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

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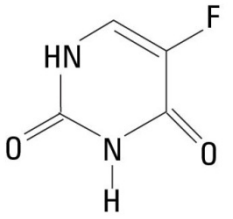
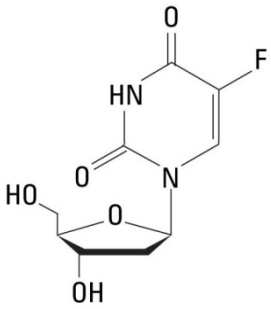
Introduction

- 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 inhibitor[1].
- 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′-deoxyuradine



[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.)

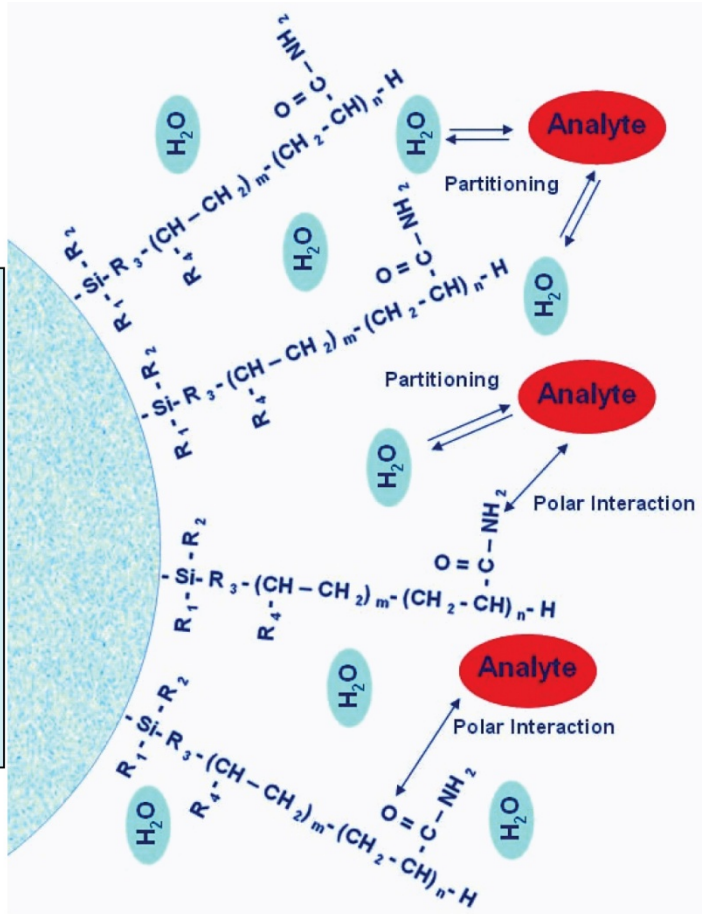
- This study demonstrates that a TSKgel NH_2-100 HILIC column effectively retains 5-FU and allows for its separation from several derivatives.
- The developed method was validated by a system suitability test.
- Limit of detection and limit of quantitation of the method were also determined.
- A column 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 NH_2-100 column for the analysis of 5-fluorouracil using a conventional HPLC system.

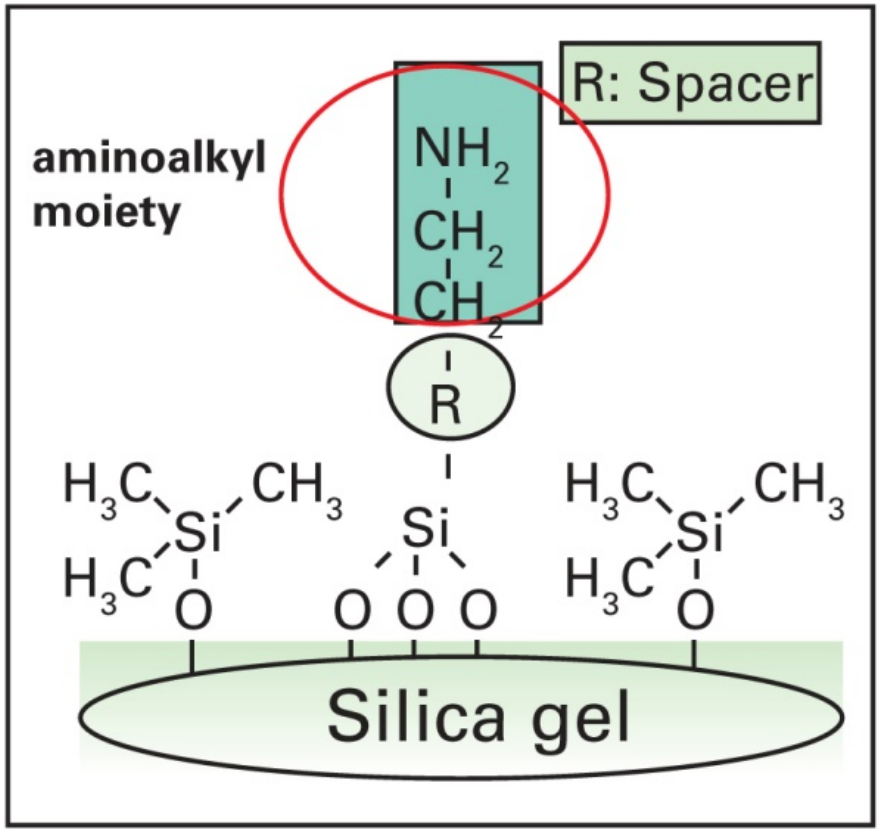
Structure and Mechanism

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



Mechanism of Hydrophilic Interaction Liquid Chromatography (HILIC)

Schematic diagram of stationary phase of TSKgel NH_2-100, 3µm columns



Properties of TSKgel NH_2-100, 3µm columns

Base material	Silica
Particle size (nominal)	3µm
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	>= 6,000	0.90 - 1.30
4.6mm ID x 15cm	>= 18,000	0.90 - 1.30

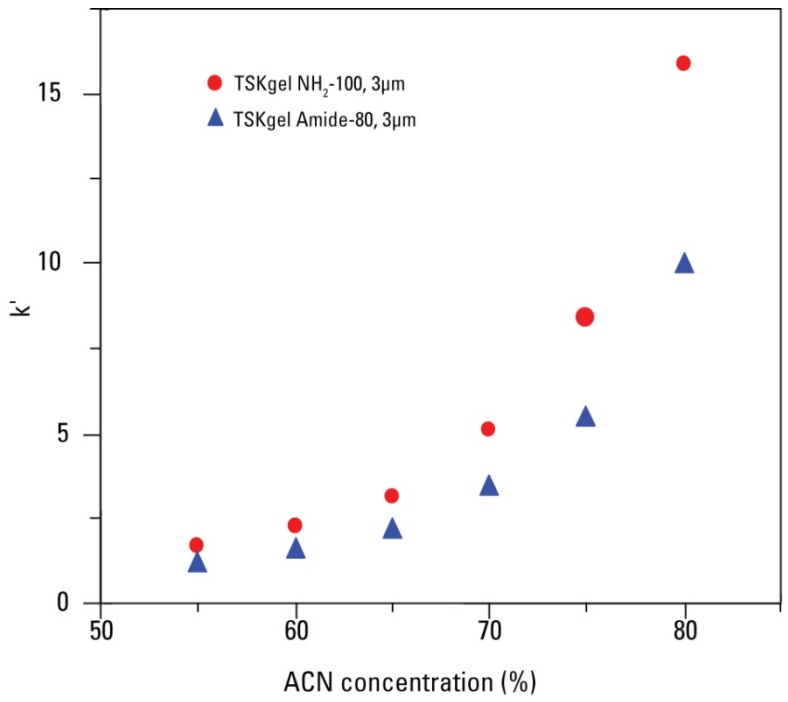
Characteristics of TSKgel NH_2-100, 3µm columns

- TSKgel NH_2-100 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 NH_2-100, 3µm 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 3µm material is ideal for use in LC/MS applications for the analysis of active pharmaceutical ingredients and their metabolites.

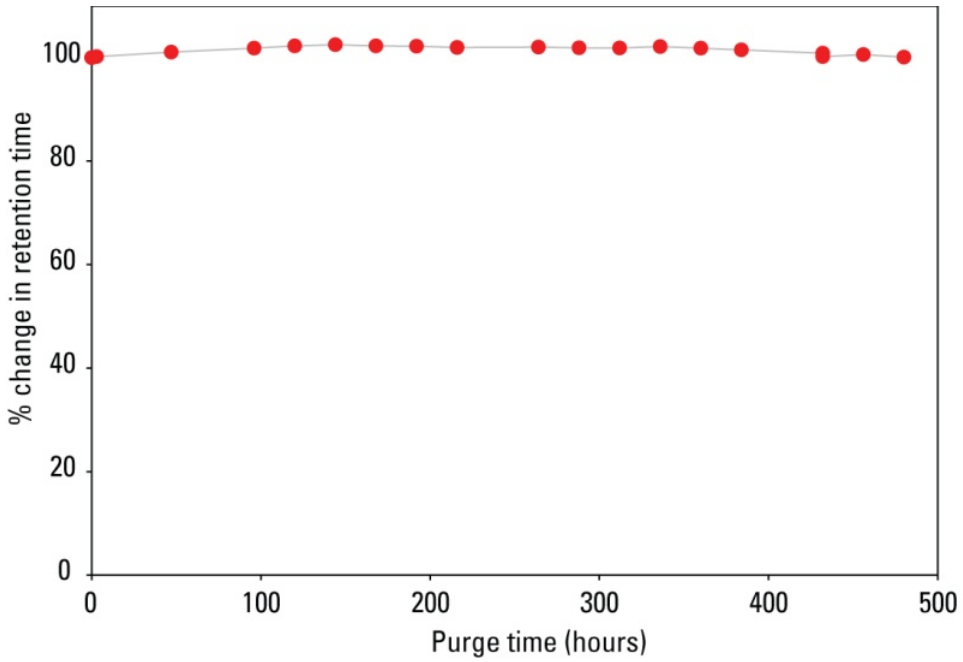
Retention of TSKgel NH_2-100 HILIC Columns



Columns:	TSKgel NH_2-100, 3µm, 4.6mm ID x 15cm
	TSKgel Amide-80, 3µm, 4.6mm ID x 15cm
Mobile phase:	H_2O/ACN = 10/90
Flow rate:	1.0mL/min
Detection:	RI
Temperature:	40°C
Injection Vol.:	10µL
Sample:	inositol

Amino-based TSKgel NH_2-100 columns expand retention & selectivity in HILIC while offering higher chemical stability, a pre-requisite for reproducible results.

Column Stability



Column:	TSKgel NH_2-100, 3µm, 4.6mm ID x 5cm
Mobile phase:	H_20/ACN = 25/75
Flow rate:	1.0mL/min
Detection:	RI
Temperature:	40°C
Injection vol.:	10µL
Sample:	inositol

After flushing a TSKgel NH_2-100 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.

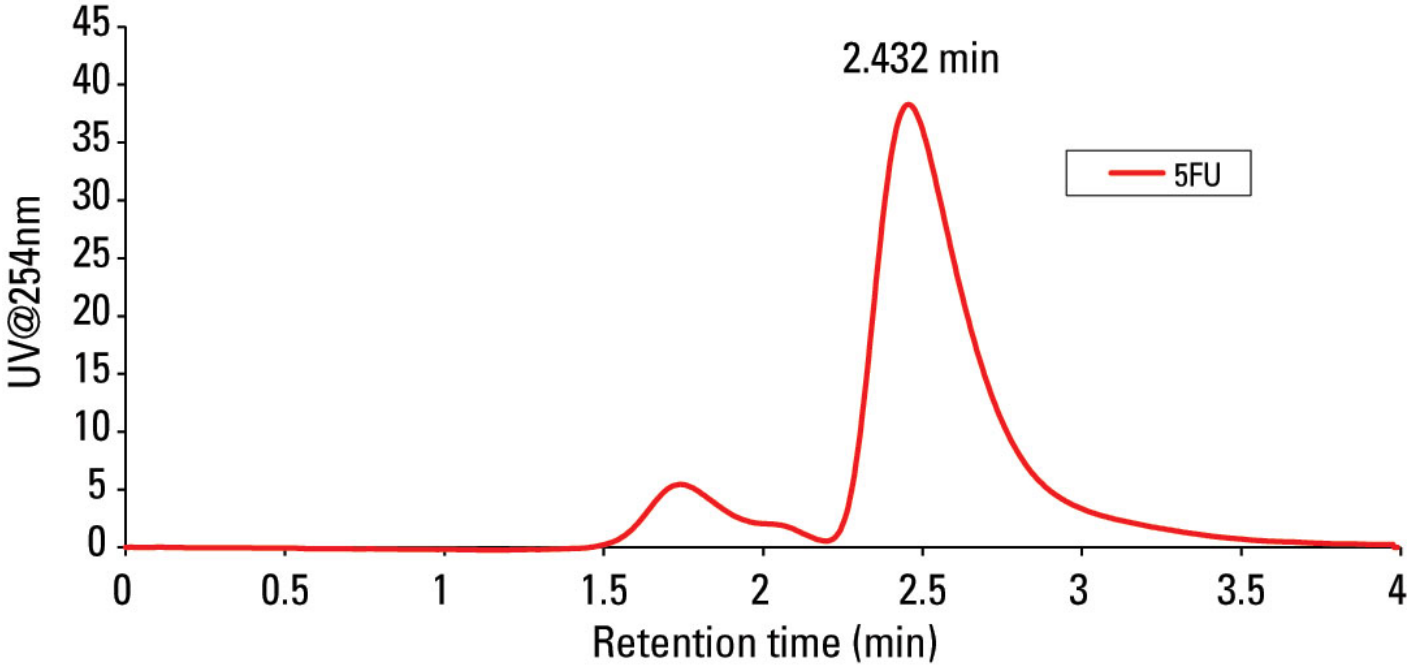
Optimal chromatographic conditions :

Columns:	TSKgel NH_2-100, 3µm, 2.0mm ID x 5cm Competitor P – 2.5µm, 2.1mm ID x 5cm Competitor Z – 3.5µm, 2.1mm ID x 5cm
Mobile phase: (Isocratic)	80% acetonitrile and 20% 5mmol/L ammonium acetate in water
Flow rate:	0.1mL/min
Detection:	UV@254nm
Temperature:	ambient
Injection vol.:	10µL

Chemicals

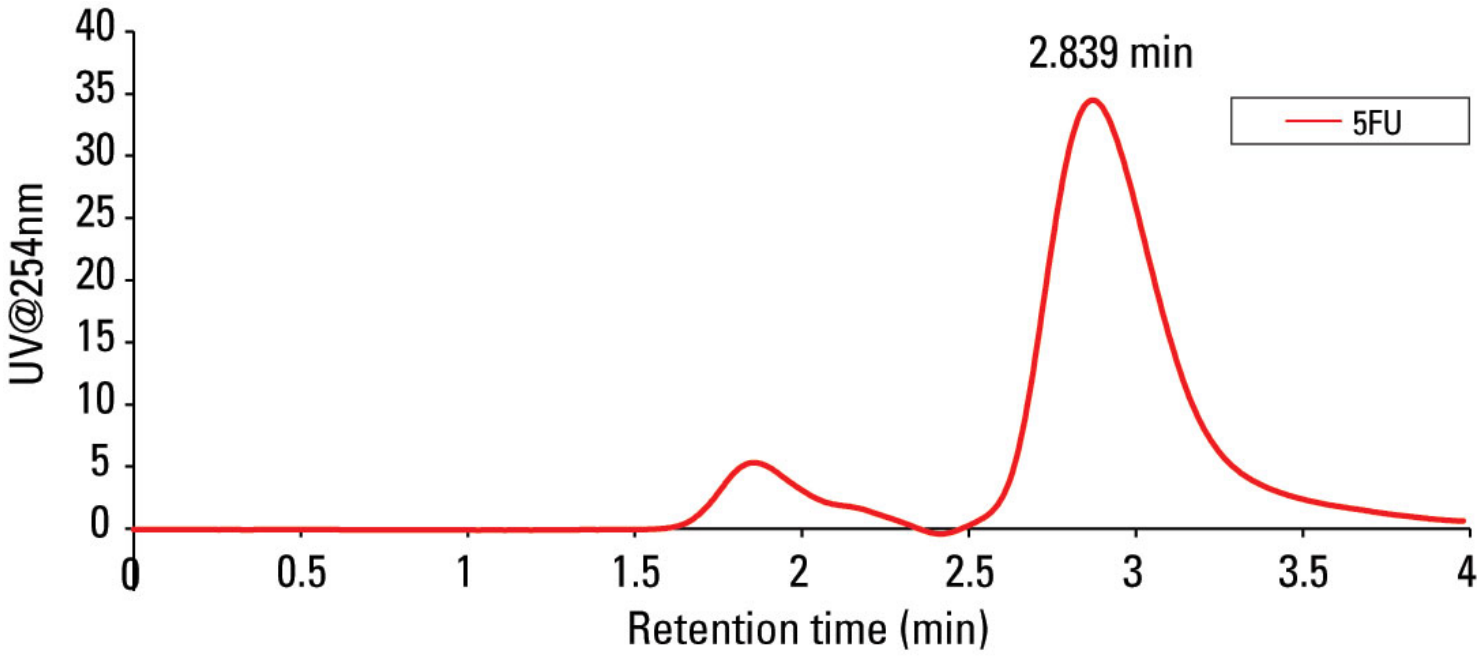
- Ammonium acetate (Fisher A637-500)
- 5-fluorouracil (Sigma)
- 5-fluoro-2’-deoxyuridine (Sigma F0503-250MG, Lot# 029K1232)
- High purity HPLC grade solvents were used for the preparation of stock standards, samples, and mobile phases.

Separation of 5-fluorouracil using TSKgel Amide-80 column



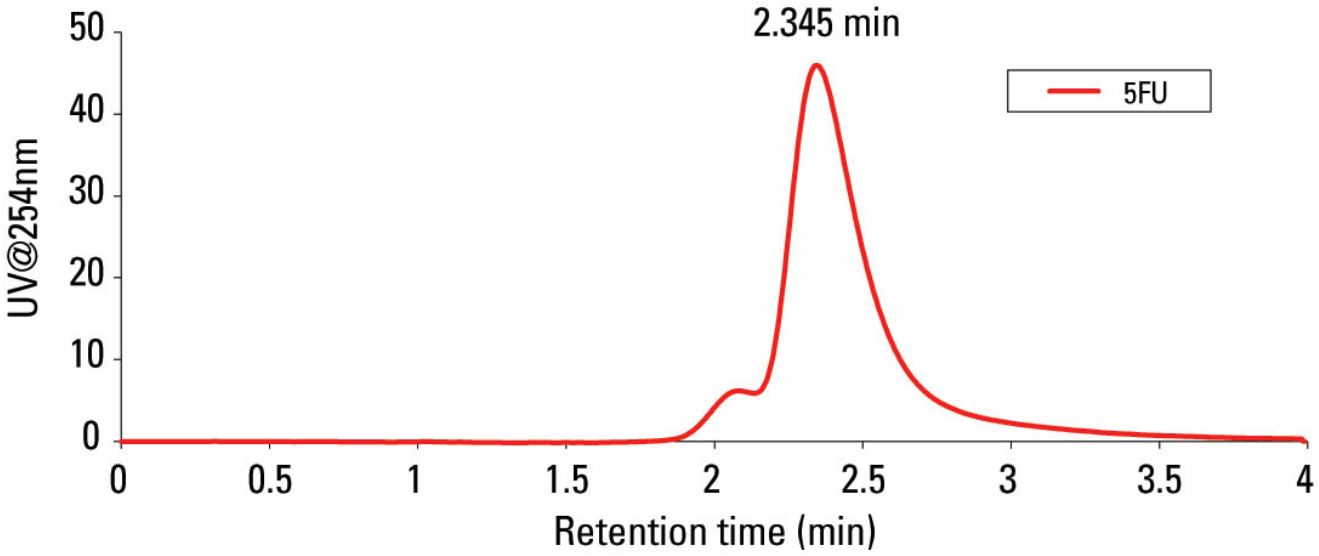
	RT (min)	k	Area	S	Plates
%RSD (5 consecutive injections)	1.3	1.9	3.9	4.3	1

Separation of 5-fluorouracil using TSKgel NH_2-100 column



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

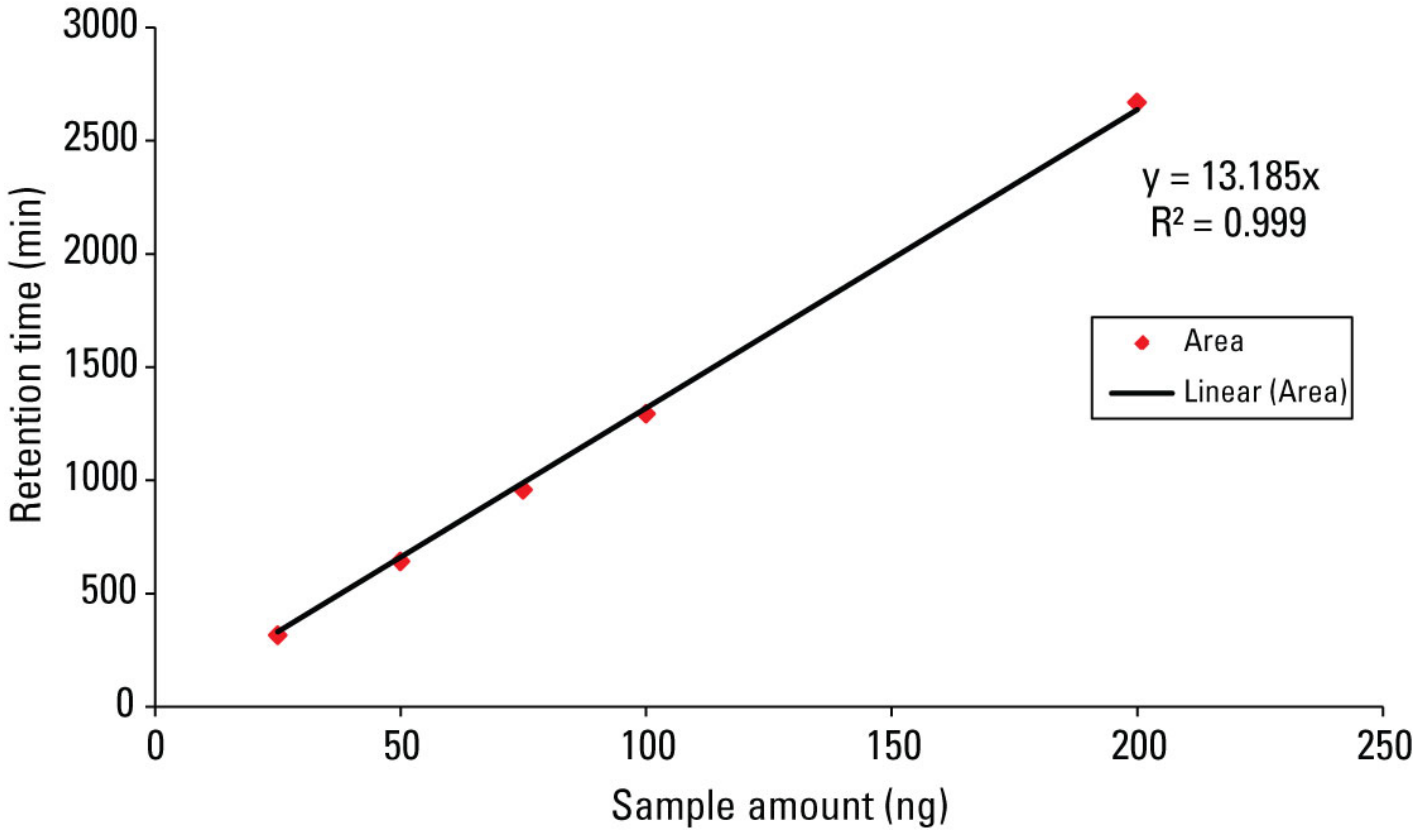
Separation of 5-fluorouracil using competitive HILIC column P



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

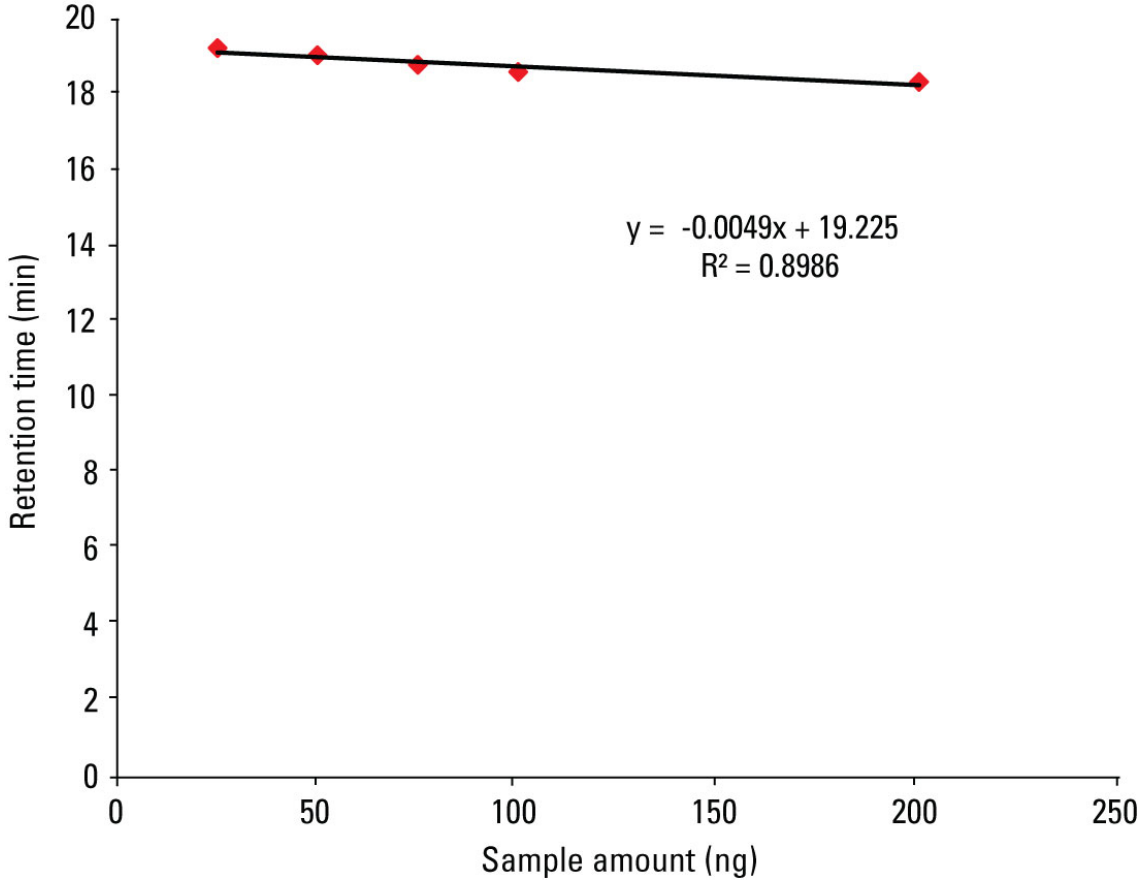
	RT (min)
Competitor Z	2.319

Test of linearity – 5-fluorouracil



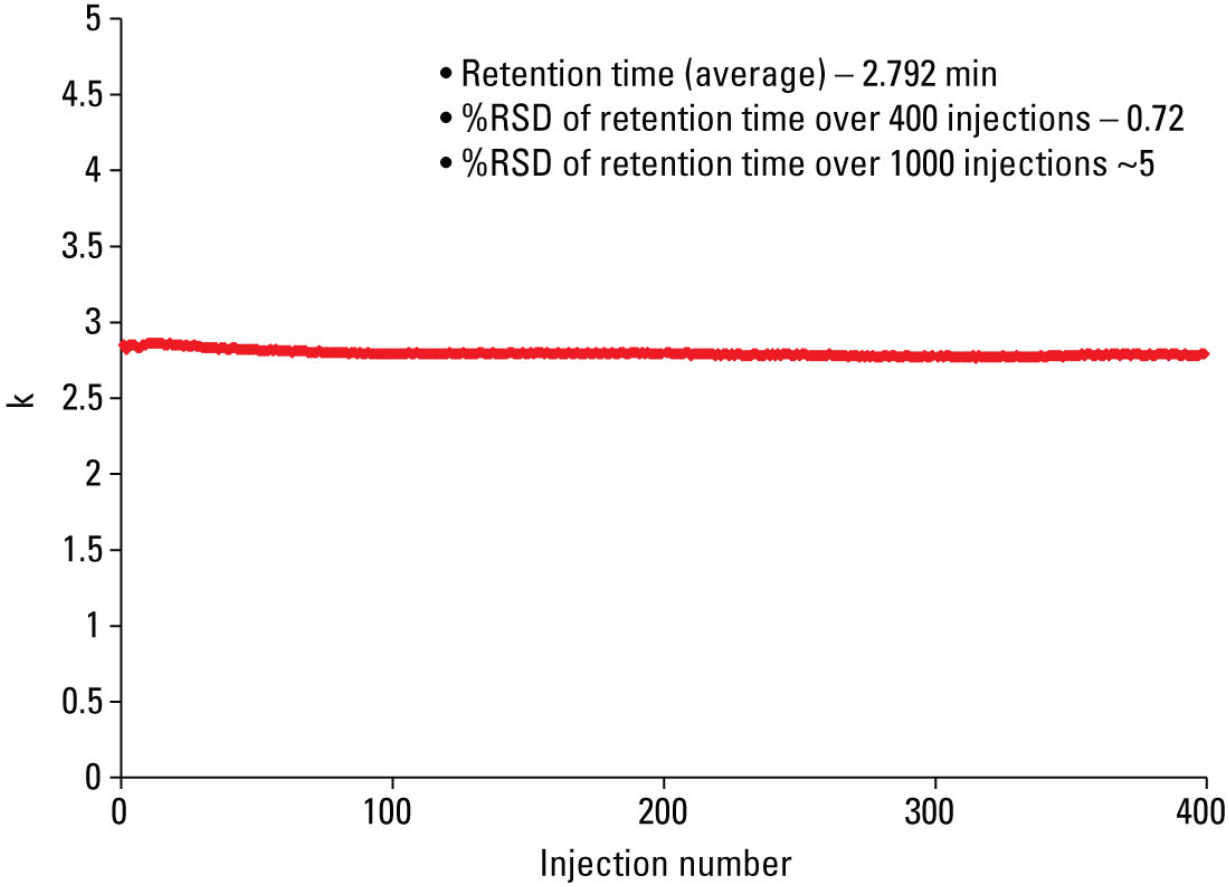
The analysis was linear within the experimental range.

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



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

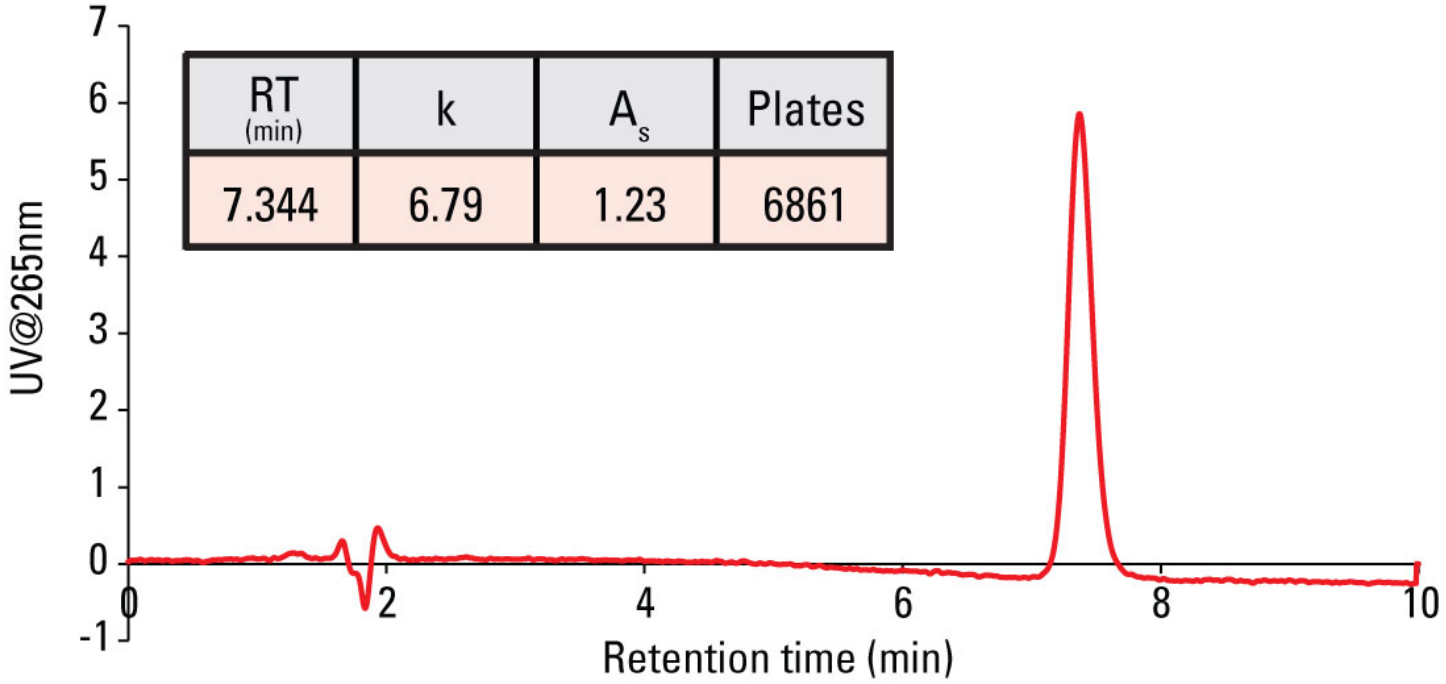
Reproducibility



The TSKgel NH_2-100 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 NH_2-100 column

RT (min)	k	A_s	Plates
7.344	6.79	1.23	6861



	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 NH_2-100 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 NH_2-100 columns packed with spherical 3µm 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.