

New Paradigm for HPLC Method Development

Reverse-phase high performance liquid chromatography (RP-HPLC) remains the preferred analytical tool for pharmaceuticals (1-5). This is true for all stages of drug development: from high-throughput screening, purification of final product, to metabolite studies. Method development must then address the gamut of these applications, whether designed for rapid generic analysis of combinatorial libraries or highly optimized for the particular sample set. As such, method development is a vital part of the entire drug discovery process.

Achieving a desired and adequate selectivity is perhaps the primary criterion in evaluating a given method. Consideration of parameters that govern selectivity in RP-HPLC has long held that changing solvent strength and/or solvent type is the most practical approach to optimizing selectivity (6-7). This has become even more attractive with the advent of computer software, in which a person need only perform a pair of isocratic or gradient runs, for which the software can then model reasonably accurate optimized conditions. However, the potential role of the stationary phase and possible initial selection thereof, is often overlooked (8). Stationary phases do fundamentally impact selectivity by their solvation, conformation, polarity, and mechanisms of sample sorption. Most recently, the polar embedded phases introduced by several manufacturers display unique characteristics. This is exemplified by Supelco's Discovery® RP-AmideC16, in which it exhibits multiple possible interactions through polar and hydrogen bonding properties as well as hydrophobic interactions of the alkyl chain, all of which in turn directly effect the affinity of the bonded phase for solvent. In sum, polar-embedded bonded phases have realized dramatic improvements in chromatography of polar analytes, typical of pharmaceutical compounds containing multiple ionic functionalities.

With ever-increasing constraints to bring pharmaceutical products to market in minimal time, analytical method development is particularly subject to such pressures, since as stated above, it prevails in all stages of the drug discovery process. Furthermore, many of the analytical methods are increasingly making use of LC-MS. LC-MS itself, however, places considerable constraints on conditions that may be used for the LC side. As such, regardless of the specific reason, the impetus to utilize bonded phase chemistry as a tool for optimizing selectivity has become most attractive (8). It is in fact, an approach to method development whose time has come, and which Supelco currently actively promotes.

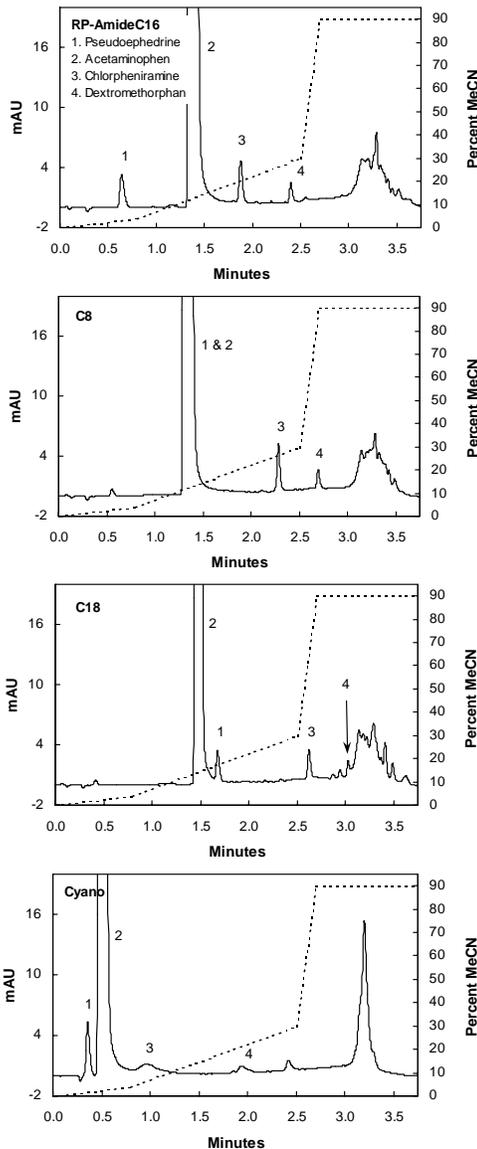
In this Reporter article we demonstrate by example, the advantages and power with which this paradigm of method development brings to the analytical chemist. First we'll take a look at chromatography of an over-the-counter (OTC) drug under gradient conditions not unlike that done routinely at walk-up stations. Last, we'll consider a sample of seven

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Figures 1 – 4.
Fast Gradients: Tylenol® Cold, Complete Formula

Column: *Discovery, 5cm x 4.6mm ID, 5µm particles*
Mobile Phase: *A: 0.1% HCO₂H (pH 2.5)*
B: MeCN
Gradient: *0 - 4% in 0.8 min., 4 - 30% in 1.7 min.*
Flow Rate: *3mL/min*
Temperature: *35°C*
Detection: *UV, 254nm*



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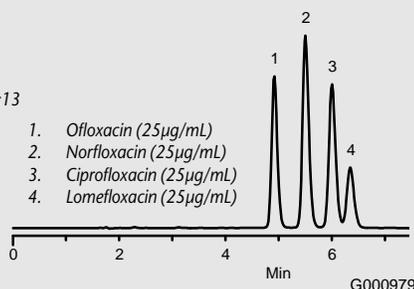
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Figure 6. on a Discovery RP-AmideC16 Column

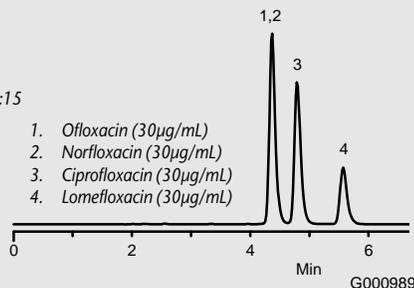
Column: 15cm x 4.6mm ID, 5µm particles
 Cat. No.: 505013
 Mobile Phase: 25mM KH₂PO₄/H₃PO₄, pH 3: MeCN, 87:13
 Flow Rate: 1mL/min
 Temperature: 35°C
 Detection: UV, 254nm
 Injection: 20µL



1. Ofloxacin (25µg/mL)
2. Norfloxacin (25µg/mL)
3. Ciprofloxacin (25µg/mL)
4. Lomefloxacin (25µg/mL)

Figure 7. on a Discovery C18 Column

Column: 15cm x 4.6mm ID, 5µm particles
 Cat. No.: 504955
 Mobile Phase: 25mM KH₂PO₄/H₃PO₄, pH 3: MeCN, 85:15
 Flow Rate: 1mL/min
 Temperature: 35°C
 Detection: UV, 254nm
 Injection: 10µL



1. Ofloxacin (30µg/mL)
2. Norfloxacin (30µg/mL)
3. Ciprofloxacin (30µg/mL)
4. Lomefloxacin (30µg/mL)

Figures 8 & 9. From Tablets

Column: 15cm x 4.6mm ID, 5µm particles
 Cat. No.: 505013 (RP-AmideC16), 504955 (C18)
 Mobile Phase: (A) 25mM KH₂PO₄/H₃PO₄, pH 3
 (B) MeCN
 10% B to 35% B over 15 min
 Flow Rate: 1mL/min
 Temp.: 35°C
 Detection: UV, 220nm
 Injection: 10µL

1. Levofloxacin
2. Ciprofloxacin
3. Lomefloxacin
4. Sparfloxacin
5. Grepafloxacin
6. Trovafloxacin

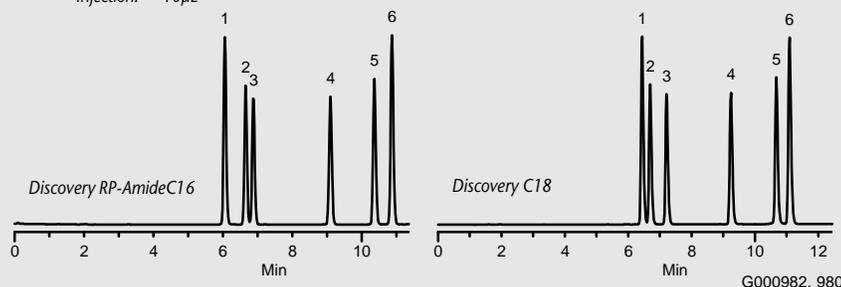
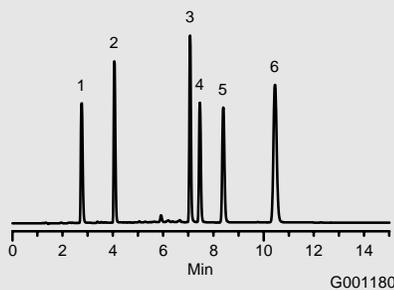


Figure 10. on a Discovery RP-AmideC16 HPLC Column

Column: 15cm x 4.6mm ID, 5µm particles
 Cat. No.: 505013
 Mobile Phase: A=0.01% TFA in water
 B=0.01% TFA in MeCN
 Flow Rate: 1.5mL/min
 Temperature: 35°C
 Detection: UV, 220nm

Gradient Program	Time (min)	%B
	0	5
	5	35
	20	35

1. Amoxicillin
2. Ampicillin
3. Piperacillin
4. Penicillin G
5. Penicillin V
6. Cloxacillin



CRITICAL PAIR SEPARATIONS MADE EASY

Fluoroquinolone Antibiotics

New antibiotics continue to be synthesized in an effort to stay ahead of the resistance that bacteria so quickly acquire. Fluoroquinolones are potent synthetic agents active against a variety of bacterial species and have emerged as one of the more important classes of antibiotics.

Here, a class of fluoroquinolones – ofloxacin, norfloxacin, ciprofloxacin, lomefloxacin – are separated by reversed phase HPLC, using isocratic elution (Figures 6 & 7), on Discovery RP-AmideC16 and Discovery C18 columns (each 15cm x 4.6mm ID, 5µm particles). The RP-AmideC16 column produced a baseline separation of ofloxacin and norfloxacin, which co-eluted on the Discovery C18. Selectivity differences for ofloxacin and norfloxacin could be explained by the hydrogen bonding between the free NH of the piperazine ring on norfloxacin and the amide functionality of RP-AmideC16. This clearly validates the discussed method development strategy (lead article) as providing a means to quickly select a column of choice for a given method.

Levofloxacin, sparfloxacin, grepafloxacin, and trovafloxacin were recently approved by the US Food and Drug Administration (FDA) and are active against many pathogenic Gram-negative and Gram-positive bacteria and other atypical pathogens. A mixture of six fluoroquinolones, was chromatographed on the same Discovery columns (Figures 8 & 9) using a phosphate buffer and gradient elution by acetonitrile. It can be seen that the critical pair changes from peaks 2 & 3 in the case of RP-AmideC16 to peaks 1 & 2 in the case of C18, due to the shifting of the relative position of peak 2. Again, this can be explained by hydrogen bonding between the the free NH of the piperazine ring on ciprofloxacin (peak 2) and the amide functionality of the RP-AmideC16. Thus, simple column switching provides a convenient means to alter critical pair selectivity.

See *Supelco App Note 153* for further details.

β-Lactam Antibiotics

Penicillin is perhaps the oldest β-lactam antibiotic prepared commercially as a drug. Because of massive overprescription at least 30 years ago, bacterial resistance has posed a serious problem to its continued use and has prompted development of new viable derivatives thereof. A mixture of penicillin analogs (penicillin G, penicillin V, cloxacillin, piperacillin, amoxicillin and ampicillin) was prepared specifically to examine selectivities of the different Discovery bonded phases for these similar structures. Penicillins G & V differ by the insertion of an additional oxygen as an ether in the case of penicillin G. Cloxacillin and piperacillin both contain bulky side chains, and amoxicillin differs from ampicillin by the addition of a phenolic hydroxyl. The chromatographic profile of the mixture is similar for C8, C18, and RP-AmideC16, giving baseline resolution of all 6 components. A gradient was necessary to keep run time reasonable and yet allow for resolution of the early eluting peaks. In the case of the Cyano

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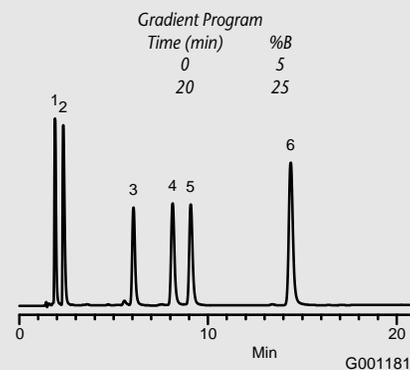
Discovery - Sigma-Aldrich
 Tylenol - McNeil Consumer Healthcare

column, under the same conditions, peaks 4 & 5 were not baseline resolved. The relatively small difference in hydrophobicity between penicillins G & V (an etheric oxygen) is not sufficient for the short cyanopropyl ligand to resolve them. Therefore an overall shallower gradient was required to resolve the fourth and fifth peaks (Figure 11). A similar situation is observed for amoxicillin and ampicillin: the short ligand of the Cyano bonded phase doesn't discriminate subtle hydrophobic differences as do the longer alkyl ligands. Again, this directly illustrates the utility of changing bonded phases to alter selectivity; results may not be readily predicted, but the small investment in time and hardware is recovered in greatly reduced method development time.

Figure 11. on a Discovery Cyano HPLC Column

Column: **15cm x 4.6mm ID, 5µm particles**
 Cat. No.: **59356-U**
 Mobile Phase: **A=0.01% TFA in water**
B=0.01% TFA in MeCN
 Flow Rate: **1.5mL/min**
 Temperature: **35°C**
 Detection: **UV, 220nm**

1. Amoxicillin
2. Ampicillin
3. Piperacillin
4. Penicillin G
5. Penicillin V
6. Cloxacillin



NEW PRODUCTS

The Discovery Family of HPLC Columns

Discovery RP-AmideC16

- Unique selectivity
- Excellent retention and resolution of polar compounds
- Less hydrophobicity than C18 phases
- Different elution profiles compared to C18
- Excellent reproducibility

Discovery C8

- Faster separation of strongly hydrophobic analytes
- Exceptional peak shapes for basic and acidic compounds
- Excellent stability from pH2 to pH8 or above
- Compatible with low organic/highly aqueous mobile phases
- Suitable for LC/MS applications
- Excellent reproducibility

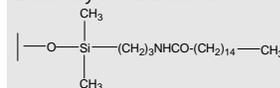
Discovery C18

- Exceptional peak shape for basic and acidic analytes
- Lower hydrophobicity than many comparable C18 columns, providing faster analysis
- Resolution of geometrical isomers and other structurally closely related compounds
- Separation of peptides and small proteins
- Suitable for LC/MS applications
- Excellent reproducibility

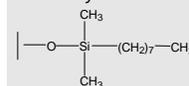
Discovery Cyano

- Low hydrophobicity, for rapid elution of hydrophobic molecules
- Retention and separation of strongly basic analytes, including quaternary ammonium salts, with excellent peak shapes
- Compatible with highly aqueous mobile phases
- Exceptional stability and column lifetime, from pH2 to pH8
- Excellent reproducibility

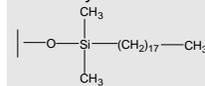
Discovery RP-AmideC16



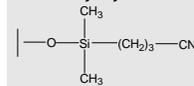
Discovery C8



Discovery C18



Discovery Cyano



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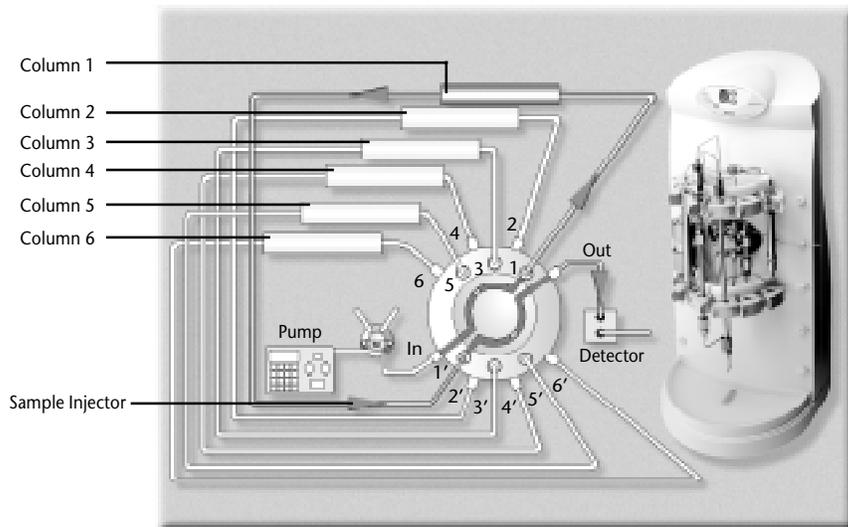
For more information, request these documents by number: **RP-AmideC16:** T498019. **C8:** T499128. **C18:** T497291. **Cyano:** T499133.

Rheodyne LabPro Column Selector

As stated in the lead article of this reporter, one useful method development technique is to compare separations obtained on several different bonded phases using the same mobile phase conditions. This technique allows the method development chemist to exploit selectivity differences between bonded phases and is easily automated by the use of a column-switching valve.

The Rheodyne LabPro Column Selector allows for automatic selection between up to 3 or 6 columns (depending on model) and is available with either a stainless steel or PEEK valve. The LabPro unit incorporates Rheodyne's patented TeleFLO design to minimize unswept internal volume and dispersion.

Refer to the HPLC accessory section of the Supelco catalog for more information.



References for "New Paradigm..."

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2. J. Zweigenbaum, K. Heinig, S. Steinborner, T. Wachs and J. Henion. (1999) *Anal. Chem.*, **71**, 2294-2300.
3. L. Huber. (1999) *Biopharm*, **12**, 64-66.
4. R.E. Majors. (1999) *LC-GC*, **17**, 212-228.
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7. L.R. Snyder, J.J. Kirkland and J.L. Glajch, *Practical HPLC Method Development*, pp. 233-265, Wiley, New York, 1997.
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New Paradigm...

(continued from page 1)

structurally related drugs, chromatographed under isocratic conditions for optimal resolution typical of metabolite or degradation studies.

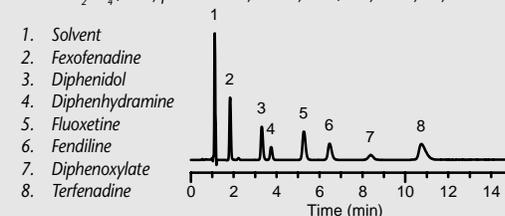
The OTC drug selected contains typical ingredients to treat cough, cold, and flu symptoms: acetaminophen, pseudoephedrine, dextromethorphan and chlorpheniramine. The tablet was crushed, extracted with 50% acetonitrile, clarified, filtered, and diluted with water as necessary. Four bonded phases of the Discovery product line were used: C18, C8, RP-AmideC16, and Cyano, each of the dimensions 4.6 mm x 50 mm ID, 5µm. The chromatograms are shown in Figures 1—4, each of a different bonded phase run under identical conditions. The gradient profiles are depicted along with the subsequent column wash following each run. The RP-AmideC16 column provides good band spacing of all four components; C8 does not resolve pseudoephedrine from acetaminophen while dextromethorphan elutes after the gradient is complete; C18 does resolve pseudoephedrine from acetaminophen, but both chlorpheniramine and dextromethorphan elute subsequent to completion of the gradient; Cyano displays inadequate retention of pseudoephedrine and acetaminophen an poor peak shape of chlorpheniramine and dextro-methorphan. It is readily apparent from these quick gradient runs, that the bonded phases do display significantly differing selectivity. These chromatograms are of a method that was optimized and finalized on RP-AmideC16 following an initial screening of the bonded phases by a fast generic gradient. Of particular note is the reversal of elution order of pseudoephedrine and acetaminophen in comparing RP-AmideC16 and C18.

In the second example, seven basic drugs were selected (diphenoxylate, diphenidol, diphenhydramine, terfenadine, fexofenadine, fluoxetine, & fendiline) which, with one exception (fluoxetine), are structured around a diphenylmethane skeleton. Discovery Cyano resolved all components within 12 min (Figure 5). Under the same conditions, the other three Discovery bonded phases do exhibit different selectivity but the run times are prohibitively long (> 1 hr) (data not shown). This latter example is further discussed in an upcoming journal article. This simply illustrates once again, that with a typical mobile phase system, different bonded phases can exhibit markedly different selectivity, and thus presents itself as a tool for further method optimization.

For further information, request **T199926**, or visit our website at www.sigma-aldrich.com/TheReporter

Figure 5.

Discovery Cyano, 15cm x 4.6mm ID, 5µm particles, (Cat. No. 59356-U), 50mM KH₂PO₄ / TEA, pH 7: MeCN, 70:30, 2mL/min, 25°C, UV, 254nm



CASE STUDY 1

Mass Overload

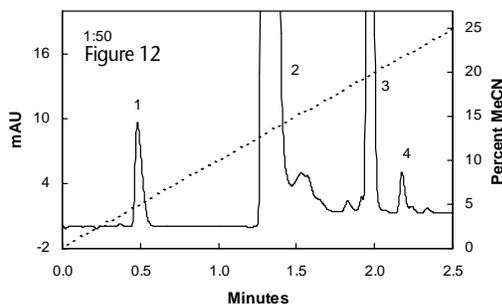
Whether in the interest of speed for high-throughput screening or for increased sensitivity with LC-MS applications, smaller columns continue to get more use. While sample capacity is directly proportional to column length, it is geometrically proportional to column diameter. Therefore it is necessary to be all the more cognizant of mass loading effects and limits.

As an example, a sample from a liquid OTC cough/cold medication was diluted 50-fold with water and then 10µL injected on a Discovery RP-AmideC16 column (4.6 mm x 50 mm, 5µm). A fast generic gradient was

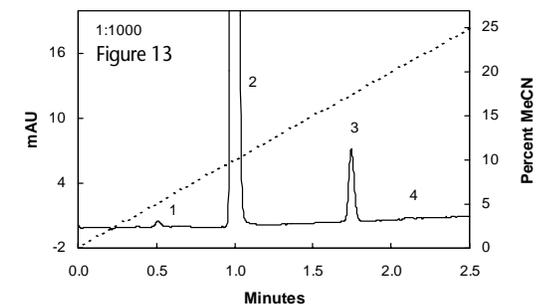
Figures 12 & 13.

Discovery RP-AmideC16, 5cm x 4.6mm ID, 5µm particles, (Cat. No. 505005), A: 0.1% HCO₂H (pH 2.5), B: MeCN, 0 - 25% in 2.5 min., 4mL/min, 35°C, UV, 254nm

1. Pseudoephedrine
2. Acetaminophen
3. Guaifenesin
4. Dextromethorphan



applied to elute all four components (Figure 12). A second sample was prepared which was a 1000-fold dilution of the original medication. This too was applied (10µL) and eluted in the same fashion (Figure 13). It is readily apparent from a comparison of the two chromatograms that the first displays distorted retentions arising from intermolecular interactions due to mass overloading. It is therefore particularly critical in method development on small columns (short and/or narrow) to confirm that sample overload has not occurred, so that resolution is not compromised.



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