RESTEK pharmaceutical

Applications note

Analyze Nucleotides, Nucleosides, Purine, and Pyrimidine Bases Simultaneously with the Ultra IBD Column

Mixtures of nucleotides, nucleosides, and their respective purine or pyrimidine bases are difficult to analyze by reversed phase/high performance liquid chromatography (RP/HPLC). These compounds cover a wide range of polarities and functionalities, from the acidic nucleotides to the basic purines and pyrimidines, making it very difficult to retain and resolve all of them with conventional alkyl stationary phases. Traditional HPLC analysis of these compounds often uses a combination of reversed phase-ion pairing (RP-IP) and/or ion exchange (IEX) mode. Nucleotides often are analyzed by anion exchange, while nucleosides sometimes are analyzed by cation exchange. These methods are not compatible with all the solutes in the mixtures and they lack ruggedness.

This Applications Note demonstrates that all three classes of compounds (nucleotides, nucleosides, and bases) can be analyzed by RP/HPLC using one column and the same, simple isocratic mobile phase. This provides greater convenience, reproducibility, and ruggedness in developing methods for these mixtures. By using a unique, intrinsically base-deactivated stationary phase (i.e., the Ultra IBD column), simple RP/HPLC conditions were identified that can resolve any common purine or pyrimidine base from its related ribonucleoside and mono-, di-, and triphosphate nucleotides.

Nucleotides and nucleosides are derived from the nitrogenous bases shown in Figure 1. These nitrogenous bases are either purines (adenine and guanine) or pyrimidines (cytosine, uracil, and thymine). A nucleoside consists of a purine or pyrimidine base linked to a five-carbon sugar (pentose). A nucleotide is composed of a nucleoside plus one or more phosphate groups. Figure 2 shows the structures of a nucleoside and three nucleotides derived from adenine.

Figure 1

Nitrogenous purine and pyrimidine bases form nucleotides and nucleosides

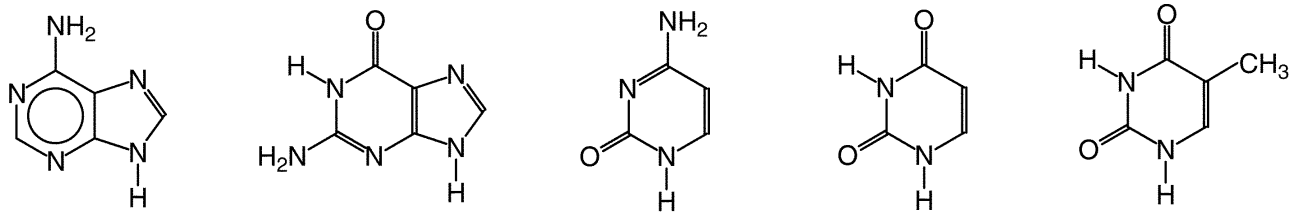
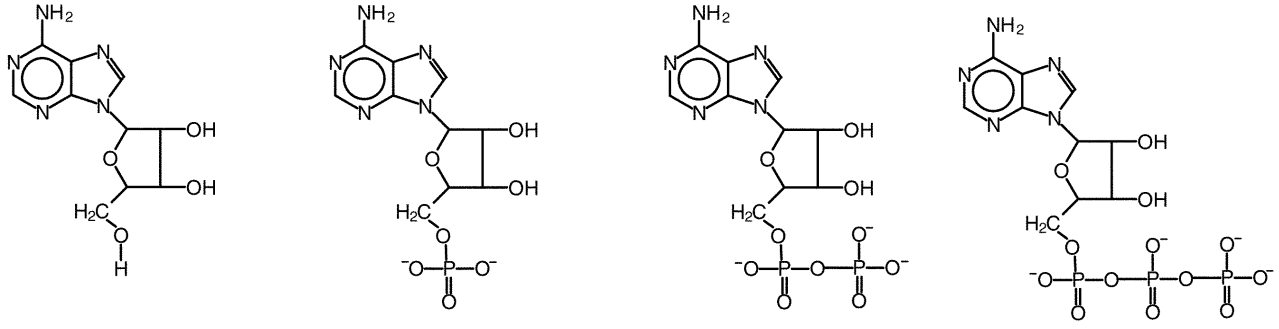


Figure 2

A nucleoside and three nucleotides derived from adenine



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Adenosine is a ribonucleoside (adenine + ribose). ATP is a particularly important nucleotide, serving as a universal source of energy for biological processes.

The Ultra IBD column is particularly effective for retaining and resolving complex mixtures of nucleotides, nucleosides, and purine and pyrimidine bases. The unique Ultra IBD stationary phase is composed of a polar group within, or intrinsic to, an alkyl chain. The polar group gives extra retention for many polar analytes as well as unique selectivity, a very high level of base deactivation, and compatibility with highly aqueous mobile phases. The Ultra IBD column is ideal for LC/MS because it often can resolve acidic, basic, zwitterionic and/or neutral compounds in a single analysis using simple mobile phases.

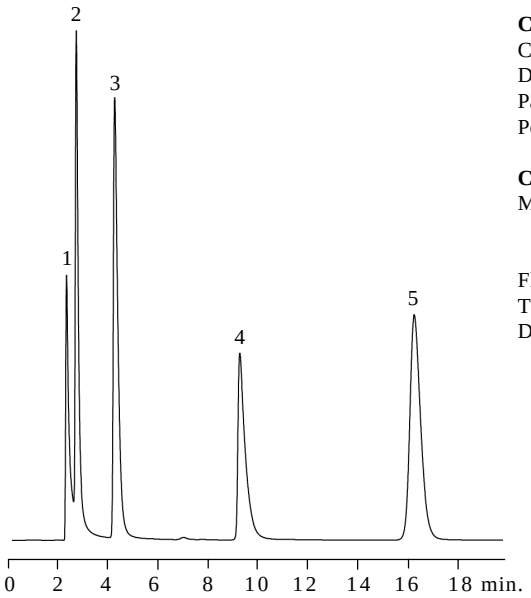
Figures 3 through 7 each show separations of one of the major purines or pyrimidines from its respective ribonucleo-

Figure 3

Ultra IBD separates adenine, adenosine, ATP, ADP, and AMP with simple isocratic reverse phase conditions.

Peak List:	Conc. (µg/mL):
1. ATP	258
2. ADP	320
3. AMP	274
4. adenine	84
5. adenosine	254

Sample:
Inj.: 20µL
Solvent: 20mM ammonium acetate, pH 5.8



Column:	**Ultra IBD**
Catalog #:	9175565
Dimensions:	150 x 4.6mm
Particle Size:	5µm
Pore Size:	100Å

Conditions:
Mobile Phase: 20mM ammonium acetate, pH 5.8: methanol (97.5:2.5)
Flow: 1.0mL/min
Temp.: 35°C
Det.: UV @ 260nm

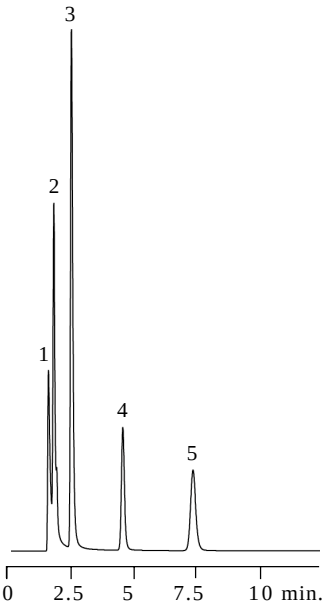
LC_0129

Figure 4

Ultra IBD separates guanine, guanosine, GTP, GDP, and GMP with simple isocratic reverse phase conditions.

Peak List:	Conc. (µg/mL):
1. GTP	212
2. GDP	260
3. GMP	362
4. guanine	80
5. guanosine	226

Sample:
Inj.: 20µL
Solvent: 20mM ammonium acetate, pH 5.8



Column:	**Ultra IBD**
Catalog #:	9175565
Dimensions:	150 x 4.6mm
Particle Size:	5µm
Pore Size:	100Å

Conditions:
Mobile Phase: 20mM ammonium acetate, pH 5.8: methanol (97.5:2.5)
Flow: 1.0mL/min
Temp.: 35°C
Det.: UV @ 260nm

LC_0131

side and mono-, di-, and triphosphate nucleotide. Guanosine, uridine, cytidine, and thymidine are ribonucleosides derived from guanine, uracil, cytosine, and thymine, respectively. Note that each of these separations was achieved using the same conditions and that, in each case, the order of elution is the same: the triphosphate, the diphosphate, then the monophosphate nucleotide, followed by the base, and lastly the nucleoside. There are slight "shoulders" on the peaks for GDP (Figure 4), TTP (Figure 6), and UMP (Figure 7). These compounds were present in the standards and were presumed to be impurities or degradation products.

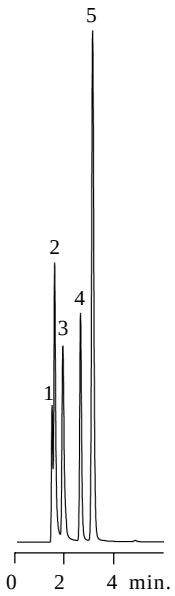
Table 1 lists the typical retention times obtained for all 25 of the compounds separated in Figures 3–7. While not all 25 compounds can be resolved in a single HPLC analysis, it is possible to analyze all of them using these chromatographic conditions and MS or MS/MS detection. Note that the mobile phase is compatible with MS detection, as all of its

Figure 5

Ultra IBD separates cytosine, cytidine, CTP, CDP, and CMP with simple isocratic reverse phase conditions.

Peak List: Conc. (µg/mL):
1. CTP 248
2. CDP 322
3. CMP 248
4. cytosine 100
5. cytidine 340

Sample:
Inj.: 20µL
Solvent: 20mM ammonium acetate, pH 5.8



Column: **Ultra IBD**
Catalog #: 9175565
Dimensions: 150 x 4.6mm
Particle Size: 5µm
Pore Size: 100Å

Conditions:
Mobile Phase: 20mM ammonium acetate, pH 5.8: methanol (97.5:2.5)
Flow: 1.0mL/min
Temp.: 35°C
Det.: UV @ 260nm

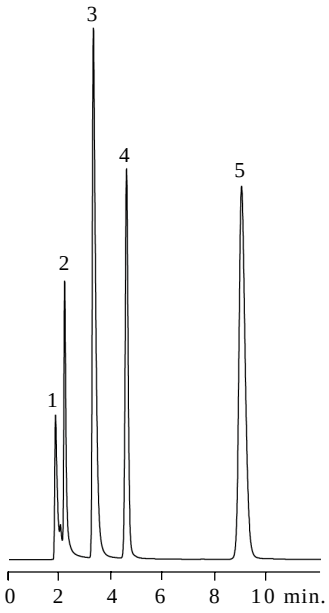
LC_0130

Figure 6

Ultra IBD separates thymine, thymidine, TTP, TDP, TMP with simple isocratic reverse phase conditions.

Peak List: Conc. (µg/mL):
1. TTP 224
2. TDP 152
3. TMP 346
4. thymine 88
5. thymidine 318

Sample:
Inj.: 20µL
Solvent: 20mM ammonium acetate, pH 5.8



Column: **Ultra IBD**
Catalog #: 9175565
Dimensions: 150 x 4.6mm
Particle Size: 5µm
Pore Size: 100Å

Conditions:
Mobile Phase: 20mM ammonium acetate, pH 5.8: methanol (97.5:2.5)
Flow: 1.0mL/min
Temp.: 35°C
Det.: UV @ 260nm

LC_0132

components are volatile. Figure 8 shows that the Ultra IBD column can resolve a mixture of 11 various nucleotides, nucleosides, and bases using ultraviolet (UV) detection.

The unique stationary phase of the Ultra IBD column can retain and resolve mixtures of nucleotides, nucleosides, and purine and pyrimidine bases by RP/HPLC, using isocratic elution with a simple, volatile mobile phase. A single set of chromatographic conditions can resolve any of the common purine or pyrimidine bases from its respective ribonucleoside and mono-, di-, and triphosphate nucleotides.

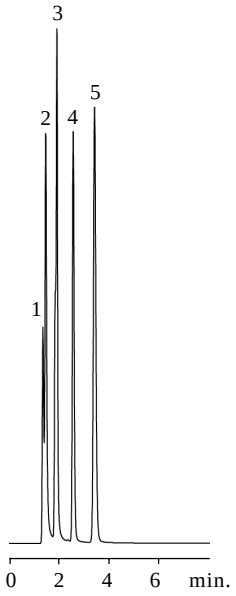
Figure 7

Ultra IBD separates uracil, uridine, UTP, UDP, UMP with simple isocratic reverse phase conditions.

Peak List: Conc. (µg/mL):

1. UTP	360
2. UDP	360
3. UMP	284
4. uracil	90
5. uridine	230

Sample:
Inj.: 20µL
Solvent: 20mM ammonium acetate, pH 5.8



Column: **Ultra IBD**
Catalog #: 9175565
Dimensions: 150 x 4.6mm
Particle Size: 5µm
Pore Size: 100Å

Conditions:
Mobile Phase: 20mM ammonium acetate, pH 5.8: methanol (97.5:2.5)
Flow: 1.0mL/min
Temp.: 35°C
Det.: UV @ 260nm

LC_0133

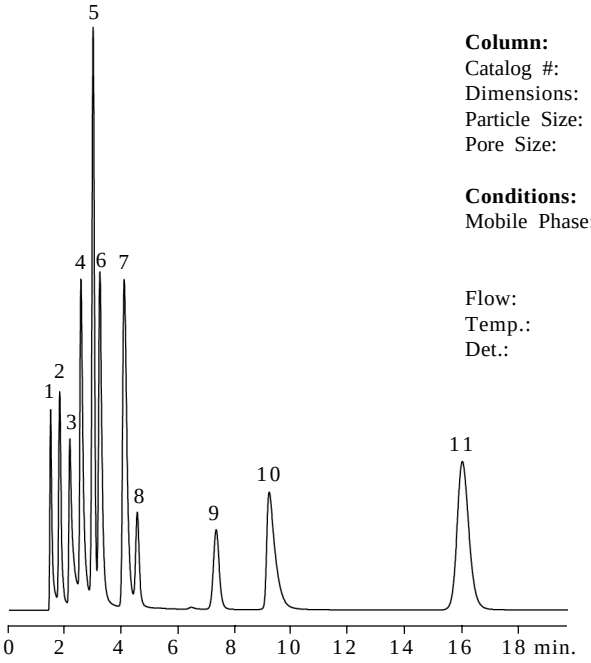
Figure 8

Ultra IBD separates an 11-component mixture of nucleotides, nucleosides, and nitrogenous bases.

Peak List: Conc. (µg/mL):

1.	CDP	146
2.	CMP	113
3.	ATP	117
4.	ADP	145
5.	cytidine	155
6.	TMP	157
7.	AMP	125
8.	guanine	36
9.	guanosine	103
10.	adenine	38
11.	adenosine	115

Sample:
Inj.: 20µL
Solvent: 20mM ammonium acetate, pH 5.8



Column: **Ultra IBD**
Catalog #: 9175565
Dimensions: 150 x 4.6mm
Particle Size: 5µm
Pore Size: 100Å

Conditions:
Mobile Phase: 20mM ammonium acetate, pH 5.8: methanol (97.5:2.5)
Flow: 1.0mL/min
Temp.: 35°C
Det.: UV @ 260nm

LC_0128

Table 1

Typical retention times for common nucleotides, nucleosides, and purine and pyrimidine bases.

Compound	Retention Time[1] (min.)
CTP	1.5
UTP	1.5
CDP	1.6
UDP	1.6
GTP	1.7
TTP	1.8
CMP	1.9
GDP	1.9
UMP	2.0
TDP	2.2
ATP	2.3
GMP	2.6
Cytosine	2.6
ADP	2.7
Uracil	2.7
Cytidine	3.1
TMP	3.3
Uridine	3.6
AMP	4.2
Guanine	4.6
Thymine	4.6
Guanosine	7.4
Thymidine	9.0
Adenine	9.3
Adenosine	16.2

1. Retention times are for 150x4.6mm column; Flow rate: 1.0mL/min; Mobile phase: 20mM ammonium acetate, pH 5.8: Methanol. (97.5:2.5, v/v).

Ultra IBD, 3µm Columns

Particle Size: 3µm	1.0mm ID cat.#	2.1mm ID cat.#	3.2mm ID cat.#	4.6mm ID cat.#
30mm length	9175331	9175332	9175333	9175335
50mm length	9175351	9175352	9175353	9175355
100mm length	9175311	9175312	9175313	9175315
150mm length	9175361	9175362	9175363	9175365

Ultra IBD, 3µm Columns with Trident™ Inlet

Particle Size: 3µm	2.1mm ID cat.#	3.2mm ID cat.#	4.6mm ID cat.#
30mm length	9175332-700	9175333-700	9175335-700
50mm length	9175352-700	9175353-700	9175355-700
100mm length	9175312-700	9175313-700	9175315-700
150mm length	9175362-700	9175363-700	9175365-700

Ultra IBD, 5µm Columns

Particle Size: 5µm	1.0mm ID cat.#	2.1mm ID cat.#	3.2mm ID cat.#	4.6mm ID cat.#
30mm length	9175531	9175532	9175533	9175535
50mm length	9175551	9175552	9175553	9175555
100mm length	9175511	9175512	9175513	9175515
150mm length	9175561	9175562	9175563	9175565
200mm length	9175521	9175522	9175523	9175525
250mm length	9175571	9175572	9175573	9175575

Ultra IBD, 5µm Columns with Trident™ Inlet

Particle Size: 5µm	2.1mm ID cat.#	3.2mm ID cat.#	4.6mm ID cat.#
30mm length	9175532-700	9175533-700	9175535-700
50mm length	9175552-700	9175553-700	9175555-700
100mm length	9175512-700	9175513-700	9175515-700
150mm length	9175562-700	9175563-700	9175565-700
200mm length	9175522-700	9175523-700	9175525-700
250mm length	9175572-700	9175573-700	9175575-700

Ultra IBD Guard Cartridges

Dimensions	cat.#	qty.
10 x 2.1mm	917550212	3
10 x 4.0mm	917550210	3
20 x 4.0mm	917550220	2