPLC and MS analysis of glycans. The ladder contains glucose homopolymer species from degree of polymerization (dp) 1 to dp 22 (i.e. the glucose monomer GU1-2AB to GU22-2AB).

FIGURE 5

Separation of a 2-AB-labeled dextran ladder on TSKgel Amide-80



Column: TSKgel Amide-80 (3 µm, 2.0 mm ID × 15 cm L)
Eluent: A) 50 mM Ammonium formate (pH 4.3), B) Acetonitrile;
Gradient: 0 - 35 min (75 - 35 % B); Flow rate: 0.22 mL/min;
Detection: Fluorescence Ex @ 360 nm, Em @ 425 nm; Temperature: 50 °C;
Injection vol: 3 µl; Sample: CAB-GHP dextran ladder (Ludger; ~300 fmol for GU2)

* Courtesy of K. Darsow & H. Lange, Institute of Bioprocessing, University of Nürnberg/Erlangen

HILIC

High-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) has become a powerful tool when detection sensitivity is an issue. HILIC offers unique advantages for MS detection of very polar compounds when compared to reversed phase mode. The higher organic content of the eluent in HILIC mode supports efficient evaporation of the solvent thus enhancing sensitivity and altering ion suppression.

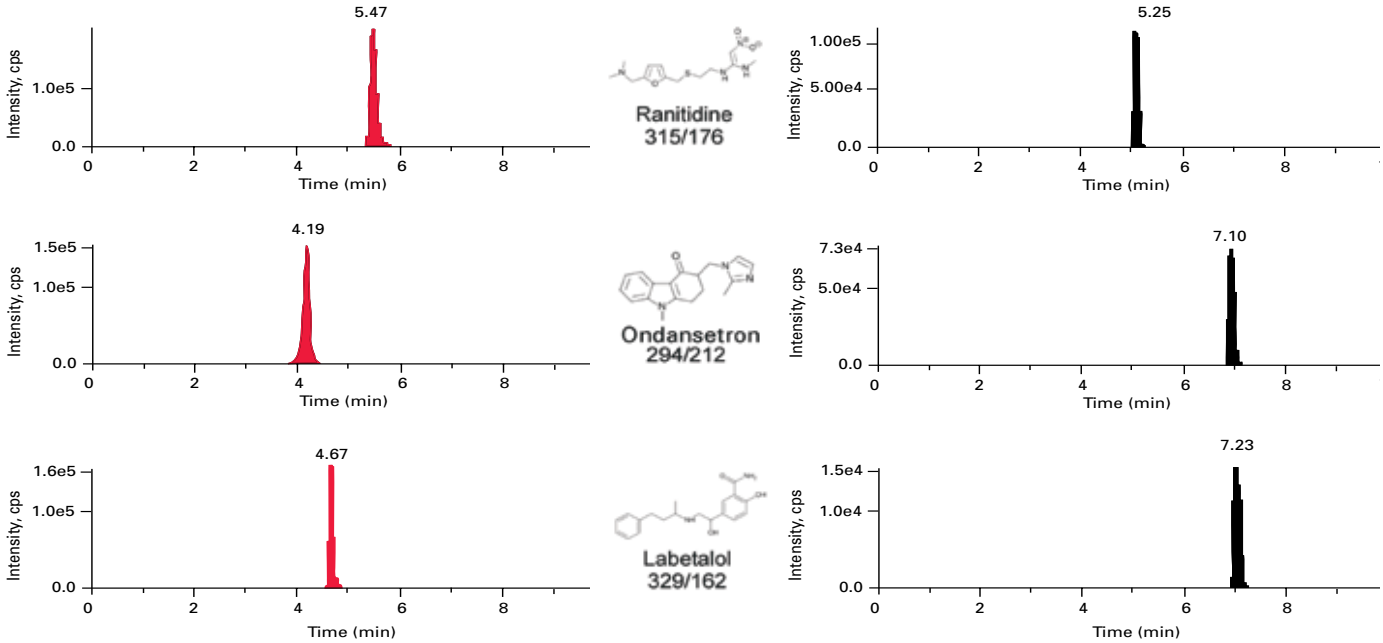
HILIC separations are performed with gradients starting with high percentage of organic solvent and ending with a high portion of aqueous solvent - opposite to typical reversed phase gradients. The elution order of compounds is usually inversed as well. As a result polar compounds are very well separated according to increased polarity in HILIC mode. At the same time the portion of organic solvent in the mobile phase is relatively high.

FIGURE 6 shows the analysis of basic drug substances using a TSKgel Amide-80 3 µm column compared to a reversed phase TSKgel ODS-100V 3 µm column. Ranitidine, a histamine H2 receptor antagonist, Ondansetron, an antiemetic serotonin receptor antagonist, and Labetalol, an alpha-1 and beta adrenergic blocker were selected to demonstrate the differences in selectivity and MS-signal response when applying different chromatographic modes.

Ranitidine has the highest number of polar groups among these molecules and as a result shows the highest retention in HILIC and the lowest retention in RPC mode. Signal intensity is almost doubled for Ranitidine in HILIC mode. For Labetalol a tenfold increase in signal height can be achieved by using HILIC instead of RPC.

FIGURE 6

LC-MS/MS Analysis of basic drugs in HILIC and RPC mode



Column: TSKgel Amide-80 3 µm (2.0 mm ID x 15 cm L)
Eluent : A: 10 mM Ammoniumformiate (pH 3.75); B: ACN
Gradient: 0 min (B 90%) -> 10 min (B 40%) ->13 min (B 40%)
Flow rate: 0.2 mL/min; Inj. volume : 5 µL (50 µg/L)
Detection : QTrap® LC-MS/MS (Applied Biosystems), ESI+

Column: TSKgel ODS-100V 3 µm (2.0 mm ID x 15 cm L)
Eluent: A: 10 mM Ammoniumformiate (pH 3.75); B: ACN
Gradient: 0 min (B 0%) -> 10 min (B 80%) ->13 min (B 80%)
Flow rate: 0.2 mL/min; Inj. volume: 5 µL (50 µg/L)
Detection: QTrap® LC-MS/MS (Applied Biosystems), ESI+

APPLICATIONS OF TSKgel NH2-100 COLUMNS

SEPARATION OF WATER SOLUBLE VITAMINS

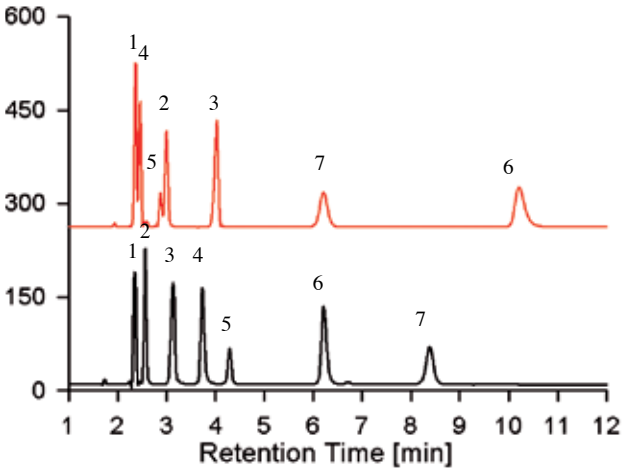
FIGURE 7 shows the separation of a standard solution of water soluble vitamins on a TSKgel NH2-100 column compared to a TSKgel Amide-80 column. Dimension (4.6 mm ID x 15 cm L), particle size (3 µm), flow rate and mobile phase were identical for both columns. The elution order of the compounds changes when applying the same mobile phase to both columns: The TSKgel NH2-100 column shows stronger retention for nicotinic acid, vitamin C, and vitamin B12, while retention of vitamin B1, B2, and pyridoxine is reduced.

SEPARATION OF METHOTREXATE AND DERIVATIVES

FIGURE 8 compares the separation of methotrexate and its derivatives (MTXPG2~7) on TSKgel NH2-100, 3µm HILIC and TSKgel ODS-100V, 3µm reversed phase narrow bore columns. Methotrexate, abbreviated MTX and formerly known as amethopterin is an inhibitor of the folic acid metabolism. It is used in cancer chemotherapy and as a treatment of autoimmune diseases. The MTX and polyglutamate derivatives were eluted in the order of the number of glutamate groups in their molecules on the TSKgel NH2-100 HILIC column, but eluted in reverse order on the TSKgel ODS-100V column. Despite the early elution of MTX and MTXPG2 on the TSKgel NH2-100 HILIC column, the overall separation is better than what can be accomplished on the C18 column.

FIGURE 7

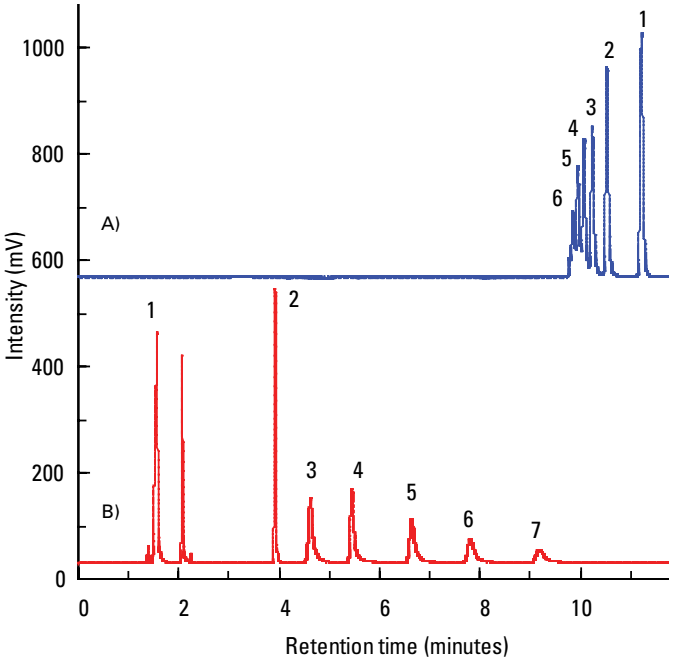
Separation of water soluble vitamins



Columns: TSKgel Amide-80 3 µm, 4.6 mm ID x 15 cm L;
TSKgel NH2-100 3 µm, 4.6 mm ID x 15 cm L;
Eluent: 25 mM phosphate buffer (pH 2.5)/ACN=30/70
Flow: 1 mL/min; Temp.: 40°C; Detection: UV @ 254 nm
Sample: Vitamin standard mixture: 1 = Nicotinamide, 2 = Vitamin B2, 3 = Pyridoxine, 4 = Nicotinic acid, 5 = Vitamin C, 6 = Vitamin B1, 7 = Vitamin B12
Injection: 5 µL

FIGURE 8

Separation of MTX and derivatives



Column: A) TSKgel ODS-100V, 3 µm, 2.0 mm ID x 15 cm L;
Mobile phase: a) H_2O/ACN (90/10) + 0.1% TFA, b) ACN + 0.1% TFA;
B) TSKgel NH2-100, 3 µm, 2.0 mm ID x 15 cm L;
Mobile phase: a) H_2O/ACN (10/90) + 0.1% TFA, b) H_2O + 0.1% TFA;
Gradient: 0 % B (0 min), 40 % B (15 min), 0 % B (17 min);
Flow rate: 0.20 mL/min; Detection: UV @ 313 nm; Temperature: 40°C;
Injection vol.: 10 µL; Sample: 1. MTX (MTXPG) 2. MTXPG2 3. MTXPG3 4. MTXPG4 5. MTXPG5 6. MTXPG6 7. MTXPG7

HILIC

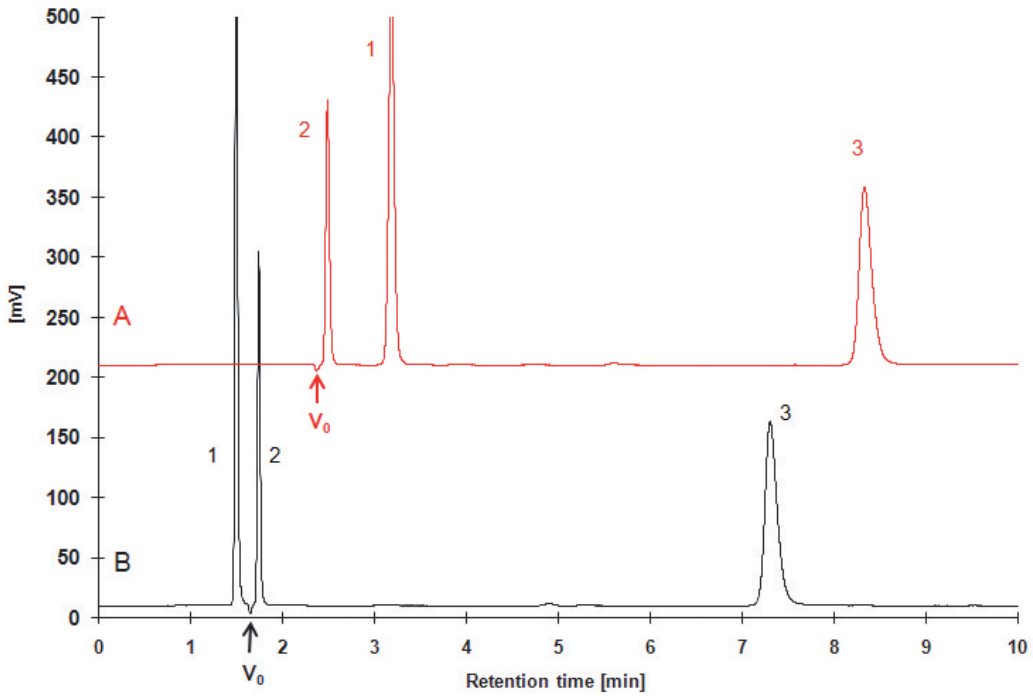
DIRECT CONNECTION HILIC COLUMN FOR DEDICATED APPLICATIONS

The TSKgel NH_2-100 DC column connects directly to other TSKgel HPLC columns. This can be used to combine separations based on polar interactions and non-polar interactions e.g. HILIC/ion exchange and reversed phase without the need of connectors or capillaries. The DC in the name 'TSKgel NH_2-100 DC' emphasizes this 'direct connect' aspect. A male-type outlet end fitting enables the direct connection to the normal end fitting of a TSKgel reversed phase column. This allows for the simultaneous gradient separation of hydrophobic and hydrophilic/acidic compounds - e.g. an active pharmaceutical ingredient (API) and its counter ion - without the loss of column efficiency normally experienced when connecting two columns with capillary tubing. Hydrophilic compounds and anions are retained strongly on the amino-alkyl bonded 3 µm silica phase of the TSKgel NH_2-100 DC 3 µm column. When coupled to a reversed phase column the overall retention of these compounds is thereby shifted from other unretained peaks.

FIGURE 9 demonstrates the use of the TSKgel NH_2-100 DC column in the separation of drug and counter ion. Maleic acid and p-toluene sulfonic acid are commonly used as counter ions in pharmaceutical preparations. Both of these organic acids are hydrophilic and are not retained on a TSKgel ODS-100V reversed phase column at pH 7.0 in 70 % methanol eluent (Chromatogram B). With the connection of a TSKgel NH_2-100 DC column prior to the TSKgel ODS-100V column, the simultaneous determination of maleic acid and the active pharmaceutical ingredient (API) desipramine becomes possible (Chromatogram A). Maleic acid is slightly retained on the TSKgel NH_2-100 DC column by an anion exchange interaction. Desipramine, on the other hand, does not interact with the protonated amino groups as it is positively charged.

FIGURE 9

Simultaneous analysis of maleic acid and desipramine



Columns: A: TSKgel NH_2-100 DC 3 µm + TSKgel ODS-100V 3 µm
B: TSKgel ODS-100V 3 µm
Mobile phase: 50 mmol/L phosphate buffer, pH 7.0/MeOH (30/70); Flow rate: 1.0 mL/min; Detection: UV @ 210 nm; Temperature: 40°C; Injection vol.: 5 µL;
Samples: 1. maleic acid (50 mg/L); 2. p-toluene sulfonic acid (50 mg/L); 3. desipramine (50 mg/L)

ORDERING INFORMATION

Part #	Description	ID (mm)	Length (cm)	Particle size (µm)	Number theoretical plates	Flow rate (mL/min) range	Maximum pressure drop (MPa)
TSKgel Stainless Steel Columns							
0021864	Amide-80	2.0	5.0	3	≥ 3,500	0.2	20.0
0021865	Amide-80	2.0	15.0	3	≥13,000	0.2	20.0
0021866	Amide-80	4.6	5.0	3	≥ 6,000	1.0	20.0
0021867	Amide-80	4.6	15.0	3	≥18,500	1.0	20.0
0020009	Amide-80	1.0	5.0	5	≥ 300	0.03 - 0.05	3.0
0020010	Amide-80	1.0	10.0	5	≥ 600	0.03 - 0.05	6.0
0021486	Amide-80	1.0	15.0	5	≥ 4,000	0.03 - 0.05	9.0
0021487	Amide-80	1.0	25.0	5	≥ 6,000	0.03 - 0.05	12.0
0019694	Amide-80	2.0	5.0	5	≥ 1,000	0.15 - 0.20	4.0
0019695	Amide-80	2.0	10.0	5	≥ 2,000	0.15 - 0.20	8.0
0019696	Amide-80	2.0	15.0	5	≥ 4,000	0.15 - 0.20	10.0
0019697	Amide-80	2.0	25.0	5	≥ 6,000	0.15 - 0.20	15.0
0019532	Amide-80	4.6	5.0	5	≥ 2,500	0.8 - 1.0	5.0
0019533	Amide-80	4.6	10.0	5	≥ 4,000	0.8 - 1.0	5.0
0013071	Amide-80	4.6	25.0	5	≥ 8,000	0.8 - 1.0	15.0
0021982	Amide-80 HR	4.6	25.0	5	≥18,000		15.0
0014459	Amide-80	7.8	30.0	10	≥ 5,000	1.0 - 2.0	7.0
0014460	Amide-80	21.5	30.0	10	≥ 8,000	4.0 - 6.0	3.0
0021967	NH_2-100	2.0	5.0	3	≥ 4,000	0.2	15.0
0021968	NH_2-100	2.0	15.0	3	≥15,000	0.2	20.0
0021969	NH_2-100	4.6	5.0	3	≥ 6,000	1.0	5.0
0021970	NH_2-100	4.6	15.0	3	≥18,000	1.0	15.0
0021999	NH_2-100 DC	4.6	5.0	3	≥ 6,000	1.0	5.0
Guard column products							
0021862	Amide-80 Guard cartridge, pk 3	2.0	1.0	3	For 2.0 mm ID columns		
0021863	Amide-80 Guard cartridge, pk 3	3.2	1.5	3	For 4.6 mm ID columns		
0021941	Amide-80 Guard cartridge, pk 3	2.0	1.0	5	For all 2 mm ID columns		
0019021	Amide-80 Guard column	4.6	1.0	5	For all 4.6 mm ID columns		
0019010	Amide-80 Guard cartridge, pk 3	3.2	1.5	5	For all 4.6 mm ID columns		
0014461	Amide-80 Guard column	21.5	7.5	10	For 21.5 mm ID column		
0021971	NH_2-100 Guard cartridge, pk 3	2.0	1.0	3	For all 2 mm ID columns		
0021972	NH_2-100 Guard cartridge, pk 3	3.2	1.5	3	For all 4.6 mm ID columns		
0019308	Guard cartridge holder				For 2 mm ID x 1 cm L guard cartridges		
0019018	Guard cartridge holder				For 3.2 mm ID x 1.5 cm L guard cartridges		

NOTE: Tosoh Bioscience offers guard columns and guard cartridges to protect your analytical column. Guard cartridges are usually delivered in packages of three and require the appropriate cartridge holder.
In general cartridges for 4.6 mm ID columns are produced in 3.2 mm ID and 1.5 cm length. They require the cartridge holder 19018. Guard cartridges for 2 mm ID columns are 2 mm ID x 1 cm L and require holder 0019308.

HILIC