



EXCERPT FROM:

# AN INTRODUCTION TO HPLC FOR PHARMACEUTICAL ANALYSIS

BY OONA MCPOLIN

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An  
Introduction to  
**HPLC**  
for  
Pharmaceutical  
Analysis

OONA McPOLIN



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# Preface

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This book is aimed at the analyst who is new to HPLC. The content is written specifically for HPLC applied to pharmaceutical analysis. The purpose of the book is to provide the necessary information which will enable the reader to confidently follow a HPLC analytical method. Therefore, the content is based around the information that an analyst needs to be able to use HPLC in a pharmaceutical analysis environment. The concepts of how the technique works and how it is applied in practice are introduced in a logical order, the intention being to build up an understanding of the whole technique gradually.

After an introduction to the topics of both HPLC and pharmaceuticals in chapter 1, the next three chapters deal with the equipment and instrumentation required for the technique. Chapter 2 describes the stationary phase and enables the reader to make sense of the many parameters used to describe a HPLC column. Chapter 3 provides all the necessary information to enable correct preparation and use of HPLC mobile phase. Chapter 4 introduces the equipment which is used to implement the technique of HPLC. The key features of each of the separate parts of the system are described to provide an understanding of how they combine to perform analysis. The second half of the book, chapters 5 to 8, concentrates on the application of HPLC for pharmaceutical analysis. Chapter 5 describes the analytical method and how to interpret its contents. Chapter 6 is a step-by-step guide on how to follow a method and set up a HPLC analysis. Chapter 7 explains the meaning of system suitability criteria and how to interpret the values obtained during an analysis. The final chapter explains the common methods of calibration and quantification used for pharmaceutical analysis, this will enable the reader to calculate the results from a HPLC analysis correctly.

In addition to providing an introduction to HPLC for pharmaceutical analysis it is intended that this book will be a useful resource. At the end of each chapter there is a list of references and/or further reading which will help the reader to develop their expertise in the technique. Useful data is provided throughout the book, such as: buffers and their pK<sub>a</sub>s; conversion tables for units of pressure; and lists of the UV cutoffs for common solvents and buffers. There is a glossary and a list of abbreviations at the back of the book to help the reader become familiar with the terminology used in HPLC and pharmaceutical analysis. When a new term is introduced it is shown in **bold** to indicate to the reader that a definition is available in the glossary.

Due to the applied nature of this topic it is necessary to mention the names of chromatography products, suppliers and manufacturers throughout the book. No

endorsement of these products is intended. Every effort has been made to ensure that brands and trademarks are accurately assigned.

I hope that this book provides the reader with the information they need to get started in HPLC. When writing the text and deciding what to include, my approach was to include the information that I wished someone had told me at the beginning of my career in HPLC. I have also included recent developments in HPLC technology to ensure that the content is fully up to date. There is a lot of information to take in at a first reading in these eight chapters, my wish is that this book will be a useful reference for the reader as they gain experience in HPLC analysis.

Oona McPolin

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## Introduction

The analytical technique of High Performance Liquid Chromatography (HPLC) is used extensively throughout the pharmaceutical industry. It is used to provide information on the composition of drug related samples. The information obtained may be **qualitative**, indicating what compounds are present in the sample or **quantitative**, providing the actual amounts of compounds in the sample. HPLC is used at all the different stages in the creation of a new drug, and also is used routinely during drug manufacture. The aim of the analysis will depend on both the nature of the sample and the stage of development. HPLC is a chromatographic technique, therefore it is necessary to have a basic understanding of chromatography to understand how it works.

### What is Chromatography?

A Russian botanist, Mikhail Tswett (1872 - 1919), is credited with the first use of chromatography in 1906 when he separated plant pigments such as chlorophylls and xanthophylls. He passed them through a glass column packed with calcium carbonate. These pigments are coloured and thus the technique was named using the Greek terms, 'chroma' meaning 'colour', and 'graphein' meaning 'to write'. This explains why the name seemingly bears little relation to the use of the technique today.

Chromatography is a technique which separates components in a mixture due to the differing time taken for each component to travel through a stationary phase when carried through it by a mobile phase. The possible mixtures of phases give rise to the types of chromatography listed in Table 1.

Table 1 Types of chromatography

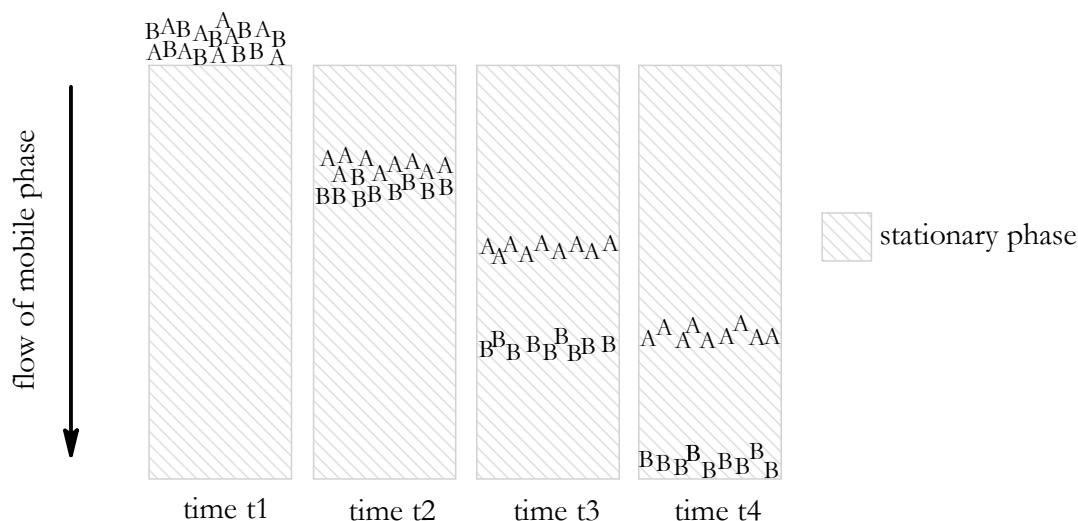
Type of chromatography	Mobile Phase	Stationary Phase
Gas Chromatography	Gas	Solid/Liquid
Liquid Chromatography	Liquid	Solid/Liquid
Supercritical-fluid chromatography	Supercritical fluid	Solid/Liquid

The stationary phase is fixed in place either in a column (a hollow tube made out of a suitable material, e.g. glass) or on a planar surface and the mobile phase moves over or through the stationary phase carrying with it the sample of interest. In practice the stationary phase can be a solid, a liquid adsorbed on a solid or an organic species (e.g. a C<sub>18</sub> alkyl chain) bonded to a solid surface. In gas chromatography and supercritical-fluid chromatography the stationary phase is fixed in place in a column. In liquid chromatography the stationary phase may be fixed in place either in a column or on a planar surface. In HPLC a column is used. The name given to liquid chromatography on a planar surface is Thin Layer Chromatography (TLC).

Figure 1 shows a very simplistic representation of how the separation is achieved. A mixture of component A and component B is introduced to the mobile phase. A and B are travelling at the same rate as the rate of flow of the mobile phase. At time t1 they encounter the stationary phase. Both A and B are attracted to the stationary phase and this slows down their rate of travel in relation to the rate of the mobile phase. This occurs because both A and B are in an equilibrium between time spent on the stationary phase and time spent in the mobile phase. Time spent on the stationary phase does not result in travel of the component through the stationary phase, only time spent in the mobile phase allows travel.

A has a slightly greater affinity for the stationary phase than B. This means that relative to B, A spends more time on the stationary phase and travels at a slower rate than B. At time t2 A and B are beginning to separate. At time t3 they are fully separated. The extra time taken for B to reach the end of the stationary phase at t4 results in further separation of A and B.

Figure 1 Representation of the separation of two components, A and B

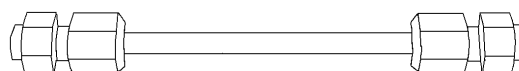


The time taken for a component to travel through the stationary phase is referred to as the **retention time**, thus the retention time of B is equal to time t4. The process of

## The Stationary Phase

It is the combination of a suitable stationary phase and mobile phase that enables the separation of a mixture and thus the analysis of the components in the mixture. In this chapter the stationary phase for HPLC is discussed. HPLC is characterised by the use of very small particles of stationary phase which are fixed in place in a HPLC **column**, often made of a material such as stainless steel. A typical column is shown in Figure 3.

Figure 3 Typical HPLC column



### Normal phase and reversed phase HPLC

In order to describe the different stationary phases available for HPLC it is necessary to explain the concept of normal and reversed phase HPLC, which was introduced in Chapter 1. These types of HPLC vary due to the polarity of the stationary phase and mobile phase in each as shown in Table 3.

Table 3 Polarity of stationary phase and mobile phase used in normal phase and reversed phase HPLC

	Stationary phase	Mobile phase
Normal phase	Polar	Non-polar
Reversed phase	Non-polar	Polar

### Normal phase HPLC

In a mixture of components to be separated those analytes which are relatively more polar will be retained by the polar stationary phase longer than those analytes which are relatively less polar. Therefore the least polar component will elute first. The attractive forces which exist are mostly **dipole**-dipole and hydrogen bonding (polar) interactions.

## Reversed Phase HPLC

In a mixture of components to be separated those analytes which are relatively less polar will be retained by the non-polar stationary phase longer than those analytes which are relatively more polar. Therefore the most polar component will elute first. The attractive forces which exist are mainly non-specific **hydrophobic** interactions. The exact nature of these interactions is still under discussion<sup>1</sup>.

## Parameters to describe a HPLC column

The parameters used to describe a HPLC column refer to the nature, type and size of its contents, the dimensions of the column and the materials used in its construction. A list of parameters is detailed in Table 4.

Table 4 Parameters used to describe a HPLC column

Parameter	Description
Packing/matrix	The finely divided material with which the column is packed, usually silica. It can be used as the stationary phase in adsorption chromatography or a bonded phase is attached for use in partition chromatography.
Bonded Phase	The stationary phase is chemically bonded to the packing/matrix.
Particle size	The size of the particles in the column (if applicable), usually measured in microns.
Pore Size	The size of the pores in the particles/monolith, usually measured in angstroms.
Length	The length of the column, usually measured in cm or mm.
Diameter	The internal diameter of the column, usually measured in mm.
Hardware	The material used to construct the external tubing and end fittings of the column.
Manufacturer	The name of the manufacturer of the column.

## Packing/Matrix of the HPLC column

### Silica

The most common packing material used in HPLC columns is silica. It is physically robust and chemically stable in virtually all solvents and at low pH (it begins to dissolve around pH7). The manufacturing technology for silica production has improved substantially since the early days of HPLC. Irregular shaped particles contaminated with metal impurities have given way to spherical particles with low levels of impurities. This purer silica is known as type B silica and the less pure material is known as type A silica. Silica packed columns may also be produced as

## The Mobile Phase

The mobile phase for HPLC is the liquid phase which is continually flowing through the stationary phase and which carries the analyte through with it. The composition of the mobile phase which is used is dependent on both the stationary phase and the nature of the compounds being analysed. The different properties of solvents define whether they are suitable for use as a mobile phase either under reversed phase or normal phase conditions.

### Solvents

The most common solvents used for HPLC are listed below in order of increasing polarity:

↓  
n-hexane  
methylene chloride  
chloroform  
methyl-t-butyl ether  
tetrahydrofuran (THF)  
isopropanol (IPA)  
acetonitrile (MeCN or ACN)  
methanol (MeOH)  
water

A blend of two (or more) of these solvents is used as the mobile phase in a HPLC analysis. The proportions of the different solvents in the blend act to adjust the polarity of the mobile phase. This is combined with a suitable stationary phase to achieve the separation of a mixture. Ideally, the components in the mixture will be separated fully and will all elute within a practical time scale.

By convention, chromatographers usually refer to the strong solvent in a mobile phase as the 'B' solvent and the weak solvent as the 'A' solvent. Generally, solvent strength is related to polarity, with non-polar solvents being 'strong' solvents for reversed phase HPLC and polar solvents being 'strong' for normal phase HPLC.

A binary mixture is a mixture of two solvents and is the most common type of mobile phase. However ternary mixtures, where three solvents are blended, are also used. The choice of the solvents in the mobile phase, and the proportions of each, will be selected during method development.

The most important property of the solvent is its ability to interact with both the stationary phase and the analytes in the mixture, resulting in the desired separation. However, there are other important properties that need to be considered. An ideal solvent will be readily available in high purity, relatively inexpensive, safe to use routinely, and compatible with the entire HPLC system including the detector.

### Reversed phase HPLC solvents

In reversed phase HPLC the solvents used for the mobile phase are those towards the end of the list on the previous page, which are relatively more polar. Water is always used together with an organic solvent which is miscible with water in all proportions. Increasing the proportion of the organic solvent (%B) in the mobile phase will reduce the retention time of the analyte. This is because the analyte will usually be more soluble in the organic solvent and therefore will spend more time in the mobile phase thus reducing the time spent on the stationary phase.

#### 'Rule of 3'

As a general rule for small molecules in reversed phase, an increase of the organic solvent component in the mobile phase of approximately 10% will result in the retention time being reduced by a factor of 3.

The two most common organic solvents, which may be combined with water to prepare a mobile phase, are acetonitrile and methanol. These are water miscible and have good properties (i.e. readily available, safe to use and compatible with HPLC systems.) Of these acetonitrile is usually the first choice, it has lower viscosity and lower **UV cutoff** than methanol (refer to chapter 4 for a full discussion of UV cutoff and UV detectors). Tetrahydrofuran (THF) may occasionally also be used, but only as a last resort, since it degrades to form peroxides, it has a high UV cutoff, it results in high backpressures and it reacts with PEEK fittings.

A combination of water with each of these three solvents may result in different separations for a given mixture of analytes. This difference in selectivity is primarily based on the polar characteristics of the solvents. The solvent selected for the separation will be the one that gives the best separation. If the separation cannot be achieved using a binary system, i.e. one organic solvent combined with water, then intermediate selectivity may be obtained by blending binary systems. The resulting mobile phase will be a combination of three components, a ternary blend.

## The HPLC System

Instrumentation is required to enable the flow of the mobile phase through the stationary phase and also to convert the separated components into meaningful information. A typical configuration of a HPLC system is shown in Figure 12 and the main components of a HPLC system are described in Table 9.

Figure 12 Configuration of a typical HPLC System

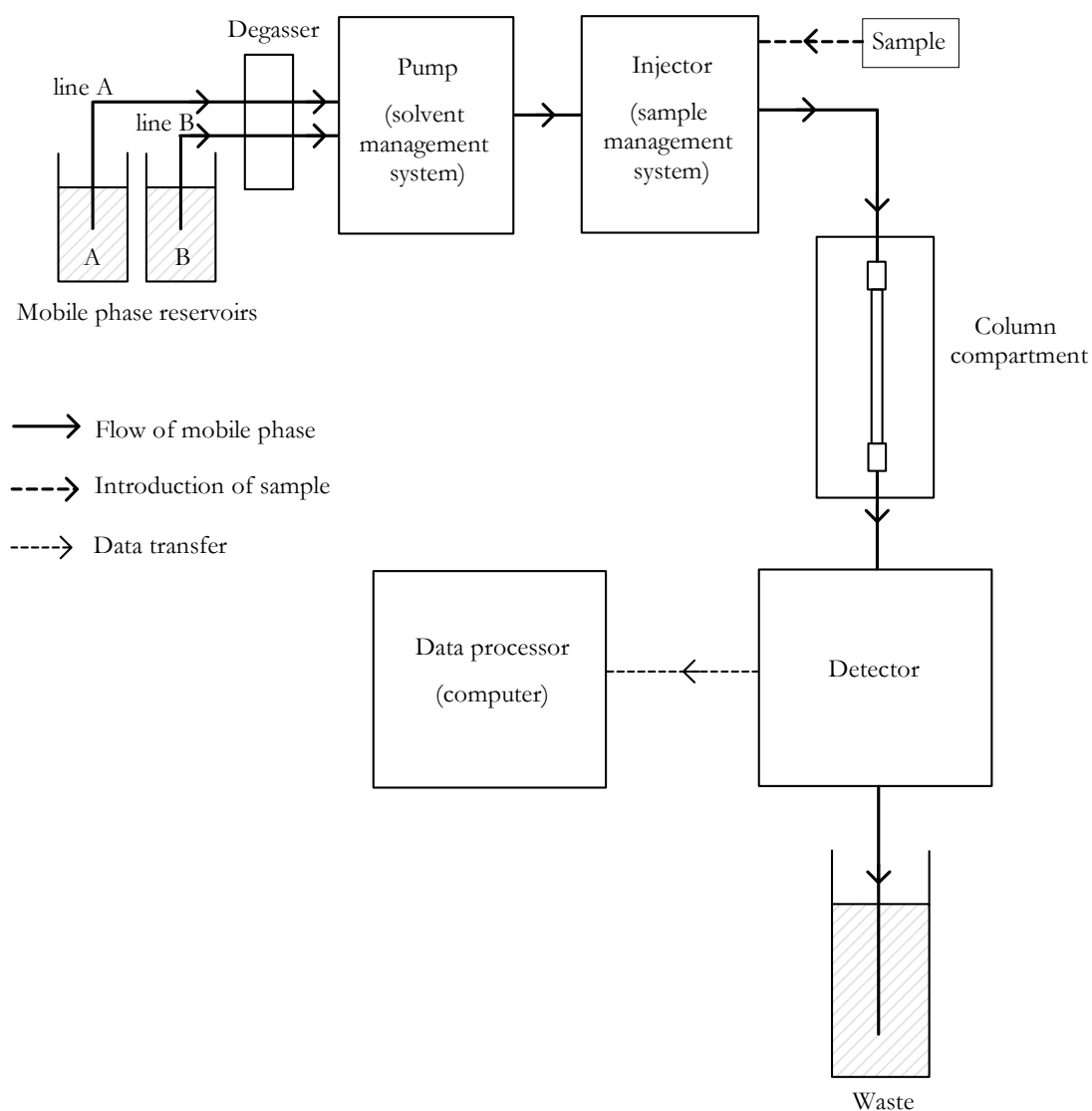


Table 9 Main components of a HPLC system

System component	Description
Mobile phase reservoir	Stores the mobile phase required for analysis
Degasser	Degasses the mobile phase
Pump	Solvent delivery system, enables the flow of the mobile phase through the system
Injector	Sample delivery system, introduces the sample to the system
Column compartment	Used to control the temperature of the column, if required
Detector	Detects each component in a separated mixture after it has eluted from the column
Data processor	Converts the data from the detector into meaningful results
Waste	Collection of the liquid waste

Each component of the HPLC system is discussed below in the order of the flow path of the mobile phase.

### Mobile phase reservoir

The mobile phase is usually stored in glass containers, often these are plastic coated as a safety measure. Plastic containers are not used since additives in the plastic may leach into the mobile phase. The container needs to be of an appropriate size so that it contains enough mobile phase for the analysis being performed (e.g. 1, 2 and 5 litre flasks are often used). PTFE tubing (or a similarly inert tubing material) connects the contents of the reservoir with the HPLC system. This tubing is typically of outer diameter (OD)  $1/8$  inch and of inner diameter (ID)  $1/16$  inch. The size of the tubing in a HPLC system is usually measured using the imperial system of inches (contrasting with the column which uses metric measurements).

At the end of the tubing which is in contact with the mobile phase there is usually a filter (10  $\mu\text{m}$ ) to remove any particulate matter, this also acts as a 'sinker' to hold the tubing at the bottom of the container. This is commonly glass, stainless steel or PEEK. A diagram of a typical mobile phase reservoir is shown in Figure 13. A lid on the container needs to allow a space for the tubing, purpose made lids can be purchased. It is important not to seal the reservoir too tightly to avoid the creation of a vacuum.

The number of mobile phase reservoirs will depend on the number of lines available on the instrumentation. To perform reversed phase gradient elution more than one line is required so that the proportion of the organic component in the mobile phase can be increased throughout the analysis. Isocratic (1 line), binary (2 lines), ternary (3

## The HPLC Analytical Method

In chapters 1 to 4 the system required to perform HPLC analysis comprising the stationary phase (the column), the mobile phase and the instrumentation was described. To define how all these elements will be used for a particular application requires the use of a HPLC analytical method. This contains all the necessary information that an analyst will need to perform the analysis.

### Applications of HPLC in pharmaceutical analysis

To understand the purpose of the HPLC analytical method it is necessary to consider the applications of HPLC in pharmaceutical analysis. There are a wide variety of applications throughout the process of creating a new drug, from initial drug discovery to the manufacture of formulated products which will be administered to patients. This process<sup>1,2</sup> is summarised below and the applications of HPLC are discussed at each stage.

#### The creation of a new drug

The process to create a new drug can be divided into three main stages. These are drug discovery, drug development and manufacturing.

#### Drug discovery

The first step in the drug discovery stage is to select the disease which the drug will treat. This decision will be based on unmet medical needs and also on financial considerations since a huge investment is needed to bring a new drug to market (this has been estimated at almost £500,000).

#### Drug target

The next step is to identify a suitable **drug target**. Drug targets are areas in the cells of the body where drugs attach and result in a therapeutic effect. The major drug targets in the body are normally large molecules (macromolecules) such as proteins, nucleic acids, lipids and carbohydrates. An understanding of which of these is involved in a particular disease state is an important starting point for a new drug project. The human genome project which has mapped the DNA of humans, and also proteomics which studies the role of proteins and their effects on diseases, are

revealing an ever increasing number of new proteins which may act as potential drug targets. Typically a number of targets will be tested in a new drug project.

When a potential drug target has been identified the challenge is to find a chemical which will interact with the target. To evaluate the effect of chemicals on the target a **bioassay** is required. This is a test to check if the desired interaction is taking place. **In vitro** tests, where specific tissues, cells or enzymes are used, are performed which are designed to produce an easily measurable effect when interaction occurs, such as cell growth or an enzyme catalysed reaction which produces a colour change.

**High throughput screening** (HTS) involves the automated testing of large numbers of compounds versus a large number of targets, typically several thousand compounds can be tested at once in 30 - 50 biochemical tests. Assays are run in a parallel fashion using multi-well assay plates (e.g. 96-, 384- and 1536-well). While the actual screen may only take a few days the design of the bioassay and automation may take much longer. The compounds screened in HTS may be from an existing compound 'library', a collection of compounds which have been synthesised by a pharmaceutical company over many years of research. They may also be sourced from **combinatorial synthesis**, a method of producing a large number of compounds in a short period of time, using a defined reaction route and a large variety of starting materials and reagents. It is usually performed on a very small scale to allow automation of the process.

The result of screening is the identification of 'hits', compounds active in the screens that have the potential to be made into drugs. These hits (numbering in the hundreds) are further analysed and screened to reduce their number, the result being lead compounds. A **lead compound** is a structure which shows a useful pharmacological activity and can act as the starting point for drug design.

### Applications

LC-MS is a useful tool for compound identification and characterisation. It may be used as a measurement tool during high throughput screening. Preparative HPLC is also used to isolate and purify hits and lead compounds as required, e.g. from combinatorial synthesis.

### Drug design

Once the structure of a lead compound is known its **structure-activity relationships** (SARs) are studied using biological testing. This defines the functional groups or regions of the lead compound which are important to its biological activity. At this stage a range of compounds which are similar to the lead compound are synthesised and tested to optimise the drug target interaction. The study of how the drug interacts with the target is called **pharmacodynamics**.

## Performing HPLC Analysis

To perform a HPLC analysis it is necessary to set up the instrumentation and prepare the appropriate test solutions following the HPLC analytical method. In this chapter the procedure for following a method is described step-by-step using the example of a HPLC analytical method provided in Figure 23 (page 78). This guide can be modified as required to provide the analyst with a checklist to use when performing HPLC analysis.

### Step 1 – Collect the required materials

It is necessary to read through the method carefully both to familiarise with the contents and also to ensure that all the items specified are available. Ideally this should be done several days before performing the analysis so that any materials that are not available can be ordered. In Table 20 the items that are typically required for a HPLC analysis are detailed alongside those required for the example.

Table 20 Required materials for HPLC analysis

Required materials	Example
Column	Symmetry C18, 5 micron, 15cm x 4.6mm i.d.
Mobile phase solvents	Water; Acetonitrile
Mobile phase reagents	Potassium phosphate; Orthophosphoric acid/ammonium hydroxide (to adjust pH if required)
Solvent for standard and sample preparation	Water; Acetonitrile
Analytical instruments	PH meter; Balance
Standard(s) (if required)	'MiracleCure' analytical reference standard; Impurity X analytical reference standard
Sample	'MiracleCure' drug substance sample to be analysed
Glassware etc. for preparation of test solutions	100mL volumetric flasks; 1mL volumetric pipette
Suitable HPLC system including the detector specified in the HPLC analytical method	HPLC system with UV detector

HPLC columns which have the same bonded phase do not necessarily have the same selectivity, as discussed in previous chapters. Therefore, the column specified in a method cannot be replaced with any column of the same bonded phase, e.g. a Hypersil C18 may not give the same chromatography as a Symmetry C18 for a given mixture. If the column specified in the analytical method is not available then extra work may be required to prove that the replacement column is suitable. Replacement of columns was discussed in Chapter 6.

For HPLC analytical methods that are performed regularly it is a good idea to dedicate an individual column to the method. This means that the column is only exposed to the mobile phase system for that method and reduces the risk of a change in column chemistry that could affect the chromatography. This is a common practice in many pharmaceutical analysis laboratories where routine analysis is performed. Several columns are usually dedicated to the method and new columns are ordered regularly to ensure that a spare is always available. When walk up HPLC systems are used with generic HPLC analytical methods an adequate supply of spare columns is essential.

**Action**

Collect all the necessary materials and order any that are unavailable.

**Step 2 – Record the analysis**

When working in a GMP/GLP environment it is essential that the analysis be recorded at the same time as it is performed. The recording of the analysis needs to be such that every action performed by the analyst can be traced. For non-GMP/GLP analysis it is advisable to follow similar principles of data recording since this will give confidence in the results obtained and also make it easier to troubleshoot if required.

The format of the 'write up' will depend on the way of working in a given analytical laboratory. The options include: handwritten notebook entries, electronic recording of data or perhaps a combination of the two. The Chromatography Data Systems (CDS) attached to most modern HPLC systems allow the electronic capture of most of the data associated with a HPLC analysis plus they are able to calculate the results and generate reports. They may be linked to other data handling systems, e.g. Laboratory Information Management Systems (LIMS).

Typical information recorded for a HPLC analysis will include: name of the analyst; date the analysis was performed; brief description of the aim of the analysis; details of weighings; identifiers for the reference standards and samples and an identifier for the HPLC system used. Prior to performing any practical work in the laboratory any available information should be detailed in the 'write up'.

## System Suitability

An important part of any HPLC analytical method is the system suitability test. The purpose of this is to ensure that the HPLC system is performing as expected. The test measures the performance of the HPLC system as a whole, including the mobile phase, pump, injector, column, detector, data processor and the operator. A satisfactory system suitability test provides assurance in the quality of the results produced from the analysis. This is particularly important for quantitative analysis.

### System Suitability Criteria

The system suitability test (SST) is made up of a number of measurable criteria which when combined provide assurance that the system is performing satisfactorily. The typical criteria which may be applied are detailed in Table 22. The criteria which will be applied for a particular method will depend on what is appropriate for that method, therefore not all the criteria in Table 22 will necessarily be applied.

Table 22 System suitability criteria

Criteria	Description
Retention	A measure of the time at which the peak of interest elutes
Injection repeatability	A measure of the reproducibility of the system throughout the chromatographic analysis
Resolution	A measure of how well the peaks in a chromatogram are separated
Tailing	A measure of the asymmetry of a peak
Efficiency	A measure of the dispersion of a peak
Capacity factor	A measure of the how well the analyte is retained on the column

The system suitability measurements are performed at the time of the analysis and the necessary injections are included in the injection sequence. Every time a chromatographic run is carried out system suitability must be evaluated. The system

suitability testing for a given method will be designed during the method development and method validation phases. System suitability testing is included as a validation parameter in the ICH guidelines on analytical method validation<sup>1</sup>. A cleverly designed system suitability test should get the most information out of a minimum number of injections. The requirements will be set in such a way that they will be met easily if the method is working properly but will fail if there is a method problem.

A full discussion of system suitability and the requirements associated with it are contained in pharmacopoeias. The most important pharmacopoeias worldwide and therefore those most likely to be followed are the United States Pharmacopeia<sup>2</sup> (USP), The European Pharmacopoeia<sup>3</sup> (EP) and the Japanese Pharmacopoeia<sup>4</sup> (JP). When performing an analysis from a monograph contained in any of these the requirements for system suitability stated must be adhered to. Since pharmacopoeias are approved by regulatory authorities the system suitability criteria stated in these are used as a guide for all HPLC methods used in pharmaceutical analysis. Although efforts to harmonise the USP, EP and JP are ongoing, there are slight differences between these in the calculation of the system suitability criteria. These are highlighted in the following discussion of each criterion.

## Retention

The time at which the peak of interest elutes from the HPLC column should be constant for a given method. Therefore a deviation from the expected retention time may indicate a problem with the system. In practice a range may be specified within which it is known that the method will perform satisfactorily. This information will be gathered during method development and method validation. For gradient methods there is the added complication of different dwell volumes on different instruments and alterations may have to be made to adjust for this. This was discussed in detail in Chapter 6. The comparison of the retention time of the peak of interest for injections throughout the course of the chromatographic run may also be included in the system suitability test to ensure that the performance has remained consistent throughout the analysis.

Alterations to the chromatographic conditions to adjust the retention time are allowed by the pharmacopoeias and the flow rate may be defined in terms of the retention time in some monographs.

## Injection Repeatability

Measuring the repeatability of the injections carried out during a chromatographic analysis ensures that the performance of the system is consistent. Repeatability is usually measured by calculating the relative standard deviation (%RSD). The standard deviation for a set of data points provides a measure of the spread of the individual data points. Converting this value into a percentage of the mean for the dataset enables the comparison of different sets of data. What this means for the non-

## Calibration and Quantification

The reason for the widespread use of HPLC in pharmaceutical analysis is that it enables the quantification of drug and drug-related molecules in a sample. All analytical results depend on the measurement of a physical property of an analyte which varies in a known way with the concentration of the analyte. In the case of HPLC analysis the physical property is the peak detected due to the analyte and the measurement used is either the peak area or the peak height. There are a number of different approaches to quantification for HPLC analysis, the most appropriate approach will depend on the method being used.

### Calibration Types

The most common types of calibration used for HPLC pharmaceutical analysis are external standard and internal standard.

#### External standard

This is the most straightforward calibration type. A standard of known concentration is prepared and analysed alongside the sample by HPLC. The response for the standard is compared to that of the sample, and thus the concentration of the sample can be determined. There are a number of requirements when using this type of calibration:

1. An analytical reference standard of known purity is required. Usually the drug molecule will be used as the standard and should be available in sufficient amounts, a purity determination will have to be carried out. For established products certified reference standards may be purchased.
2. The detector should have a linear response over the concentration range expected. The detectors discussed in Chapter 4 have a large linear range, the concentrations used in the method are selected to suit the detector.
3. The concentration of the standard should be similar to that of the sample. This ensures that the sample response is within the linear range of the method. For samples where the concentration is unknown a suitable dilution may be estimated and then the sample injected. If the response is out of range the dilution can then be modified to suit.

4. The dilutions for preparation of the test solutions and the injection volume should be reproducible. The use of volumetric glassware and modern HPLC injection systems enable high reproducibility and accuracy.
5. The recovery of the samples should be 100%. Drug substance samples and straightforward drug products usually have 100% recovery. However, with complex drug products and biological samples it may be difficult to achieve 100% recovery.

### Internal standard

This type of calibration similarly requires the preparation of external standard solutions but in addition a constant concentration of a second compound is added to each sample. The sample concentration is directly proportional to the ratio of the analyte to internal standard. The requirements 1, 2 and 3 for external standard also apply to internal standard. In addition the internal standard component should:

1. Be chromatographically resolved from the analyte but elute closely.
2. Produce a detector response similar to that produced by the analyte for a given weight.
3. Be stable in the test solutions.

Internal standard calibration is used in other chromatographic techniques to correct for differences in the injection volume. For the modern injection systems used with HPLC this correction is not required and in this case the internal standard is used to correct for differences in the extraction procedure of the sample. Therefore it is often used for difficult sample matrices such as biological samples and complex formulations such as ointments and creams. The internal standard is added prior to extraction.

### Single point calibration

During the validation of the HPLC analytical method the linearity of the method will be established. A series of standard concentrations covering an appropriate range will be analysed and the results subjected to linear regression analysis. This results in the generation of the equation of the line,

$$y = mx + c$$

where  $y$  is the detector response and  $x$  is the concentration and 'm' and 'c' are constants. Figure 30 shows an example of the type of plot which is generated during method validation. The 'x' markers indicate the response obtained for a series of standards injected and the best straight line is drawn through them using regression analysis. Due to small differences in the system and the detector response this calibration cannot be used for all subsequent analyses. The standard needs to be injected alongside the samples each time the analysis is performed.

# Glossary

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## A

<b>Accuracy</b>	Expresses the closeness of agreement between the true value and the value found.
<b>Active Pharmaceutical Ingredient (API)</b>	The 'active' or the 'active pharmaceutical ingredient' is the substance in a drug preparation that is pharmaceutically active.
<b>Adsorbent</b>	Packing used in adsorption chromatography.
<b>Adsorption</b>	The process of interaction between the solute and the surface of an adsorbent, e.g. silica.
<b>Analyte</b>	The compound of interest to be analysed by injection onto and elution from a HPLC column.
<b>Assay</b>	An analytical method to analyse or quantify a substance in a sample.

## B

<b>Back pressure</b>	The pressure experienced when mobile phase is pumped through the column.
<b>Band broadening</b>	The process of increasing width of the chromatographic band as it moves down the column. Sometimes called band dispersion or band spreading.
<b>Baseline</b>	The region in a chromatogram where no peaks are present and the detector response is due only to the mobile phase.
<b>Bioanalysis</b>	The chemical analysis of biological samples, e.g. plasma, urine etc.
<b>Bioassay</b>	A biological test, measurement or analysis to determine whether compounds have the desired effect either in a living organism, outside an organism, or in an artificial environment.
<b>Biopharmaceutical</b>	A drug produced by biotechnology.
<b>Biotechnology</b>	The application of scientific and engineering principles to the processing of materials by biological agents.

# List of Abbreviations

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<b>ACN</b>	Acetonitrile
<b>ADME</b>	Absorption, distribution, metabolism and excretion
<b>APCI</b>	Atmospheric pressure chemical ionisation
<b>API</b>	Active Pharmaceutical Ingredient
<b>AUFS</b>	Absorption Units Full Scale
<b>CDS</b>	Chromatography Data System
<b>CN</b>	Cyano
<b>CSP</b>	Chiral Stationary Phase
<b>DAD</b>	Diode Array Detector
<b>DMPK</b>	Drug Metabolism and Pharmacokinetics
<b>ECD</b>	Electrochemical Detector
<b>ELSD</b>	Evaporative Light Scattering Detector
<b>EMA</b>	European Medicines Agency
<b>EP</b>	European Pharmacopoeia
<b>ESI</b>	Electrospray ionisation
<b>FDA</b>	Food and Drug Administration
<b>GC</b>	Gas Chromatography
<b>GCP</b>	Good Clinical Practice
<b>GFC</b>	Gel Filtration Chromatography
<b>GLP</b>	Good Laboratory Practice
<b>GMP</b>	Good Manufacturing Practice
<b>GPC</b>	Gel Permeation Chromatography

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