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The Reporter

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Optimizing HPLC Separations: Samples with Widely Differing Polarities

When confronted with a sample containing widely varying polarities, the LC chromatographer will typically rely on gradient elution. However, if gradient elution is not an option, isocratic elution is generally quite time consuming, and for late eluting peaks, issues of sensitivity may arise. We address this with an example of ten water-soluble vitamins [thiamine (B1), riboflavin (B2), pyridoxine (B6), pantothenic acid (B5), cyanocobalamin (B12), biotin, niacin, niacinamide, folic acid, and ascorbic acid (C)] which are eluted isocratically in a two step process. From the drawings of these compounds (Figure 1) it is clear that they represent a diverse array of structures, functional groups, polarities, and size.

We developed an isocratic method for their elution, employing a simple mobile phase system devoid of ion pair reagents or other additives. These sample components were resolved by use of a pH-7 phosphate buffer/methanol mobile phase system. To illustrate (Figure 2) the wide variation in polarities or hydrophobicities of sample components, the sample was initially chromatographed on Discovery® C18 under gradient elution conditions. This particularly demonstrates the greater hydrophobicities of cyanocobalamin and riboflavin. Isocratic conditions that provide separation of cyanocobalamin and riboflavin in reasonable time will not permit adequate resolution of the other eight vitamins. Therefore, a set of two mobile phase conditions with different organic compositions would be a practical solution.

Figure 3, pg. 4, shows the chromatogram of eight well-resolved water-soluble vitamins on a Discovery C18 column using 1% methanol. Under this condition, cyanocobalamin and riboflavin are strongly retained on the column. Further study has confirmed that the presence of cyanocobalamin and riboflavin has no effect on elution of the other eight vitamins even with multiple injections. Cyanocobalamin and riboflavin can be eluted from a Discovery C18 column using a mobile phase containing 20% methanol (Figure 4, pg. 4). Note also the elution order change of the folic acid and biotin under isocratic conditions. In Figure 2, folic acid preceedes biotin, however under isocratic conditions biotin preceeds folic acid. Both conditions (1% or 20% methanol) provide excellent peak shape and resolution on Discovery C18. For those who prefer isocratic methods, these two sets of conditions can be run sequentially to provide complete separation of all water-soluble vitamins. As an alternative, a column switching system can be used with 2 columns, each kept equilibriated with the appropriate mobile phase. Either approach provides a simpler methodology than gradient elution in a reasonable run time.

LC issue

See Supelco App Note 148 for further details

Figure 1. Structures of Ten Water-Soluble Vitamins

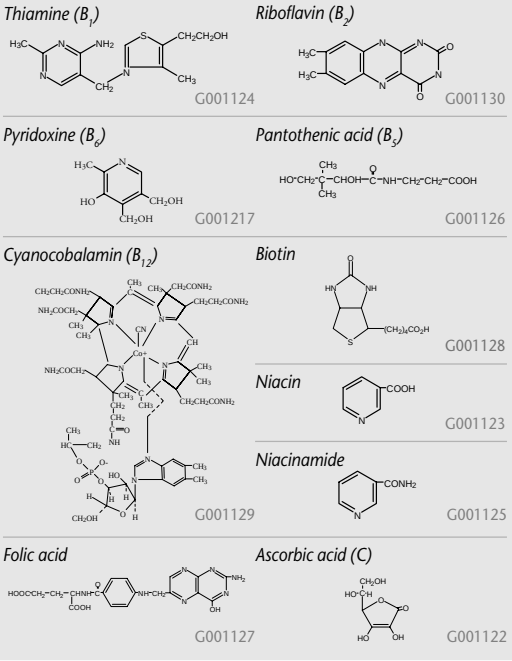

Figure 2. Gradient Elution of Ten Water-Soluble Vitamins

Column: ***Discovery C18****, 15cm x 4.6mm ID, 5µ*
Mobile Phase: *A: 50 mM K_2HPO_4, pH 7; B: MeOH*
Gradient: *1%B for 5 min, 1-30%B (linear gradient) over 15 min, 30%B for 5 min.*
Flow rate: *1 mL/min*
Temperature: *35°C*
Detection: *UV, 220 nm*
Injection: *10 µL*

1. *Ascorbic acid (Vitamin C), 30 µg/mL*
2. *Niacin, 6 µg/mL*
3. *Pantothenic acid (Vitamin B_5), 100 µg/mL*
4. *Pyridoxine (Vitamin B_6), 3 µg/mL*
5. *Niacinamide, 6 µg/mL*
6. *Thiamine (Vitamin B_1), 12 µg/mL*
7. *Folic acid, 10 µg/mL*
8. *Biotin, 120 µg/mL*
9. *Cyanocobalamin (Vitamin B_{12}), 12 µg/mL*
10. *Riboflavin (Vitamin B_2), 30 µg/mL*

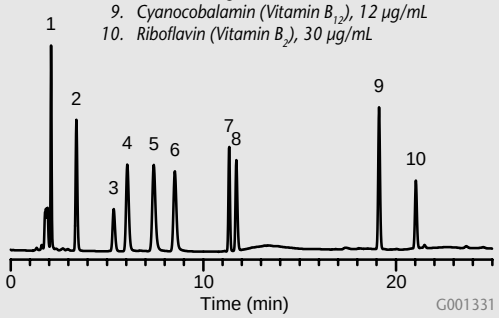

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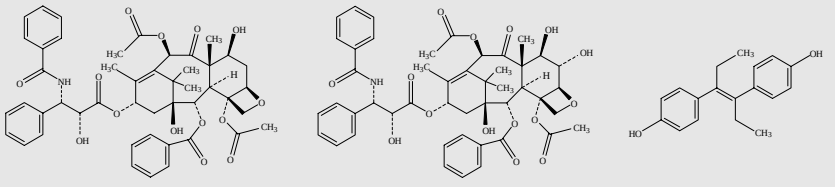
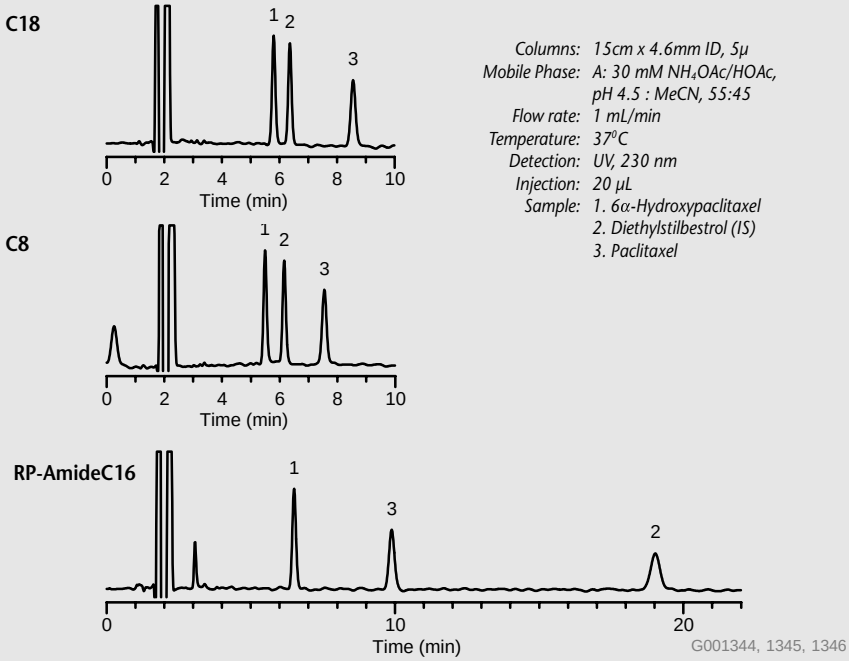
Figure 5. Structures of paclitaxel, 6α-hydroxypaclitaxel, and diethylstilbestrol.



Paclitaxel *6α-Hydroxypaclitaxel* *Diethylstilbestrol*

G001341,1342,1343

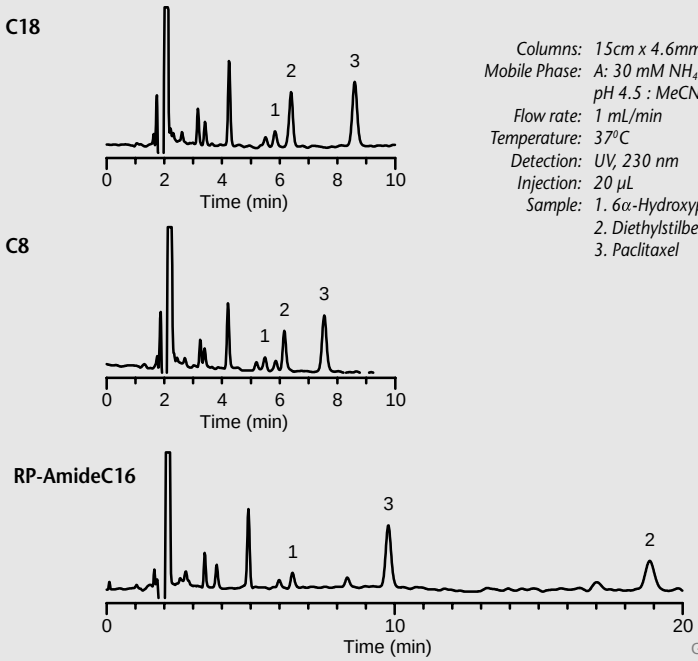
Figure 6. Chromatograms of a standard mixture of paclitaxel, 6α-hydroxypaclitaxel, and internal standard diethylstilbestrol on Discovery C18, C8, and RP-AmideC16 columns.



Columns: 15cm x 4.6mm ID, 5µ
Mobile Phase: A: 30 mM NH_4OAc/HOAc, pH 4.5 : MeCN, 55:45
Flow rate: 1 mL/min
Temperature: 37°C
Detection: UV, 230 nm
Injection: 20 µL
Sample: 1. 6α-Hydroxypaclitaxel
2. Diethylstilbestrol (IS)
3. Paclitaxel

G001344, 1345, 1346

Figure 7. Isocratic elution of Paclitaxel sample incubated by enzyme CYP2C8 using Discovery C18, C8, and RP-AmideC16 columns.



Columns: 15cm x 4.6mm ID, 5µ
Mobile Phase: A: 30 mM NH_4OAc/HOAc, pH 4.5 : MeCN, 55:45
Flow rate: 1 mL/min
Temperature: 37°C
Detection: UV, 230 nm
Injection: 20 µL
Sample: 1. 6α-Hydroxypaclitaxel
2. Diethylstilbestrol (IS)
3. Paclitaxel

G001347, 1348, 1349

APPLICATION REPORT

More Optimization: Are Application Specific Columns Necessary?

Manufacturers often recommend application-specific columns for difficult separations, and show separtions of standards rather than actual metabolites. However, we've developed a simple isocratic method for separation of paclitaxel and its metabolites, with general purpose reversed-phase HPLC columns–Discovery C18, C8, and RP-AmideC16 columns.

Paclitaxel is a novel anticancer drug originating from the bark of the Pacific yew tree *(Taxus brevifolia)* proven to be effective in the treatment of a variety of cancers, including ovarian, breast, lung, and leukemia. 6 α-Hydroxypaclitaxel is the major paclitaxel metabolite produced by the P450 enzyme, CYP2C8. Quantitative determination of this metabolite in paclitaxel treated cancer patients is critical in pharmacology and toxicology. With the increased demand for testing, the requirement for more stable and reproducible analytical testing methods has also emerged.

Paclitaxel, 6 α-hydroxypaclitaxel, and internal standard diethylstilbestrol were well separated by a reversed-phase isocratic elution (NH_4Ac buffer : MeCN, 55 : 45) on three Discovery Columns: C18, C8 and RP-AmideC16 (Figure 6). A unique selectivity difference was observed on Discovery RP-AmideC16 column. The elution order of diethylstilbestrol and Paclitaxel was reversed while diethylstilbestrol was retained much longer than Paclitaxel on this column. This phenomenon can be attributed to the hydrogen bonding between the aromatic hydroxyl group on diethylstilbestrol and the amide functionality of RP-AmideC16.

One important metabolite of paclitaxel is 6 α-hydroxy-paclitaxel, which is generated through paclitaxel 6 α-hydroxylation when paclitaxel is incubated with enzyme CYP2C8 (one form of cytochrome P450 enzymes). Diethylstilbestrol was again used as the internal standard, and the degraded sample run on Discovery C18, C8, and RP-AmideC16 columns (Figure 7). It is clear that the biological matrix introduces additional peaks, but all three Discovery columns gave good resolution without interference from the matrix peaks. Discovery RP-AmideC16 exhibits a selectivity and resolution that is particularly noteworthy, in that all matrix peaks are clearly baseline resolved from the three peaks of interest.

This simple isocratic elution method, particularly with Disocvery RP-AmideC16, is well suited for qualitative and quantitative analysis of paclitaxel and its metabolite 6 α-hydroxypaclitaxel in real biological samples. The volatile ammonium acetate buffer used in this method also makes it attractive and convenient for LC- MS applications if desired.

See Supelco App Note 162 for further details.

NEW PRODUCT

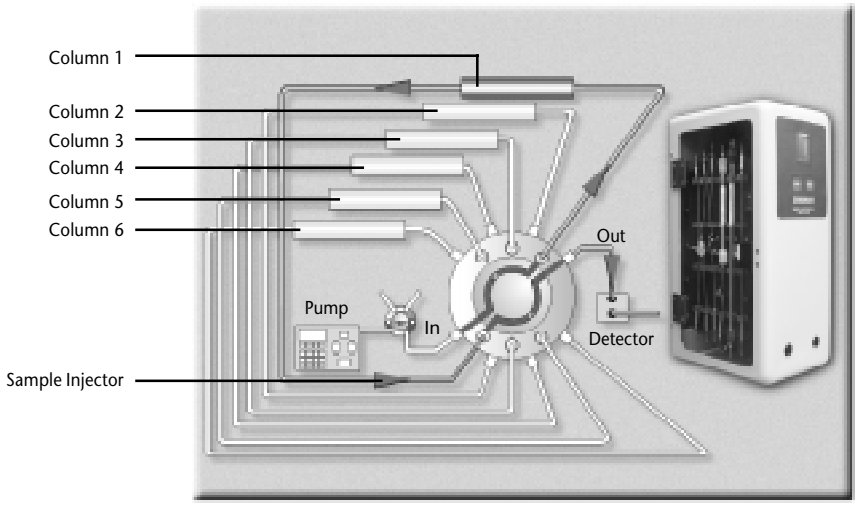
Timberline Heated 6 Column Selector

Comparison of separations on various HPLC columns is a useful tool for the methods development chemist. This technique allows the chemist to exploit selectivity differences between bonded phases. The use of an automated column switching device allows these comparisons to be run unattended.

The Timberline column selector holds up to 6 columns in an oven that may be programmed from 30°C to 50°C. The unit uses a Valco column selector and includes an integrated heat exchanger to pre-heat mobile phase.

For additional information, call Supelco at 800-359-3041 (US), 814-359-3014 (Intl.), or your local Sigma-Aldrich representative.

Note: This product is a custom product for Supelco. CE certification of this product is pending.



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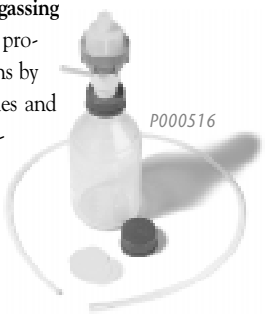
Easy-to-use Aura filter/degasser protects your instrument and columns by simultaneously removing particles and gases from your mobile phases. Filter/degass your solution directly into your mobile phase reservoir without intermediate step.

Cat. No. 58094



Inline Prefilters

Protect your column from particulates originating from pump seal and injection valve wear. Simple and cost-effective; replace only filter (2µ or 0.5µ).

(2.0µ)....**Cat. No. 55078,** (0.5µ)....**Cat. No. 55079**

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- Custom resin processing and packaging

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NEW DISCOVERY LITERATURE

Discovery HPLC Columns and SPE Tubes

An introduction to the Discovery family of products. Topics covered include phases, selectivity, reproducibility, and column lifetime.

Request T499126

Discovery HPLC Method Development Guidelines

An explaination of the principles of column selectivity, efficiency, and resolution that can be exploited for faster and easier method development. Included are 7 product profiles offering information on each Discovery HPLC and SPE phase.

Request T199926

Pharmaceutical Applications

Pharmaceutical applications including antibiotics, antidepressants, antihypertensives, antiulcers, anti-inflammatorys, and barbiturate related compounds. Many applications are shown on all four of the main Discovery phases for comparison.

Request T499127

Agricultural Applications

This brochure contains 10 agricultural pesticide applications. Comparisons between Discovery phases demonstrate the utility column selectivity brings to method development.

Request T499147

LC PERFORMACE TIP - GOOD CHROMATOGRAPHIC PRACTICE

Buffer Preparation

Aqueous pH buffers are essential for chromatographing ionic analytes. The ionization state of the analyte will dramatically alter retention, and therefore working at a defined pH is a key parameter in method development. Criteria for buffer selection and preparation include the desired final pH, pK_a of the buffering species, concentration of buffer, and other considerations of sample collection/recovery/detection: how is the analyte to be handled subsequent to the chromatographic separation? With the popularity of LC/MS, limitations on buffer selection are placed by consideration of buffer volatility and specific effects on ionization in the mass spectrometer. Standardizing buffer preparation is necessary for rugged and robust methods: is the solution prepared with the salt of the buffer, or the free acid/base? What base or acid is used to titrate it to the final pH? What about mixing of an organic phase? These sorts of questions need to be addressed in a laboratory SOP. Buffers should be filtered (0.45µ minimum) to remove particulates and reduce problems of bacterial growth. Filtration also usually provides for some measure of degassing, even if only temporary.

For an SOP addressing proper buffer and mobile phase preparation, see our web site at ***www.sigma-aldrich.com/TheReporter***

Hillel Brandes,

References for "Optimizing HPLC Separations..."

1. *Machlin, L.J. ed.* Handbook of Vitamins, *Marcel Dekker, Inc., New York,* ***1991***.
2. *Ottaway, P.B. ed.* The Technology of Vitamins in Food, *Chapman & Hall, Inc., New York,* ***1993***.
3. *De Leenheer, A.P.; Lambert, W.E.; De Ruyter, M.G.M Ed.* Modern Chromatographic Analysis of the Vitamins, Chromatographic Science Series *Vol 30, Marcel Dekker, Inc., New York,* ***1985***.

Figures 3 & 4 from "Optimizing HPLC Separations"…

(continued from page 1)

Figure 3. Isocratic Elution of Water-Soluble Vitamins (**I**)

Column: ***Discovery C18****, 15cm x 4.6mm ID, 5µ*
Mobile Phase: 50 mM K_2HPO_4, pH 7: MeOH, 99:1
Flow rate: 1 mL/min
Temperature: 35°C
Detection: UV, 220 nm
Injection: 10 µL

1. *Ascorbic acid (Vitamin C), 30 µg/mL*
2. *Niacin, 6 µg/mL*
3. *Pantothenic acid (Vitamin B_5), 100 µg/mL*
4. *Pyridoxine (Vitamin B_6), 3 µg/mL*
5. *Niacinamide, 6 µg/mL*
6. *Thiamine (Vitamin B_1), 12 µg/mL*
7. *Biotin, 120 µg/mL*
8. *Folic acid, 10 µg/mL*

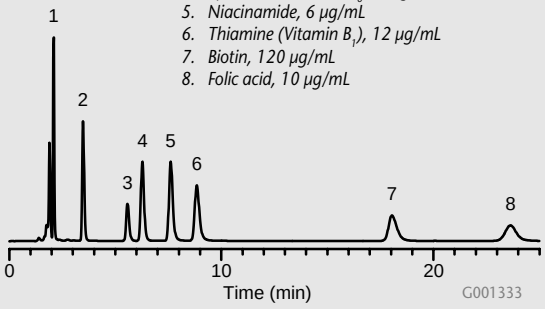
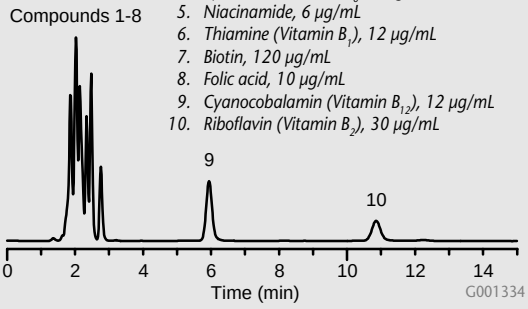


Figure 4. Isocratic Elution of Water-Soluble Vitamins (**II**)

Column: ***Discovery C18****, 15cm x 4.6mm ID, 5µ*
Mobile Phase: 50 mM K_2HPO_4, pH 7: MeOH, 80:20
Flow rate: 1 mL/min
Temperature: 35°C
Detection: UV, 220 nm
Injection: 10 µL

1. *Ascorbic acid (Vitamin C), 30 µg/mL*
2. *Niacin, 6 µg/mL*
3. *Pantothenic acid (Vitamin B_5), 100 µg/mL*
4. *Pyridoxine (Vitamin B_6), 3 µg/mL*
5. *Niacinamide, 6 µg/mL*
6. *Thiamine (Vitamin B_1), 12 µg/mL*
7. *Biotin, 120 µg/mL*
8. *Folic acid, 10 µg/mL*
9. *Cyanocobalamin (Vitamin B_{12}), 12 µg/mL*
10. *Riboflavin (Vitamin B_2), 30 µg/mL*



CASE STUDY 2

Consider Guard Column Capacity

The purpose of employing a guard column is to extend the useful lifetime of the analytical column by trapping sample matrix components that would otherwise foul the column. Those components that do not elute even under stringent column washing remain on the guard column instead of the analytical column. This advantage is significant because the guard column costs considerably less to replace than the analytical column. Of course, the addition of the guard column will delay elution of the sample since it increases the total column length and volume.

Columns of a 2.1mm ID are increasingly popular, often for purposes of enhanced sensitivity. The use of a 4.0mm ID guard column with a 2.1mm analytical column does not necessarily cause efficiency to suffer (Figures 9 & 10), but peak sharpness (height/width) may. This could be an issue for critical pair separation. The 4.0 mm guard column gives increased capacity to trap matrix components over and above the capacity of a 2.1 mm guard column. Increasing the ID from 2.1mm to 4.0mm increases the total theoretical capacity by a factor of 3.6. This significantly extends the guard column's useful life, reducing both the cost of replacement guards and analytical columns, and minimizing system downtime with minimal loss of performance.

Figure 9. Analytical Column without Guard

Column: ***Discovery C18****, 15cm x 2.1mm ID; 5µ*
Sample: formoterol
Sample size: 2 µL
Mobile Phase: 0.1% HCO_2H: MeCN, 80:20
Flow rate: 0.2 mL/min
Detection: 254 nm
Temperature: 25° C

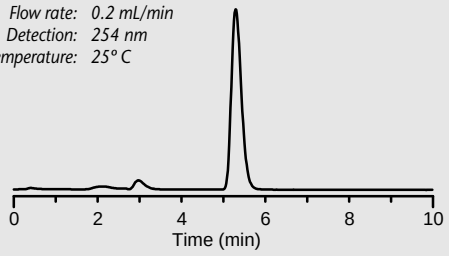
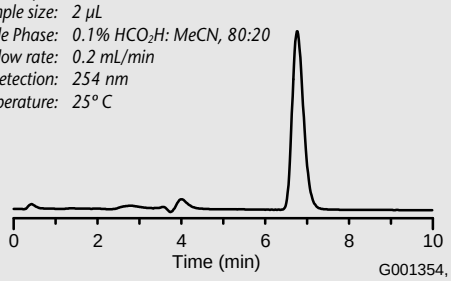


Figure 10. Analytical Column with Guard

Column: ***Discovery C18****, 15cm x 2.1mm ID with guard, 2cm x 4.0mm ID; 5µ*
Sample: formoterol
Sample size: 2 µL
Mobile Phase: 0.1% HCO_2H: MeCN, 80:20
Flow rate: 0.2 mL/min
Detection: 254 nm
Temperature: 25° C



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