Gradient Operation in HPLC

Dr. Shulamit Levin
Medtechnica
levins@medtechnica.co.il

http://www.forumsci.co.il/HPLC
http://shulalevin.tripod.com
Gradient Operation in HPLC

Introduction - Optimizing Gradient Separations

The following diagram illustrates the cycle time parameters that are used in a typical gradient.

Outline

- Introduction
- Strategies for Higher Throughput Gradient Separations to Achieve Maximum Throughput and Optimal Resolution
  - system solutions
  - method solutions

Typical Problems Encountered in Gradient Chromatography

- Non-reproducible retention times
- Difficulties to transfer from analytical to narrowbore columns
- Long reequilibration times
- Long cycle times (injection to injection)

More efficient analyses desired
Introduction - Options to Improve Sample Throughput

- **System Solutions:**
  - Reduce Gradient Delay Volume
  - Decrease Re-equilibration time
  - Reduce Injection Cycle time
  - Modify Instrument
  - Use Multiple Parallel Columns
  - Adjust Detector Sampling Rate

- **Method Solutions:**
  - Use Shorter Gradients
  - Use Higher Flow Rates
  - Use Shorter Columns
  - Use a Smaller Particle Size
  - Decrease Re-equilibration Time
  - Increase Temperature

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Test Probe Structures

- 1-hydroxy-7-aza-benzotriazole
- methyl 3-amino-2-thiophenecarboxylate
- 4-methylbenzene sulfonamide
- 4-aminobenzophenone

Initial Separation and Conditions

**Conditions:**
- Column: Symmetry® C18, 5 µm, 4.6 X 50 mm
- Mobile phase: A=0.1% TFA in water, B=0.1% TFA in acetonitrile
- Gradient: 0-60% B in 8 minutes
- Column temperature: 30.0 °C
- Flow rate: 1 mL/min
- Detector: 254 nm
- Injection volume: 1 µL

H. Weller, Bristol-Myers Squibb Pharmaceutical Research Institute
**Volumes in an HPLC System**

- **System Volume**
  - Waters 2690 <650 µL

- **Column Volume**

- **Extra Column Volume**

- **Detector**

**Effect of Precolumn Volume**

- **Reducing Delay Volume**

**Gradient Shape and Precolumn Volume**

- **Theoretical Gradient**
  - No column, 1.00 mL/min
  - 3.9x150 mm, 1.00 mL/min
  - 2.1x150 mm, 0.29 mL/min

**Determination of System Precolumn Volume**

- **Definition:** Delay volume is the volume of plumbing between the point the gradient is formed and the inlet of the column.

**System components affecting dwell volume:**
- Pump
- Gradient Mixers
- Injector

**Volumes in an HPLC System**

- **Proportioning Valve**
- **Solvent delivery**
- **Injector**
- **Column**
- **Detector**

**Effect of Precolumn Volume**

- **Reducing Delay Volume**

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Calculation of Gradient Equilibration Volume

- Re-equilibration is a necessary part of gradient chromatography. Both the HPLC system and the column must be at initial conditions at the beginning of each run to ensure reproducible chromatographic separations.
- The re-equilibration volume can be divided into two parts, the system washout and the column re-equilibration.
- For good system/column equilibration

\[ t_r = \frac{(3V_I + 5V_C)}{F} \]

where:
- \( t_r \) is the re-equilibration time in minutes,
- \( V_I \) is the total system volume,
- \( V_C \) is the column volume in mL,
- \( F \) is the flowrate in mL/min.

Column volume = \( 0.7 \pi r^2 L/2 \)

System volume = 650-3000 µL

- Theoretical Gradient
  - No column, 1.00 mL/min
  - 3.9x150 mm, 1.00 mL/min
  - 2.1x150 mm, 0.29 mL/min

Delay Volume

Re-equilibration Time

Theoretical Gradient

3x System
5x Column

3.9 x 150 mm
2.1 x 150 mm
1.0 x 150 mm

Volume, mL

0 2 4 6 8 10

1.0 mL/min
0.290 mL/min
0.066 mL/min

Time, min

0 10 20 30 40

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**Reduction of Re-equilibration Time**

Reduce re-equilibration time, two approaches:

1. increase flow rate
2. reduce column volume

**Reduction of Re-equilibration Time**

(Approach 1 - Increase Flow Rate)

Column: 3.9 X 50 mm

- Column volume (c.v.) = 0.60 mL
- 5 minute gradient @ 1 mL/min
- Instrument delay volume (d.v.) = 650 µL
- Gradient volume = 3 x c.v. = 3

Total re-equilibration time, \( t \)

\[
= \frac{(3(0.65) + 5(0.7)(0.6))}{1}
= 4.0/1
= 4.0 \text{ min.}
\]

Re-equilibration time is reduced by 50%

**Reduction of Re-equilibration Time**

(Approach 2 - Reduce Column Volume)

Column: 3.9 X 50 mm

- Column volume (c.v.) = 0.60 mL
- 5 minute gradient @ 1 mL/min
- Instrument delay volume (d.v.) = 650 µL
- Gradient volume = 3 x c.v. = 3

Total re-equilibration time, \( t \)

\[
= \frac{(3(0.65) + 5(0.7)(0.6))}{2}
= 2.0/2
= 2.0 \text{ min.}
\]

Re-equilibration time is reduced by 20%

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  - **method solutions**

**Shorter Cycle Time**

- **Reducing Total Cycle Time**
  - Reduce Cycle Times by:
    - Programming a system purge in the method which occurs during the injection of the sample or...
    - Employing two columns and performing column switching.

**Reduction of Re-equilibration Time (Approach 2 - Reduce Column Volume)**

- **Conditions:**
  - Symmetry® C18, 5 µm
  - Mobile phase: A=0.1% TFA in water, B=0.1% TFA in acetonitrile
  - Gradient: 0-60% B in 5 minutes
  - Column temperature: 30.0 °C
  - Detector: 254 nm
  - Injection volume: 1 µL
  - Flow rate: 1 mL/min.

- **Increase throughput by approximately 25% by reducing column volume from 0.170 (50 mm length) to 0.069 (20 mm length).**

**Samples per day**

- **320 +16%**
- **276**

**Column:**
- 3.9 x 50 mm
- 3 mL/min
- 3 min. gradient
Higher Throughput Through Column Switching

Column: Symmetry®, C18, 5 µL, 19 X 50 mm
Flow Rate: 20 mL/min.
Re-equilibration requires 5 column volumes = 150 mL = 7.5 min.
Re-equilibration period = unused time

Column switching can reduce runtimes by approx. 30%

Summary - System Solutions

- Reducing Gradient Delay Volume
  - Use 0.12 mm (0.005”) i.d. tubing instead of 0.25 mm (0.009”) to reduce system volume;
  - Shorten all tubing lengths;
  - Reduce the extra-column volume in the auto-injector by employing a smaller loop;
  - Remove gradient mixers

- Achieve Faster Gradient Chromatography By...
  - Reducing Re-equilibration Time
    - Reduce column volume
    - Increase flow rate
  - Reducing Cycle Time
    - Program injection to occur during re-equilibration
    - Implement column switching

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      - increase temperature
Factors Influencing Resolution in Gradient RP-HPLC Separations...

Resolution, Rs, between two closely resolved analytes in gradient RP-HPLC is a function of column efficiency N, selectivity a, and the retention factor:

\[
R_s = \frac{\Delta t}{W} \sim \frac{\sqrt{N}}{4} \ln \alpha \ast \frac{1}{B \cdot \frac{\Delta B}{t_0} \cdot t_0 + 1}
\]

Efficiency Selectivity Retention

\[
c = \%B/\text{minute} = \frac{\Delta \%}{t_0}
\]

Upon substitution of the actual variables (\(\Delta \%/t_0\) (gradient time)) for c, gradient slope, one can see the relationship between gradient time and resolution, and....

Resolution Dependence on Gradient Time and Flow Rate for a Gradient Method

(Symmetry® C18, 4.6 X 50 mm, 5 µm)

1. Effect of changing gradient run time, \(t_0\)
2. Effect of changing flow rate, \(F\)
Impact of Reducing Gradient Time ($t_g$) on Resolution

**Conditions:**
- Column: Symmetry® C18, 5 µm, 4.6 X 50 mm
- Mobile phase: A=0.1% TFA in water, B=0.1% TFA in acetonitrile
- Gradient: 0-60% B in noted gradient time
- Column temperature: 30.0 °C
- Flow rate: 1 mL/min.
- Detector: 254 nm
- Injection volume: 1 µL

- Longest gradient time provides best resolution
- Shortest gradient time maximizes throughput
- Reducing just gradient time sacrifices resolution

Impact of Flow Rate ($F$) on Resolution

**Conditions:**
- Column: Symmetry® C18, 5 µm, 4.6 X 50 mm
- Mobile phase: A=0.1% TFA in water, B=0.1% TFA in acetonitrile
- Gradient: 0-60% B in 4 minutes
- Column temperature: 30.0 °C
- Detector: 254 nm
- Injection volume: 1 µL

- Resolution goes through an optimum due to the combination of gradient expansion and decrease in plate count
- Optimum resolution is approximately 1 to 2 mL/min for most practical separation problems

Summary - Impact of Gradient Time on Resolution

- Resolution increases as gradient time increases
- Throughput decreases as gradient time increases

Summary - Impact of Flow Rate on Resolution

- Resolution goes through an optimum due to the combination of gradient expansion and decrease in plate count
- Optimum resolution is approximately 1 to 2 mL/min for most practical separation problems
Resolution Dependence on both Flow Rate and Gradient Time for a Gradient Method
(Symmetry® C18, 4.6 X 50 mm, 5 µm)

3. Effect of changing gradient run time, t_g, and flow rate, F

Summary - Reduction of Cycle Time

- To obtain the maximum sample throughput, the gradient time must be adjusted inversely proportionally to the flow rate.

- As shown in the previous slide the sample throughput was increased by 800% upon increasing the flow rate to 5 mL/min. and reducing the gradient time to 2 minutes.

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Impact of Column Length on Resolution

- How Short is Too Short?
  - It is not the column length which influences the separation in so much as the number of gradient volumes moving across the column.

- 2 Approaches:
  - **Approach 1**: Gradient volume in not proportion to the column volume (gradient run time constant while changing the column length).
  - **Approach 2**: Scale gradient volume in proportion to the column volume (change the gradient run time proportionally with the column length).

The Number of Column Volumes per Minute Impacts Resolution
What Factors Influence Gradient RP-HPLC Separations...

L (column length) is varied. Gradient volume is scaled in proportion to the column volume.

\[ R_s = \frac{\Delta t}{W} \sim \frac{\sqrt{N}}{4} \]

Terms are constant

\[ \ln \alpha \cdot \frac{1}{B \cdot \Delta \% \cdot \epsilon_1 \cdot \pi F_2 \cdot L/F + 1} \]

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Impact of Column Length on Resolution (Approach 2)
- Gradient volume scaled to column volume

Conditions:
- Symmetry® C18, 3.5 µm
- Mobile phase: A=0.1% TFA in water, B=0.1% TFA in acetonitrile
- Gradient: 0-60% B in noted time
- Column temperature: 30.0 °C
- Detector: 254 nm
- Injection volume: 1 µL
- Flow rate: 1 mL/min.

Reduce analysis time by >50%
- Trade-off: reduction in resolution

Summary - Impact of Column Length on Resolution
- Maximum sample throughput is realized when the gradient volume is scaled proportionally to the column volume.

Gradient Delay Time

Impact of the Number of Column Volumes on Peak Shape

Conditions:
- Symmetry® C18, 5 µm
- Mobile phase: A=0.1% TFA in water, B=0.1% TFA in acetonitrile
- Gradient: 0-60% B in 4 minutes
- Column temperature: 30.0 °C
- Detector: 254 nm
- Injection volume: 1 µL
- Flow rate: 2 mL/min.
Impact of Particle Size (dp) on Resolution

**Conditions:**
- Columns: Symmetry® C18, 5 µm, 4.6 X 50 mm and Symmetry® C18, 3.5 µm, 4.6 X 50 mm
- Mobile phase: A=0.1% TFA in water, B=0.1% TFA in acetonitrile
- Gradient: 0-60% B in 4 minutes
- Column temperature: 30.0 °C
- Detector: 254 nm
- Injection volume: 1 µL
- Flow rate: 1 mL/min.

- Achieve increased resolution with the smaller particle size material in the same gradient time
- Increase throughput and resolution with smaller particle size if flow rate is increased

**Symmetry® C18, 3.5 µm, 2.1 x 50 mm**
- Rs (peak A and B) = 11.97
- Rs (peak B and C) = 8.79
- Rs (peak C and D) = 7.35

**Symmetry® C18, 5 µm, 2.1 x 50 mm**
- Rs (peak A and B) = 6.71
- Rs (peak B and C) = 7.32
- Rs (peak C and D) = 6.45

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**Reducing the Effect of Gradient Delay Volume**

- Make gradient steeper by increasing the flow rate or decreasing the gradient time

- Increase flow rate (2 to 3 mL/min.)
- Increase gradient slope by 50% (4 to 2 min. grad)

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**Comparison of Resolution Dependence on Particle Size**

**Symmetry® C18, 3.5 µm, 4.6 X 50 mm**
- Max. Resolution @ 1 mL/min., 16 min. gradient = 18.8

**Symmetry® C18, 5 µm, 4.6 X 50 mm**
- Max. Resolution @ 1 mL/min., 16 min. gradient = 16.2

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**Impact of Particle Size (dp) on Resolution**

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Summary - Impact of Particle Size on Resolution

- Resolution is increased as a result of using a smaller particle size. This is due to the increase in the number of theoretical plates.

- If the flow rate is increased as well as the particle size being decreased, an increase in sample throughput is realized with increasing resolution.

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Summary - Method Solutions

- To obtain the fastest throughput:
  - increase flow rate
  - decrease column volume
  - decrease particle size
  - scale gradient volume with decrease in column volume
  - increase temperature to reduce viscosity of mobile phase allowing increases in flow rate

Impact of Temperature

- Faster Separation
- Narrower Peaks
- Lower Backpressure
- Lower Viscosity
- Greater Sensitivity

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