APPLICATIONS

Reversed Phase Retention of Uracil, 5,6-Dihydrouracil, and 5-Fluorouracil with a Kinetex® PS C18 HPLC/UHPLC Column and MS Detection

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Introduction

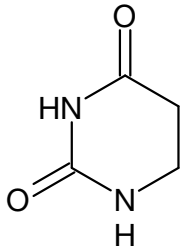
Uracil is a pyrimidine derivative and is considered a polar compound with a LogP value of -0.55. Because of uracil's diverse chemical use, the interest in the development of an efficient reversed phase HPLC method, which is able to simultaneously analyze uracil and its derivatives (5,6-dihydrouracil and 5-fluorouracil), has increased. However, uracil and its derivatives relative polarity present a unique challenge in terms of ensuring adequate chromatographic retention and compound resolution under reversed phase MS-compatible conditions.

Reversed phase mode is often used in chromatography applications because of its broad applicability for both the analysis of polar and non-polar compounds. However, the analysis of extremely polar compounds requires several considerations such as compound retention, peak shape, and selectivity. Mobile phase additive such as buffer systems, derivatization agents, or ion-pairing reagents can mitigate these challenges but the potential incompatibility with MS detection and irreproducibility issues limit the overall effectiveness of these additives. Described in this application is the reversed phase analysis of uracil, 5,6-dihydrouracil, and 5-fluorouracil with MS detection using a novel multi-mode LC selectivity. The selectivity of the Kinetex PS C18 is attributed to the unique stationary phase modification comprised of an alkyl C18 ligand with a covalently bonded polar functional group (**Table 2**). This positive (under slightly acidic conditions) functional group provides both improved peak shape for basic compounds and enhanced selectivity for polar compounds.[1] Also, the polar group ensures the phase is stable in under 100 % aqueous mobile phase condition.[2]

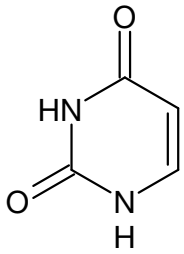
Experiment

Analytical reference standards for uracil, 5,6-dihydrouracil, and 5-fluorouracil were obtained through Sigma-Aldrich® (St. Louis, MO). The reversed phase mobile phase conditions used were Water with 0.1 % formic acid as the weak solvent A and methanol as the strong organic solvent B. An injection volume of 5 µL and a flow rate of 0.2 mL/min were used with a Kinetex 2.6 µm PS C18 HPLC/UHPLC column for this example. The column dimension of 150 x 3.0 mm was selected for use. The reference standards were prepared in the initial mobile phase as the diluent which was comprised of Water with 0.1 % formic acid/methanol (93:7) and diluted to the concentration of 200 ng/mL for all three compounds.

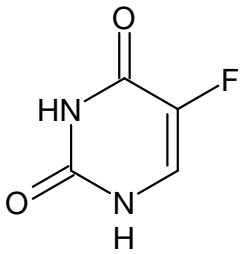
Uracil
Molar mass: 112.088 g/mol
Acidic pK_a: 8.8
LogP: -0.86



5,6-Dihydrouracil
Molar mass: 114.104 g/mol
Acidic pK_a: 11.73
LogP: -1.21



5-Fluorouracil
Molar mass: 130.078 g/mol
Acidic pK_a: 7.18
LogP: -0.66



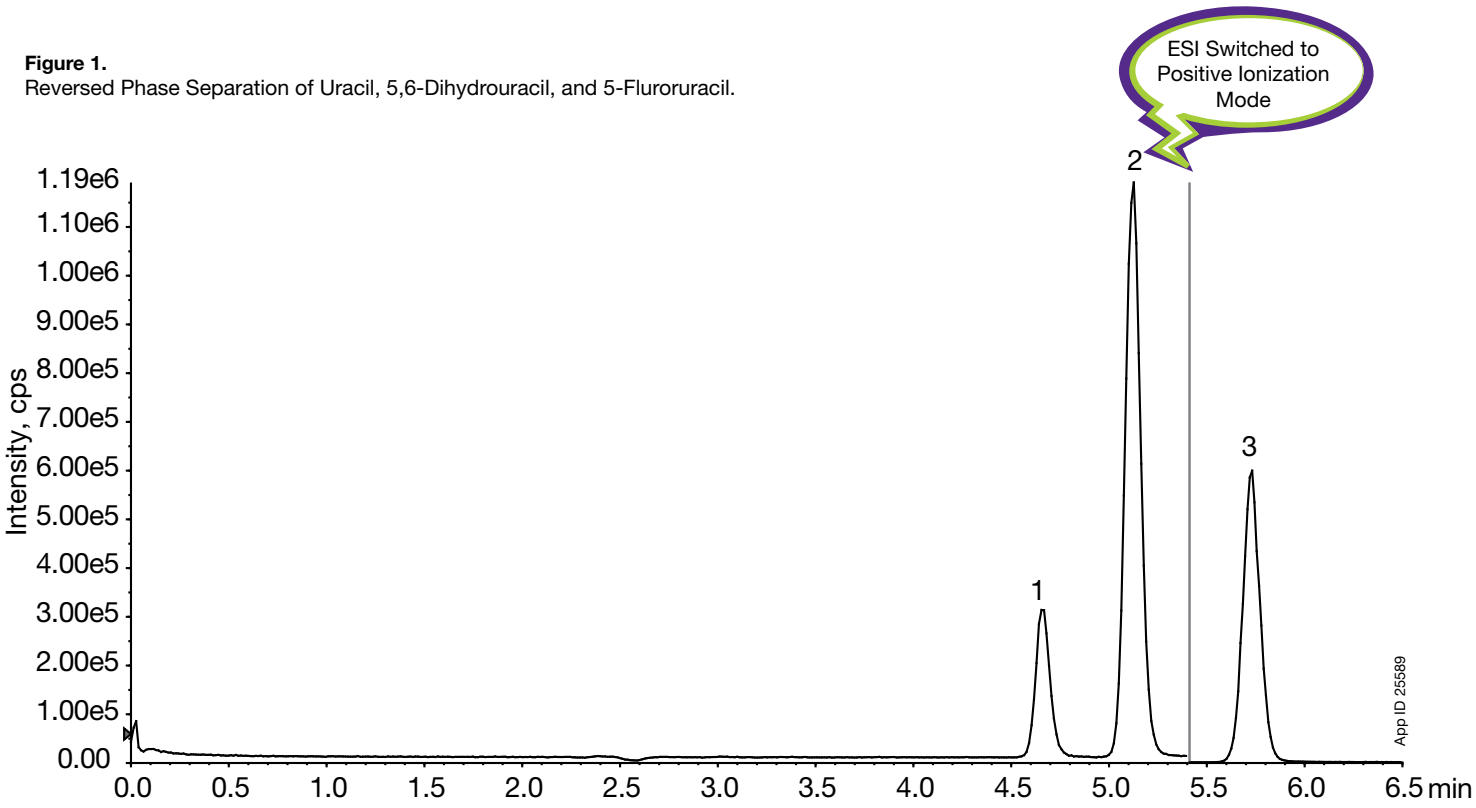
The column oven temperature was set to 15 °C on an Agilent® 1260 Infinity HPLC system, which was used for this investigation. For detection, a SCIEX® Triple Quad™ 4500 MS/MS was used. The Triple Quad 4500 was equipped with an Electrospray-Ionization (ESI) source capable of in-analysis polarity switching and eQ™ electronics which can polarity switch in 50 ms and has scan speeds of 20,000 Da/s. This allowed for simultaneous ionization and detection of dihydrouracil and uracil in positive mode (+ve) and 5-fluorouracil negative mode (+ve) (**Table 1**). The ESI polarity was +ve from 0.0 to 5.4 minutes and switched to -ve from 5.4 to 6.0 minutes in order to accomplish this analysis. The MS scan mode used for this example was a full scan acquisition resulting in a typical total ion current (TIC) plot (**Figure 1**).

Table 1.
ESI Ionization Source Parameters.

Source/Gas Parameters	NEGATIVE ION	POSITIVE* ION
Curtain Gas (CUR):	25	25
Collision Gas (CAD):	7	7
Temperature (TEM):	650	650
Ion Source Gas 1 (GS1):	60	60
Ion Source Gas 2 (GS2):	55	55
IonSpray Voltage (IS):	-4500	4500
Entrance Potential (EP):	-10	10
Collision Cell Exit Potential (CXP):	-6	13

*5-fluorouracil was analyzed in positive ionization mode.

Figure 1.
Reversed Phase Separation of Uracil, 5,6-Dihydrouracil, and 5-Fluroruracil.



Column: Kinetex® 2.6 µm PS C18
Dimensions: 150 x 3.0 mm
Part No.: 00F-4780-Y0
Mobile Phase: A: Water with 0.1 % Formic Acid
B: Methanol

Gradient:

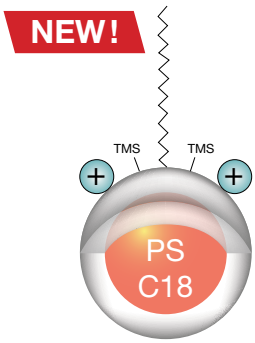
Time (min)	% B
0	7
6	7
6.01	50
7	50
7.01	7
12	7

Flow Rate: 0.2 mL/min
Temperature: 15 °C
Injection Volume 5 µL
Detector: MS/MS SCIEX® API 4500™
Sample:
1. 5,6-Dihydrouracil (4.66 min)
2. Uracil (5.12 min)
3. 5-Fluorouracil (5.73 min)

Table 2.
Material Characteristics.

Packing Material	Available Particle Size (µm)	Pore Size (Å)	Effective Surface Area (m^2/g)	Effective Carbon Load (%)	pH Stability	Pressure Stability (bar)
PS C18	2.6	100	200	9	1.5 – 8.5*	1,000/600†

* pH stability under gradient conditions. pH stability is 1.5 - 10 under isocratic conditions.
† 2.1 mm ID Kinetex® columns are pressure stable up to 1000 bar.



Kinetex 2.6 µm PS C18
A C18 with a positive surface charge modification that provides both polar and hydrophobic selectivity, is stable in 100 % aqueous conditions, and demonstrates improved peak shape for basic compounds.

Conclusion

In this application, the simultaneous reversed phase retention, separation, and MS detection of the extremely polar compounds uracil, 5,6-dihydrouracil, and 5-fluorouracil are displayed. The unique multi-interaction selectivity of the Kinetex PS C18 column circumnavigated the associated reversed phase challenges with the analysis of these extremely polar compounds. The Kinetex PS C18 demonstrated good chromatographic performance and retention without the use of ion-pairing or derivatization reagents. The selected mobile phase components were volatile, and therefore, compatible with MS detection.

The Triple Quad™ 4500 MS/MS that was used for this example, was equipped with an ESI source capable of in-analysis polarity switching. This allowed for simultaneous ionization and detection of 5,6-dihydrouracil and uracil in positive mode and 5-fluorouracil in negative mode. Uracil, 5,6-dihydrouracil, and 5-fluorouracil were retained, separated, and detected in under 6 minutes (**Figure 1**).

References

1. Zeshan Aqeel, Waleed Afaq, and Ryan Splitstone. The Effectiveness of Polar Stationary Phase Modification on Peak Shape for Basic Compounds Under General Reversed Phase Conditions – A Comparison of Four Alkyl C18 Phases. [Website] www.phenomenex.com. Available at: https://www.phenomenex.com/ViewDocument?id=the+effectiveness+of+polar+stationary+phase+modification+on+peak+shape+for+basic+compounds+under+-general+reversed+phase+conditions+-+a+comparison+of+four+alkyl+c18+phases_ [Accessed 30 July, 2019].
2. Sy Do, Lawrence Loo, Ryan Splitstone. Demonstrating the Kinetex PS C18 HPLC/UHPLC Column's Resistance to Dewetting and 100 % Aqueous Stability. [Website] www.phenomenex.com. Available at: https://www.phenomenex.com/ViewDocument?id=demonstrating+the+kinetex%C2%AE+ps+c18+hplc_uhplc+column%E2%80%99s+resistance+to+dewetting+and+100%25+aqueous+stability [Accessed 30 July, 2019].

Kinetex® Core-Shell LC Column Ordering Information

2.6 µm Minibore Columns (mm)					SecurityGuard™ ULTRA Cartridges‡
Phases	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	3/pk
PS C18	00A-4780-AN	00B-4780-AN	00D-4780-AN	00F-4780-AN	AJ0-8951
					for 2.1 mm ID

2.6 µm MidBore™ Columns (mm)				SecurityGuard ULTRA Cartridges‡
Phases	50 x 3.0	100 x 3.0	150 x 3.0	3/pk
PS C18	00B-4780-Y0	00D-4780-Y0	00F-4780-Y0	AJ0-8950
				for 3.0 mm ID

2.6 µm Analytical Columns (mm)					SecurityGuard ULTRA Cartridges‡
Phases	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	3/pk
PS C18	00B-4780-E0	00D-4780-E0	00F-4780-E0	00G-4780-E0	AJ0-8949
					for 4.6 mm ID

‡ SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000.

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