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SUPELCO

Bulletin 938

Utility of Pentafluorophenylpropyl Stationary Phases for RP-HPLC Analyses: Biogenic Amines on Discovery HS F5

Small, polar molecules are particularly difficult to analyze by RP-HPLC because of poor retention on traditional C18 columns. Methods to enhance retention, such as derivatization and ion-pairing, are often time consuming, inhibit LC/MS analysis, and can adversely affect the method's robustness and reproducibility. In the studies reported here, the unique retention and selectivity of the polar, pentafluorophenylpropyl Discovery HS F5 phase toward basic compounds are exploited to produce efficient separations for several classes of biogenic amines. The results show superior retention and selectivity of the Discovery HS F5 for these analytes when compared to traditional methods on C18 columns. Additionally, the Discovery HS F5 stationary phase was shown to be resistant to loss of hydrophobic retention under 100% aqueous conditions.

Key Words

- RP-HPLC • LC/MS • polar compounds • biogenic amines
- selegiline • amphetamines • catecholamines
- norepinephrine and metabolites

The Problem of RP-HPLC Retention of Small, Polar Molecules

The vast majority of reversed-phase HPLC (RP-HPLC) separations of small, organic molecules utilize columns packed with C18-modified, porous, spherical silica particles. However, conventional C18 columns lack the ability to adequately retain small, polar molecules for reliable quantification. This is a significant limitation since many drugs, drug metabolites, and other molecules of biological interest are polar in nature. Indeed, the metabolic tendency is to increase the polarity of the molecule over the parent compound to facilitate excretion.

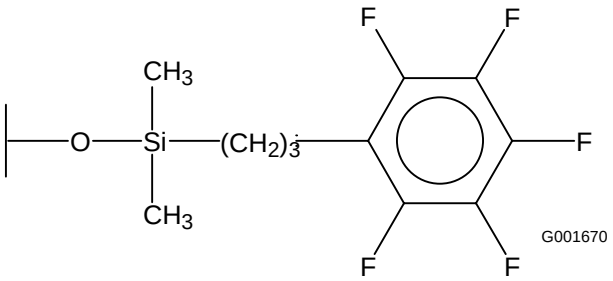
There are two primary approaches used to increase RP-HPLC retention of polar compounds on C18 columns. First, the polar functional groups on the compounds can be derivatized and converted into less polar moieties. Derivatization procedures are often time-consuming and can cause extraneous peaks in the chromatogram due to excess reagents and side products. Second, ion-pair reagents can be added to the mobile phase that form non-polar complexes with the polar analytes. Both ion-pairing and derivatization often result in methods that lack reproducibility and robustness. In addition, the poor volatility and ion-suppressing effects of ion-pair reagents make methods employing them less amenable to mass spectral (MS) analyses.

Improving on Traditional RP-HPLC Methodology for Biogenic Amines

Biogenic amines, an ubiquitous group of compounds with wide-reaching biological activity, are a particularly difficult class of small, polar molecules to analyze by RP-HPLC. The majority of chromatographic methods published on these compounds utilize the retention-enhancing techniques of ion-pairing or derivatization described previously.

We sought to determine if retention of underivatized biogenic amines could be achieved without ion-pairing by using an RP-HPLC stationary phase that exhibit enhanced polar retention and selectivity. RP-HPLC phases that contain polar groups as part of their structure often succeed in retaining and resolving compounds that C18 phases do not because they can interact with analytes via mechanisms not available with the C18 alkyl chains. Of many such phases currently available, the pentafluorophenylpropyl phases have shown wide applicability. Earlier work (1) demonstrated that Discovery HS F5 pentafluorophenylpropyl phase shows excellent retention properties for basic compounds. It was chosen for these studies to provide improved retention for biogenic amines over a conventional C18 phase without the need for ion-pairing or derivatization. The structure and properties of the Discovery HS F5 column are presented in Figure A. The three examples that follow show how simply changing the HPLC stationary phase can dramatically improve the retention and selectivity of these difficult compounds while improving LC/MS compatibility.

Figure A. Structure and Properties of Pentafluorophenylpropyl Discovery HS F5



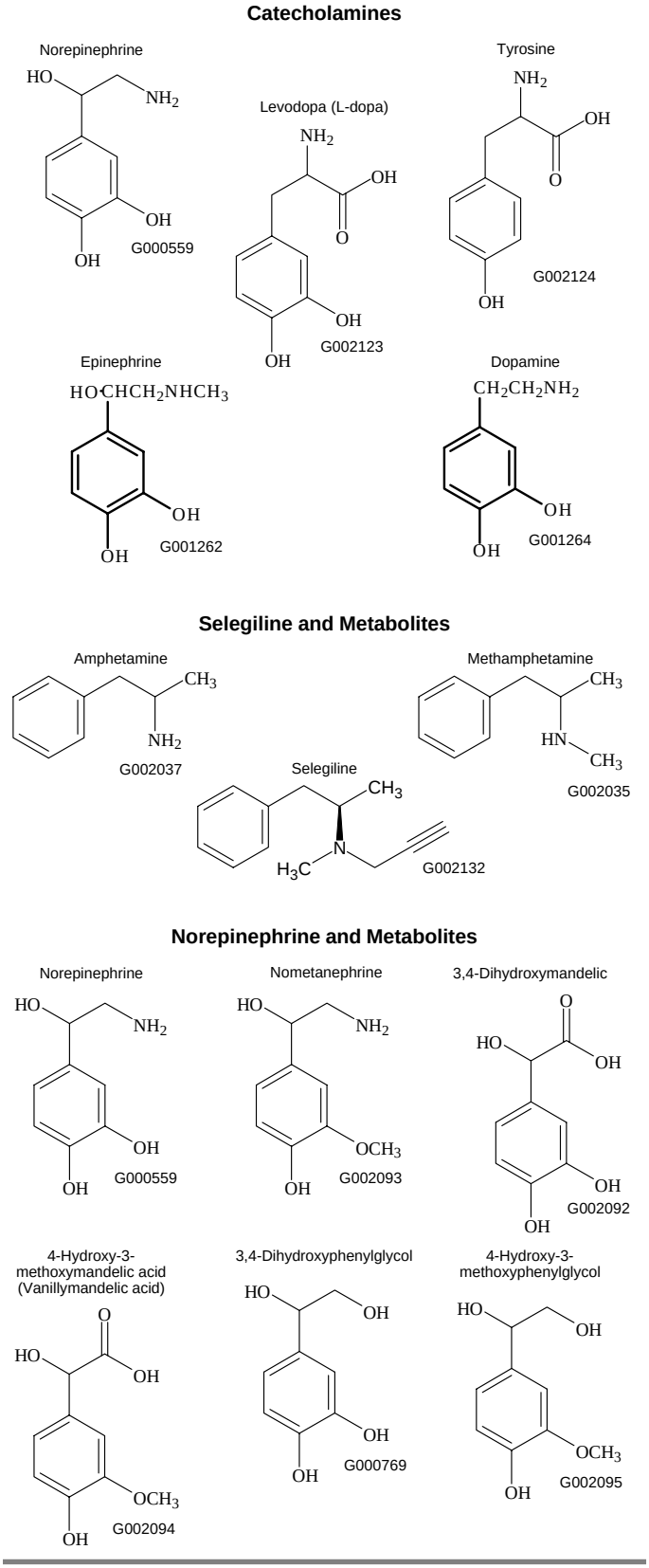
Properties of Discovery HS F5:

USP Code:	L43
Bonded Phase:	Pentafluorophenylpropyl
Endcap (yes / no):	Yes
Particle Platform:	Silica
Particle Shape:	Spherical
Particle Purity:	<10ppm metals
Particle Sizes (µm):	3, 5, and 10
Pore Size (Å):	120
Surface Area (m^2/g):	300
Packing Density (g/mL):	0.58
%C:	12
Coverage (µmoles/m^2):	4
pH Range:	2 to 8
Temperature Range:	≤70°C

Example 1: Enhanced Polar Retention and Selectivity of Catecholamines on Discovery HS F5 Columns

Catecholamines are involved in regulation of response to stress, psychomotor activity, emotional processes, learning, sleep, and memory (2). Members of the catecholamine family include norepinephrine, dopamine, levodopa (L-dopa), epinephrine, and tyrosine. The structures of the catecholamines used in this study are found in Figure B.

Figure B. Structures of Biogenic Amine Analytes



Traditional Chromatographic Analysis of Catecholamines

Analysis of catecholamines is often accomplished by GC/MS. The main disadvantage of this technique is that it requires time-consuming derivatization. HPLC analyses using C18 stationary phases have been reported where ion-pair modifiers were used to impart retention and improve selectivity (3, 4). Chan and Ho recently reported the separation and atmospheric pressure chemical ionization (APCI) MS detection of several catecholamines (5). However, the short retention times reported in this system are likely to result in interferences from unresolved endogenous species from sample matrices.

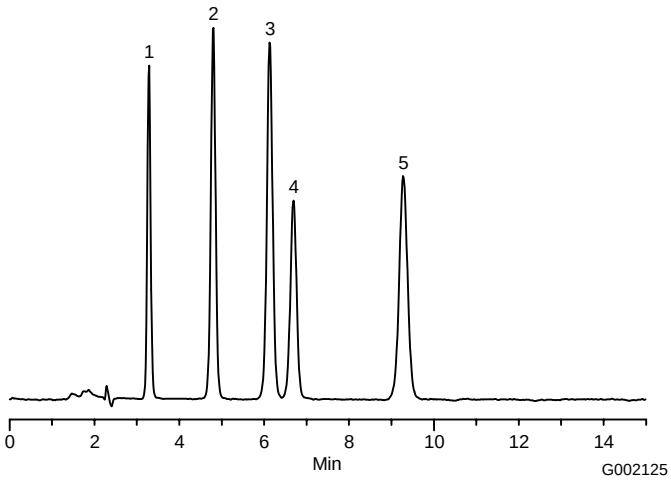
Optimized RP-HPLC of Catecholamines on Discovery HS F5

By employing a pentafluorophenylpropyl phase (Discovery HS F5) that has added polar retention and selectivity, significant improvements over the traditional GC and LC methods were accomplished. The results and the analysis conditions are shown in Figure C. Retention and resolution was achieved without ion-pairing or derivatization. Even on the polar Discovery HS F5 stationary phase, 100% aqueous conditions were required to provide retention of norepinephrine. (See Example 3 for a discussion of 100% aqueous compatibility of Discovery HS F5.) These conditions present a significant improvement over ion-pair and unmodified C18 systems in terms of robustness and LC/MS suitability. The chromatogram in Figure C also shows that the catecholamines, although basic in nature, elute with excellent peak shape on the Discovery HS F5 column.

Figure C. Separation of Catecholamines on Discovery HS F5

Column:	**Discovery HS F5, 15cm x 4.6mm ID, 5µm particles**
Cat. No.:	**567516-U**
Mobile Phase:	50mM ammonium formate, adjusted to pH 3 with formic acid
Flow Rate:	1.0mL/min
Det.:	UV, 266nm
Temp.:	35°C
Inj.:	10µL
Sample:	Each compound 50µg/mL in mobile phase (all compounds were obtained from Sigma Chemicals, St. Louis, MO)

1. Norepinephrine
2. Levodopa (L-dopa)
3. Epinephrine
4. Tyrosine
5. Dopamine



Example 2: Reversed- and Normal-Phase Mechanisms of Discovery HS F5 Resolves Selegiline and Amphetamine Metabolites

Selegiline is an adjunct drug used in the treatment of Parkinson and Alzheimer's neurological disorders. It is believed to act as an inhibitor to monoamine oxidase, an enzyme found throughout the body; two different forms being found in liver, kidney, stomach, intestinal wall, and brain. Selegiline has three common metabolites: N-desmethylselegiline, amphetamine, and methamphetamine. Over a period of 48 hours, 45% of administered selegiline appears in the urine as these three metabolites (6,7). The structures of the analytes in this study are presented in Figure B.

Traditional RP-HPLC Analysis of Selegiline and Metabolites

Due to the very polar nature of amphetamine and methamphetamine, their retention on traditional C18 phases usually requires highly aqueous mobile phases. However, selegiline is relatively non-polar making it difficult to chromatograph the three compounds simultaneously without gradient conditions or excessively long run times. By using ion-pairing, amphetamine and methamphetamine retention can be increased relative to selegiline. However, ion-pairing is not desirable because it reduces the robustness, reproducibility, and LC/MS-compatibility of the method.

Discovery HS F5 Provides Unique Retention and Selectivity

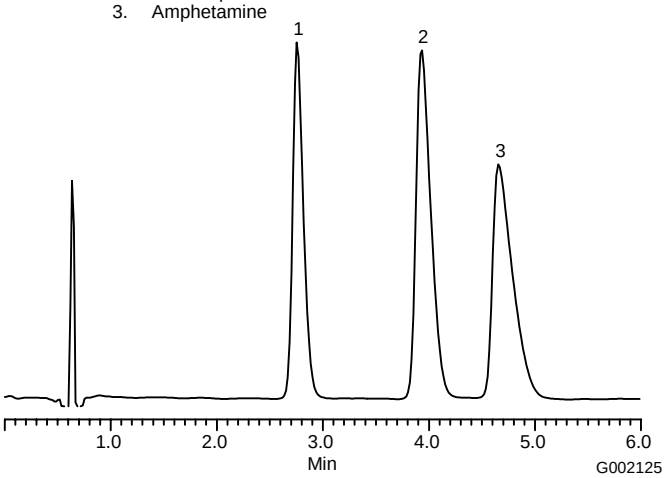
The objective of this study was to develop conditions suitable for the isocratic RP-HPLC analysis of selegiline and two of its metabolites: amphetamine and methamphetamine. The Discovery HS F5 pentafluorophenylpropyl phase typically exhibits greater retention for basic compounds in comparison to traditional C18 phases. Often, retention and resolution can be achieved on the Discovery HS F5 that are not possible on a C18. The intent of the present study was to exploit these traits to produce an efficient, isocratic separation of selegiline and its metabolites without ion-pairing.

The resulting chromatographic separation is shown in Figure D. Note that even though selegiline is more hydrophobic than its amphetamine metabolites, it elutes before them. A possible explanation of this phenomenon is the ability of pentafluorophenylpropyl phases (like the Discovery HS F5) to interact via both normal- and reversed-phase mechanisms. Under the conditions in this study, the more basic amphetamine metabolites are retained under a normal-phase type retention mechanism (adsorption); increasing organic modifier concentration in the mobile phase increases their retention. Conversely, selegiline is retained via a reversed-phase (partition) mechanism; increasing the organic modifier decreases its retention. The proper utilization of both mechanisms of retention on the Discovery HS F5 phase leads to the highly efficient separation shown in Figure D. The ability to retain polar compounds with high organic mobile phases presents advantages for LC/MS as well. Further discussion of this unique property of the Discovery HS F5 phase is found in references 1 and 8.

Figure D. Retention of Selegiline and its Amphetamine Metabolites on Discovery HS F5

Column:	**Discovery HS F5, 5cm x 4.6mm ID, 5µm particles**
Cat. No.:	**567513-U**
Mobile Phase:	25:75 water (10mM ammonium acetate, pH 4.0 with acetic acid):CH_3CN
Flow Rate:	1.0mL/min
Det.:	UV, 210nm
Temp.:	40°C
Inj.:	10µL
Sample:	Each compound 50µg/mL in mobile phase (all compounds were obtained from Sigma Chemicals, St. Louis, MO)

1. Selegiline
2. Methamphetamine
3. Amphetamine



Example 3: Norepinephrine and Metabolites Demonstrate Stability of Discovery HS F5 in 100% Aqueous Mobile Phases

Metabolism of catecholamine neurotransmitters like norepinephrine plays an important role in regulating their synaptic levels (9). Simultaneous determination of basic norepinephrine and its acidic metabolites presents a formidable chromatographic challenge. In this study the separation of norepinephrine and its metabolites 3,4-dihydroxymandelic acid, 3,4-dihydroxyphenylglycol, normetanephrine, 4-hydroxy-3-methoxymandelic acid (vanillylmandelic acid), and 4-hydroxy-3-methoxyphenylglycol (Figure B) was achieved using a Discovery HS F5 pentafluorophenylpropyl phase. The same 100% aqueous conditions that were used in the catecholamine separation described in Example 1 was found to be suitable for this set of analytes.

Aqueous Conditions Cause Loss of Hydrophobic Retention on C18 Phases

Under highly aqueous conditions, alkyl stationary phases, such as C18, are known to undergo a reduction or complete loss of hydrophobic retention. The mechanism behind this loss of retention has been variously attributed to phase collapse, dewetting, pore exclusion, or a combination thereof (10-12). For analyses using C18 phases it is therefore prudent to always include a small percentage of organic modifier in the mobile phase. However, small, polar molecules often require the use of 100% aqueous mobile phases to obtain suitable retention. Even small concentrations of organic modifier can reduce retention sufficiently to interfere with reliable quantification. The ability of a stationary phase to operate reliably in 100% aqueous mobile phases is therefore desirable. In this study, the stability of Discovery HS F5 pentafluorophenylpropyl phase in 100% aqueous conditions was compared to a traditional C18 phase. Chromatographic retention and resolution were compared initially and following static storage in 100% aqueous mobile phase.

Discovery HS F5 Exhibits No Loss of Retention Compared to C18

Initial separation results on the C18 and Discovery HS F5 columns are shown in Figure E. Following overnight static storage of the columns in the 100% aqueous mobile phase, the columns were equilibrated for approximately five minutes and the analysis was repeated. These results are shown in Figure F. Note that the short equilibration time was necessary to ensure the phase did not have time to recover from any effect of the aqueous storage conditions. The short equilibration was also the cause for the small peaks eluting before T_0 on the Discovery HS F5 column. These peaks disappear upon proper equilibration. While the chromatograms obtained for both Day 1 and Day 2 on the Discovery HS F5 columns are nearly identical, the Day 2 C18 chromatogram shows a loss of retention due to the high-aqueous storage. Comparison of the results for both columns on Day 1 (Figure E) also demonstrates the superior retention and selectivity of Discovery HS F5 over the C18; the HS F5 column resolved all six catecholamines whereas the C18 column did not.

Summary of Unique Properties of Discovery HS F5

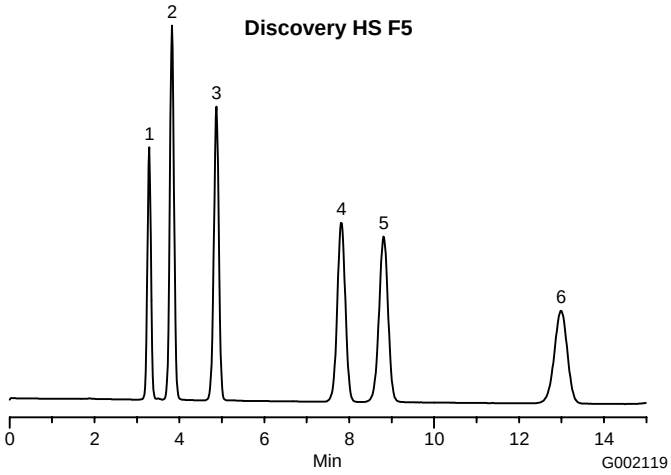
The studies reported here show that the Discovery HS F5 pentafluorophenylpropyl phase has properties that make it ideally suited for the HPLC analysis of the basic, polar biogenic amines:

- Enhanced retention and selectivity toward polar compounds allows analysis without ion-pairing or derivatization.
- Mixed reversed- and normal-phase retention mechanisms give unique selectivity allowing isocratic separation of polar and non-polar compounds in the same run, and providing benefits for LC/MS.
- Compatibility with 100% aqueous mobile phases allows operation without loss of hydrophobic retention encountered on C18 phases.

Figure E. Initial (Day 1) Separation of Norepinephrine Metabolites on Discovery HS F5 and Discovery HS C18 Columns

Column: **Discovery HS F5, 15cm x 4.6mm ID, 5µm particles, (or C18 of the same dimensions)**
Cat. No.: **567516-U**
Mobile Phase: 50mM ammonium formate, pH 3 with formic acid
Flow Rate: 1.0mL/min
Det.: UV, 266nm
Temp.: 35°C
Inj.: 10µL
Sample: Each compound 50µg/mL in mobile phase (all compounds were obtained from Sigma Chemicals, St. Louis, MO)

1. Norepinephrine
2. 3,4-Dihydroxymandelic acid
3. 3,4-Dihydroxyphenylglycol
4. Normetanephrine
5. 4-Hydroxy-3-methoxymandelic acid (vanillylmandelic acid)
6. 4-Hydroxy-3-methoxyphenylglycol



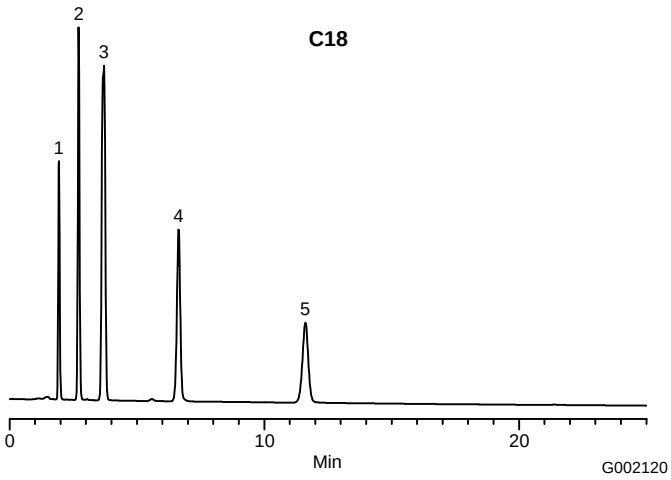
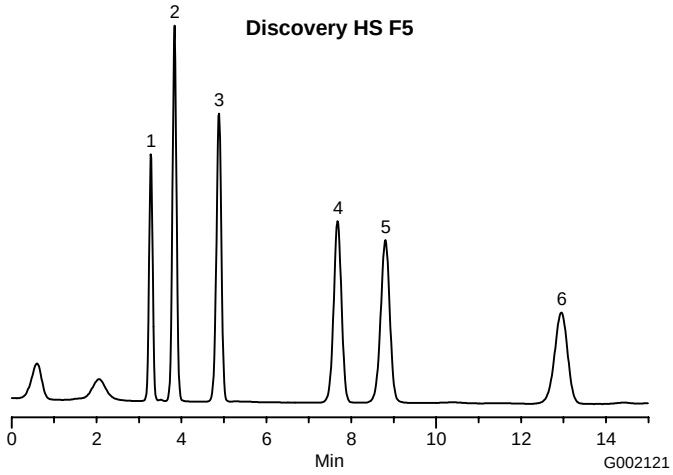
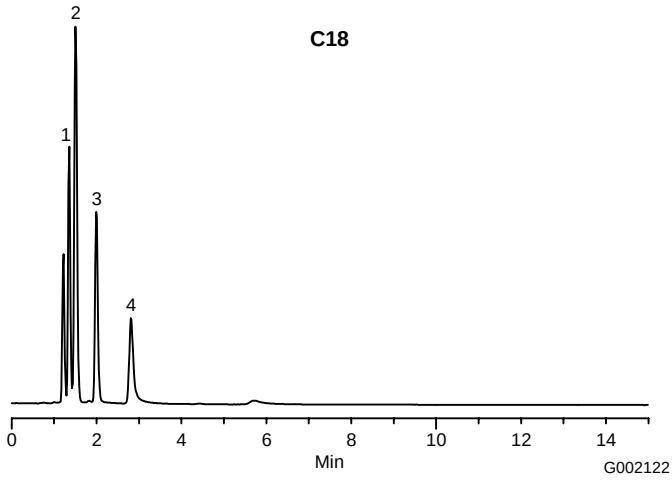


Figure F. Separation of Norepinephrine Metabolites on Discovery HS F5 and Discovery HS C18 Columns After Static Aqueous Storage

Column: **Discovery HS F5, 15cm x 4.6mm ID, 5µm particles, (or C18 of the same dimensions)**
Cat. No.: **567516-U**
Mobile Phase: 50mM ammonium formate, pH 3 with formic acid
Flow Rate: 1.0mL/min
Det.: UV, 266nm
Temp.: 35°C
Inj.: 10µL
Sample: Each compound 50µg/mL in mobile phase (all compounds were obtained from Sigma Chemicals, St. Louis, MO)

1. Norepinephrine
2. 3,4-Dihydroxymandelic acid
3. 3,4-Dihydroxyphenylglycol
4. Normetanephrine
5. 4-Hydroxy-3-methoxymandelic acid (vanillylmandelic acid)
6. 4-Hydroxy-3-methoxyphenylglycol





Conclusion

The unique retention and selectivity of Discovery HS F5 pentafluorophenylpropyl phase provides separations of biogenic amines that are superior to methods employing traditional C18 phases. Discovery HS F5 does not require detrimental ion-pair reagents, gradient elution, nor the time-consuming derivatization required by GC/MS protocols. Additionally, the presence of both normal- and reversed-phase retention mechanisms on the Discovery HS F5 can be employed to develop highly efficient separations for compounds with widely differing properties. The use of 100% aqueous mobile phases on Discovery HS F5 was been shown to provide stable retention and selectivity for norepinephrine metabolites; conditions where C18 phases exhibit loss of hydrophobic retention. Discovery HS F5 can reliably retain and resolve polar compounds under conditions not possible on traditional C18 phases.

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Particle Size	ID (mm)	Length (cm)	Cat. No.
Discovery HS F5			
3µm	2.1	5.0	**567500-U**
3µm	2.1	15.0	**567503-U**
3µm	4.0	15.0	**567532-U**
3µm	4.6	15.0	**567507-U**
5µm	2.1	5.0	**567508-U**
5µm	2.1	15.0	**567511-U**
5µm	4.0	15.0	**567535-U**
5µm	4.6	5.0	**567513-U**
5µm	4.6	15.0	**567516-U**
5µm	4.6	25.0	**567517-U**

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