

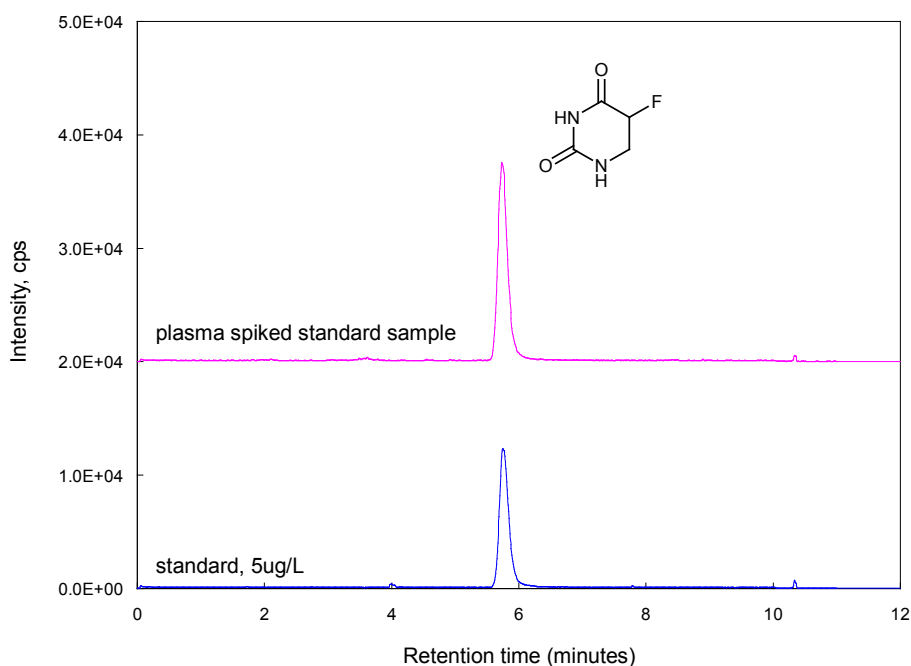
Analysis of 5-Fluorouracil in Plasma by LC-MS/MS

5-Fluorouracil (5-FU) belongs to the pyrimidine antimetabolite class of anticancer drugs. Although it is used broadly for various types of cancer, it is particularly effective against cancers of the digestive system, such as colon cancer and stomach cancer. An application of 5-FU in a plasma sample using hydrophilic interaction chromatography (HILIC) is shown here. Water containing formic acid/acetonitrile was used to elute 5-FU. After elution, the column was washed using a stepwise gradient. Analysis of a standard sample confirmed linearity of the calibration curve over the concentration range of 1.0 to 100µg/L. Recovery of $\geq 87\%$ was obtained for a plasma sample that was spiked at a concentration of 50µg/L, diluted four times with acetonitrile containing 1% formic acid, followed by protein extraction.

Table 1. Analytical conditions

Column:	TSKgel Amide-80, 3µm, 2.0mm ID x 15cm
Mobile phase:	A: 0.1% formic acid in water/acetonitrile = 3/97 B: 0.1% formic acid in water
Gradient:	0min (0%B) → 10min (0%B) → 10.1min (50%B) → 11.1min (50%B) → 11.2min (0%B)
Flow rate:	0.2mL/min
Temperature:	40°C
Injection vol.:	2µL
Instrument:	Agilent 1200SL series QTRAP [®] (AB SCIEX)
Ion source:	ESI (Negative) <i>m/z</i> 129>42

Figure 1: Chromatograms of standard sample and plasma-spiked standard sample





Separation of 5-fluorouracil and its derivatives using a stable bonded phase HILIC column

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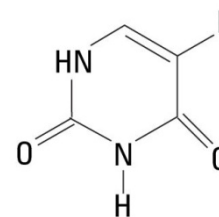
Tosoh Bioscience LLC, King of Prussia, PA 19406



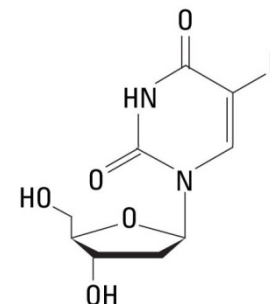
Introduction

- The pyrimidine analog 5-fluorouracil (5-FU) is an anticancer ("antineoplastic" or "cytotoxic") drug.
- The chemotherapy agent 5-FU has been in use for the treatment of cancer for about 40 years.
- This compound acts in several ways, but principally as a thymidylate synthase inhibitor¹.
- 5-fluoro-2'-deoxyuridine, another polar compound in the metabolic pathway of 5-FU, also inhibits DNA synthesis by prior inhibition of thymidylate synthetase.

5-fluorouracil



5-fluoro-2'-deoxyuridine



¹ Dawson, R. M. C., et al., Data for Biochemical Research, 3rd ed., p. 267., Oxford University, Press, New York, (1986).



Introduction (contd.)

- 5-FU is highly polar and elutes in the void volume when separated by reversed phase chromatography (RPC), even in 100% aqueous mobile phase.
- Thus RPC, the most widely used mode of retention in HPLC, cannot be used for the analysis of 5-FU.
- Ion Pair reagents cannot be employed due to the non-ionizable nature of this compound.
- By using an amide or amino bonded phase column, polar compounds can be retained by hydrophilic interaction chromatography (HILIC).
- The HILIC mode of separation offers ~10-fold increase in the sensitivity of detection when used in conjunction with electrospray-ionization mass spectrometry (ESIMS).



Introduction (contd.)

- This study demonstrates that a TSKgel NH₂-100 HILIC column effectively retains 5-FU and allows for its separation from several derivatives.
- The developed method was validated by a system suitability test.
- Limit of detection and limit of quantitation of the method were also determined.
- A column lifetime study using 5-FU yielded about 1000 injections without any significant change in the capacity factor of the column.

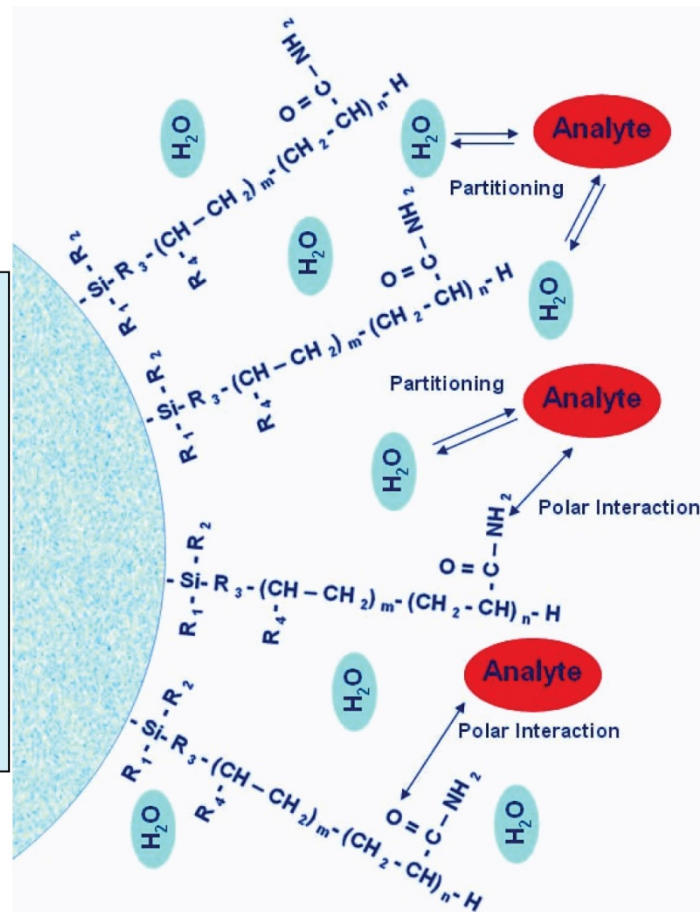


Objective

To show the usefulness of the silica-based TSKgel NH₂-100 column for the analysis of 5-fluorouracil using a conventional HPLC system.

Structure and Mechanism

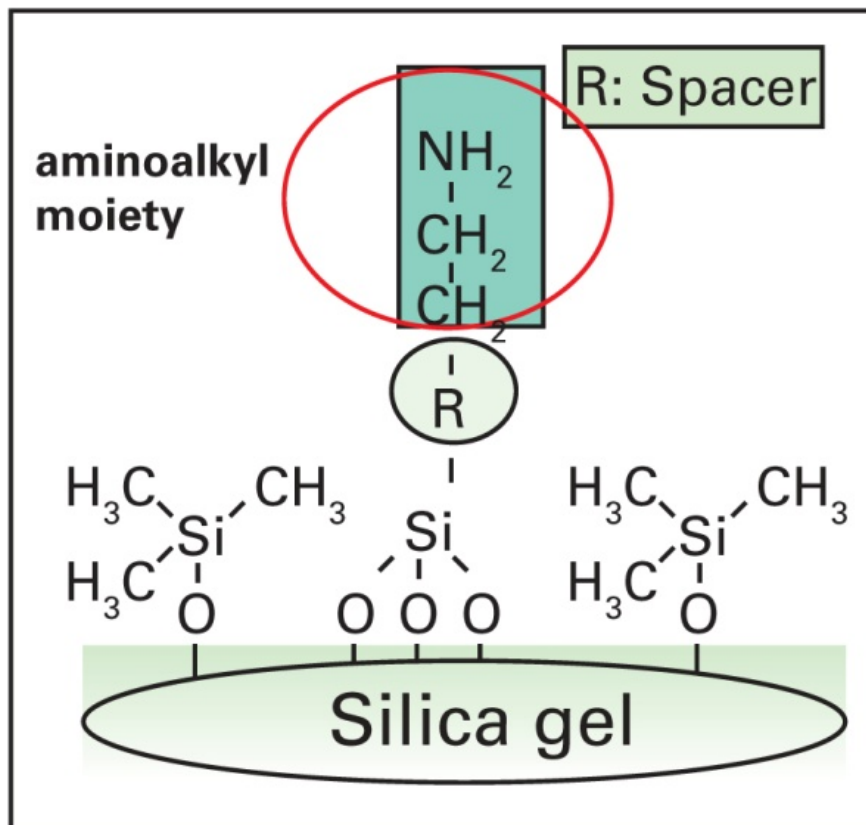
- Polar stationary phase as in normal phase LC
- Mobile phase similar to reversed phase (high organic)
- Elution in order of increasing hydrophobicity



Mechanism of Hydrophilic Interaction Liquid Chromatography (HILIC)



Schematic diagram of stationary phase of TSKgel NH₂-100, 3μm columns





Properties of TSKgel NH₂-100, 3 μ m columns

Base material	Silica
Particle size (nominal)	3 μ m
Pore size (nominal)	10nm
Specific surface area (nominal)	450m ² /g
Ligand *	Alkylamino
End-capping reagent	Trimethylsilyl groups

* Alkyl spacer also incorporates 2nd and 3rd amino groups

Column size	Theoretical plates	A _s
4.6mm ID x 5cm	$\geq 6,000$	0.90 - 1.30
4.6mm ID x 15cm	$\geq 18,000$	0.90 - 1.30



Characteristics of TSKgel NH₂-100, 3μm columns

- TSKgel NH₂-100 columns are packed with spherical 3μm silica particles containing 100Å pores.
- The internal and external surfaces of the particles are derivatized with a proprietary alkylamino silane reagent, while remaining and accessible silanol groups are endcapped with trimethylsilane.
- This novel bonding strategy provides expanded selectivity and a better safeguard against hydrolysis of the underlying silica.

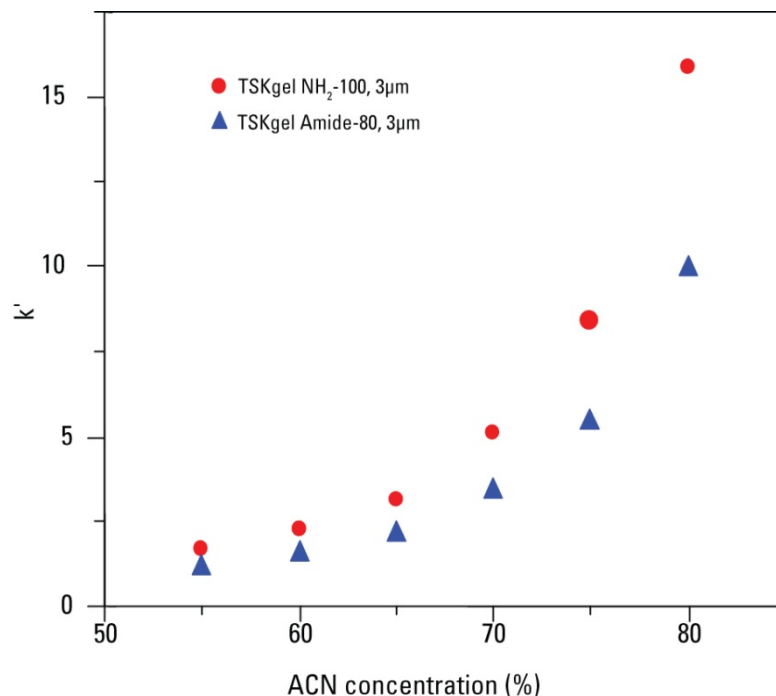


Characteristics of TSKgel NH₂-100, 3 μ m columns

- The chemical stability of the bonded phase was greatly enhanced by first using an endcapping reaction followed by the actual amino-ligand attachment.
- These columns can be used with evaporative light scattering (ELS) and mass spec (MS) detectors.
- The 3 μ m material is ideal for use in LC/MS applications for the analysis of active pharmaceutical ingredients and their metabolites.



Retention of TSKgel NH₂-100 HILIC Columns

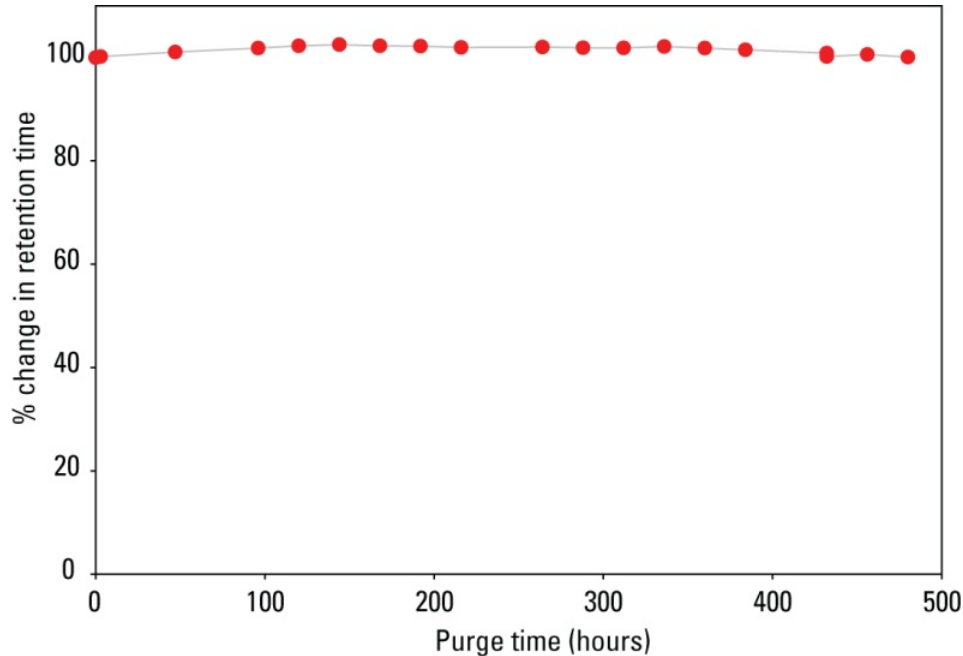


Columns: TSKgel NH₂-100, 3 μ m, 4.6mm ID x 15cm
TSKgel Amide-80, 3 μ m, 4.6mm ID x 15cm
Mobile phase: H₂O/ACN = 10/90
Flow rate: 1.0mL/min
Detection: RI
Temperature: 40°C
Injection Vol.: 10 μ L
Sample: inositol

Amino-based TSKgel NH₂-100 columns expand retention & selectivity in HILIC while offering **higher chemical stability**, a pre-requisite for reproducible results.



Column Stability



Column: TSKgel NH₂-100, 3 μ m, 4.6mm ID x 5cm
Mobile phase: H₂O/ACN = 25/75
Flow rate: 1.0mL/min
Detection: RI
Temperature: 40°C
Injection vol.: 10 μ L
Sample: inositol

After flushing a TSKgel NH₂-100 column with 18L mobile phase (500 hours), retention of inositol barely changed.



Materials and Methods

All analyses were carried out using an Agilent 1200 HPLC system run by Chemstation (ver B.04.01) unless mentioned otherwise.

Optimal chromatographic conditions :

Columns: TSKgel NH₂-100, 3 μ m, 2.0mm ID x 5cm
Competitor P – 2.5 μ m, 2.1mm ID x 5cm
Competitor Z – 3.5 μ m, 2.1mm ID x 5cm

Mobile phase: 80% acetonitrile and 20% 5mmol/L ammonium acetate
(Isocratic) in water

Flow rate: 0.1mL/min

Detection: UV@254nm

Temperature: ambient

Injection vol.: 10 μ L

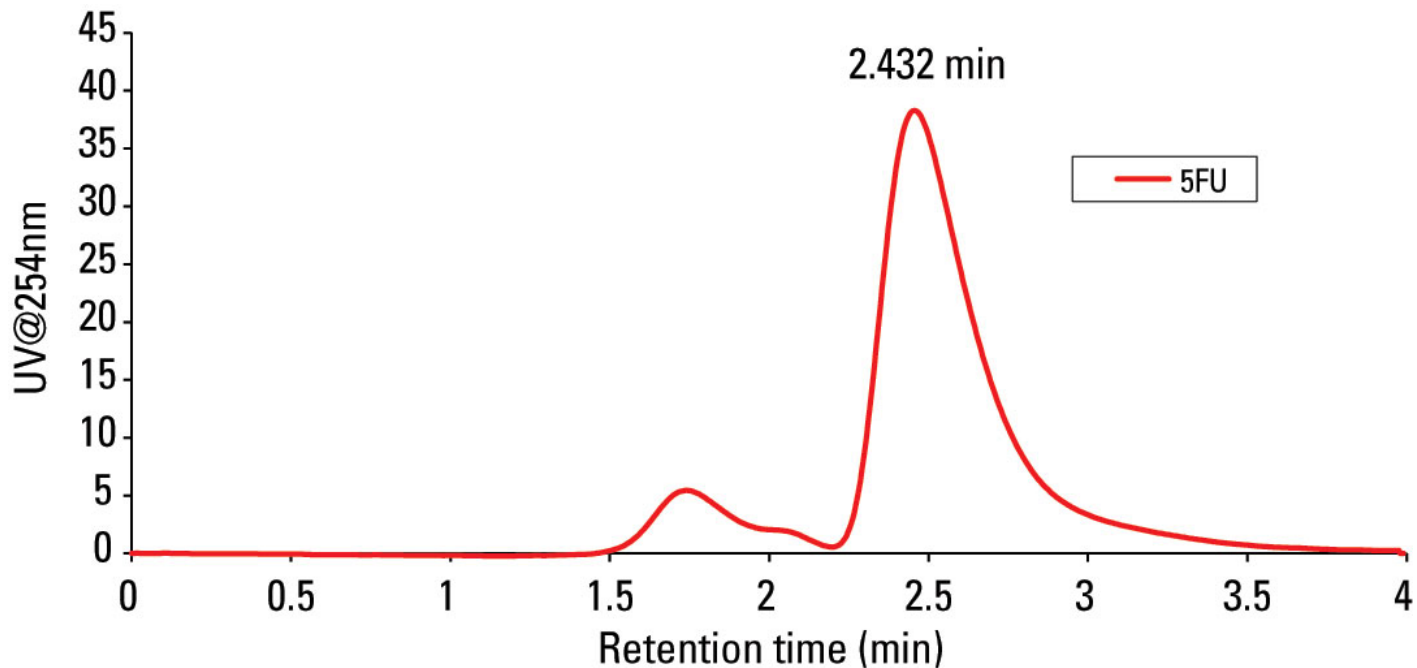


Chemicals

- Ammonium acetate (Fisher A637-500)
- 5-fluorouracil (Sigma)
- 5-fluoro-2'-deoxyuridine (Sigma F0503-250MG, Lot# 029K1232)
- High purity HPLC grade solvents were used for the preparation of stock standards, samples, and mobile phases.



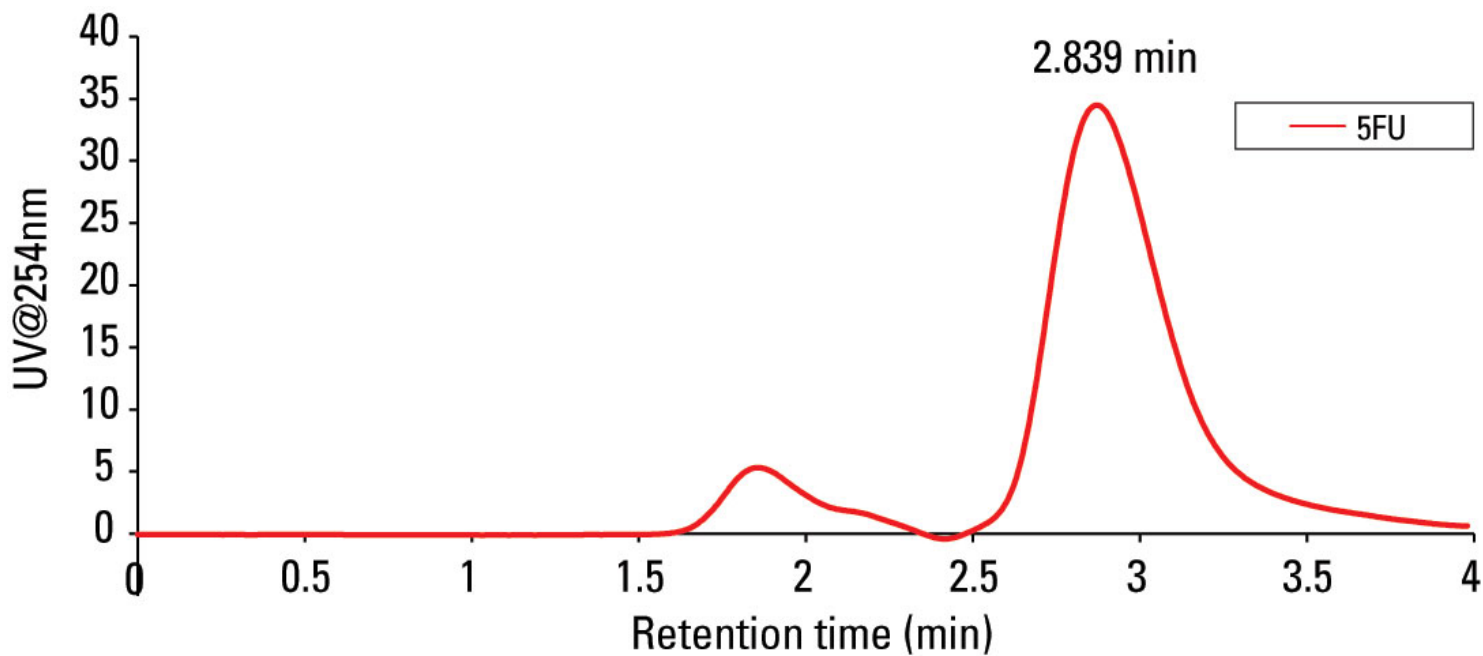
Separation of 5-fluorouracil using TSKgel Amide-80 column



	RT (min)	k	Area	S	Plates
%RSD (5 consecutive injections)	1.3	1.9	3.9	4.3	1



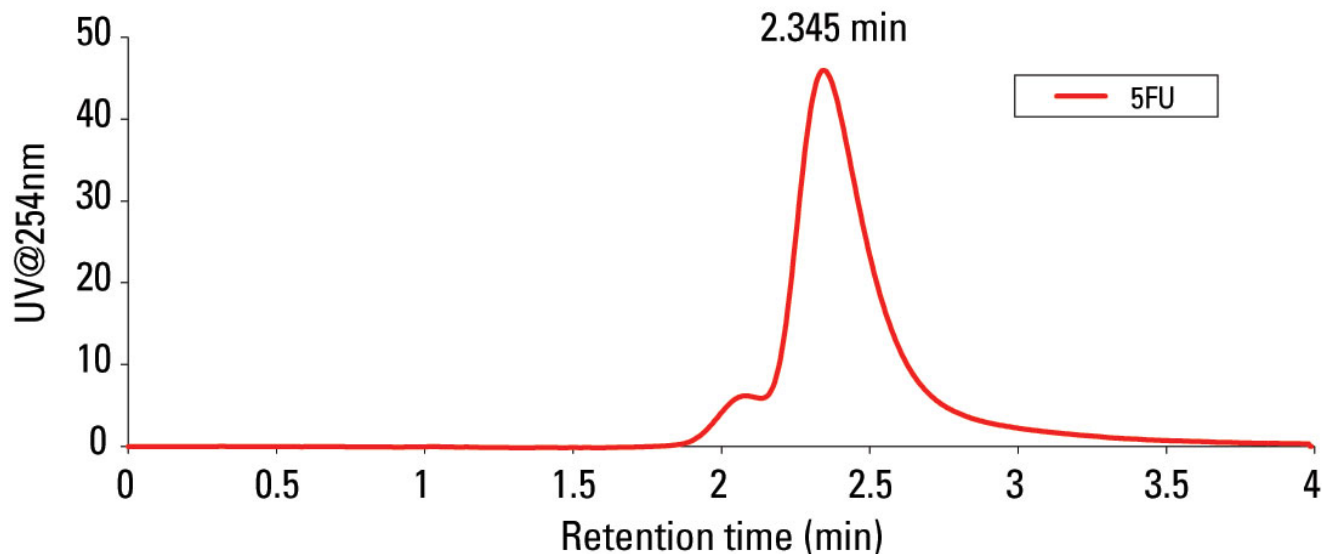
Separation of 5-fluorouracil using TSKgel NH₂-100 column



	RT (min)	k	Area	S	Plates
%RSD (5 consecutive injections)	1.6	2.4	1.12	2.26	5



Separation of 5-fluorouracil using competitive HILIC column P

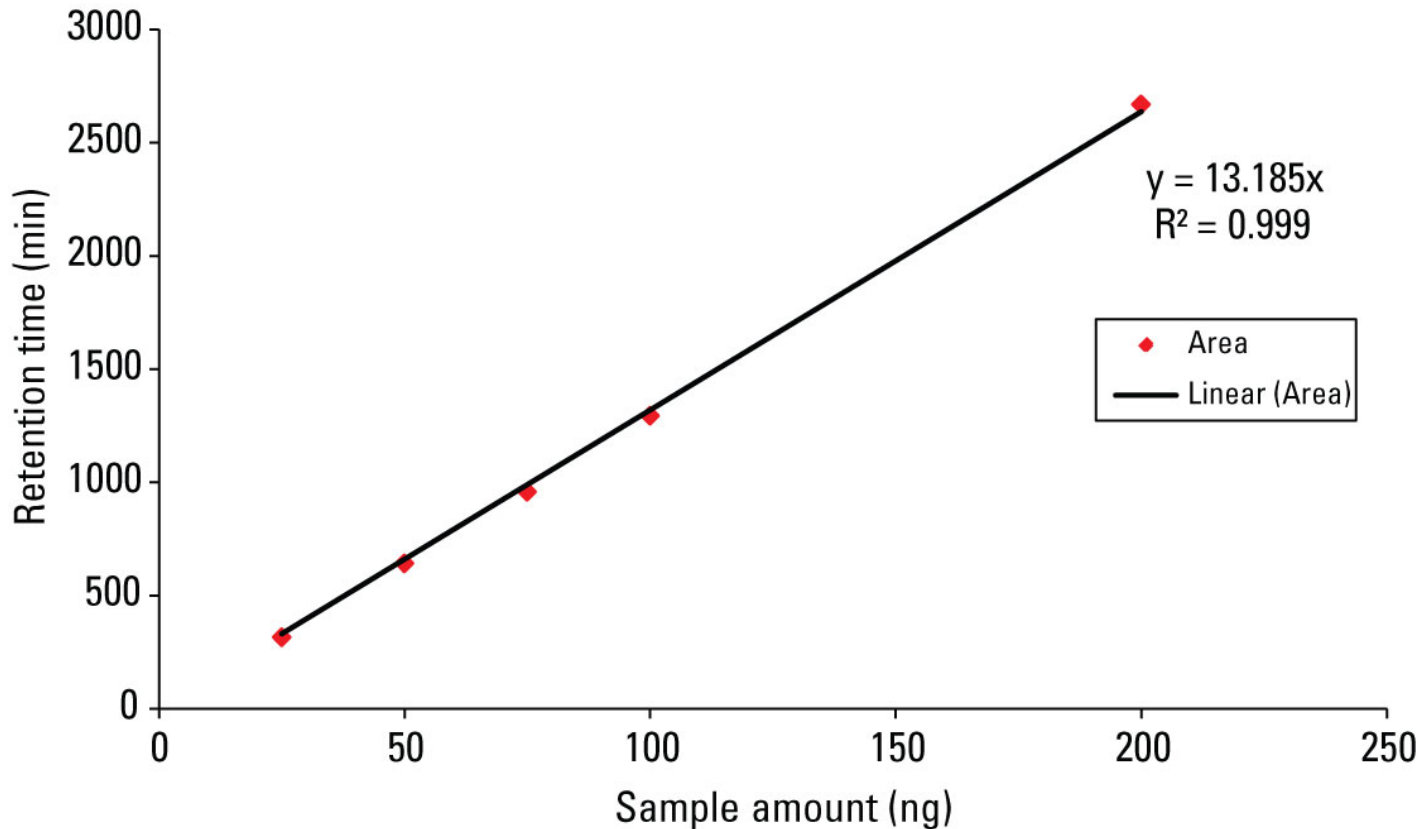


Separation of 5-fluorouracil using other competitive HILIC column (chromatograms not shown here).

	RT (min)
Competitor Z	2.319



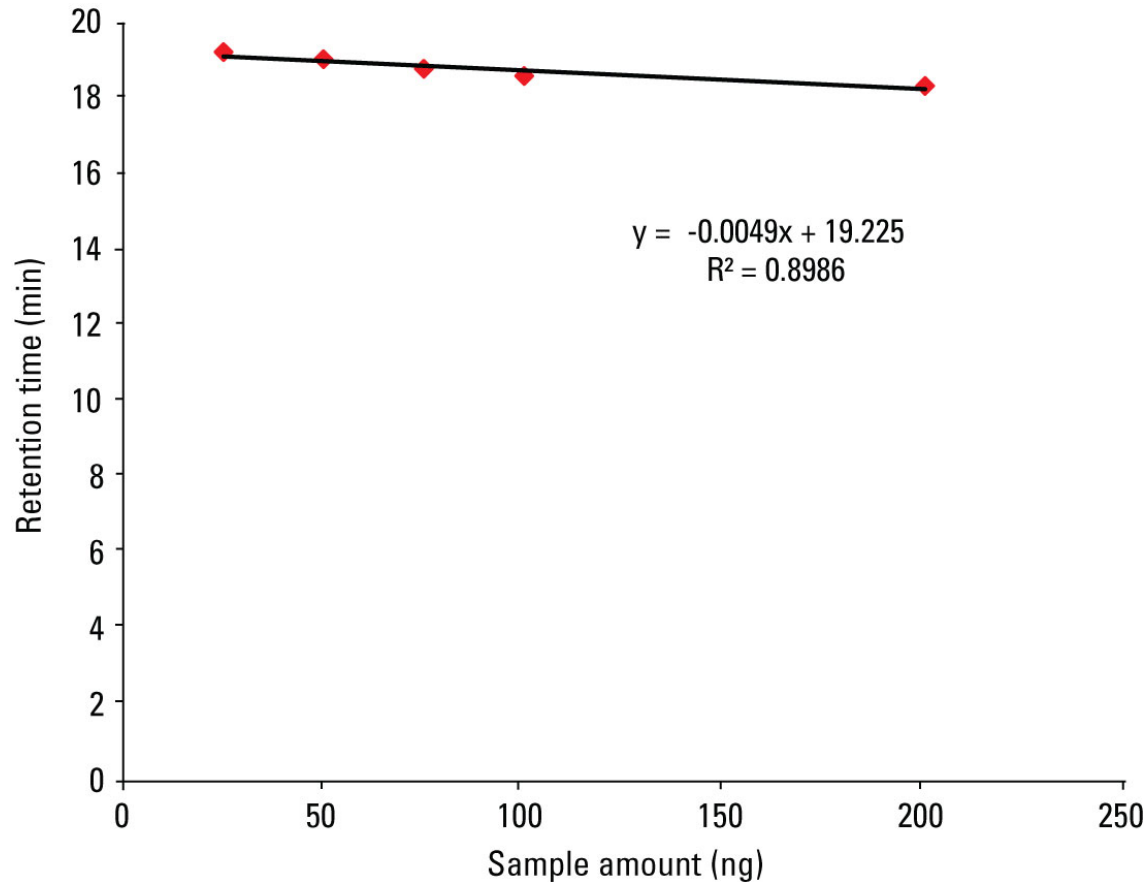
Test of linearity – 5-fluorouracil



The analysis was linear within the experimental range.



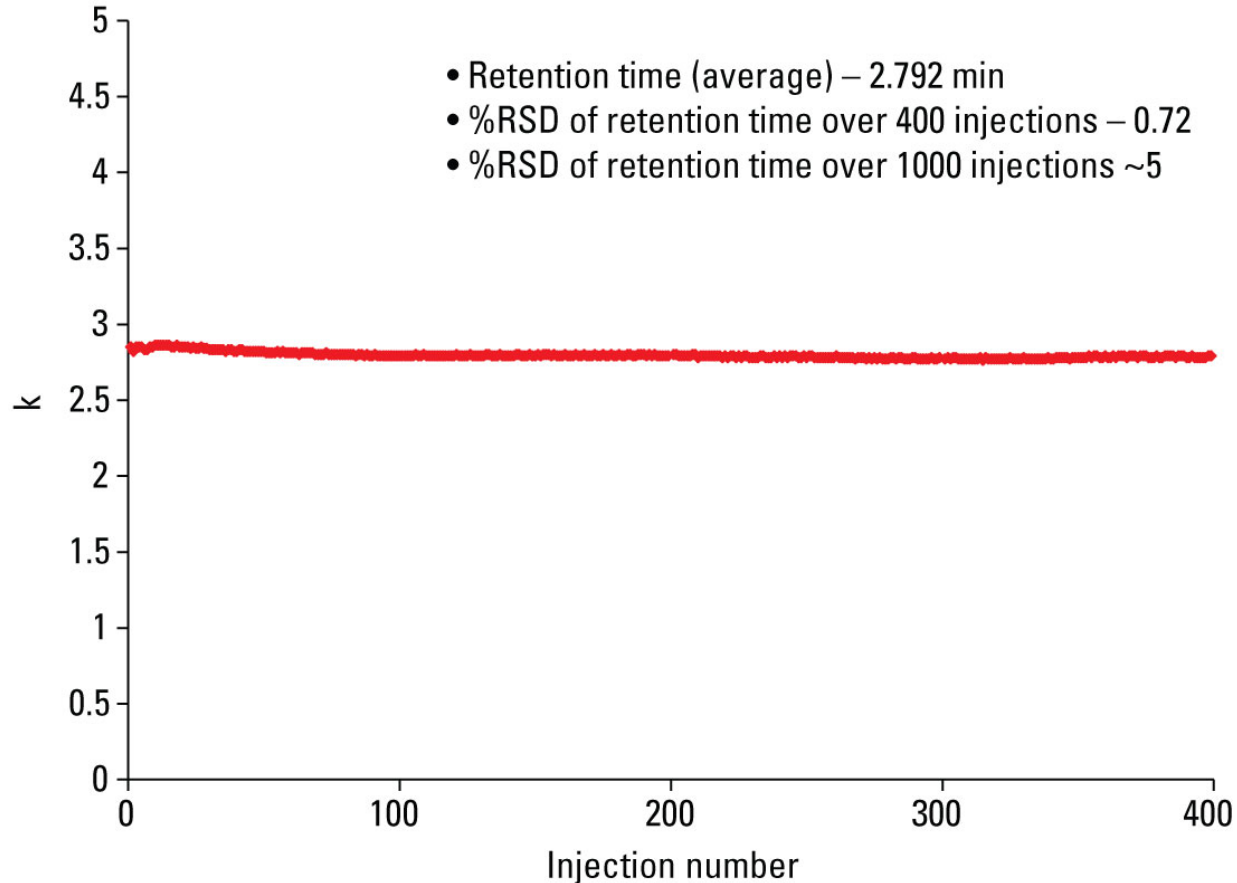
Effect of sample (5-FU) load on retention time



Shift in retention time was <5% within the experimental range of loading.



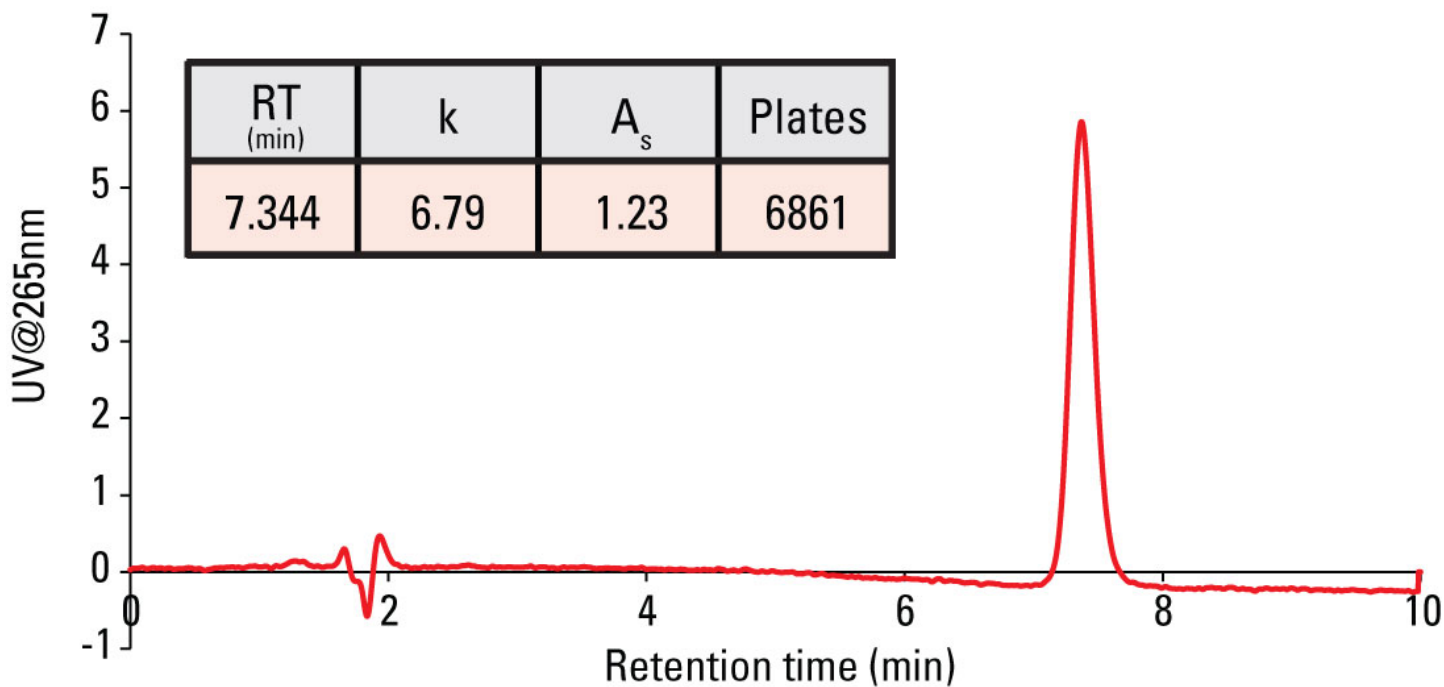
Reproducibility



The TSKgel NH₂-100 column is very stable. The novel bonding strategy provided a better safeguard against hydrolysis of the underlying silica. The chemical stability of the bonded phase was greatly enhanced by first using an endcapping reaction followed by the actual amino-ligand attachment.



Separation of 5-fluoro-2'-deoxyuridine using TSKgel NH₂-100 column



	RT (min)	k	Area	S	Plates
%RSD (5 consecutive injections)	0.453	0.487	0.198	2.32	3.84



System Suitability

	RT (min)	k	Area (mAU*S)	S	Plates
1	7.371	6.82	80.35	0.85	7245
2	7.334	6.78	80.66	0.83	7005
3	7.344	6.79	80.30	0.81	6861
Average	7.335	6.78	80.45	0.83	6930
Stdev	0.033	0.03	0.16	0.02	266
%RSD	0.447	0.49	0.20	2.32	3.84

%RSD values of the peak parameters over 3 consecutive injections show high reproducibility in the analysis.



Separation of 5-fluoro-2'-deoxyuridine using TSKgel NH₂-100 column

Flow rate (mL/min)	RT (min)	k	A_s	Plates
0.3	2.457	1.61	1.37	2317
0.2	3.667	1.59	1.43	2134
0.1	7.344	6.79	1.23	6861

The flow rate of 0.1mL/min yielded the best efficiency under the chromatographic conditions of the analysis.



Conclusions

- TSKgel NH₂-100 columns packed with spherical 3 μ m silica particles containing 100Å pores can be used for the separation of polar molecules such as 5-fluorouracil (Sigma) and 5-fluoro-2'-deoxyuridine with good resolution and consistency.
- The column yielded better separation compared to the competitive columns.