Selectivity and Detectability Optimizations in HPLC

SATINDER AHUJA

Development Department
Pharmaceuticals Division
CIBA-GEIGY Corporation
Suffern, New York
## CONTENTS

### CHAPTER 1  THE SCOPE OF SELECTIVITY AND DETECTABILITY OPTIMIZATION IN HPLC

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1. Selectivity and Detectability</td>
<td>1</td>
</tr>
<tr>
<td>1.2. Modes of HPLC</td>
<td>2</td>
</tr>
<tr>
<td>1.3. Theoretical Considerations</td>
<td>3</td>
</tr>
<tr>
<td>1.4. Various Routes to High Resolution</td>
<td>4</td>
</tr>
<tr>
<td>1.4.1. Optimum Column Selection (Conventional versus Small-Particle</td>
<td>5</td>
</tr>
<tr>
<td>versus Microbore Columns)</td>
<td></td>
</tr>
<tr>
<td>1.4.2. Optimum Analysis Time</td>
<td>6</td>
</tr>
<tr>
<td>1.5. High-Resolution Evaluations</td>
<td>7</td>
</tr>
<tr>
<td>1.6. HPLC Optimization</td>
<td>8</td>
</tr>
<tr>
<td>1.7. Multifactor Optimization</td>
<td>9</td>
</tr>
<tr>
<td>References</td>
<td>13</td>
</tr>
</tbody>
</table>

### CHAPTER 2  PHYSICOCHEMICAL BASIS OF RETENTION

*By L. R. Snyder*

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1. General Aspects of Retention</td>
<td>15</td>
</tr>
<tr>
<td>2.1.1. Partition Chromatography</td>
<td>16</td>
</tr>
<tr>
<td>2.1.2. Retention Involving the Displacement</td>
<td>18</td>
</tr>
<tr>
<td>of Mobile-Phase Molecules</td>
<td></td>
</tr>
<tr>
<td>2.1.3. Adsorption of Mobile Phase by the Stationary Phase</td>
<td>18</td>
</tr>
<tr>
<td>2.1.4. Intermolecular Interactions between</td>
<td>19</td>
</tr>
<tr>
<td>Solute and Solvent Molecules</td>
<td></td>
</tr>
<tr>
<td>2.2. Ion-Exchange Chromatography</td>
<td>21</td>
</tr>
<tr>
<td>2.3. Adsorption (Normal-Phase) Chromatography</td>
<td>24</td>
</tr>
<tr>
<td>2.3.1. Mobile-Phase Strength</td>
<td>24</td>
</tr>
</tbody>
</table>

vii
2.3.2. Solute Retention and Localization
2.4. Reversed-Phase Chromatography
   2.4.1. Mobile-Phase Strength
   2.4.2. Silanol Effects
   2.4.3. Solute Retention
2.5. Ion-Pair Chromatography
   2.5.1. Retention as a Function of Mobile-Phase Composition
2.6. Size-Exclusion Chromatography
2.7. Macromolecular Samples
2.8. Other Chromatographic Procedures
   2.8.1. Ion Chromatography
   2.8.2. Hydrophobic-Interaction Chromatography (HIC)
   2.8.3. Ligand-Exchange Chromatography
References

CHAPTER 3  PROBING SEPARATION MECHANISM IN HPLC

3.1. Separations in RPLC versus Octanol Partition Data
3.2. Impact of Other Physicochemical Parameters on Retention
3.3. Retention in HPLC versus Solubility
3.4. Stationary-Phase Effects
   3.4.1. Adsorption/Normal Phase
   3.4.2. Reversed Phase
3.5. Stationary-Phase Dynamics
3.6. Retention Mechanism Investigations
3.7. Molecular Probes/Retention Index
References

CHAPTER 4  CONVENTIONAL APPROACHES TO MOBILE-PHASE SELECTION AND OPTIMIZATION

4.1. General Considerations
   4.1.1. Properties of Sample or Solute
# CONTENTS

4.1.2. Column Selection 75  
4.1.3. Column Evaluations 77  
4.1.4. Mobile-Phase Selection in HPLC 78  
4.1.5. Solvent Classification 79  
4.1.6. Mobile-Phase Additives 81  

4.2. Modes of Chromatography 82  
4.2.1. Adsorption Chromatography 82  
4.2.2. Normal-Bonded Phases 85  
4.2.3. Reversed-Phase Chromatography 87  
4.2.4. Ion-Exchange Chromatography 92  

References 100

---

## CHAPTER 5 IMPROVING SEPARATIONS IN ADSORPTION FOR NORMAL-PHASE SEPARATIONS 103

5.1. Adsorption Chromatography 104  
5.1.1. Column Packings 104  
5.1.2. Mechanism of Adsorption Chromatography 107  
5.1.3. Solvent Selection in LSC 111  
5.1.4. Solvent Strength in LSC 112  
5.1.5. Role of Modulators 115  
5.1.6. Modulators and Selectivity 117  
5.1.7. Mobile-Phase Optimization Strategies 119  
5.1.8. Effect of Sample Structure on Retention 120  
5.1.9. Applications of Adsorption Chromatography 123  

5.2. Normal-Phase Chromatography 130  
5.2.1. Theory 133  
5.2.2. Column Packings 134  
5.2.3. Mobile-Phase Effects 136  
5.2.4. Applications of Normal-Phase Chromatography 140  

5.3. Miscellaneous Approaches 155  

References 156
## CHAPTER 6  SELECTIVITY OPTIMIZATION IN REVERSED-PHASE SEPARATIONS  \(\text{Page 161}\)

6.1. Stationary Phase  \(\text{Page 162}\)
   6.1.1. Pore Size and Volume  \(\text{Page 167}\)
   6.1.2. Nature of Bonded Phases  \(\text{Page 168}\)
   6.1.3. Column Efficiency  \(\text{Page 170}\)
   6.1.4. Columns  \(\text{Page 171}\)
   6.1.5. Problems with HPLC Columns  \(\text{Page 173}\)
   6.1.6. Column Evaluations  \(\text{Page 174}\)
6.2. Retention Mechanism  \(\text{Page 186}\)
6.3. Eluotropic Solvent Scale  \(\text{Page 190}\)
6.4. Mobile-Phase Selection and Optimization  \(\text{Page 196}\)
   6.4.1. Effect on Selectivity  \(\text{Page 200}\)
   6.4.2. Enthalpy of Binding  \(\text{Page 202}\)
6.5. Applications  \(\text{Page 204}\)
References  \(\text{Page 223}\)

## CHAPTER 7  ION-EXCHANGE CHROMATOGRAPHY OF IONIC AND IONIZABLE COMPOUNDS  \(\text{Page 229}\)

7.1. Ion-Exchange Chromatography  \(\text{Page 230}\)
   7.1.1. Column Packings  \(\text{Page 231}\)
   7.1.2. Mobile-Phase Selection  \(\text{Page 237}\)
   7.1.3. Retention Mechanism  \(\text{Page 242}\)
   7.1.4. Applications of IEC  \(\text{Page 248}\)
7.2. Ion Chromatography  \(\text{Page 256}\)
   7.2.1. Applications of IC  \(\text{Page 259}\)
References  \(\text{Page 261}\)

## CHAPTER 8  OPTIMIZING SELECTIVITY OF ION-PAIR SEPARATIONS  \(\text{Page 265}\)

8.1. Nature of Ion Pairs  \(\text{Page 265}\)
8.2. Theory  \(\text{Page 267}\)
8.3. Column Packings  \(\text{Page 271}\)
8.4. Mobile-Phase Selection and Optimization  \(\text{Page 272}\)
   8.4.1. Role of the Counterion  \(\text{Page 272}\)
   8.4.2. Solvent Effects  \(\text{Page 275}\)
CONTENTS

8.4.3. Ionic Strength and Secondary Ion Effects 278
8.4.4. Association/Dissociation Effects 280
8.4.5. pH Effects 281
8.4.6. Other Separation Variables 283
8.5. Retention Mechanism 284
8.6. Applications 293
References 310

CHAPTER 9 MACROMOLECULAR SEPARATIONS 315

9.1. Types of Macromolecules 317
9.1.1. Proteins 317
9.2. Purity Assessment 318
9.2.1. Recombinant-DNA-Derived Proteins 318
9.2.2. Polypeptides 319
9.2.3. Monoclonal Antibodies 320
9.2.4. Nucleic Acids 320
9.3. Chromatographic Methods 322
9.4. Retention Mechanism 329
9.4.1. Adsorption 329
9.4.2. Reversed Phase 330
9.4.3. Ion Exchange 338
9.4.4. Size-Exclusion Chromatography 339
9.5. Mechanistic Studies 341
9.5.1. Proteins 341
9.5.2. Nucleotides 354
9.5.3. Enzymes 356
9.5.4. Miscellaneous Compounds 358
9.6. Applications 361
References 387

CHAPTER 10 ISOMERIC SEPARATIONS 395

10.1. Chiral Columns 400
10.2. Chiral Additives to HPLC Eluents 403
10.3. Modes of Separation 407
10.3.1. Chromatography of Diastereomeric Derivatives 408
CONTENTS

10.3.2. Enantiomeric Resolution Using Chiral Mobile-Phase Additives 409
10.3.3. Enantiomeric Resolution Using Chiral Stationary Phases 412

10.4. Applications 419
10.4.1. Reversed-Phase/Normal-Phase Adsorption Columns 420
10.4.2. Ligand Chromatography 425
10.4.3. Pirkle-Type Columns 430
10.4.4. Cyclodextrin Columns 442
10.4.5. Protein Columns 444
10.4.6. Miscellaneous Applications 451

References 455

CHAPTER 11 COMPUTER OPTIMIZATION OF SELECTIVITY 461

11.1. Experimental Design 461
11.2. Optimization Methods 465
11.2.1. Window Diagram Methods 466
11.2.2. Simplex Methods 474
11.2.3. Gradient Elution 483
11.2.4. Interactive Methods 485
11.3. Practical Approaches 488
11.3.1. Computer Simulation and Solvent-Strength Optimization 488
11.3.2. Instrumentation 497
11.4. Optimum Method for Optimization 501

References 502

CHAPTER 12 SELECTIVE DETECTORS IN HPLC 505

12.1. UV Detectors 507
12.1.1. Applications 511
12.2. Fluorescence Detectors 513
12.2.1. Applications 518
12.3. Miscellaneous Luminescent Detectors 519
12.4. Electrochemical Detectors 522
12.4.1. Applications 525
12.5. Refractive Index Detectors 534
12.6. Mass Spectrometric Detectors 537
   12.6.1. Applications 542
12.7. Miscellaneous Detectors 544
References 548

CHAPTER 13 DETECTABILITY OPTIMIZATION 555
13.1. Noise and Detection Limits 556
13.2. Column Evaluations for Detectability Optimization 558
13.3. Limit of Detection 565
13.4. Detector Evaluation and Optimizations 568
   13.4.1. Ultraviolet Detectors 568
   13.4.2. Fluorescence Detectors 573
   13.4.3. Novel Detectors and Detection Methods 578
13.5. Derivatization 588
13.6. Miscellaneous Approaches 595
References 598

INDEX 605