Several ZORBAX RRHD 1.8 µm Selectivities Facilitate Method Development

Application Note

Pharmaceuticals

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Abstract

Agilent Rapid Resolution High Definition columns offer new levels of productivity for HPLC because they are made with 1.8 µm particles, they are stable to 1200 bar and they are available in a variety of bonded phases for large potential selectivity differences. This application note describes the ways in which RRHD columns allow for selectivity refinement of methods for selected endocannabinoids.

Introduction

Rapid Resolution High Definition (RRHD) columns, used on UHPLC instruments, provide significant productivity enhancements because they are stable to 1200 bar and can withstand higher flow rates. The Eclipse Plus phase features a double end-capped process and unique bonding that delivers exceptional peak shapes across a broad range of analytes. This makes it an exceptional column for method development. Many users of UHPLC are working with analyses that require the highest level of sensitivity and resolution. Sometimes, alternate C18 phases are desirable because of the additional selectivity refinements they allow.

Endogenous cannabinoids are neurotransmitters that naturally occur in animal organs, especially the brain, and have a role as intercellular messengers similar to the well-known acetylcholine, gamma aminobutyric acid (GABA), or dopamine. They are quite different, however, because endocannabinoids are lipophilic and found in cell membranes, whereas acetylcholine, GABA, and dopamine are highly water soluble and are found in the vesicles inside cells.

Anandamide or arachidonoylethanolamide (AEA) was the first endocannabinoid discovered in 1992. Since then, research has shown that these neurotransmitters play significant roles in many life functions, including memory, sleeping and eating patterns, and even implantation of the blastocyst (embryonic stage) in the uterus. Structures of anandamide and other endocannabinoids analyzed in this application are represented in Figure 1.

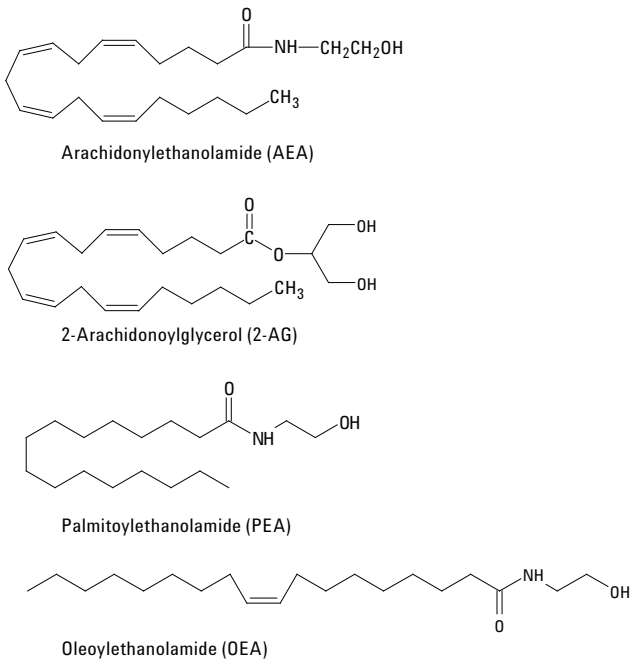


Figure 1. Encocannabinoid-related compounds.

Experimental

Four endocannabinoid fatty amides were obtained from Sigma-Aldrich (Bellefonte, PA, USA):

- Arachidonoylethanolamide (AEA)
- 2-Arachidonoylglycerol (2-AG)
- Palmitoylethanolamide (PEA)
- Oleoylethanolamide (OEA)

These were diluted in methanol to a concentration of about 1 to 5 mg/mL each component, then diluted 1:100 in 50% methanol/water for a final sample concentration of 0.01 to 0.05 mg/mL. The 2.1-mm ID columns are ideal for electrospray ionization-mass spectrometry (ESI-MS) because low flow rates (<1 mL/min) allow for optimal electrospray ionization and introduction to the high-vacuum mass spectrometer.

Long, 100-mm RRHD columns were used for this analysis to further improve efficiency and resolution. The longer RRHD columns coupled with Agilent's 1290 Infinity LC system and high linear velocity flow rates exploit the system and column's UHPLC pressure limits (1200 bar).

The following Agilent ZORBAX RRHD columns were used:

- Agilent ZORBAX Rapid Resolution High Definition (RRHD) Eclipse Plus C18, 2.1 mm × 100 mm, 1.8 µm
  Agilent Part Number: 959758-902
- Agilent ZORBAX Rapid Resolution High Definition (RRHD) Eclipse XDB-C18, 2.1 mm × 100 mm, 1.8 µm
  Agilent Part Number: 981758-902
- Agilent ZORBAX Rapid Resolution High Definition (RRHD) StableBond SB-C18, 2.1 mm × 100 mm, 1.8 µm
  Agilent Part Number: 858700-902
- Agilent ZORBAX Rapid Resolution High Definition (RRHD) Extend C18, 2.1 mm × 100 mm, 1.8 µm
  Agilent Part Number: 758700-902

The HPLC system was an Agilent 1290 Infinity LC with an Agilent 6410 Triple Quadrupole Mass Spectrometer.

– G4220A Binary Pump, with mobile phase A: H_2O and B: CH_3CN, each with 0.1% HCOOH. Analysis was isocratic at 1 mL/min, with varying amounts of CH_3CN.

– G4226A Automatic Liquid Sampler (ALS), with injection volume set to 1 µL.

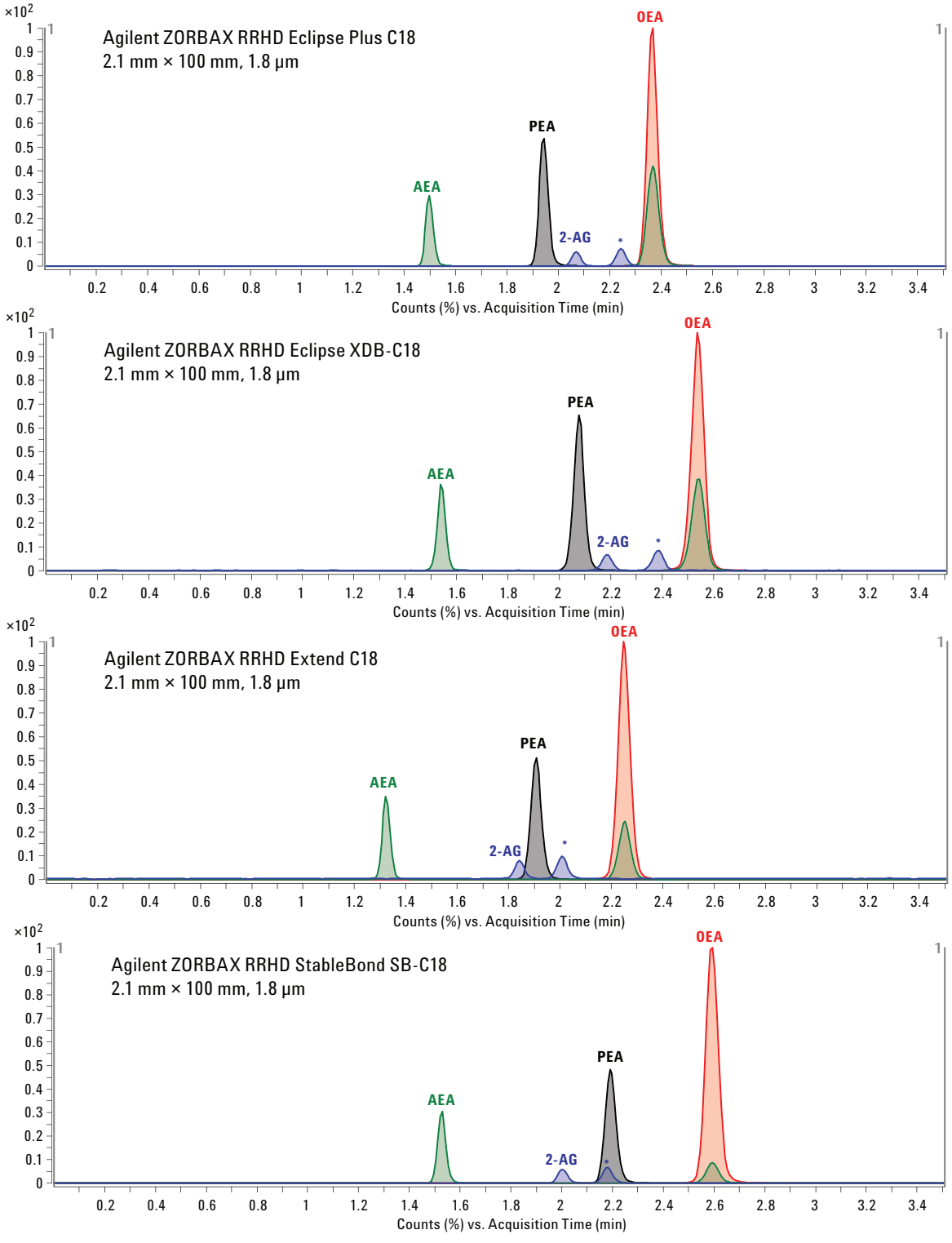
– G1316C Thermostatted Column Compartment (TCC), with temperature set to 30 °C.

– G6410A Triple Quadrupole Mass Spectrometer (QQQ), with MS source: electrospray AP-ESI; drying gas temperature and flow: 325 °C, 12 L/min; nebulizer gas pressure: 35 psi; capillary voltage: 3000 V; in MS2Scan mode from 290 to 390. Individual components were monitored at 348, 300, 379 and 326 for AEA, PEA, 2-AG and OEA respectively.

Discussion of Results

Examining Selectivity (α) for Best Resolution

The variety of stationary phases available on ZORBAX columns makes them useful for method development, especially for changing selectivity. Having a variety of bonded phases (columns) available to sequentially try in method development analyses demonstrates the different selectivity easily gained from the columns. Figure 2 is an overlay of four different C18 bonded phases available on ZORBAX RRHD 1.8 µm particles. All have a symmetrical peak shape and similar retention. Notice however, that although only four compounds comprise the endocannabinoid sample, a fifth peak is detected. This impurity has a mass of 379, and is believed to be 1,3-arachidonolyglycerol, a rearrangement of 2-AG. This is based on extracted ion MSD data.



*The second blue peak is an impurity, believed to be 1,3-arachidonolyglycerol, a rearrangement of 2-AG

Figure 2. The selectivtiy of four Agilent ZORBAX RRHD C18 columns is compared using a method for endocannabinoids (see Experimental section for detailed method parameters).

The differences in selectivity between the four columns are due to the subtle, yet important differences in bonding, such as the type of bonding, the endcapping, or the amount and type of silanols on the silica. Other factors that influence selectivity including mobile phase composition, temperature, and pH are identical.

These four C18 bonded phases differ slightly, all based on 1.8 µm ZORBAX Rx-SIL silica, though the silica is modified to improve peak shape with the Eclipse Plus C18 column. Each column has its strengths. The Eclipse Plus is the most inert of the four. The Eclipse XDB and the Extend have slightly more silanols and the StableBond has the most exposed silanols. Silanols are often thought to be deleterious but can be used to provide selectivity.

Another way to alter selectivity is to change the mobile phase. In MSD applications, volatile mobile phases (MS friendly) are preferred. Here, a popular aqueous acetonitrile and formic acid mixture was used. However making different mobile phases for experimentation followed by flushing/equilibrating the HPLC system consumes more time than simply substituting columns. The goal here was rapid method development.

Examining Retention (k) for Best Resolution

Because Eclipse Plus C18 offers the most promising selectivity for the endocannabinoids, it will be used to address the retention factor of the resolution equation. The mobile phase strength (percent organic) was changed in 5% increments to alter the retention factor, and hopefully improve resolution. The entire sequence of runs to determine the best organic strength lasted less than two hours, including each run in triplicate with a 10-min equilibration between mobile phase compositions. This comparison used 100-mm RRHD columns; if 50-mm columns were used, the screening time could have been halved.

Figure 3 shows the effect of changing the organic solvent concentration on resolution. Typically, in reversed phase chromatography, k increases as the organic strength weakens. The 2-AG and 1,3-AG peaks are retained at a different rate (move faster than the other peaks). At 75% CH_3CN, the 2-AG coelutes with the PEA, and the 1,3-AG is resolved. At 60% CH_3CN, the two peaks have shifted so the 2-AG is completely resolved, but the 1,3-AG coelutes with the OEA peak. With this 100-mm RRHD Eclipse Plus C18 column, all five peaks are completely resolved with 70% CH_3CN.

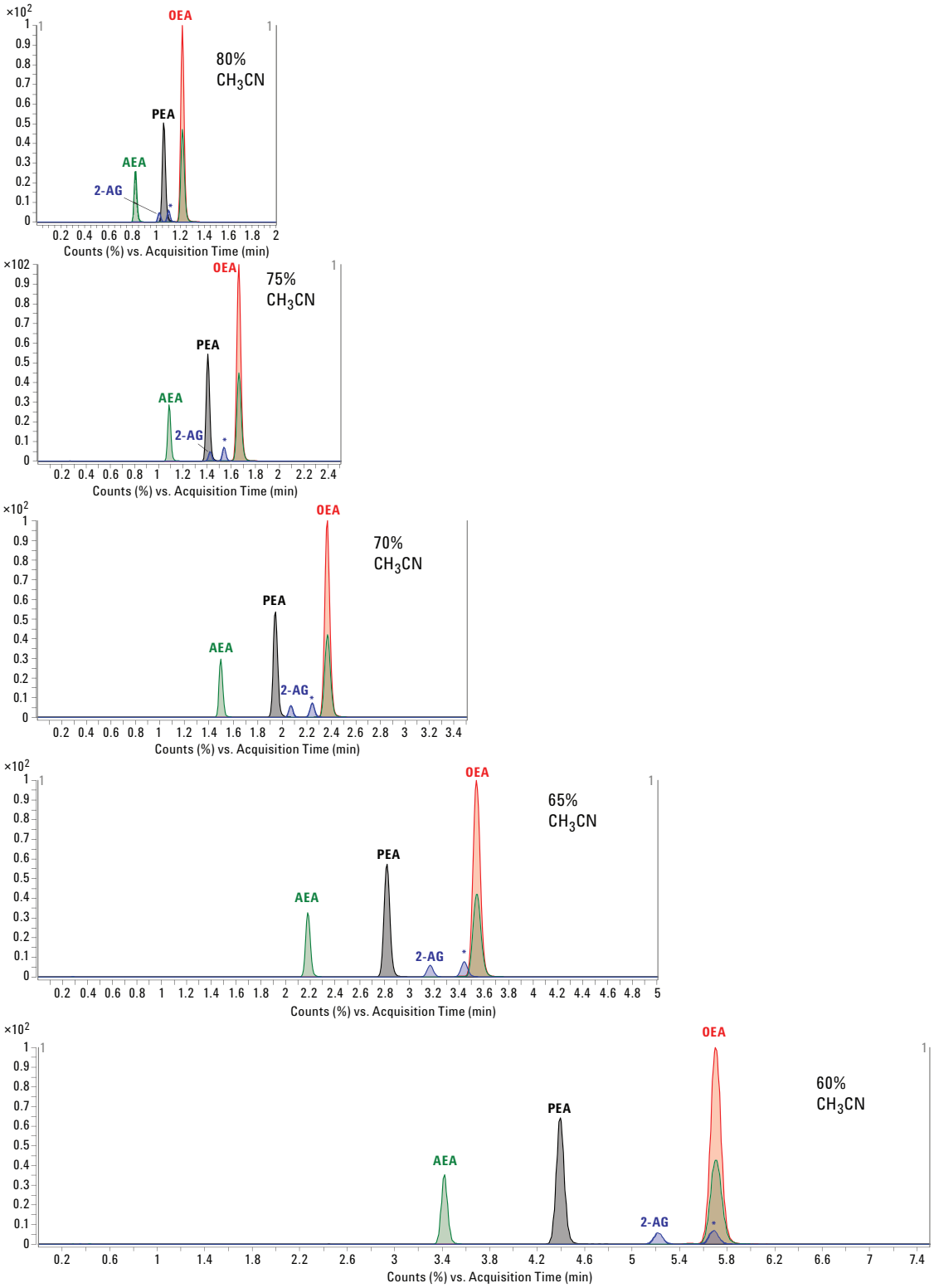


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Figure 3. The strength of the mobile phase is adjusted with this endocannabinoid method on Agilent ZORBAX Eclipse Plus to show the influence of k on resolution (see Experimental section for detailed method parameters).

Conclusions

The variety of bonded phases (selectivities) available on Agilent ZORBAX 1.8 µm packing adds to the strength of the column's 1200 bar stability. In addition to the four C18 phases described in this work, a variety of other phases are available, or in development, for RRHD columns, making them a flexible, scalable option for pharmaceutical labs that need to ensure method compatibility throughout the development cycle.

Reference

1. Henderson, J., Long W. "Exploiting RRHT Columns with Different C18 Selectivities to Quickly Develop Methods for Endocannabinoids," 2007, Agilent Technologies publication 5989-6128EN.

For More Information

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