PART I  METHODOLOGY

1.  Introduction to Mass Spectrometry  3
   Scott A. Smith, Ruth Waddell Smith, Yu Xia, and Zheng Ouyang

   1.1.  History  3
      1.1.1.  Atomic Physics  4
      1.1.2.  Early Applications  7
      1.1.3.  Organic Structural Analysis  7
      1.1.4.  The Biological Mass Spectrometry Revolution  8

   1.2. Ionization Methods  9

   1.3.  Mass Spectrometer Types  10
      1.3.1.  Magnetic Sector Mass Spectrometers  10
      1.3.2.  Quadrupole Mass Filter and Quadrupole Ion Trap
         Mass Spectrometers  14
      1.3.3.  Time-of-Flight Mass Spectrometers  19
      1.3.4.  Fourier Transform Ion Cyclotron Resonance
         Mass Spectrometers  22
      1.3.5.  Orbitrap Mass Spectrometers  25

   1.4.  Tandem Mass Spectrometry  28
      1.4.1.  Ion Isolation  29
      1.4.2.  Ion-Molecule Collisions and Collision-Induced
         Dissociation  30
      1.4.3.  Electron Capture Dissociation and Electron Transfer
         Dissociation  32

   1.5.  Separation Techniques Coupled to Mass Spectrometry  35
      1.5.1.  Gas Chromatography–Mass Spectrometry  35
      1.5.2.  Liquid Chromatography–Mass Spectrometry  37
      1.5.3.  Capillary Electrophoresis–Mass Spectrometry  42
      1.5.4.  Ion Mobility Spectrometry–Mass Spectrometry  45
## CONTENTS

1.6. Prospects for Mass Spectrometry 48  
References 51

2. LC Method Development and Strategies 59  
*Gang Xue and Yining Zhao*  
2.1. Introduction 59  
2.2. Column, pH, and Solvent Screening 60  
  2.2.1. Resolution: Goal of Separation 60  
  2.2.2. Screening: Systematic Approach to Seeking Selectivity 60  
  2.2.3. Screening Instrumentation and Controlling Software 67  
2.3. Gradient and Temperature Optimization 69  
2.4. Orthogonal Screening 70  
  2.4.1. Method Orthogonality 71  
  2.4.2. Selection of Orthogonal Methods 72  
  2.4.3. Impurity Orthogonal Screening 74  
2.5. High-Efficiency Separation 76  
2.6. Conclusions 78  
References 78

3. Rapid Analysis of Drug-Related Substances using Desorption Electrospray Ionization and Direct Analysis in Real Time Ionization Mass Spectrometry 81  
*Hao Chen and Jiwen Li*  
3.1. Introduction 81  
3.2. Ionization Apparatus, Mechanisms, and General Performance 83  
  3.2.1. Desorption Electrospray Ionization (DESI) 83  
  3.2.2. Direct Analysis in Real Time (DART) 85  
3.3. Drug Analysis in Biological Matrices using DESI and DART 87  
  3.3.1. DESI Application 88  
  3.3.2. DART Application 89  
3.4. High-Throughput Analysis 92  
3.5. Chemical Imaging and Profiling 94  
3.6. Future Perspectives 101  
References 101

4. Orbitrap High-Resolution Applications 109  
*Robert J. Strife*  
4.1. Historical Anecdote 109  
4.2. General Description of Orbitrap Operating Principles 110  
4.3. The Orbitrap is a “Fourier Transform” Device 112
4.4. Performing Experiments in Trapping Devices

4.4.1. “Raw” HPLC Data Look Like Infusion Data
4.4.2. How Much Mass Resolution Should Be Used During HPLC

4.5. Determining Elemental Compositions of “Unknowns” Using an Orbitrap

4.6. Orbitrap Figures of Merit in Mass Measurement

4.6.1. Accuracy
4.6.2. Precision
4.6.3. Discussion

4.7. HPLC Orbitrap MS: Accurate Mass Demonstration and Differentiation of Small Molecule Formulas Very Proximate in Mass/Charge Ratio Space

4.8. Determination of Trace Contaminant Compositions by Simple Screening HPLC-MS and Infusion Orbitrap MS

4.9. Determining Substructures: Orbitrap Tandem Mass Spectrometry (MS^n)

4.10. Multianalyzer (Hybridized) System: The Linear Ion Trap/Orbitrap for MS/MS and Higher-Order MS^n, n > 2

4.11. Mass Mapping to Discover Impurities


4.13. Conclusion

References

5. Structural Characterization of Impurities and Degradation Products in Pharmaceuticals Using High-Resolution LC-MS and Online Hydrogen/Deuterium Exchange Mass Spectrometry

Guodong Chen and Birendra N. Pramanik

5.1. Introduction

5.2. Characterization of Impurities
5.2.1. Mometasone Furoate
5.2.2. Enol Tautomer Impurity in Hepatitis C Virus (HCV) Protease Inhibitor

5.3. Characterization of Degradation Products
5.3.1. Everninomicin
5.3.2. Posaconazole

5.4. Conclusions

References
6. Isotope Pattern Recognition on Molecular Formula Determination for Structural Identification of Impurities 183

Ming Gu

6.1. Introduction 183

6.2. Three Basic Approaches to Isotope Pattern Recognition 184

6.2.1. With Centriod Data 185

6.2.2. With Profile Data without Peak Shape Calibration 187

6.2.3. With Profile Data with Peak Shape Calibration 189

6.3. The Importance of Lineshape Calibration 190

6.3.1. Lineshape Calibration Using Standards 191

6.3.2. Lineshape Self-Calibration 193

6.4. Spectral Accuracy 194

6.5. Formula Determination with Quadrupole MS 194

6.5.1. Impurity Identification with LC-MS 195

6.5.2. Impurity Identification with GC-MS 200

6.5.3. Pros and Cons of Determination of Elemental Decomposition (DEC) with Quadrupole MS 201

6.6. Formula Determination with High-Resolution MS 203

6.7. Conclusions and Future Directions 208

References 208

PART II APPLICATION

7. Practical Application of Very High-Pressure Liquid Chromatography Across the Pharmaceutical Development–Manufacturing Continuum 215

Brent Kleintop and Qinggang Wang

7.1. Introduction 215

7.2. Theory and Benefits of VHPLC 217

7.3. VHPLC Method Development 220

7.3.1. Adapting Existing HPLC Methods to VHPLC 220

7.3.2. Developing New VHPLC Methods 224

7.4. Other Practical Considerations 226

7.5. VHPLC Method Validation 227

7.6. Summary 229

References 229

8. Impurity Identification for Drug Substances 231

David W. Berberich, Tao Jiang, Joseph McClurg, Frank Moser, and R. Randy Wilhelm

8.1. Introduction 231
8.2. Case Studies

8.2.1. Identification of Impurities in Each Synthetic Step of Drug Substance during Process Development 232
8.2.2. Impurity ID by LC/MS during Exploratory Chemistry: Evaluation of New Raw Materials 237
8.2.3. Impurity Identification during Accelerated Stability Studies 243

8.3. Conclusions 249
References 250

9. Impurity Identification in Process Chemistry by Mass Spectrometry 251
David Q. Liu, Mingjiang Sun, and Lianming Wu

9.1. Introduction 251
9.2. Experimentation 252

9.2.1. Liquid Chromatography Conditions 252
9.2.2. LC-MS Systems 253
9.2.3. GC-MS System 253
9.2.4. Accurate Mass 253
9.2.5. Online H/D Exchange LC-MS 254

9.3. Applications 254

9.3.1. Identification of Reaction Byproducts by Data-Dependent LC/MS\textsuperscript{n} 254
9.3.2. Online H/D Exchange Aids Structural Elucidation of Process Impurities 257
9.3.3. LC-MS for Chemical Reaction Impurity Fate Mapping 260
9.3.4. GC-MS for Impurity Profiling of Small-Molecule Starting Materials 262
9.3.5. Identification of a Process Impurity that Impacts Downstream Formulation 265
9.3.6. Differential Fragmentation between Sodiated and Protonated Molecules as a Means of Structural Elucidation 267

9.4. Concluding Remarks 275
Acknowledgments 275
References 276

10. Structure Elucidation of Pharmaceutical Impurities and Degradants in Drug Formulation Development 279
Changkang Pan, Frances Liu, and Michael Motto

10.1. Importance of Drug Degradation Studies in Drug Development 279
10.2. Drug Degradation Studies in Formulation Development 281
10.2.1. Drug Substance–Excipient Interaction 281
10.2.2. Small Unknown Peaks (~0.1%) (Low-Dose Drugs <1 mg per Dose) 282
10.2.3. “Busy” LC Chromatogram with Multiple Peaks (Combination Drug Products) 282
10.2.4. Modification of Non-MS-Compatible LC Methods 282
10.2.5. Uncontrollable Multiple Chemical Reactions in Stability Samples 283
10.2.6. Separation Interference and Contamination Induced by Excipients 283
10.2.7. Peak Isolation and NMR Confirmation for Late-Phase Projects 284

10.3. Complexity of Impurity Identification in Drug Development 284
10.3.1. Drug Substance (DS) Degradation 284
10.3.2. DS–Excipient Interaction 285
10.3.3. DS–Residual Solvent Interaction 287
10.3.4. DS–Solvent Impurity Interaction 287
10.3.5. Metal Ion–Catalyzed Reaction 289
10.3.6. DS–Excipient Impurity Interaction 289
10.3.7. DS–Salt Interaction 291
10.3.8. DS–Preservative Interaction 291
10.3.9. Preservative–Excipient Interaction 292
10.3.10. Excipient Degradation 292
10.3.11. Leachables and Extractables 293

10.4. Strategy for Structure Elucidation of Unknowns 295
10.4.1. Non-MS-Compatible Method versus MS-Compatible Method 295
10.4.2. Selection of Ionization Mode (ESI or APCI, Positive or Negative) 298
10.4.3. Multiple Approaches for Structure Elucidation 298
10.4.4. Structure Confirmation 299

10.5. Hyphenated Analytical Techniques Used in Drug Development 300
10.5.1. LC-MS/MS for Fragmentation Pathways 302
10.5.2. High-Resolution MS for Chemical Formula/Elemental Composition 302
10.5.3. SEC/CLND or HPLC/CLND: Nitrogen-Specific Detection 304
10.5.4. GC-MS with EI-CI Combination 305
10.5.5. Headspace GC-MS: Volatile Compounds 305
10.5.6. NMR and LC-NMR 306
10.5.7. TD-GC/MS: Chemical Reactions Attributing to Weight Loss in TGA 307

10.6. Case Studies 307
10.6.1. LC-MS, GC-MS, and LC-NMR Studies of a Drug Degradation Product 307
  10.6.1.1. LC-MS Analysis 308
  10.6.1.2. GC-MS Analysis 308
  10.6.1.3. LC-NMR Analysis 308

10.6.2. Strategy for Identification of Leachables in Packaged Liquid Formulation 313

10.6.3. Characterization of Methionine Oxidation in Parathyroid Hormone Formulation 316
  10.6.3.1. Oxidation, Isolation, and Digestion of PTH1-34 316
  10.6.3.2. Mass Assignment of PTH1-34 Oxidized Variants 317
  10.6.3.3. Mass Assignment of CNBr Digested Peptide Fragments 318
  10.6.3.4. LC-MS/MS Studies of Ion Fragments from Oxidized Peptides 322

Acknowledgment 326
References 326

11. Investigation of Degradation Products and Extractables in Developing Topical OTC (Over the Counter) and NCE (New Chemical Entity) Consumer Healthcare Medication Products 337
   Fa Zhang

11.1. Introduction 337

11.2. Oxidatively Induced Coupling of Miconazole Nitrate with Butylated Hydroxytoluene in a Topical Ointment 338
  11.2.1. HPLC-MS Screening 339
  11.2.2. Organic Synthesis 341
  11.2.3. Degradation Mechanism 344

11.3. Extractables from Rubber Closures of a Prefilled Semisolid Drug Applicator 347
  11.3.1. Isolation of the Extractables 348
  11.3.2. Structural Identification of Extractables 5 and 6 348
  11.3.3. Structural Identification of Extractables 7 and 8 349
  11.3.4. Structural Identification of Extractable 9 351

11.4. New Degradation Products and Pathways of Vitamin D and Its Analogs 352
  11.4.1. Thermal Isomerization of Vitamin D₃ in DMSO 355
  11.4.2. Autoxidation of Isotachysterol 356
11.4.2.1. Mechanism of Isotachysterol Autoxidation 362
11.4.3. Thermal Degradation of Ecalcidene 364
11.4.4. Acid-Induced Degradation of Ecalcidene 368
11.4.5. Iodine-Induced Degradation of Ecalcidene 370
  11.4.5.1. cis/trans-Isomerization of Ecalcidene 371
  11.4.5.2. cis/trans-Isomerization of Previtamin D3–Type Isomer 24 372

11.5. Reductive Degradation of a 1,2,4-Thiadiazolium Derivative 376
11.6. Conclusions 382
References 383

12. Characterization of Impurities and Degradants in Protein Therapeutics by Mass Spectrometry 391
Li Tao, Michael Ackerman, Wei Wu, Peiran Liu, and Reb Russell

12.1. Introduction to Therapeutic Proteins 391
12.2. Recent Advances in Mass Spectrometry 392
12.3. Impurities 393
  12.3.1. Endotoxin 394
  12.3.2. Residual DNA 394
  12.3.3. Residual HCP 395
12.4. Degradation Products 395
  12.4.1. Chemical Degradation 396
    12.4.1.1. Deamidation/Isomerization 396
    12.4.1.2. Protein Fragmentation 400
    12.4.1.3. Oxidation 401
  12.4.2. Variants Caused by Posttranslational Modification 404
    12.4.2.1. Case Study: Characterization of S-Thiolation on Secreted Proteins from E. coli 406
    12.4.2.2. TM307 408
    12.4.2.3. TM485 408
    12.4.2.4. TM358 and TM687 410
12.5. Conclusions 413
References 413

13. Identification and Quantification of Degradants and Impurities in Antibodies 427
David M. Hambly and Himanshu S. Gadgil

13.1. Introduction to Antibodies and Protein Drugs 427
13.1.1. Antibody Classification and Subtypes 427
13.1.2. Antibody Structure 428
13.1.3. Antibody-Domain Structure 429
13.1.4. Recombinant Antibody Production 429
13.1.5. Methods for Characterizing Antibody Degradation and Impurity 430

13.2. Overview of Degradations and Impurities in Protein Drugs and Antibodies 431
13.2.1. Chemical Degradations and Impurities 431
  13.2.1.1. Methionine Oxidation 431
  13.2.1.2. Disulfide Bonds or Reduced Cysteine 432
  13.2.1.3. Deamidation of Asparagine and Glutamine 432
  13.2.1.4. Isomerization of Aspartic Acid and Glutamic Acid 433
  13.2.1.5. Amide Backbone Hydrolysis Reactions 433
  13.2.1.6. Glycation of Lysine Residues 433
  13.2.1.7. C-Terminal Lysine Variants 434
  13.2.1.8. Carbohydrate Variants 434

13.3. Methods Used to Identify and Quantitate Degradations and Impurities 435
13.3.1. Whole-Protein Mass Analysis Methods 435
  13.3.1.1. Carbohydrate Variation 435
  13.3.1.2. Detection of Lysine C-terminal Variants and Glycated Lysine 437
  13.3.1.3. Detection of Disulfide Bond Variants in IgG2 Antibodies 437
13.3.2. Methods for Evaluating the Mass of Protein Fragments 438
  13.3.2.1. Limited Digestion Method for Antibodies 438
  13.3.2.2. Limited and Reduced Method for Antibodies 440
  13.3.2.3. Reduced Protein Mass Analysis 441
13.3.3. Methods for Evaluating Peptides for Impurities and Degradations 443
  13.3.3.1. Reduced and Alkylated Peptide Mapping 443

13.4. Conclusions 450

Appendix 450
References 453

INDEX 461