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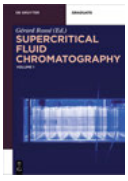

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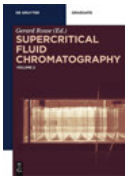

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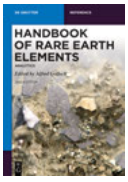

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High Performance Liquid Chromatography

Theory, Instrumentation and Application in Drug Quality Control

DE GRUYTER

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Preface

HPLC is the premier analytical technique in pharmaceutical analysis, which is predominantly used in the Pharmaceutical Industry and Drug Quality Control Laboratories for a large variety of samples. It is the method of choice for checking the purity of new drug candidates, monitoring changes or scale up of synthetic procedures, evaluating new formulations, and scrutinizing quality control/assurance of final drug products (DPs). To support each new drug application or commercial product, tens of thousands of HPLC tests are conducted by a host of dedicated scientists to assure the potency and quality of the new drug product.

Our goal was to provide the pharmaceutical analysts who constitute a significant fraction of all HPLC users in Drug Quality Control Laboratories with an updated view (new trends) of the concepts and practices application of modern HPLC, illustrated with many figures and case studies. Note that this basic book for practitioners was written at both an introductory and intermediate level.

The main focus of this book is on small drug molecules and pharmaceutical dosage forms. This book provides practical guidelines using case studies on sample preparation, column and instrument selection. It captures the essence of major pharmaceutical applications in drug quality control laboratories (assays, content uniformity and dissolution testing). In addition the book highlights novel approaches in HPLC and the latest developments in hyphenated techniques, such as LC-MS, and data handling.

Each of the 11 chapters (see table of contents), provides the reader with an in-depth understanding of HPLC theory, hardware, methodologies, regulations, applications, and new developments.

This book can be broadly classified into 5 major sections:

1. Overview, theory, instrumentation, and columns including ion chromatography and size exclusion (Chapters 1–5). The HPLC-column is the heart of the HPLC instrument and essential to its success. The book provides an extensive collection for technical information about HPLC-columns (physico-chemical properties and chromatographic characteristics) from various manufacturers, and help the analyst for decide on the ideal approach for their analysis according to the requirements of drug manufacturers specifications or/and the desired Pharmacopeia. In addition the authors give practical advices on how to prepare mobile phases, choose a suitable detector and set up an HPLC analysis.

2. HPLC methods applications and practices, including sample preparation and assays for Active Pharmaceutical Ingredients (API) in drug substances (DS) and drug products (DP), content uniformity and dissolution testing for DPs pharmaceutical dosage forms (Chapters 6–8). Also Chapter 8 highlights the regulatory aspects of ICH and USP guidelines for selection which analytical method can be use by the analyst in Drug Quality Control Laboratories, instrumental calibration, HPLC system qualifications,

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analytical method validation and checking for suitability of HPLC system before its using in analysis.

3. HPLC–Mass Spectrometry (HPLC–MS) and its applications in Drug Quality Control Laboratories (Chapter 9). In this Chapter and based on HPLC–MS we have presented result from study the adulteration of some natural herbal products with undeclared some synthetic PDE-5 inhibitors (Sildenafil, Tadalafil and Varddenafil).

4. Safety in HPLC laboratories (Chapter 10). This Chapter addresses the OSHA and EPA concerns about safety laboratory protocol when using HPLC equipment, solvents, samples and waste disposal in HPLC laboratories. Also we presents in this Chapter preventive measures and procedures which are related to safety in HPLC laboratories.

5. Glossary (Chapter 11)
Furthermore the book will serve as a definitive reference source for laboratory analysts, researchers, managers, and executives in industry, academe, and government agencies, who are engaged in various phases of using HPLC as analytical tool.

The book was written to be self sufficient interims of the needs of the average professional or technicians who plans to work with modern HPLC. We believe this book will prove its useful in most Drug Quality Control Laboratories where modern HPLC is practiced.

Following a hands-on approach, the book gives insight into the key pharmaceutical applications of HPLC and the latest requirements of the major regulatory agencies such as ICH, FDA, USP.

It is with a great deal of pleasure that I would like thank those who have contributed in so many ways to be completion of this book. First and foremost the authors wishes thank De Gruyter for giving chance and publishing this book. Most important, we would like to acknowledge the professionalism of my editor Stella Mueller, De Gruyter, Germany, whose enthusiasm and support made this a happy project, for her encouragement and keeping track of all the paper work. We also owe much to the reviewer (reading for proof), who has given me many insights and valuable advice. Special thanks go to Ulla Schmidt, Data Group / De Gruyter, Germany, for well organization of my book draft during the production and for her fast response for my questions.

The authors also are indebted to thank Professor Dr Moustafa Abbassy, Damanhur University, Professor Dr Ahmed Massoud, Kafer Elsheikh University, Egypt for given me many insights and valuable advice during the preparation of the book and Eng. Rana Marwan, Al Sharhan Industries, Kuwait for her technical support during the preparation of the manuscript.

Numerous manufacturers of analytical instrumentation and other products and services related to HPLC have contributed to the writing of this book by providing diagrams, applications notes and photos of their products. We are especially grateful

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Finally, I am professor Dr Moustafa A. Khalifa (2nd author) and I would like to thank my wife Howida, my sons Dr Mohammed and Dr Amr, my grandsons Hesham, Moustafa and Hady and my granddaughter Howida for their continue to put up with the inconveniences brought about by rushed deadline for the book project science 2 years ago. To them, I pledge more quality time to come after 2021 for playing and enjoying.

The Authors
10-12-2021
Kuwait

Contents

Preface —— V

1 High-performance liquid chromatography (HPLC): theoretical principles —— 1
1.1 Introduction —— **1**
1.2 What is HPLC in relation to other chromatographic techniques? —— **2**
1.3 Components of an HPLC system —— **2**
1.3.1 Mobile phase pumping system (mobile phase supply unit) —— **2**
1.3.2 Injection system (injector) —— **4**
1.3.3 Separating system (HPLC column) —— **4**
1.3.4 Detection system (detector) —— **5**
1.3.5 Data acquisition and analysis (computer) —— **6**
1.3.6 Fraction Collector —— **6**
1.3.7 Connection tubing —— **6**
1.4 History of HPLC —— **6**
1.5 Types of separation in HPLC —— **8**
1.6 Mechanism of separation in HPLC —— **9**
1.7 Where HPLC can be used as an analytical tool? —— **11**
1.8 Advantages of modern HPLC —— **14**
References —— **15**

2 HPLC columns for pharmaceutical analysis —— 17
2.1 Normal- and reversed-phase columns —— **17**
2.1.1 Introduction —— **17**
2.1.2 Packing materials or stationary phase for HPLC columns —— **17**
2.1.3 Silica as a packing material for NP-HPLC columns —— **20**
2.1.4 Silica as a base material for other HPLC packing materials —— **22**
2.1.4.1 Silica gel structure —— **22**
2.1.4.2 Important properties of silica —— **25**
2.1.5 Silica-based RP-HPLC (bonded silica) as a packing material for HPLC columns —— **27**
2.1.6 Modern packing materials for HPLC columns —— **33**
2.1.6.1 Symmetry packing —— **34**
2.1.6.2 Symmetry shield reversed-phase (SSRP) packing materials for HPLC columns (embedded polar group technology) —— **37**
2.1.6.3 XTerra packing (hybrid particle technology) —— **40**
2.1.7 HPLC column hardware (container) —— **43**
2.1.8 HPLC column efficiency —— **47**
2.1.9 HPLC column back pressure —— **49**

2.1.10 HPLC column storage — **51**
2.1.11 Column temperature — **52**
2.1.12 Column protection — **53**
2.1.13 HPLC column, cleaning and regeneration — **56**
2.1.14 Specification of HPLC packings — **59**
2.1.14.1 Physicochemical properties — **59**
2.1.14.2 Chromatographic characteristics — **63**
2.1.15 HPLC column troubleshooting — **63**
2.2 Ion-exchange (IC, IEC or IEX) and size-exclusion (SEC) columns for HPLC — **64**
2.2.1 Introduction — **64**
2.2.2 Ion Chromatography (IC, IEC and IEX) — **65**
2.2.2.1 Principles of ion chromatography — **67**
2.2.2.2 Packing materials (stationary phase) for IC columns — **71**
2.2.2.3 Mobile phase for IC — **73**
2.2.2.4 IC instrumentation — **73**
2.2.2.5 Principles of ion suppressors — **75**
2.2.2.6 IC applications for ion analysis — **76**
2.2.2.6.1 IC applications for anion analysis — **76**
2.2.2.6.2 IC applications for cation analysis — **77**
2.2.3 Ion-pair chromatography (IPC) — **78**
2.2.4 Ion-exclusion chromatography (IEXC) — **79**
2.2 4.1 Introduction — **79**
2.2.4.2 Principles of ion-exclusion chromatography (IEXC) — **80**
2.2.4.3 Application of IEXC for analysis of pharmaceutical products — **81**
2.2.5 Size-exclusion chromatography (SEC) — **84**
2.2.5.1 Introduction — **84**
2.2.5.2 Principles of SEC — **85**
2.2.5.3 Packing materials for SEC columns — **86**
2.2.5.4 Applicatuon of SEC for analysis of pharmaceutical products in drug quality control laboratories — **87**
References — **89**

3 HPLC mobile phase and HPLC pumping system — 95
3.1 Introduction — **95**
3.2 Solvent selection for mobile phase preparation — **95**
3.2.1 Polarity — **96**
3.2.2 Viscosity — **97**
3.2.3 UV transparency — **97**
3.2.4 Refractive index — **97**
3.2.5 Boiling point — **98**
3.2.6 Inertness to the sample — **98**

3.2.7 Corrosion resistant — **99**
3.2.8 Toxicity — **99**
3.3 Mobile phase preparation and handling — **100**
3.3.1 Mobile phase preparation — **100**
3.3.2 Buffered mobile phase — **100**
3.3.2.1 Acidic mobile phases — **102**
3.3.2.2 Ion-paired mobile phase — **103**
3.3.2.3 Basic mobile phase — **103**
3.3.3 Mobile phase filtration — **103**
3.3.3.1 Off-line filtration (vacuum filtration) — **104**
3.3.3.1.1 Filtration procedures — **106**
3.3.3.1.2 Use the filtered mobile phase — **107**
3.3.3.1.3 Maintenance and storage of the Millisolve system — **109**
3.3.3.2 In-line mobile phase filtration — **109**
3.3.4 Mobile phase degassing — **110**
3.3.4.1 Sparging — **113**
3.3.4.2 Heat — **113**
3.3.4.3 Vacuum — **113**
3.3.4.4 Sonication — **113**
3.3.4.5 Mobile phase degasser (in-line degassing of mobile phase) — **116**
3.3.5 Water for mobile phase (water HPLC grade; ultra pure water) — **117**
3.3.5.1 *Directions for use of* Norganic cartridge (trace organic removal cartridge) — **118**
3.3.6 Important notices about mobile phase selection and preparation — **119**
3.4 HPLC mobile phase pumping system — **121**
3.4.1 HPLC mobile phase pumping system components and operating guides — **121**
3.4.2 Mobile phase pressure and flow relationships — **123**
3.4.3 HPLC mobile phase flow control and programming systems — **125**
3.4.3.1 Gradient elution — **125**
3.4.3.2 Isocratic elution — **128**
3.5 Solvent miscibility — **128**
3.6 Optimization of the mobile phase — **128**
References — **130**

4 HPLC injection system (injector) — 133
4.1 Introduction — **133**
4.2 Loop injector for HPLC — **133**
4.3 Manual injection — **135**
4.4 Automation of HPLC injection valve (autosamplers) — **138**
4.5 Operating principles the HPLC autosampler works on — **139**

4.5.1 Advantages of using HPLC autosamplers — 139
4.5.2 Practical guidelines for operation of HPLC autosampler — 140
References — 141

5 HPLC detectors — 143
5.1 Introduction — 143
5.2 Types of HPLC detectors — 143
5.2.1 UV absorbance detectors (spectrophotometric detectors) — 144
5.2.1.1 Principles of UV and UV/VIS absorbance detection — 147
5.2.1.2 UV/UV–VIS detector flow cell — 147
5.2.1.3 Characteristics of a UV/UV–VIS detectors — 149
5.2.2 Diode array detector (DAD) or photodiode array detector (PDA) — 151
5.2.3 Conductivity detectors — 152
5.2.3.1 Characteristics of conductivity detectors — 155
5.2.4 Electrochemical detector (ED) — 155
5.2.5 Fluorescence detector — 157
5.2.5.1 Major application for FL detectors — 160
5.2.5.2 Characteristics of fluorescence detector — 163
5.2.6 Refractive index detector — 163
5.2.6.1 Characteristic of RI detectors — 165
5.2.7 Mass Spectrometric Detectors — 165
5.3 HPLC detector performance — 166
References — 167

6 HPLC as analytical tool in drug quality control laboratories: getting started — 169
6.1 Introduction — 169
6.2 HPLC hardware component selection — 170
6.2.1 HPLC pumping system for mobile phase (pumps) — 170
6.2.1.1 Isocratic and gradient HPLC system — 171
6.2.1.2 Types of HPLC pumping systems — 172
6.2.2 HPLC – sample introduction system (HPLC injectors/ autosamplers) — 173
6.2.3 Columns — 174
6.2.4 Detectors — 174
6.2.4.1 HPLC detector performance — 175
6.2.4.2 HPLC-UV/Vis/PDA detector operating guides — 178
6.2.5 Miscellaneous items — 178
6.3 HPLC manufacturers and equipment selection — 179

6.4 HPLC – components (units) connection (tubing, fittings and instillation of an HPLC system) without internal dead volume and leak free — **180**
6.4.1 HPLC tubing — **180**
6.4.2 HPLC fittings — **182**
6.4.2.1 Compression fittings — **182**
6.4.2.2 HPLC tubing connectors — **183**
6.4.2.3 Teflon fittings — **183**
6.4.2.4 Finger-tightened fittings (polyether ether ketone) — **186**
6.5 HPLC column installation (column connection) — **187**
6.5.1 Use the following procedure to install your column — **189**
6.6 Running of an HPLC system — **191**
6.7 HPLC – instillation quality — **191**
6.7.1 Measurement of HPLC instrument band spreading (IBW) — **192**
References — **193**

7 Preparation of pharmaceutical samples for HPLC analysis in drug quality control laboratories — 197
7.1 Introduction — **197**
7.2 Methods for preparations of pharmaceutical dosage forms for analysis using HPLC — **200**
7.2.1 Extraction — **200**
7.2.1.1 Liquid–liquid extraction — **200**
7.2.1.2 Solid–liquid extraction — **201**
7.2.1.3 Soxhlet extraction — **202**
7.2.1.4 Ultrasound-assisted extraction (sonication) — **204**
7.2.1.5 Solid-phase extraction (SPE) — **205**
7.2.2 Distillation — **207**
7.2.3 Dissolution — **207**
7.2.4 HPLC column switching (online SPE) as a sample preparation for pharmaceutical products for HPLC analysis — **207**
7.2.5 Sample size reduction — **208**
7.2.6 Clean up of sample extracts — **211**
7.2.6.1 Freezing and centrifugation — **211**
7.2.6.2 Filtration or centrifugation — **211**
7.2.7 Preconcentration of sample extract before HPLC analysis — **212**
7.3 Extraction efficiency (API recovery) — **214**
7.4 Sample stability — **214**
7.5 Choice of sample solvent — **214**
References — **215**

8 HPLC as analytical tool in drug quality control laboratories: qualitative and quantitative analysis — 217
8.1 Introduction — **217**
8.2 HPLC safety in drug quality control laboratories — **217**
8.3 What analytical methods can be used by analysts in drug quality control laboratories — **218**
8.4 Qualitative and quantitative analysis of pharmaceutical products (drug products) and drug substances in drug quality control laboratories — **220**
8.4.1 Stationary phase (packing materials) and their selection for HPLC columns to be used in drug quality control laboratories for analysis of DP and DS — **221**
8.4.1.1 Conventional stationary phase (packing materials) for HPLC columns to be used in drug quality control laboratories for analysis of DP and DS — **221**
8.4.1.2 Modern packing materials for HPLC columns — **222**
8.4.1.2.1 Symmetry packing — **223**
8.4.1.2.2 Symmetry shield reversed-phase packing materials for HPLC columns (embedded polar group technology) — **224**
8.4.1.2.3 XTerra packing (hybrid particle technology) — **225**
8.4.1.2.4 Sterically hindered group and polyfunctional silane chemistry — **226**
8.4.1.2.5 Modern HPLC columns trends — **226**
8.4.2 HPLC column hardware — **227**
8.4.3 HPLC columns selection for analysis of pharmaceutical products and drug substances in drug quality control laboratories — **229**
8.4.3.1 HPLC columns selection for United States Pharmacopeia analytical methods — **229**
8.4.4 Column installation (column connection) to an HPLC instrument — **231**
8.4.5 Recommended operating guides for RP-HPLC and NP-HPLC columns — **231**
8.4.6 Mobile phase preparation and connection to HPLC instrument — **233**
8.4.7 Set up parameters for HPLC instruments — **233**
8.4.8 HPLC column equilibration — **234**
8.4.9 Checking for HPLC system qualifications and calibration — **237**
8.4.9.1 Checking for HPLC system qualifications — **237**
8.4.9.2 Design qualification (DQ) — **237**
8.4.9.3 Installation qualification (IQ) — **237**
8.4.9.4 Operational qualification (OQ) — **237**
8.4.9.5 Performance qualification (PQ) — **238**

8.4.9.6 HPLC system calibration — **238**
8.4.9.6.1 Wave length accuracy testing of UV-Vis detector in HPLC — **238**
8.4.9.6.2 HPLC injector (autosampler) accuracy — **239**
8.4.9.6.3 HPLC injector (autosampler) precision (sampling precision) — **240**
8.4.9.6.4 Mobile phase flow rate accuracy and precision — **241**
8.4.9.6.5 Column oven temperature accuracy — **241**
8.4.9.6.6 Injector or autosampler carryover — **241**
8.4.9.6.7 Detector linearity (linearity response of the HPLC UV-Vis detector) — **242**
8.4.9.6.8 Injector linearity — **243**
8.4.9.7 Documentation — **244**
8.4.10 Preparation of reference standard solution — **244**
8.4.11 Checking for suitability of HPLC system (system suitability test) (SST) — **245**
8.4.11.1 SST acceptance limit (acceptance criteria) — **249**
8.4.11.2 Reproducibility injection of the HPLC system (injection precision) — **250**
8.4.11.3 Column efficiency and plate number (theoretical plates number), height equivalent to a theoretical plate, plates per meter (N/m) and reduced plate height (h) = H/dp = L (µm)/N × dp (µm) — **250**
8.4.11.3.1 Height equivalent to a theoretical plate or plate height — **252**
8.4.11.3.2 Plates per meter (N/m) — **252**
8.4.11.3.3 Reduced plate height — **253**
8.4.11.4 Peak asymmetry factor — **254**
8.4.11.5 Tailing factor (T_f) — **254**
8.4.11.6 Capacity factor (k') or retention factor — **256**
8.4.11.7 Separation factor (selectivity) — **256**
8.4.11.8 Resolution — **258**
8.4.11.9 Resolution and peak separation — **258**
8.4.12 Sampling and sample preparation — **260**
8.4.12.1 Sampling — **260**
8.4.12.2 Sample preparation — **262**
8.4.12.3 Sample analysis — **264**
8.4.12.3.1 Sample solution injection — **264**
8.4.12.3.2 Qualitative analysis (identification) — **267**
8.4.12.3.3 Quantitative analysis — **269**
8.4.13 Application for HPLC as analytical tool for analysis of pharmaceutical substances and pharmaceutical products in drug quality control laboratories — **277**
8.4.13.1 Assay for potency — **280**
8.4.13.1.1 Assay potency for risperdal tablets (LC = 2 mg/tablet) — **281**

8.4.13.1.2 Assay potency based on a portion of the composite ground powder taken from tablet or capsules equal to the average tablet weight or average capsule weight — **282**
8.4.13.1.3 Assay potency for APIs in liquid form of pharmaceutical products — **284**
8.4.13.2 Assay of preservatives — **289**
8.4.13.3 Content uniformity test — **291**
8.4.13.4 Dissolution testing — **294**
8.4.13.4a Choice of the dissolution apparatus — **295**
8.4.13.4b Dissolution medium — **299**
8.4.13.4c Dissolution procedures — **299**
8.4.13.4d Sampling and sample analysis — **299**
8.4.13.4e Dissolution Criteria — **303**
References — **304**

9 HPLC–Mass Spectrometry (HPLC–MS) and its applications in drug quality control laboratories — 309
9.1 Introduction — **309**
9.2 Principles of mass spectrometry — **309**
9.3 High Performance Liquid Chromatography – Mass Spectrometry (HPLC–MS) — **312**
9.3.1 Principles of High Performance Liquid Chromatography – Mass Spectrometry (HPLC–MS) — **312**
9.3.2 Interfacing MS and HPLC — **314**
9.3.2.1 Atmospheric pressure chemical ionization (HPLC–MS–APCI) interface for MS — **314**
9.3.2.1a Operating conditions for atmospheric pressure chemical ionization (HPLC–MS–APCI) interface for MS — **315**
9.3.2.2 Electrospray ionization — **316**
9.3.2.2a Operating conditions for electrospray ionization interface of HPLC–MS — **318**
9.4 Mobile phase selection for HPLC–MS — **319**
9.5 Design of API interface for HPLC–MS (ion source design) — **320**
9.6 The analyzer — **321**
9.6.1 The quadrupole mass analyzer — **321**
9.6.2 The triple quadrupole analyzer (HPLC–MS–MS technique) — **324**
9.7 The detector — **325**
9.7.1 Electron multiplier — **326**
9.7.2 Dynolyte photomultiplier — **326**
9.8 Performance criteria for HPLC–MS system — **327**
9.9 Combination between HPLC–MS and PDA detectors (HPLC–PDA–MS) — **328**

9.9.1 HPLC–PDA–ESI(+)–MS as analytical tool in drug quality control laboratories (screening for presence of some approved synthetic drugs—PDE5 inhibitors, such as Sildenafil, Tadalafil and Vardenafil—as illegal adulterants for natural hebbal products–herbal medicines and dietary supplementary food — **328**
References — **339**

10 Safety in HPLC laboratories — 343
10.1 Introduction — **343**
10.2 Safety handling for solvents, mobile phase preparation and its supply for HPLC equipment during analysis — **344**
10.2.1 Safety handling for solvent and mobile phase preparation — **344**
10.2.2 Safety caps for safe handling of mobile phase supply to HPLC equipment during analysis — **345**
10.3 Safety waste caps for safe disposal of HPLC wastes (safety handling for waste in HPLC laboratories) — **348**
10.4 Safety funnels for filling the liquid waste safely in waste container — **352**
10.4.1 Safety funnel with ball valve — **352**
10.4.2 Safety funnels with hinged lid — **352**
10.4.3 Safety waste caps with included funnel — **353**
10.5 Waste container with filling level control and monitoring devices to avoid any risk of overflow — **356**
10.6 Waste container with filling level control and safety funnel for waste collection in HPLC laboratories (disposal unit) — **357**
10.7 Complete safety set for HPLC — **358**
10.8 Pressured liquids safety in HPLC laboratories — **359**
10.9 Electrical safety in HPLC laboratories — **360**
10.9.1 Electrical fire precautions — **362**
10.10 Physical injuries and safety in HPLC laboratories — **362**
10.11 HPLC detectors handling and safety in HPLC laboratories — **364**
10.11.1 UV precautions — **364**
10.12 General safety rules in HPLC laboratories — **364**
References — **366**

11 Glossary — 369

Index — 377

1 High-performance liquid chromatography (HPLC): theoretical principles

1.1 Introduction

High-performance liquid chromatography (HPLC, formerly referred to high-pressure liquid chromatography) is, for many scientists, an essential piece of apparatus for the separation, identification, purification and quantification of various compounds, especially very polar (organic) in their complex mixtures. Users of HPLC work in a variety of fields, including analysis of pharmaceuticals, foods, waters, cosmetics, biochemicals and environmental samples [1–6]. For HPLC analysis, mixtures from interested compounds are first dissolved in a liquid solvent and then forced to flow with mobile phase through a stationary phase in the form of column (usually stainless steel column) under high pressure. On this stationary phase (column), the mixture of chemical compounds is resolved into its components. The amount of resolution is important and is dependent upon the extent of interaction between the solute components and the stationary phase. The stationary phase is defined as the immobile packing material of the column. The moving part of the system is the mobile phase, which is a liquid. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of solvents and columns. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems. Thus, HPLC has the ability to easily separate a wide variety of chemical mixtures, especially which contains thermally labile or easily oxidized compounds.

In the opposite, gas chromatography (GC) is limited in its applications, and the analysis of compounds which are thermally labile or easily oxidized is not possible. So, HPLC can fill this gap and has thus become an essential addition to GC in every analytical laboratory. Advances in HPLC technology have been assisted by the continual development of new stationary phases, improvements in instrumentation and the facility the method offers for the application of computer technology and developments in automation techniques.

It is, therefore, not surprising that the number of publications which are now dealing with HPLC exceeded than GC [7].

In this chapter we will discuss the following topics:

- **What is HPLC in relation to other chromatographic techniques?**
- **Components of an HPLC system**
- **History of HPLC**
- **Types of separation in HPLC**
- **Mechanism of separation in HPLC**

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- **Where HPLC can be used as an analytical tool?**
- **Advantages of modern HPLC**

In this part, we will discuss the previously mentioned topics in detail.

1.2 What is HPLC in relation to other chromatographic techniques?

In chromatographic technique, the components of a chemical mixture are separated based upon the rates at which they are carried through a stationary phase (solid or liquid on solid support) by gaseous or liquid phases (mobile phase). Chromatographic method is categorized into three types based on the nature of the mobile phase [7]. The three types of phases include liquid, gases, and supercritical fluids as shown in Fig.1.1. Liquid chromatography (LC) can be performed in columns and on planar surfaces, but GC and supercritical fluid chromatography are restricted to column procedures. Column chromatography is often described as elution chromatography in which solutes are washed through stationary phase by the movement of the mobile phase (eluent).

The chromatographic separation of a mixture of various compounds depends primarily on the fact that each of them is physicochemically different from two nonmiscible phases (the stationary phase and the mobile phase). Thus, thermodynamic *effects such as partition and absorption are mostly involved. Depending on the* physical state of the mobile phase, a distinction is made between GC and LC (as shown in Fig.1.1). A further distinction depends on the form of stationary phase, layer or column, which leads to other classifications, thin-layer chromatography and column chromatography. The stationary phase may be solid, porous chemically modified or liquid film on solid support.

1.3 Components of an HPLC system

Schematic representation of the essential components of an HPLC system is found in Fig.1.2. We shall discuss briefly these following components.

1.3.1 Mobile phase pumping system (mobile phase supply unit)

It consists of high-pressure pump to force the mobile phase through the HPLC system and provides flow rates from 100 μL/min for small bore columns and 1–3 mL/min for other columns. Suitable pressure gauge (manometer) over pressure protection and flow meter is placed in pump to measure the system pressure and flow rate of the

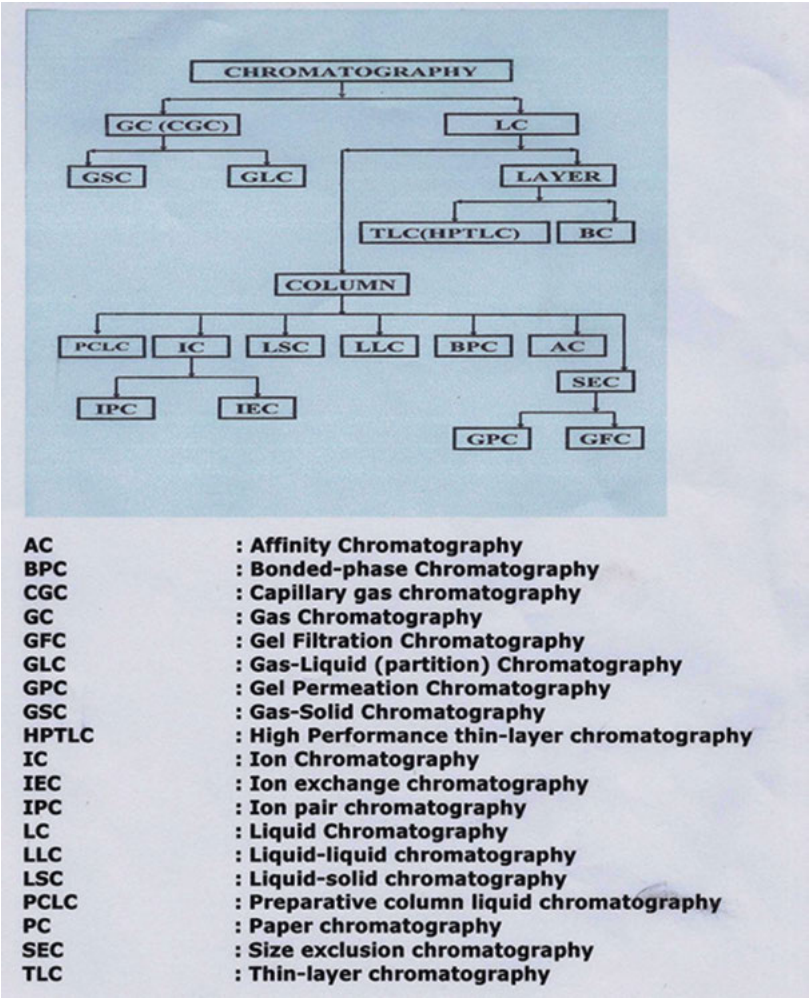

Fig.1.1: Categories of chromatography and their relationship to each other.

mobile phase. Mobile phase reservoir presents a part of the system and it holds the mobile phase that will be pumped into the HPLC system through mobile phase inlet line (Teflon tubing) which is supported by metal frit (mobile phase inlet filter) for in-line filtration of mobile phase. Two basic elution modes for the mobile phase are used in HPLC analysis for pharmaceutical products in drug quality control laboratories. The first is called isocratic elution. In this mode, the mobile phase, either a pure solvent or a mixture, *remains the same during the separation and sample run.* The second type is called gradient elution, wherein, as its name implies, the mobile phase composition changes during the separation and sample run (for details, see Chapters 3 and 6).

1.3.2 Injection system (injector)

Sampling valves and loops injectors to inject the sample into the mobile phase just at the head of separation column (see Chapter 4 for details).

1.3.3 Separating system (HPLC column)

HPLC column (Fig.1.3) consists of two components: the column packing materials (stationary phase) and the hardware (container) in which the stationary phase is found (as shown in Fig.1.4). On separation column, the sample component can be separated to their individuals. Since the stationary phase inside the column is composed of micro-size particle that is irregular or spherical (as shown in Fig.1.5), a high-pressure pump is required to move the mobile phase through the column. In fact the heart of the HPLC is considered to be the column (for details, see Chapters 2 and 8).

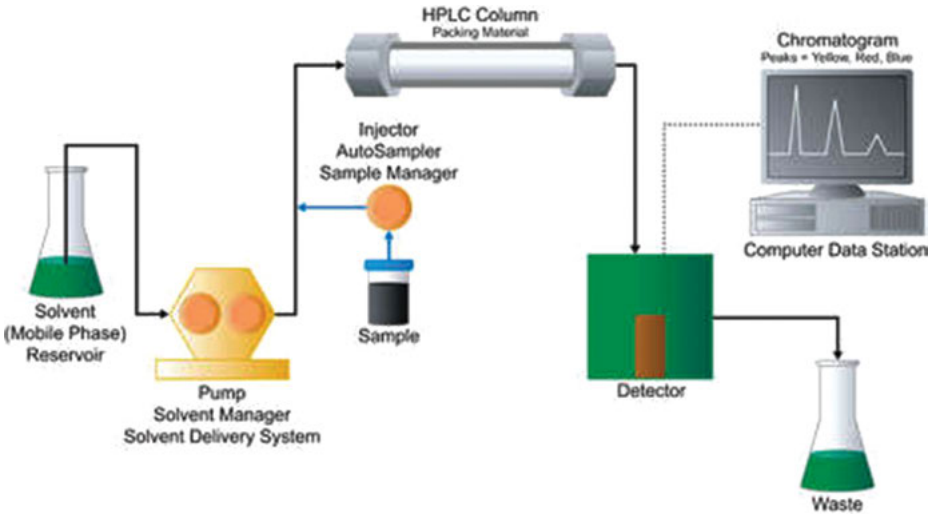

Fig.1.2: Schematic diagram of an HPLC system (permission granted to reproduce by Waters Corporation, www.waters.com).

Fig.1.3: Hardware of an HPLC column.

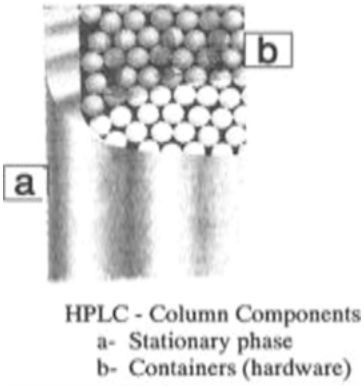

Fig.1.4: Stationary phase (a) inside the hardware and (b) of an HPLC column.

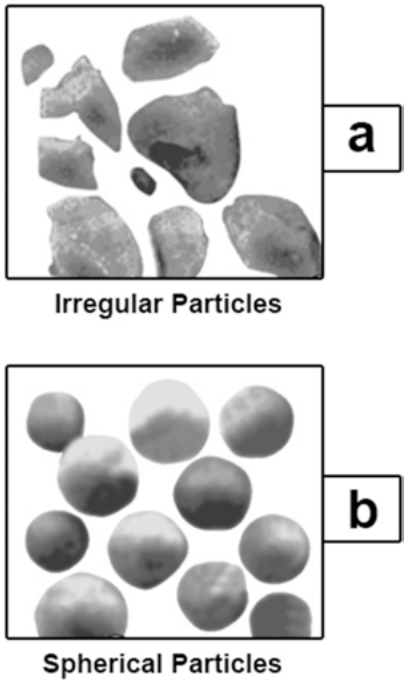

Fig.1.5: Stationary phase particles: irregular (a) or spherical (b).

1.3.4 Detection system (detector)

For detection, the presence of solutes which have been separated by column and eluted out in mobile phase (for details, see Chapter 5).

1.3.5 Data acquisition and analysis (computer)

To collect, store and analyze the chromatographic data resulting from HPLC, nowadays computers and other data processing equipments are being used more frequently.

1.3.6 Fraction Collector

HPLC can also be used to purify and collect desired amounts of each compound separated using a fraction collector downstream of the detector flow cell. This process is called preparative chromatography (see Fig.1.11).

1.3.7 Connection tubing

In HPLC, there is connection tubing for joining the parts of the instrument together (for details, see Chapter 6).

HPLC systems can be either modular or integrated as shown in Fig.1.6 and 1.7, respectively. In practice, both types are popular in drug quality control laboratories [3].

1.4 History of HPLC

The term *chromatography* means "color writing" (Chroma is the Greek word meaning color combined with graphy means writing). This was first discovered by Mikhail Tswett (1903) [8, 9], a Russian botanist who separated plant pigments, particularly the carotenoids and the chlorophylls on chalk ($CaCO_3$) packed in glass columns and eluted by organic solvent, petroleum ether. The result of this process was that the plant pigments were separated into a series of discrete colored bands on the column, divided by regions entirely free of pigments as they passed through the column (stationary phase).

Since the 1930s, chemists used gravity-fed silica columns (Fig.1.8) to purify organic materials and ion-exchange resin columns to separate ionic compounds and radionuclides [8, 9]. Lacking both speed and resolution, the technique of chromatography languished many years until the field was revolutionized by the Nobel Prize winning work of Martyn and Synge in 1941 [8, 9]. This work was important because it established a firm theoretical basis for the separation mechanism.

The first generation of HPLC was developed by researchers in the 1960s, including Horvath, Kirkland and Huber [8, 9]. With continued advances in performance during this time (smaller particles for stationary phase), the term of HPLC remained the same but the name was changed to HPLC. Commercial development of in-line

Fig.1.6: Examples of modular HPLC systems (Agilent 1100 Series System) (permission granted to reproduce by Agilent Technologies, www.agilent.com).

Fig.1.7: Examples of integrated HPLC (Waters Alliance System) (permission granted to reproduce by Waters Corporation, www.waters.com).

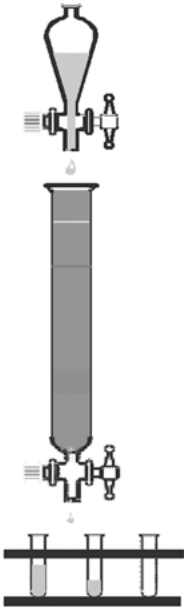

Fig.1.8: Illustrative diagram showing the traditional technique of low-pressure liquid chromatography using a glass column and gravity-fed solvent with manual fraction collection.

detectors and reliable injectors allowed HPLC to become a sensitive and quantitative technique leading to an explosive growth of applications.

The science of HPLC was revolutionized a few years ago [10–12] with the advent of ultra-HPLC (UHPLC) (such as Waters Co. Acquity UHPLC Systems [12]), which made it possible for researchers to analyze samples with greater speed, resolution and sensitivity. Today, HPLC continues to evolve rapidly toward higher speed, efficiency and sensitivity, driven by the emerging needs of life sciences and pharmaceutical applications.

The annual worldwide sales of HPLC systems and accessories approached US $3 billion in 2002 [13].

1.5 Types of separation in HPLC

According to many scientists [3, 4] and on the bases of the chromatographic modes of separation, HPLC stationary phase for HPLC columns can be categorized into four major types: normal phase (NP), reversed phase (RP), ion-exchange chromatography and size-exclusion chromatography (SEC). In addition, there are other specialized modes too (e.g., affinity, chiral or specified applications) [3, 4]. NP-HPLC means that the polarity of the stationary phase is higher than that of the mobile phase, for example, when using silica in adsorption chromatography. RP means that the polarity of the stationary phase is less than that of the mobile phase, for example, when using hydrocarbon-type bonded phases and polar mobile phase. With both modes, solutes

are eluted in order of polarity, *with normal phase least polar elute first and with reverse phase most polar elute first*. Details will be given in Chapter 2.

These previously mentioned modes of HPLC present most important ones in the field of drug quality control. In this book, we shall discuss all of these modes and their applications for pharmaceutical products analysis in drug quality control laboratories.

1.6 Mechanism of separation in HPLC

In HPLC analysis, the chromatographic process begins by injecting the sample to be analyzed (a mixture of some components A, B and C as shown in Fig.1.9) on top of the column. Components in the mixture distribute between two immiscible phases: one phase is mobile phase and the other is the stationary phase. The rate of migration of each species is determined by its distribution coefficient: species which are distributed mainly into the mobile phase move rapidly (component A in Fig.1.9(3)), and species which are distributed mainly in the stationary phase move slowly (component C in Fig.1.9(3)). Solutes that move in stationary phase will form bands (Fig.1.9(3)). Solute bands grow border as they pass through the column. As the bands of solutes emerge from the column it can be detected using selective or universal detectors depending upon the property of the component being measured. The response of the detector to the presence of each component is displayed on chart recorder or computer.

The HPLC detector's output signal should normally produce bell-shaped Gaussian peak (Fig.1.9(4)) representing concentration profile for the eluting components. Concentration profile is called a peak, and a series of peaks from the sample mixture is called chromatogram (Fig.1.9(4)). The time required to elute a peak of the chromatogram is called retention time and it is given the symbol t_R. The amount of time required for a solute that does not interact with the stationary phase is known as the *void time*, t_0. No compound can be eluted in less than the void time. Retention time is dependent on flow rate (F) and retention volume (V_r) of the mobile phase. Retention volume (V_r) is the volume of mobile phase that was passed through the column at t_R and it is simply product of the retention time and flow rate F according to the following equation: $V_r = F \times t_R$. So ($t_R \times F$) is a constant, thus changing flow rate will change t_R. Retention of solutes in HPLC system can be controlled by varying the composition of the mobile phase (mobile phase is usually a mixture of strong and weak solvents). Solvents that give low retention are called strong solvents.

Tentative identification using HPLC for a compound can be made by comparing its retention time to the retention time of known compound suspected to be present in sample under analysis (t_RA, t_RB and t_RC of components A, B and C on chromatogram of sample (Fig.1.9(4)) can be compared with t_RA, t_RB and t_RC on chromatogram of known compounds A, B and C produced at the same HPLC conditions has been used

for the analysis of sample). The most frequent application of HPLC is quantitative analysis. The area (or height) under a chromatographic peak is proportional to the amount of material present. Peak area is usually measured with an electronic integrator or computer data system. Comparing peak area in the sample and standard, the quantity of unknown compound can be determined (for details, see Chapter 8).

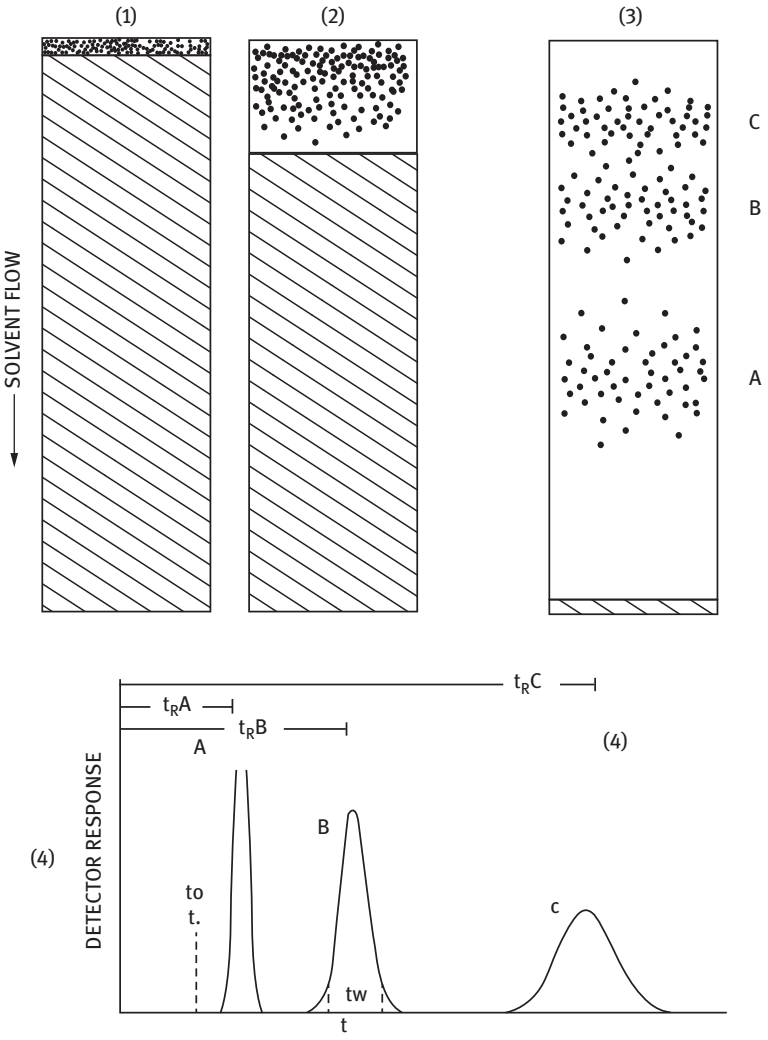

Fig.1.9: Separation of hypothetical chemical components (A + B + C) in a sample by HPLC: (1–3) separation inside the column, and (4) the resulting chromatogram contains three peaks: peak A, peak B and peak C.

The objective of using HPLC in analysis is the separation of a mixture of components, so resolution is a term used quantitatively to describe how well the objective was met. Resolution (Rs) is defined as the differences in retention time for the two adjacent peaks on the HPLC chromatogram divided by the average width of their peaks (Fig.1.10) as follows:

$$Rs = \frac{t_R B - t_R A}{1/2\,(w_B + w_A)}$$

w_B is the width of peak B and $t_R B$ is the t_R for peak B;

w_A is the width of peak A and $t_R A$ is the t_R for peak A

(as shown in Fig.1.10).

The amount of resolution (high, moderate and low as shown in Fig.1.10) is important and is dependent upon the extent of interaction between the solute component and the stationary phase and mobile phase. Thus, HPLC has the ability to separate a wide variety of chemical mixtures. The primary decision of separation is made with the selection of the stationary phase, and the separation procedure is finally tuned through mobile phase selection and manipulation. Frequently, these require changing the mobile phase composition during chromatography. When this process involves more than one solvent, it is called gradient programming and requires some specialized equipment called *HPLC gradient* (for details, see Chapters 3 and 6).

If there is no change in the composition of the mobile phase during the HPLC analysis, it is *called isocratic elution* for mobile phase, isocratic – HPLC. No matter what the mode is, a careful selection of operating parameters based upon the knowledge of the solute chemistry will usually allow separation of very similar molecular species.

1.7 Where HPLC can be used as an analytical tool?

The use of various chromatographic separation techniques such as GC, LC and SEC **is primarily limited by the molecular weight of the sample as shown in** Tab.1.1 **[7].** In comparison, HPLC is more suitable than GC for the analysis of thermally unstable, easily oxidizable and nonvolatile compounds [14–20].

In addition, advances in instrumentation design and performance and the use of smaller, more porous particles as stationary have resulted in an improvement in the theoretical plate height of the LC column.

In general, HPLC can be used whenever the sample is a liquid or can be dissolved in a liquid. Given a particular problem, the extreme versality of the HPLC technique often makes it the separation technique of first choice. Some of the more common uses are listed in Tab.1.2 [20–30]. One of the most attractive uses of HPLC

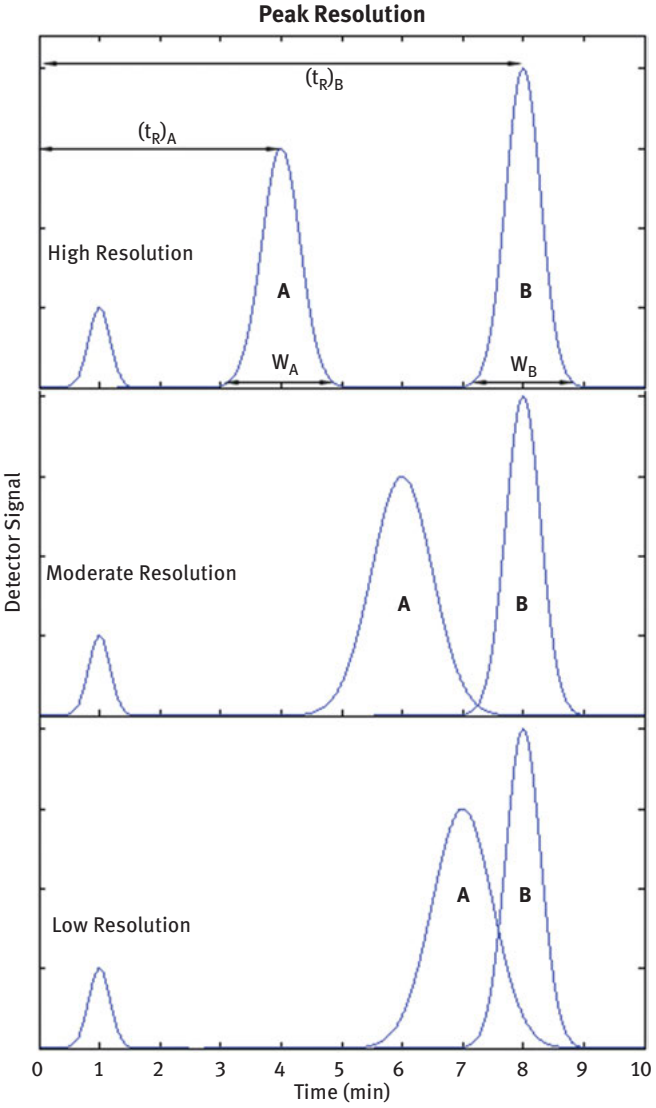

Fig.1.10: HPLC chromatogram showing two peaks and t_R for each peak and the amount of resolution (high, moderate and low).

is in the analysis of trace components [21–22], especially related substances and degradation products in pharmaceutical products [23–30]. There are a variety of sophisticated approaches, ranging from preconcentration to selective detection, that aid in trace analysis. Two applications of HPLC, which have benefitted significantly from advances in this area, are clinical chemistry and trace pollution analysis (pesticide

Tab.1.1: Applications of chromatographic separation techniques according to the molecular weight of the solute.

Chromatographic method	Molecular weight range (Da)		
GC	2	to	400
HPLC	100	to	2,000
SEC	500	to	5,000,000

residue analysis in food and water). The clinical measurement of both endogenous and exogenous compounds in body fluids is becoming increasingly practical using HPLC. The inherent sensitivity of the HPLC technique allows detection of extremely low levels of carcinogenic pollutants.

Tab.1.2: Common HPLC uses.

Life science	Industrial	Pharmaceutical
Drug monitoring	Polymers	Drug analysis
Toxicology	Oil products	Formulation testing
Amino acids	Pesticides	Trace analysis for impurities and degradation products
Proteins	Antioxidants	Quality control
Lipids	Surfactants	Raw materials
Carbohydrates	Food	Drug screening in human fluids

It was in the year 1980 HPLC methods appeared for the first time for the assay of bulk drug material [31]. HPLC has become the principal method in USP XXVII [32] and, to a lesser extent, one of the most widely used methods is also in Ph. Eur. [33].

Today, a very specialized HPLC field has developed a well-characterized approach and highly sophisticated equipment. According to most chromatographic theories, the optimum limits of separation potential are now being approached. These limits are quite impressive, and because of this, HPLC enjoys an unprecedented position of deserved popularity.

HPLC can also be used to purify and collect desired amounts of each compound separated using a fraction collector downstream of the detector flow cell. This process is called preparative chromatography (see Fig.1.11).

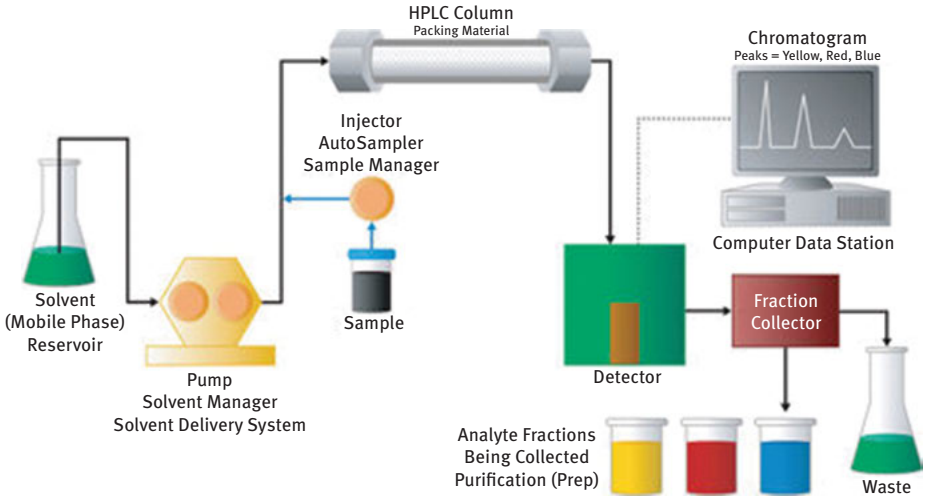

Fig.1.11: HPLC system for purification: preparative chromatography (permission granted to reproduce by Waters Corporation, www.waters.com).

1.8 Advantages of modern HPLC

The major advantages of HPLC over the other chromatographic techniques are speed, resolution and high sensitivity Rapid resolution of even difficult problems is often routine. In part, this is due to stationary phase improvements which have increased column efficiency so much that shorter columns are becoming increasingly practical. Much work is being done in this area, and the final goal is to increase efficiency so that even shorter columns can be used in HPLC analysis.

Resolution is maintained with these shorter columns by better exploiting the inherent selectivity of different interaction modes. In addition to the increased speed and resolution, standard detectors are now capable of detecting very minute component concentrations. This makes HPLC one of the best techniques for trace chemical analysis, especially analysis for degradation and related substances of pharmaceutical products in drug quality control laboratories.

GC is limited in its applications, and the analysis of compounds that are thermally labile or easily oxidized is not possible. HPLC fills this gap and has thus become an essential addition to GC in drug quality control laboratories.

In addition to the abovementioned advantages, HPLC offers further attractions. From an economic viewpoint, the stability of the equipment and the columns is important. These are now so well designed that they maintain a trouble-free existence for extended periods of time. This is in contrast to other chromatographic techniques,

where column degradation often occurs. Also important is growing automation trend in HPLC, which frees the user from some of the more mundane aspects of chromatography. In addition, automation streamlines HPLC methods development, thereby yielding better results in less time.

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2 HPLC columns for pharmaceutical analysis

2.1 Normal- and reversed-phase columns

2.1.1 Introduction

HPLC column *is called the separating system of HPLC*. It consists of two components (Fig.2.1): the column packing materials (stationary phase) and the hardware (container) in which the stationary phase is found (as shown in Fig.2.1). On separation column, the sample component can be separated to their individuals. Because of the fact that the inside phase of the column is made up of spherical or irregular micro-sized particles (Fig.2.1b1 and Fig.2.1b2), it is important that a high-pressure pump is used. This will move the mobile phase by pressure inside the column. In fact it has been mentioned by many scientists [1–11] that the heart of the HPLC is considered to be the column. In this chapter, there are details about these two components of *HPLC* columns: the column packing materials (stationary phase) and the hardware (container). In addition, this chapter discusses HPLC column specifications and selection for pharmaceutical analysis in drug quality control (QC) laboratories. In addition, column operation, handling, care, maintenance and trouble shooting are taken into consideration.

2.1.2 Packing materials or stationary phase for HPLC columns

Packing materials or the stationary phase of the HPLC columns are based either on the organic polymer or on inorganic ceramic substances [1–3]. Out of the inorganic ceramics used, alumina and silica are the most common ones. The inorganic packing is high in rigidity and cannot swell in any sort of solvent. Methacrylates and cross-linked styrene divinylbenzene make the basis for polymeric HPLC-grade packing. The best thing about it is that polymeric packing is not rigid like the inorganic ones. It is compressible. Analytes and solvents can easily enter the polymer matrix. This can cause the particles to swell. As a result, this will reduce the transfer of mass and decrease the efficiency of the column.

Based on the chromatographic modes of separation and according to many scientists [1–11], the stationary phase of HPLC columns is classified into four classes: NP-HPLC (normal phase), IEC (ion-exchange chromatography)-HPLC (ion-exchange phase), RP (reversed phase)-HPLC and SEC (size-exclusion chromatography)-HPLC (size-exclusion phase). In addition, other specialized modes like chiral and affinity exist, and other applications specified [1]. The NP in HPLC means that the stationary phase has a higher polarity when compared to the mobile phase, for instance, when using silica in adsorption chromatography (Fig.2.2). In the RP, the stationary

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phase has a lower polarity when compared to the mobile phase, for example, when using hydrocarbon-type bonded phases and polar mobile phase (Fig.2.3). In both the modes, the order of polarity is used to elute the solutes. In NP, the least polar elute comes first and in the RP the most polar elute comes first. A comparison is shown in Table 2.1, and diagramed in Fig.2.2–2.4 for the general characteristics of NP and RP mode chromatography.

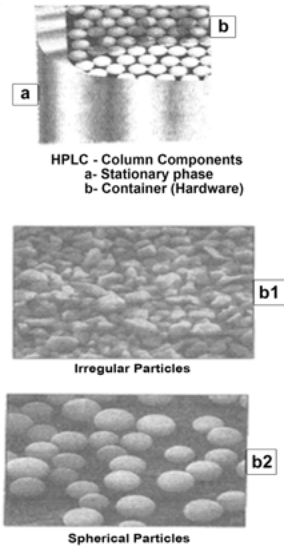

Fig.2.1: HPLC column components: (a) stationary phase and (b) container (hardware).

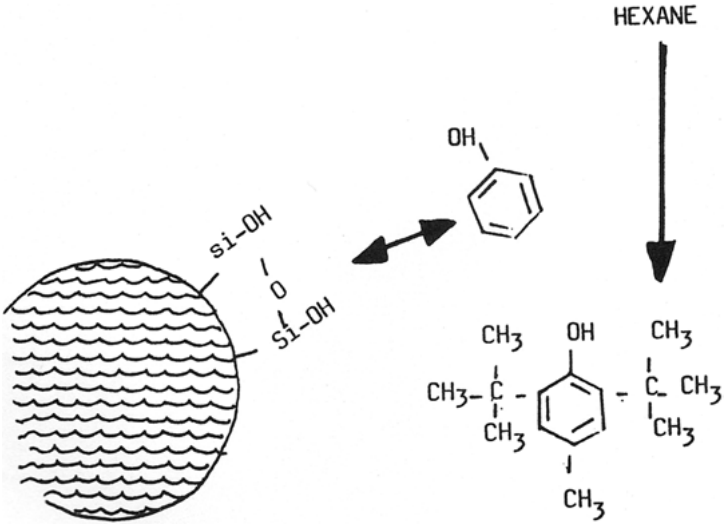

Fig.2.2: Mechanism of interaction between the solute and the stationary phase or normal-phase HPLC (NP-HPLC).

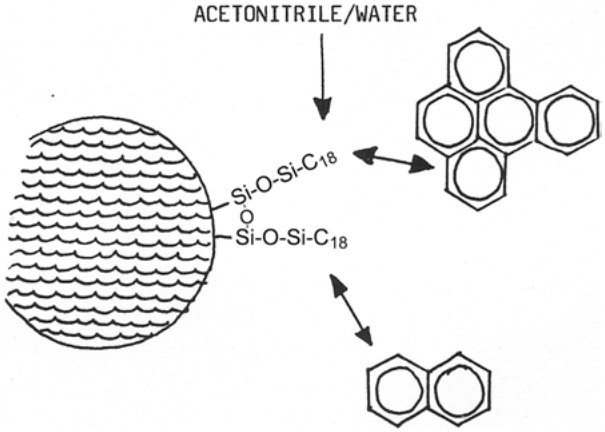

Fig.2.3: Mechanism of contact between both the stationary phase and the solute of the reversed-phase HPLC (RP-HPLC).

Tab.2.1: General properties of normal- and reversed-phase stationary phases for HPLC columns.

Properties	Normal phase	Reversed phase
1. Packing polarity	High to medium	Low to medium
2. Solvent polarity	Low polarity to medium	Medium polarity to high
3. Order of sample elution	Least polar first	Most polar first
4. Effect of increasing solvent polarity on elution time	Elution time is reduced	Elution time is increased

Silica-based RP-HPLC columns have gained wide popularity use in drug QC laboratories because of the following advantages:

- More reproducible and quicker as compared to different HPLC modes.
- Generally easier to perform experiments.
- Broader application scope allowing samples that have an extensive polarity range to be divided and separated.
- Makes use of the mobile phase which is inexpensive.
- The application includes separating ionizable or ionic organic compounds. This is done by using ion pairing technique ion pairing mode = IPC) (see Chapter 3).
- Used in 70–80% of all HPLC applications.

In this chapter, we focus on RP-HPLC columns. The reason for this is that more than 70–80% of the applications of HPLC use RP-HPLC. In addition, NP-HPLC packing for HPLC columns is considered. Followed by this, we shall give enough information about packing materials for HPLC columns (NP- and RP-HPLC columns).

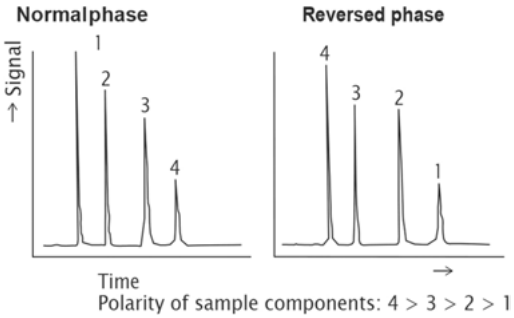

Fig.2.4: Elution of analytes with varying polarity from reversed- and normal-phase HPLC column matrices.

2.1.3 Silica as a packing material for NP-HPLC columns

Silica is the most popular material for HPLC stationary phase (packing materials for HPLC column, and it is called NP-HPLC.

NP chromatography (NPC) started with silica packing, and the name reflects that this was the first type. Silica has a high affinity for water and since chromatographic characteristics change after hydration, the reproducibility of analysis is not as easy as with other packings. NP presents a polar surface, and solvents used are nonpolar and nonaqueous; to increase the elution strength, the polarity of the eluent has to be increased.

Silica packing for HPLC columns is small porous silica particles with spherical or irregular shape (Fig.2.5) having nominal diameter of 3, 5 or 10 μm. They are manufactured so as to have a narrow particle size and pore size distribution. Silica packing is also available as porous-layer beads (Fig.2.6). They consist of an inert spherical core of glass, 30–40 μm in diameter, with a thin outer coating of silica. Silica is very soluble in solutions with a pH value over 7.5 and under 2.6.

During continuous use of silica, small quantities of the packing are washed out of the column, leading to the formation of voids over a period of time. Silica-packed columns quickly change their chromatographic behavior in storage and must be thoroughly conditioned by the mobile phase before being used in analysis.

In addition, alumina can be used as a matrix in NPC [1–3].

Also silica is the most popular base material for other HPLC stationary phases such as silica-based RP (bonded silica, RP), symmetry bonded phase (SRP), symmetry shield bonded phase (symmetry shield reversed phase, SSRP) and XTerra

packing (see Section 2.1.5). Also silica is the most popular base materials for IEC and SEC packing materials for HPLC columns (see Section 2.2).

For the previously mentioned disadvantages of using silica as packing materials for HPLC columns, it is advisable to transfer separations with this case to modified silica (RP silica, Sections 2.1.5 and 2.1.6).

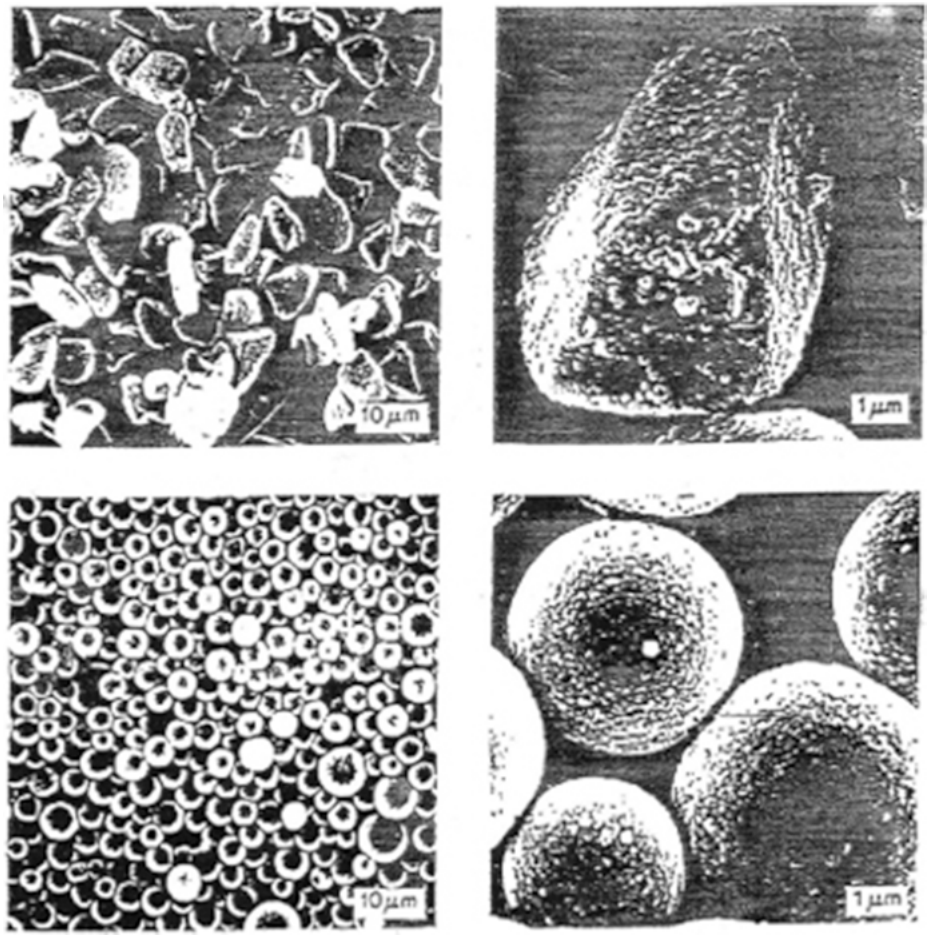

Fig.2.5: Scanning electron micrograph showing irregular (mean particle diameter 5 µm) (above) and spherical (mean particle diameter 5 µm) (below) silica particles.

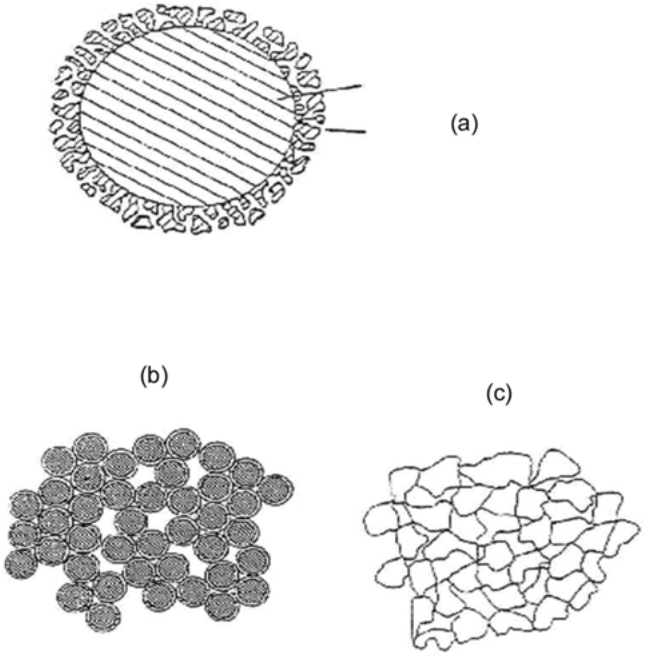

Fig.2.6: Silica particles in different forms.
(a) Porous layer, (b) Spherical, (c) Irregular

2.1.4 Silica as a base material for other HPLC packing materials

2.1.4.1 Silica gel structure

The structure of silica gel used for chromatography is in amorphous form (i.e., say, it has no regularity) and is also the *silicon dioxide* (SiO2) porous form. It is made up of unbalanced tridimensional framework of changing both the *oxygen* and silicon atoms (Fig.2.7) with the voids and pores that are *nanometer* sized. There is no hard-and-fast rule for filling the voids; they can either contain a gas, water or liquid. One predominant feature of the surface of the silica is silanol (SiOH) groups (Fig.2.8). The surface of silica is usually strongly hydrated, and the extent of hydration depends upon the thermal history of the silica. In general, there is reversible hydrolysis–dehydration process at the surface siloxane bonds, where, according to the prevailing conditions, the siloxane links can open to form silanol groups or two adjacent silanol groups can condense to form a siloxane link. Further, the degree to which the adsorbed water molecules are bound can vary and the adsorption can be very strong, and is so much difficult to distinguish between the adsorbed water and water derived from silanol condensation. It is, therefore, difficult to be precise about

the structure of silica, since it can be prescribed as a living polymer rather than as a material of fixed structure. A representation of the structure is shown in Fig.2.7. The main features of such surfaces are the silanol groups and the iloxane backbone, which, being mildly hydrophobic, has little to do with the separations.

There are three important types of silanol groups, which have been identified on the surface of silica particles: free silanol, the hydrogen bonded silanol and the gem – silanol (Fig.2.8). The hydrogen bonded silanols make up the most part of the surface, and silanols with the gem and free hydroxyls comprise around 30% of the total. The silanol groups (bound and free) contribute to about 8 μmol/m^2 of total concentration. Out of all of these, the free group of silanol makes up for the reaction sites and premier adsorption sites. In case of the process of adsorption, bound silanol groups have a secondary role to play.

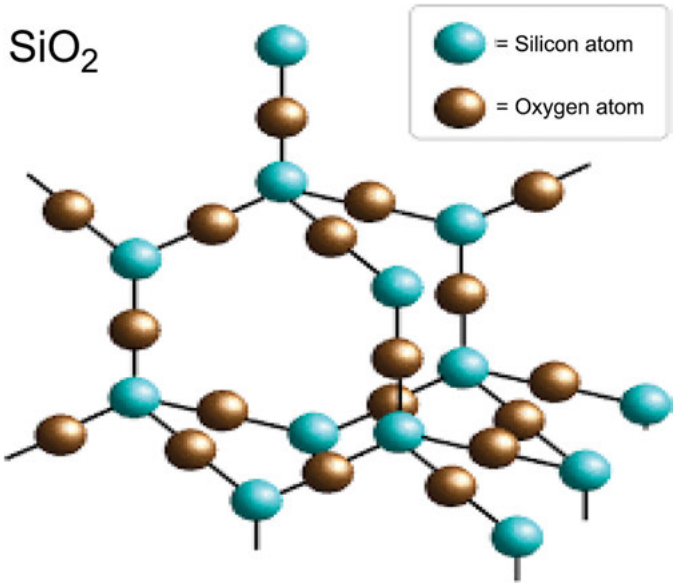

Fig.2.7: Network of silicon–oxygen–silicon linkages in silica structure.

If silica is heated, a number of changes take place. Between ambient temperature and around 120 °C, there is a reversible loss of surface adsorbed water. Workers studying the silica surface often use silica which has been heated under vacuum at 120 °C as a standard, dry but fully hydroxylated surface. As the temperature is increased, the loss in the weight is due to the condensation of hydrogen bonded silanols to form siloxane bonds. This loss in hydroxyls is reversible at temperature up to around 500 °C. As temperature increased from 500 to 850 °C, the changes begin