High Performance Liquid Chromatography (HPLC) in the pharmaceutical analysis

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Content:

Introduction

Drug manufacturing control requires high level and intensive analytical and chemical support of all stages to ensure the drug's quality and safety. The pharmacopeia constitutes a collection of recommended procedures for analysis and specifications for the determination of pharmaceutical substances, excipients, and dosage forms that is intended to serve as source material for reference or adaptation by anyone wishing to fulfill pharmaceutical requirements. The most important analytical technique used during the various steps of drug development and manufacturing is the separation technique: High Performance Liquid Chromatography (HPLC).

The key to a proper HPLC system operation is knowledge of the principles of the chromatographic process, as well as understanding the reasons behind the choice of the components of the chromatographic systems such as column, mobile phase and detectors. A scheme of an HPLC system is shown in Figure 1. A high pressure pump is required to force the mobile phase through the column at typical flow rates of 0.5-2 ml/min. The sample to be separated is introduced into the mobile phase by injection device, manual or automatic, prior to the column. The detector usually contains low volume cell through which the mobile phase passes carrying the sample components eluting from the column. There are books describing the practicality of HPLC operation. It is expected of any proper HPLC system that is used in the pharmaceutical laboratories to produce highly accurate and precise results, due to health related issues of improper measurements. Every HPLC system must be qualified to comply with the strict demands from health authorities for high quantitative performance.

Quality standards in pharmaceutics require that all instruments should be adequately designed, maintained, calibrated, and tested. The approach that has been adopted in the environment of the analytical instrument has become known as the "Four Qs": design qualification (DQ), installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ).Design qualification is performed at the vendor's site, and it is representative of the way an instrument is developed and produced, usually governed by International Organization for Standardization (ISO) criteria.

The installation qualification (IQ) process can be divided into two steps: *pre-installation* and *physical installation.* During pre-installation, all information relevant to the proper installation, operation, and maintenance of the instrument is checked. Workers confirm the site requirements and the receipt of all of the parts, pieces, and manuals necessary to perform the installation of the specific HPLC unit. During physical installation, serial numbers are recorded and all fluidic, electrical, and communication connections are made for system components. Documentation describing how the instrument was installed, who performed the installation, and other various details are archived.

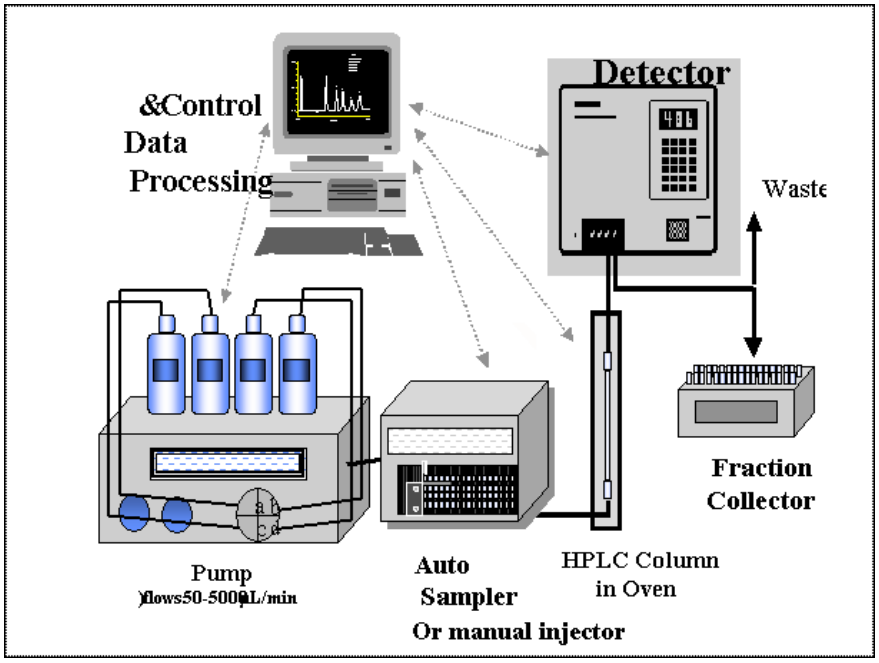

Figure 1: A Scheme of an HPLC System

The operational qualification process ensures that the separate modules of a system (pump, injector, and detector) are operating according to the defined specifications such as accuracy, linearity, and precision. Specific tests are performed to verify parameters such as detector wavelength accuracy, flow rate, or injector precision.

The performance qualification (PQ) step verifies system performance as a whole. Performance qualification testing is conducted under real operating conditions in the analytical laboratory that is going to be using the instrument. In practice, sometimes operational and performance qualification blend together, particularly for linearity and precision (repeatability) tests, which can be conducted more easily at the system level.

The performance qualification test of the HPLC system uses a method with a well-characterized analyte mixture, column, and mobile phase. It incorporates type of measurements from the system suitability section of the general chromatography chapter <621> in the *U.S. Pharmacopeia* . In the end of the process proper documentation is archived.

1. Modes of HPLC

There are various modes of operation of HPLC. The mechanism of interaction of the solutes with the stationary phases determines the classification of the mode of liquid chromatography.

Table 1 summarizes the variety of modes of liquid chromatography, of which Reversed Phase stands out as the most widely used mode in HPLC, therefore, the discussion will elaborate on this mode.

Mode	Normal Phase	Reversed Phase	Ion exchange	Chiral	Affinity	Size Exclusion
Stationary Phases chemistry	Polar-hydrophilic	Non-polar-lipophilic	Ion-bonding	Chiral recognition	Bioaffinity	Sieving by size
Typical Stationary Phases	Silica, Alumina	Alkylated silica, mostly C18	Ionic functional groups on silica or polymer	Chiral groups on silica surfaces	Either substrates or biomolecules,	Gel type polymers
Typical mobile phase	Hexane; isopropanol; methylene chloride	Water; methanol; acetonitril; buffers; ion pairing agents	Water; buffers; acid; base	Two modes: aqueous and non-aqueous	Water; buffers	Two modes: aqueous and non-aqueous
Typical solutes	Fatty and oily	Almost all organic compounds	Any ion-charged compounds	Enantiomers small and large molecules	Biomolecules or their substrates	Polymers: synthetic or biological

Reversed Phase (13)

Reversed phase liquid chromatography (RPLC) is considered as the method of choice for the analysis of pharmaceutical compounds for several reasons, such as its compatibility with aqueous and organic solutions as well as with different detection systems and its high consistency and repeatability. Sensitive and accurate RPLC analysis, whether in the pharmaceutical or bioanalytical field, necessitates the use of stationary phases which give symmetrical and efficient peaks. Therefore, manufacturers of stationary phases are continuously improving and introducing new RPLC products, and the selection of various types of reversed phase stationary phases is high. The needs for consistency as well as the globalization of the pharmaceutical companies require that the methods will be transferred from site to site, using either the same column brands or their equivalents. Therefore, an extensive categorization or characterization of the rich selection of stationary phases has been done in recent years.

The stationary phase in the Reversed Phase chromatographic columns is a hydrophobic support that is consisted mainly of porous particles of silica gel in various shapes (spheric or irregular) at various diameters (1.8, 3, 5, 7, 10 μM etc.) at various pore sizes (such as 60, 100, 120, 300). The surface of these particles is covered with various chemical entities, such as various hydrocarbons (C1, C6, C4, C8, C18, etc) as can be seen in Figure 2. There are also hydrophobic polymeric supports that are used as stationary phases when there is an extreme pH in the mobile phase. In most methods used currently to separate medicinal materials, C18 columns are used, which sometimes are called ODS (octedecylsilane) or RP-18.

The more hydrophobic are the sample components the longer they stay in the column thus they are separated. The mobile phases are mixtures of water and organic polar solvents mostly methanol and acetonitrile. These mixtures contain frequently additives such as acetate, phosphate, citrate, and/or ion-pairing substances, which are surface active substances such as alkylamines as ion-pairng of anions or alkylsulfonates, ion-pairing of cations. The purpose of using such additives is to enhance efficiency and/or selectivity of the separation, mostly due to control of their retention.

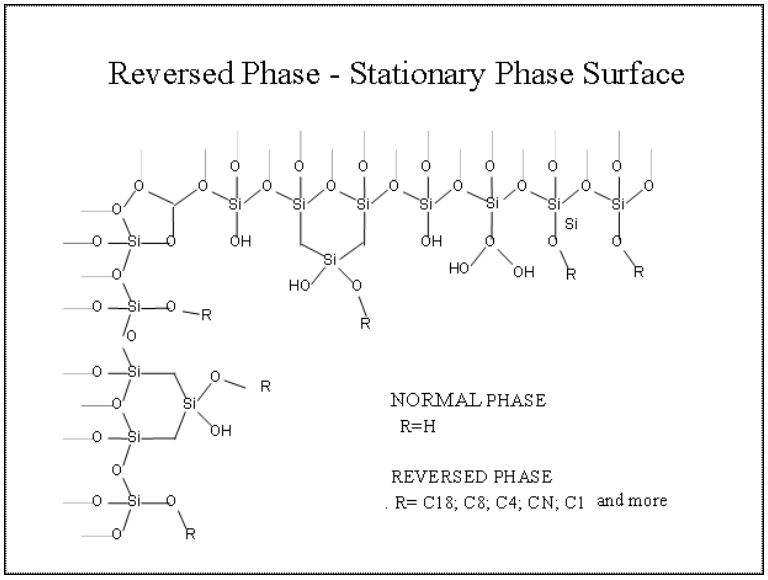

Figure 2: The surface of Reversed Phase stationary phases

The parameters that govern the retention in Reversed Phase systems are the following:

A. The chemical nature of the stationary phase surface .
B. The type of solvents that compose the mobile phase and their ratio
C. The pH and ionic strength and additives of the mobile phase

When the effect of these parameters on the retention of the solutes is understood it is possible to manipulate them to enhance selectivity.

A. The chemical nature of the stationary phase

The surface of the stationary phase is described in Figure 2. The chemical nature is determined by the size and chemistry of hydrocarbon bonded on the silica gel surface, its bonding density (units of µmole/m^2), and the purity and quality of the silica gel support. As a rule, the more carbons in a bonded hydrocarbon the more it retains organic solutes (as long as similar % coverage is compared). The higher the bonding density the longer the organic solutes are retained. A column is considered relatively hydrophobic if its bonding density exceeds 3 µmole/m^2.

Very important modifiers of the stationary phase's surface are surface-active substances used as mobile phase's additives, acting as ion-pair reagents. These are substances such as tri-ethylamine or tetrabutylamine or hexyl, heptyl, octyl sulfonate. They are distributed between the mobile phase and the hydrophobic surface and cover it with either positive (alkylamines) or negative (alkylsulfonates) charges. This change of the surface into charged surface affects the retention significantly, especially on charged species in the sample.

B. Composition of the mobile phase

As a rule, the weakest solvent in Reversed Phase is the most polar one, water. The other polar organic solvents are considered stronger solvents, where the order of solvent strength follows more or less their dielectric properties, or polarity. The less polar the solvent added to the mobile phase, the stronger it gets, shortening the retention times.

C. PH and ionic strength of the mobile phase

When the samples contain solutes of ionizable functional groups, such as amines, carboxyls, phosphates, phosphonates, sulfates and sulfonates, it is possible to control their ionization degree with the help of buffers in the mobile phase. As a rule, the change of an ionizable molecule to an ion makes it more polar and less available to the stationary phase. For example, increasing the pH of the mobile phase above 4-5, which is the typical pKa of carboxyl groups, reduces the retention of carboxyl containing compounds. On the other hand, substances that contain amines whose pKa is around 8 will retain longer when the pH will be above 8. In most of the traditional silica-gel based stationary phases it is not possible to increase the mobile phase's pH above 8 due to hydrolysis of the silica gel. During the 2000s there have been developed extended pH stationary phases.

2. HPLC Theory: System Suitability Parameters

High performance liquid chromatography is defined as a separation of mixtures of compounds due to differences in their distribution equilibrium between two phases, the stationary phase packed inside columns and the mobile phase, delivered through the columns by high pressure pumps. Components whose distribution into the stationary phase is higher, are retained longer, and get separated from those with lower distribution into the stationary phase. The theoretical and practical foundations of this method were laid down at the end of 1960s and at the beginning of 1970s. The theory of chromatography has been used as the basis for *System- Suitability* tests, which are set of quantitative criteria that test the suitability of the chromatographic system to identify and quantify drug related samples by HPLC at any step of the pharmaceutical analysis.

Retention Time (t_R), Capacity Factor k' and Relative Retention Time (RRT)

The time elapsed between the injection of the sample components into the column and their detection is known as the Retention Time (t_R). The retention time is longer when the solute has higher affinity to the stationary phase due to its chemical nature. For example, in reverse phase chromatography, the more lypophilic compounds are retained longer. Therefore, the retention time is a property of the analyte that can be used for its identification.

A non retained substance passes through the column at a time t_0, called the Void Time.

The *Retention Factor* or *Capacity Factor k'* of an analyte is measured experimentally as shown in Figure 3 and Eqn 1:

Eqn 1a $$k' = \frac{t_R - t_0}{t_o}$$

The *Capacity Factor* describes the thermodynamic basis of the separation and its definition is the ratio of the amounts of the solute at the stationary and mobile phases within the analyte band inside the chromatographic column:

Eqn 1b $$k' = \frac{C_s}{C_m}\Phi$$

Where C_s is the concentration of the solute at the stationary phase and C_m is its concentration at the mobile phase and phi is the ratio of the stationary and mobile phase volumes all within the chromatographic band.

The *Retention Factor* (Eqn 1a) is used to compare the retention of a solute between two chromatographic systems, normalizing it to the column's geometry and system flow rate. The need to determine the void time can be tricky sometimes, due to the instability of the elution time of the void time marker, t_0, therefore, when the chromatogram is complex in nature, and one known component is always present at a certain retention time, it can be used as a retention marker for other peaks. In such cases the ratio between the retention time of any peak in the chromatogram and the retention time of the marker is used ($t_{R\ (Peak)}$ / $t_{R\ (Marker)}$) and referred to as the *Relative Retention Time* (RRT). RRT is also used instead of the capacity ratio for the identification of the analyte as well as to compare its extent of retention in two different chromatographic systems.

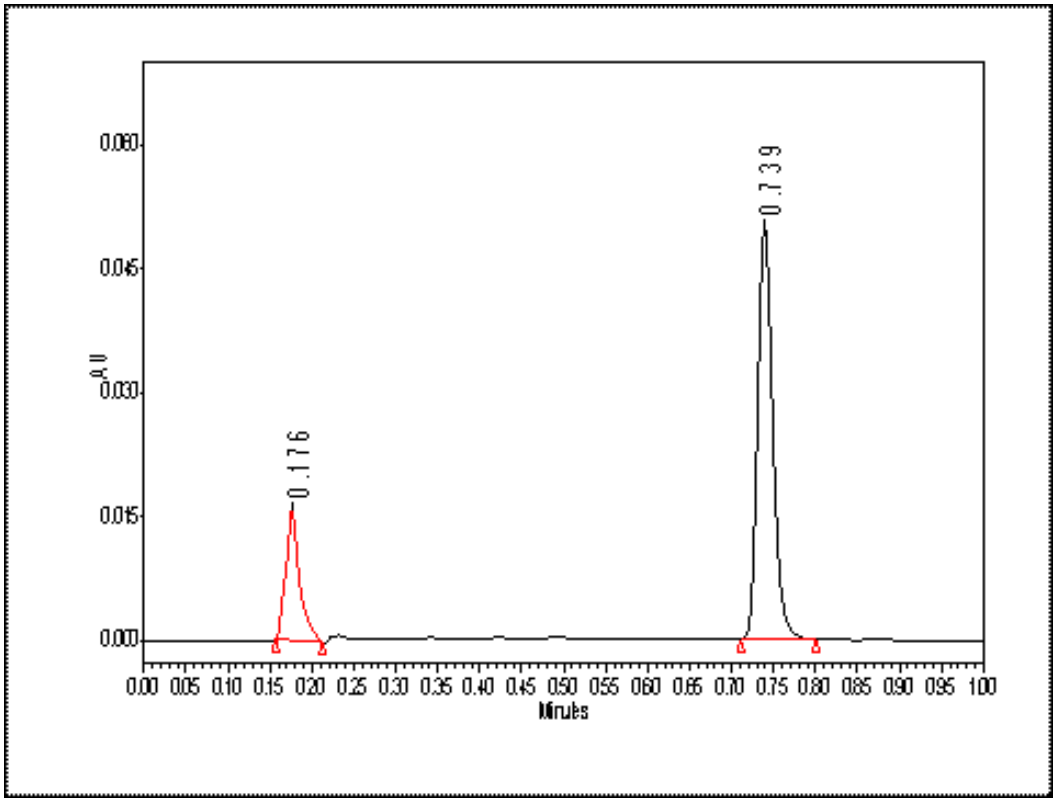

Figure 3: Example of Capacity factor calculation in LC. In this case: t_R = 0.739, t_0 = 0.17, therefore k' = (0.739-0.176)/0.176 = 3.20

Efficiency: Plate Count N and Peak Capacity P_c

Figure 4 describes a chromatogram with 4 peaks and a detected void peak. The parameters of the System Suitability are displayed in the inserted table. The sharpness of a peak relative to its retention time is a measure of the system's efficiency, calculated as N, plate count. Band-broadening phenomena in the column such as eddy diffusion, molecular diffusion, mass-transfer kinetics and extra-column effects reduce the efficiency of the separation.The sharpness of a peak is relevant to the limit of detection and limit of quantification of the chromatographic system. The

sharper the peak for a specific area, the better is its signal-to-noise; hence the system is capable of detecting lower concentrations. Therefore, the efficiency of the chromatographic system must be established by the system suitability test before the analysis of low concentrations that requires high sensitivity of the system, such as the analysis of drug impurities and degradation products.

The efficiency of the separation is determined by the Plate Count N when working at isocratic conditions, whereas it is usually measured by Peak Capacity P_c when working at gradient conditions .The following equation for the plate count is used by the United States Pharmacopoeia (USP) to calculate N (See Figure 5):

$$N = 16 \times \left(\frac{t_R}{w_{(Baseline)}}\right)^2$$

Eqn. 2

Where *w* is measured from the baseline peak width calculated using lines tangent to the peak width at 50 % height (See Figure 5). European and Japanese Pharmacopoeias use the peak width at 50% of the peak height (See Figure 5), hence the equation becomes:

$$N = 5.54 \times \left(\frac{t_R}{w_{(50\%)}}\right)^2$$

Eqn 3

Peak Capacity P_c is defined as number of peaks that can be separated within a retention window for a specific pre-determined resolution. In other words, it is the runtime measured in peak width units . It is assumed that peaks occur over the gradient chromatogram. Therefore, *Peak Capacity* can be calculated from the peak widths w in the chromatogram as follows:

$$1 + \frac{t_g}{(1/n)\sum_1^n w}$$

Eqn. 4:

Where *n* is the number of peaks at the segment of the gradient selected for the calculation, t_g. Thus peak capacity can be simply the gradient run time divided by the average peak width. The sharper the peaks the higher is the peak capacity, hence the system should be able to resolve more peaks at the selected run time as well as detect lower concentrations.

Another measure of the column's chromatographic efficiency is the *Height Equivalent to Theoretical Plate (HETP)* which is calculated from the following equation:

Eqn 5: HETP = (L/N)

Where L is column length and N is the plate count. HETP is measured in micrometer. For example, in the peak of Figure 5 the column length was 100 mm (0.1 m) therefore HETP was 8.8 µm at the flow rate of the experiment.

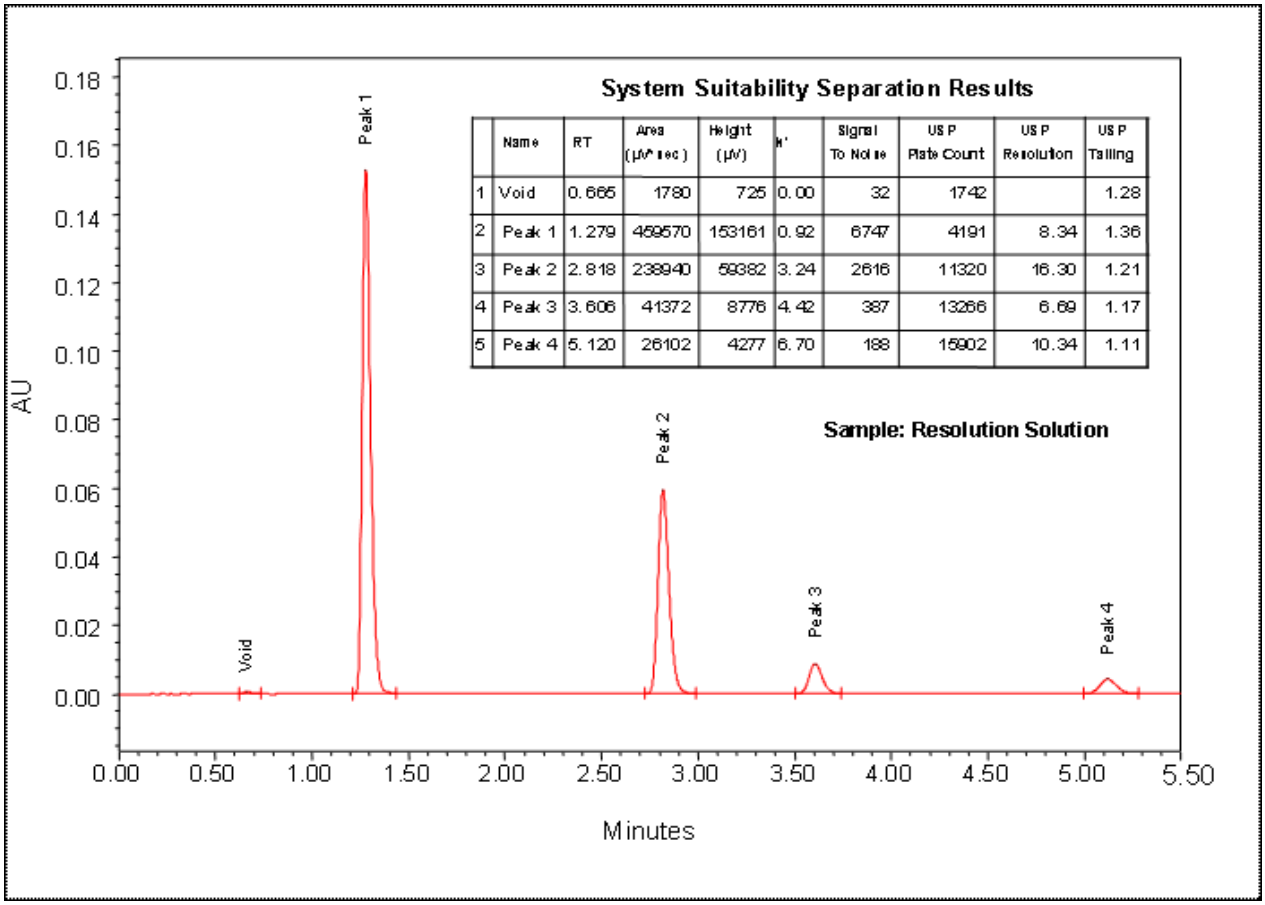

Figure 4: Example of a System Suitability Run's Result

The behavior of HETP as function of linear velocity has been described by various equations (35). It is frequently called "The Van-Deemter curve", and it is frequently used to describe and characterize various chromatographic stationary phases' performance and compare them to each other (36-39). The lower are the values of HETP, the more efficient is the chromatographic system, enabling the detection of lower concentrations due to the enhanced signal-to-noise ratio of all the peaks in the chromatogram.

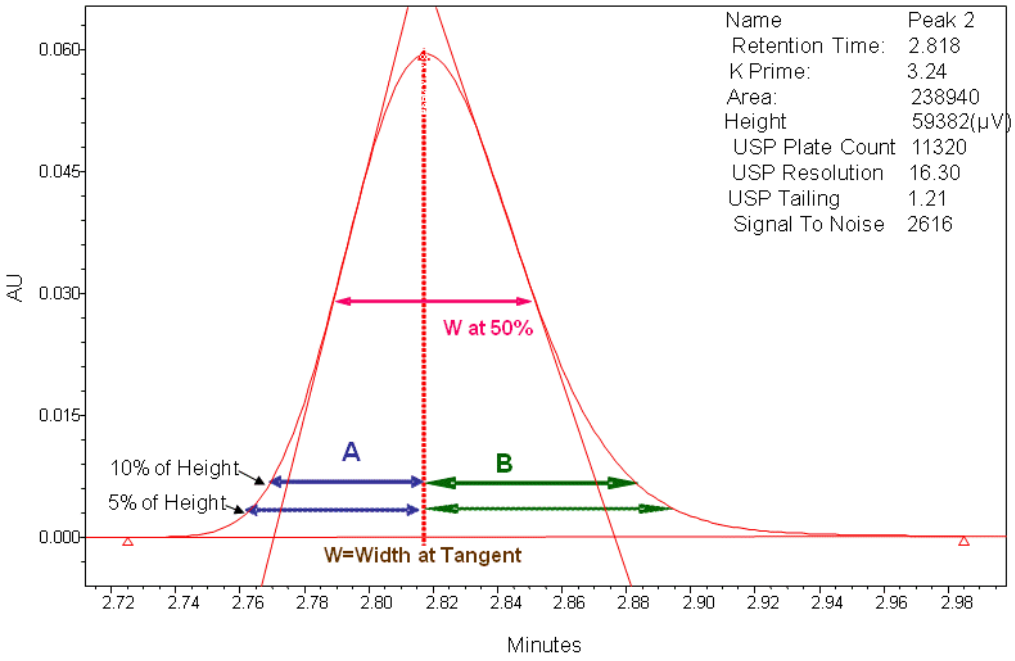

Figure 5: System Suitability measurements on a single peak

Peak *Asymmetry Factor*, A_f and *Tailing Factor T*

The chromatographic peak is assumed to have a Gaussian shape under ideal conditions, describing normal distribution of the velocity of the molecules populating the peak zone migrating through the stationary phase inside the column. Any deviation from the normal distribution indicates non-ideality of the distribution and the migration process, therefore might jeopardize the integrity of the peak's integration, reducing the accuracy of the quantitation. This is the reason why USP Tailing is a peak's parameter almost always measured in the system suitability step of the analysis.

The deviation from symmetry is measured by the *Asymmetry Factor*, A_f or *Tailing Factor T*. The calculation of *Asymmetry Factor*, A_f is described by the following equation:

Eqn 6:
$$A_f = \frac{A_{(10\%h)}}{B_{(10\%h)}}$$

Where *A* and *B* are sections in the horizontal line parallel to the baseline, drawn at 10% of the peak height as shown in Figure 5.

The calculation of *Tailing Factor*, *T*, which is more widely used in the pharmaceutical industry, as suggested by the pharmacopeias, is described by the following equation:

Eqn 7:
$$T = \frac{A_{(5\%h)} + B_{(5\%h)}}{2A_{(5\%h)}}$$

Where A and B are sections in the horizontal line parallel to the baseline, drawn at 5% of the peak height, as also shown in Figure 5. The USP suggests that *Tailing Factor* should be in the range of 0.5 up to 2 to assure a precise and accurate quantitative measurement. The peaks in Figures 4 and 5 have a tailing factor within the USP requirements.

Selectivity Factor: Alfa *and* Resolution Factor Rs

The separation is a function of the thermodynamics of the system. Substances are separated in a chromatographic column when their rate of migration differs, due to their different distribution between the stationary and mobile phases. The *Selectivity Factor, a,* and *Resolution Factor, Rs*, measure the extent of separation between two adjacent peaks. The *Selectivity Factor*accounts only for the ratio of the *Retention Factors*, *k'*, of the two peaks (k'_2/k'_1), whereas the*Resolution Factor, Rs*, accounts for the difference between the retention times of the two peaks relative to their width.

The equation that describes the experimental measurement of the *Resolution Factor, Rs*, is as follows:

Eqn 8: $$Rs = \frac{t_{R(2)} - t_{R(1)}}{0.5(w_2 + w_1)}$$

Where t_R is the retention time of peaks 1 and 2 respectively and *w* is their respective peak width at the tangents' baseline (see Figure 5). According to the pharmacopeias *Rs* should be above 1.5 for an accurate quantitative measurement. Figure 4 shows that the resolution measured between every two adjacent peaks in the chromatogram was above 1.5, therefore, it was above the minimum required.

The resolution is a critical value when working with complex samples such as drug impurities and degradation products, or when the formulation is complex and excipients might interfere with the quantitative measurements. Therefore, it is an essential part of the system suitability measurement stage before the quantitative work of these type of samples.

The sample used for the measurements of *Rs* during the system suitability runs is sometimes called *Resolution Solution*, as can be seen in Figure 4 It usually contains the components that are the most difficult to resolve.

The theoretical description of the *Resolution Factor Rs* equation is shown in Equation 9. It includes some of the above parameters, the plate count *N*, the selectivity and the average of the two peaks' capacity factors *k'*:

Eqn 9: $$Rs = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_{ave}'}{k_{ave}'+1}\right)$$

It can be clearly seen from this equation that the plate count is the most effecting parameter in the increase of the chromatographic resolution. Since the plate count increases with the reduction in particle diameter, it explains the reduction in particle diameter of the stationary phase material during the last 3 decades of HPLC. This is also the rational behind the recent trend in HPLC, the use of sub 2 micron particle columns and the development of a specially design of ultra performance HPLC systems to accommodate such columns .

3. The Role of HPLC in Drug Analysis

The most characteristic feature of the development in the methodology of pharmaceutical and biomedical analysis during the past 25 years is that HPLC became undoubtedly the most important analytical method for identification and quantification of drugs, either in their active pharmaceutical ingredient or in their formulations during the process of their discovery, development and manufacturing.

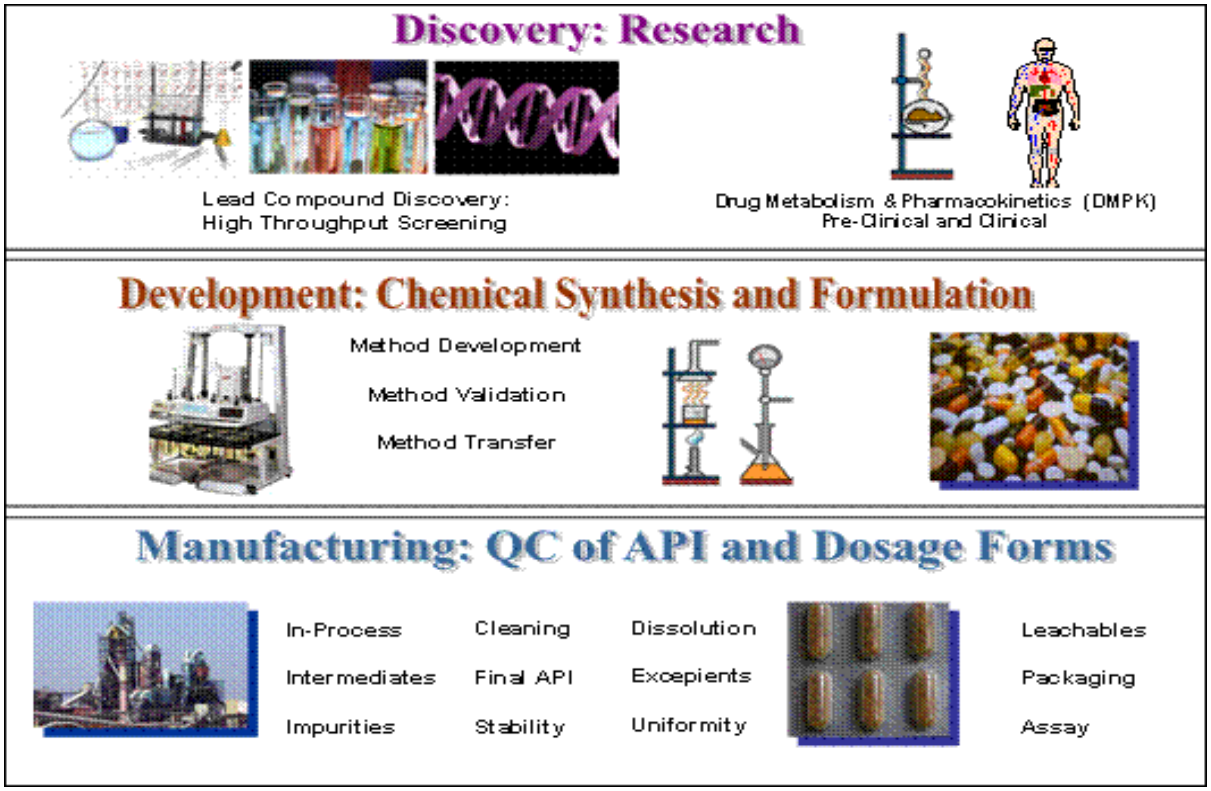

Figure 6: Steps in a drug development and manufacturing in the pharmaceutical industry

Figure 6 summarizes the various stages of the pharmaceutical streamline of drug discovery, development and manufacturing. Drug development starts with the discovery of a molecule with a therapeutic value. This can be done by high throughput screening during which separations by fast or ultra-fast HPLC are performed. At the discovery stage there can be also characterizing synthetic or natural products. Drug metabolism and pharmacokinetics (DMPK) is the step where the candidate compounds for drug are tested for their metabolism and pharmacokinetics. The studies involve use of LC-MS or LC-MS/MS.

The next stage is the development stage, where HPLC is used to characterize products of the chemical synthesis, by analyzing the active pharmaceutical ingredients (API), their impurities and/or degradation products generated by accelerated aging. The development of formulation requires also studies of the dissolution properties of solid dosage forms as well as assays of the pharmaceutical formulations. Method for the verification of system's cleanliness during the manufacturing process are developed and used at this stage. All the HPLC methods that have been finalized at the developmental stage are validated and transferred to the manufacturing laboratories for a quality control analysis.

3.1 The Discovery Stage

The goal in the discovery stage of drug development is to discover a new, safe and active chemical entity (NCE) that will become medication for diseases. During the last decade parallel synthesis of potential lead compounds, using combinatorial chemistry, has been done. The large numbers of products created by the combinatorial chemistry are then identified by fast LC-MS methods and screened by in-vitro bioassays and/or pharmacological or chemical tests to allow a selection of a few chosen drug candidates.

When the lead compounds are selected, the next steps involve pharmacological studies. Due to its high sensitivity and selectivity, HPLC coupled with tandem mass spectrometry, HPLC-MS/MS, has become the predominant method in bioassays, and pharmacokinetic and metabolic studies. In addition, another new development in this field has been the introduction of column's packing with ultra-fine particles (<2 um) enabling short columns to be used (5 cm or less) and rapid analyses (e.g., 5 min or even less than 1 min) to be carried out by UPLC (ultra performance liquid chromatography).

Bioanalytical studies

The support of bioanalytical testing includes quantitation of a target compound in various biological matrices as part of preclinical lead optimization by measurement its adsorption, distribution, metabolism and excretion (ADME).The samples are physiological fluids such as plasma, serum, tissue extracts and urine, typically from experimental animals or human subjects. During the last decade conventional HPLC methods using fluorescence or electrochemical detection, following an elaborate sample clean-up, have been replaced by HPLC-MS/MS or U-HPLC-MS/MS methodologies. Sample clean-up is simpler and more automated and usually involves protein precipitation and/or solid phase extraction techniques.

The chromatographic method is typically very rapid, gradient or isocratic, using small columns' dimensions, such as 2.1x50 mm or less. A trace of a typically stable daughter ion signal, obtained from the fragmentation of a parent analyte is used for the detection and quantitation. Such a mode is called MRM (multiple reaction monitoring) or SRM (selective reaction monitoring), which is more stable, sensitive and selective than either the fluorescence or electrochemical signal. Samples are usually ordered in 96 microplates for higher throughput and minimum variability of the sample vials. An internal standard is used typically to minimize assay variability, due to the clean-up stage and MS/MS response, which is still less stable than UV response.

3.2 The Development Stage

The development stage is the part during which some of the HPLC methods that will be used during the subsequent manufacturing stages are developed, validated and then transferred. The methods involve identification of the drug substance, its assay, impurities, stability, the dosage form content uniformity, dissolution, etc. The methods are validated first, and then transferred to the quality control laboratories. Details are given in 3.3.

Method Validation

All HPLC methods used for the development of pharmaceuticals and for the determination of their quality have to be validated. In cases whereby methods from the Pharmacopoeia's are used, it is not necessary to evaluate their suitability, provided that the analyses are conducted strictly according to the methods' intended use. In most other cases, especially in cases of modification of the drug composition, the scheme of synthesis or the analytical procedure, it is necessary to re-evaluate the suitability of the HPLC method to its new intended use .

The parameters tested throughout the method validation as defined by the ICH, USP and FDA and other health organizations are the following: Specificity or selectivity, precision (repeatability, intermediate precision, reproducibility or ruggedness), accuracy or trueness or bias, linearity range, limit of detection, limit of quantitation and robustness.

The terms ***selectivity*** **and** ***specificity*** are often used interchangeably. The USP monograph defines selectivity of an analytical method as its ability to measure accurately an analyte in the presence of interference, such as synthetic precursors, excipients, enantiomers and known (or likely) degradation products that might be present in the sample matrix. A method whose selectivity is verified is a "Stability Indicating Method", for details please see section 3.3.

Precision of a method is measured by injecting a series of standards and measuring the variability of the quantitative results. The measured standard deviation can be subdivided into three categories: repeatability, intermediate precision, and reproducibility (or ruggedness):

- Repeatability is obtained when one operator using one system over a relatively short time-span carries out the analysis in one laboratory. At least 5 or 6 determinations of three different matrices at two or three different concentrations should be done and the relative standard deviation calculated.
- Intermediate precision is a term that has been defined by ICH as the long-term variability of the measurement process and is determined by comparing the results of a method run within a single laboratory over a number of weeks. A method’s intermediate precision may reflect discrepancies in results obtained by different

operators, from different instruments, with standards and reagents from different suppliers, with columns from different batches or a combination of these. Objective of intermediate precision validation is to verify that in the same laboratory the method will provide the same results once the development phase is over.

- Reproducibility (or reggedness), as defined by ICH represents the precision obtained between laboratories. The objective is to verify that the method will provide the same results in different laboratories, preparing it for the transfer to other sites.

Typical variations affecting a method's reproducibility are:

- Differences in room temperature and humidity;
- Operators with different experience and thoroughness;
- Equipment with different characteristics, such as delay volume of an HPLC system or injection modes;
- Variations in material and instrument conditions, for example different protocols of the mobile phases preparation; changes in composition, pH, flow rate of mobile phase;
- Equipment and consumables of different ages;
- Columns from different suppliers or different batches;
- Solvents, reagents and other material with different quality

Accuracy of an analytical method is the extent to which test results are close to their true value. It is measured from the result of a quantitative determination of a well characterized known sample. The amount measured is compared to the known amount.

Linearity of an analytical method is determined by a series of three to six injections of five or more standards whose concentration's span is 80-120 percent of the expected concentration range. The response should be proportional to the concentrations of the analytes, directly or by means of a well-defined mathematical calculation. A linear regression equation, applied to the results, should have an intercept not significantly different from zero. If a significant non-zero intercept is obtained, it should be demonstrated that there is no effect on the accuracy of the method. The range of concentrations that an analytical method can be implemented on is the interval between the upper and lower levels (including these levels) that have been demonstrated to have the appropriate precision, accuracy and linearity. The range is normally expressed in the same units of the test results (e.g. percentage, parts per million) obtained by the analytical method.

Limit of detection: It is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified. In chromatography the detection limit is the injected amount that results in a peak height of at least twice or three times as high as the baseline noise level.

Limit of quantitation: It is the minimum injected amount that gives precise measurements. In chromatography it typically requires peak heights of 10 to 20 times higher than baseline noise at precision of <10-15% RSD between results.

Robustness of analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Once validated, the methods are ready for transfer to the manufacturing quality control laboratories. Method transfer is the last stage of the validation, whereby results are tested on both the development and the manufacturing sites. Technology transfer is especially important in the era of increasing globalization of the pharmaceutical companies .

3.3 The Manufacturing Stage

Identification

The identification test by HPLC is aimed to confirm the identity of the active pharmaceutical ingredient inside the samples of either drug substance or drug product. Two independent tests are needed, for example, one chromatographic and one spectroscopic. Therefore, a chromatographic run with a diode-array or MS detector will provide both parameters, the retention time in the chromatogram and the spectrum UV or MS of the eluting peak, matched against a known standard.

Assay and Content Uniformity

An assay of a sample containing either drug substance or drug product quantitatively measures the actual amount of the active ingredient compared to that expected in the drug substance assay, whereas in drug products the actual amount measured is verified against the label claim. The official assays are described in the pharmacopeias, such as United State's (USP).

When a solid dosage form such as tablet or capsule are tested, it is usually requested to sample a composite of 10-20 units to avoid tablet-to-tablet variations. The API is extracted from the dosage form in the simplest most effective way to optimize recovery. A portion of the composite group, equivalent to an average unit dosage form weight is taken for the analysis. Typical specifications for most drug products are 90-110% of the label claim.

A content uniformity test is similar to the drug product assay but an individual solid dosage form is tested instead of a pool of the tablets/capsules as in the assay tests. 10 tablets are taken for the assay, and if the specifications of uniformity are not met, more tables are taken. The typical calculations are made according to a scheme given by the USP.

Most assays and content uniformity methods involve the use of UV detection, no extensive sample preparation is needed, the standards are usually external and highly qualified, typical specifications can be 98-102% purity on dried basis. Specifications for system suitability were determined by the pharmacopeia.

Dissolution

Drug absorption from a solid dosage form after oral administration depends on its release from the drug product, its dissolution or solubilization under physiological conditions, and the permeability across the gastrointestinal tract. Because of the critical nature of the first two of these steps, in vitro dissolution may be relevant to the prediction of in vivo performance. Based on this general consideration, in vitro dissolution tests for solid oral dosage forms, such as tablets and capsules, are used to assess the lot-to-lot quality of a drug product; guide the developers of new formulations and ensure continuing product quality and performance after certain changes, such as changes in the formulation, the manufacturing process, the site of manufacture, and the scale-up of the manufacturing process

The dissolution test measures the release of the drug substance from its dosage form into a dissolution bath under standardized conditions, specified by the pharmacopeias. The US Food and Drug Administration issued a guidance for the industry on 1997 . The tests are performed usually on two types of bath apparatus, Type I is a basket method and Type II is a paddle method. Since most drugs have absorption in the UV-VIS range, their dissolution has been traditionally measured by UV-VIS spectrometry and HPLC-UV. The advantage of HPLC over UV spectrometry is its separation capabilities, providing higher specificity and sensitivity as well as its applicability in formulations with multiple API's or very low dose.

The method is typically isocratic and very short to accommodate for the high throughput needed, therefore the columns are typically very short. The sample is collected from the dissolution apparatus at certain time intervals, designed according to its release pattern, and the released substance must follow strict release specifications. A dissolution profile of Prednisone tablets, used to calibrate a dissolution bath are shown in Figure 7.

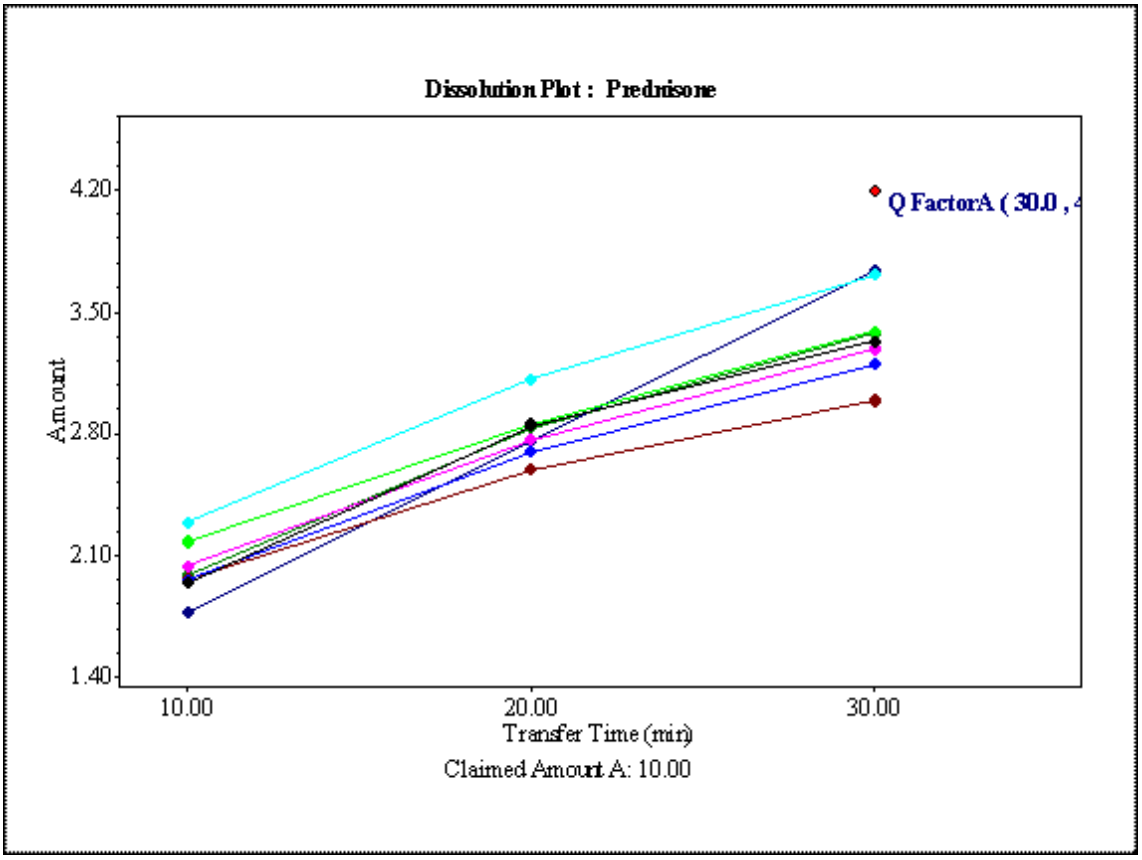

Figure 7: Dissolution profile of Prednisone tablets

Drug Impurities

Many potential impurities result from the API manufacturing process, including starting materials, isomers, intermediates, reagents, solvents, catalysts and reaction by-products, therefore, impurity control in is essential during the process of drug development and manufacturing. HPLC has become the major technique in this process.

The quality of the API starting material is tested by HPLC to qualify it for the production of API that meets its specifications, as its impurities can be carried through directly or can participate in the reaction chemistry to produce significant impurities in the final product, the API. It is likely that different synthetic routes that produce different impurities may be used by different suppliers. In some cases, the synthetic route is not disclosed to the API producer; therefore, a thorough investigation of impurities in starting materials must then be conducted. This investigation includes HPLC method development, a search for potential impurities, as well as identification of significant unknown impurities and assessment of their impact on the API quality. Results of such an investigation are used to set specifications for the starting material that will assure its suitability for use in API production .Similar considerations apply to the drug product. Strict international regulatory requirements have been enforced for several years as outlined in the International Conference on Harmonization (ICH) Guidelines Q3A(R), Q3B(R) and Q3C.

A typical method for the measurement of impurities can be rather complicated and difficult, as it requires high resolution to distinguish between closely related compounds, high sensitivity to detect low concentrations of the impurities and wide dynamic range of the system to be able to detect the major components as well as their impurities and degradation products in one run. The method should have the following important attributes:

First of all the sample preparation should be as simple as possible, to stay true to the original composition of the sample, either a drug substance or a drug product, not to lose or create an impurity during this process.

The chromatographic separation should be optimal, to resolve all the impurities from each other and from the API's.

The quantitative protocol involves frequently a calibration, using well characterized impurities standards, and/or calibration against a diluted standard of the main API, taking into account their relative response factor. Sometimes the impurities are just normalized against the main peak using % area.

The main peak is frequently at overloaded concentrations and if the normalization method of % area is used, its inclusion within the linear range should be established

According to the ICH guidelines and depending on the daily dose, the quantification limits of unspecified impurities can get as low as 0.05-0.1% of the main peaks. Specified known impurities can be reported at higher levels, provided their toxicology was determined.

Method validation must include the establishment of limit of detection (to be used for rejection of small peaks) and limit of quantitation (which is used as the minimum value for linearity range specifications)

There is a substantial effort to ensure that the main peak does not hide a co-eluted impurity by measuring peak purity (see next section).

Most methods for impurities involve a gradient of the mobile phase, to be able to resolve a complex mixture of all compounds in one run, polar as well as non-polar. In recent years the mobile phase is programmed to be mass spectrometry compatible, i.e., the buffers used are volatile, so that impurities can be identified by LC-MS at the developmental and verification stages of the analysis.

Drug Stability

Stability testing of drug substance or product is performed to ensure that their quality does not vary with time under the influence of a variety of environmental factors such as temperature, humidity, and light .In addition, stability studies are typically done using HPLC methods, to determine the re-test period of a drug substance and/or the shelf life of the drug product as well as its recommended storage conditions. Guidelines to the industry are given by the ICH.

An HPLC method used for stability studies is termed: *Stability Indicating Method* (SIM). According to the FDA guidelines, a SIM is defined as a validated analytical procedure that accurately and precisely measures active ingredients (drug substance or drug product) free from potential interferences like degradation products, process impurities, excipients, or other potential impurities. FDA recommends that all assay procedures for stability studies will be stability indicating. A typical chromatogram of a drug substance in a stability program is shown in Figure 8. The results are shown in Table 2.

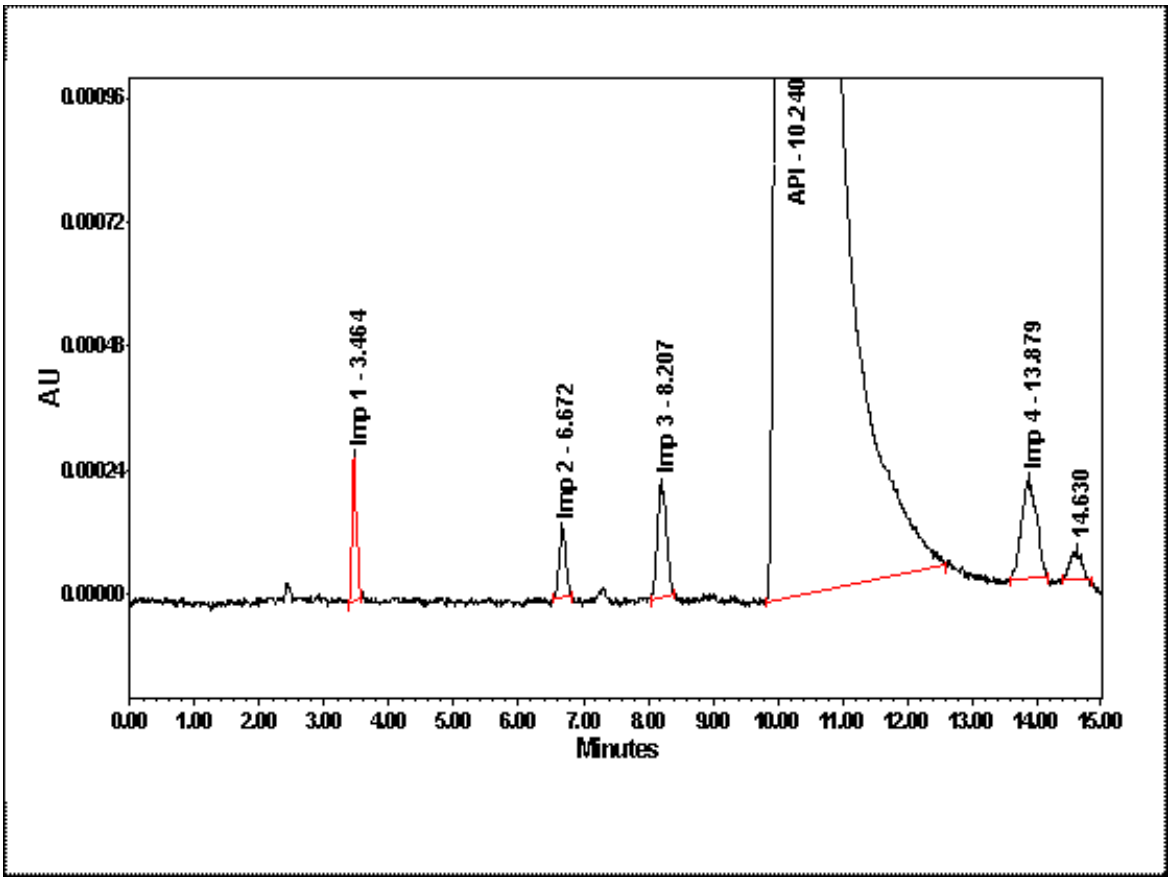

Figure 8: A chromatogram of an API sample at a stability program. Detection: UV at 286 nm, isocratic run (70:30 water:acetonitrile with 0.1% TFA); Flow rate=1 ml/min. Results are shown in Table 2.

Table 2: Results from HPLC run of an API after 3 months in heat.

Name	RT	Area	% Area	RRT	Signal to Noise	k'	USP Resolution	USP Plate Count
Imp 1	3.46	1319	0.01	0.34	18.2	0.41		13539
Imp 2	6.67	1163	0.01	0.65	8.9	1.72	18.68	14124
Imp 3	8.21	2119	0.01	0.80	14.1	2.35	6.40	17169
API	10.24	14505569	99.94	1.00	70348.3	3.18	6.65	14214
Imp 4	13.88	3243	0.02	1.36	12.4	4.66	9.52	13251
unk	14.63	861	0.01	1.43	4.1	4.97	1.84	21690

The HPLC system used for stability indicating methods development and validation must contain 3D data capabilities such as diode-array detectors and/or mass spectrometers, to be able to detect spectral non-homogeneity within the chromatographic peaks . Figures 9 and 10 present 3D data obtained by photo-diode array and mass detectors respectively. The modern UV-VIS diode-array technology is capable of evaluating specificity of the method to the analyte in question by collecting spectrum at each acquisition data point across a peak, and through software manipulations each such spectrum is compared to a reference spectrum, to determine spectral homogeneity, hence determine peak purity. Figures 11 and 12 demonstrate graphically how the software collects spectra from various points across the peaks to determine their similarity. We can see in this example that there is a minor interference only in Peak 2, as its UV spectra do not perfectly overlap. The mass spectrometer's spectra in Figure 12 were collected by combining spectra across the peak. The spectra confirm the suspicion and indicate the co-eluting interference in Peak 2, however, they also indicate that there is an interference in Peak 1 as well, although the UV spectra could not detect it!

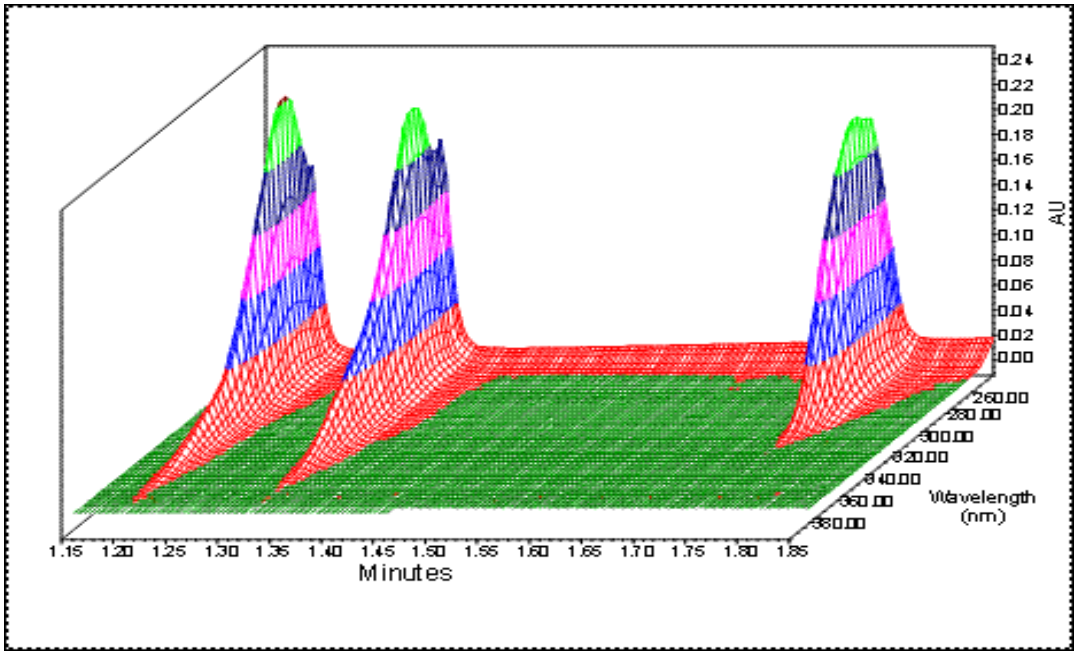

Figure 9: 3 dimensional plot of a chromatographic run of 3 sulfa drugs, using a diode-array detector

Since 2006 it has been recommended that a peak purity test, based upon photo-diode-array (PDA) detection, will also be based on mass spectrometry (MS) whenever possible, to be more definite.As already was noticed in Figures 9-12 and discussed in the previous section, the spectroscopic data obtained by the MS detector is much more reliable in evaluating the interferences co-eluting with the major chromatogram's peaks.

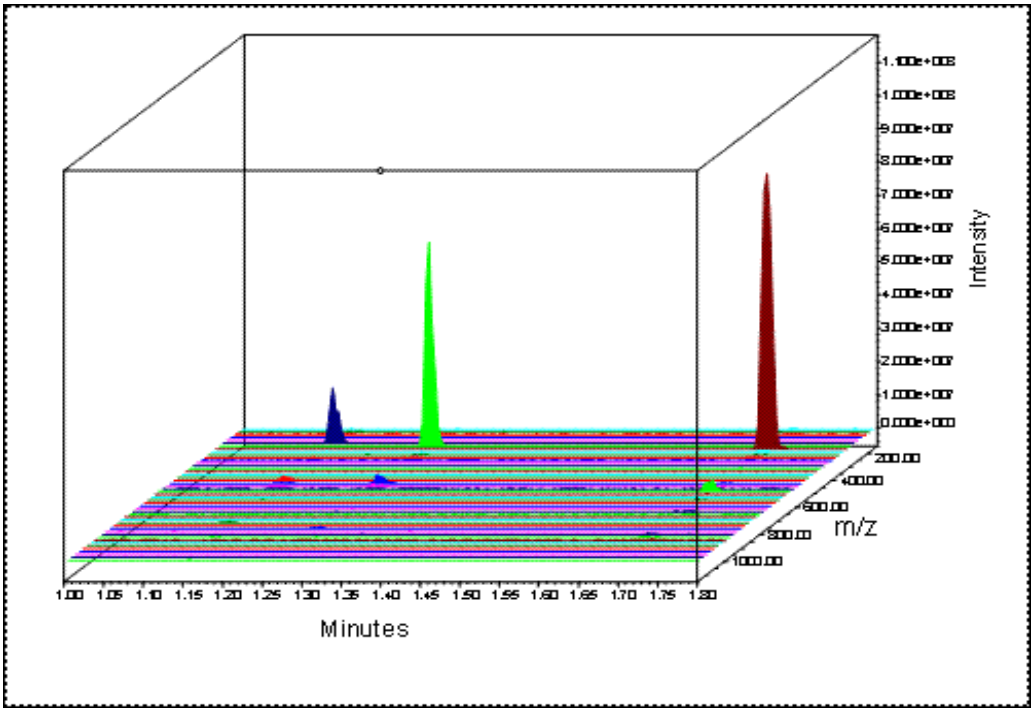

Figure 10: 3 dimensional plot of the same 3 sulfa drugs as in Figure 9, using a mass spectrometer detector

As noticed in Figures 11 and 12, the UV-VIS diode-array detectors might be limited in evaluating peak purity in cases whereby the UV-VIS spectra of the main peak and its impurities are similar and/or the noise in the system is relatively high, masking the co-eluting impurities and degradants.

Three conditions must be fulfilled for the major components and their related compounds to be effective in the peak purity determination by diode-array detectors:

- They must have a UV chromophore, or some absorbance in the wavelength range selected
- There should be a minimal degree of chromatographic resolution between the main peak and its co-eluting impurities (Chromatographic resolution between 0.3 to 0.7).
- There must be some degree of spectral difference between the analyte peak and the co-eluting impurity.

Stress testing is carried out on a single batch of the drug substance and/or the drug product, using forced degradation of the drug substance. The results of such studies can help identify the likely degradation products, which can in turn help establish the degradation pathways and the chemical stability of the molecule and to validate the stability indicating capabilities of the analytical procedures used.

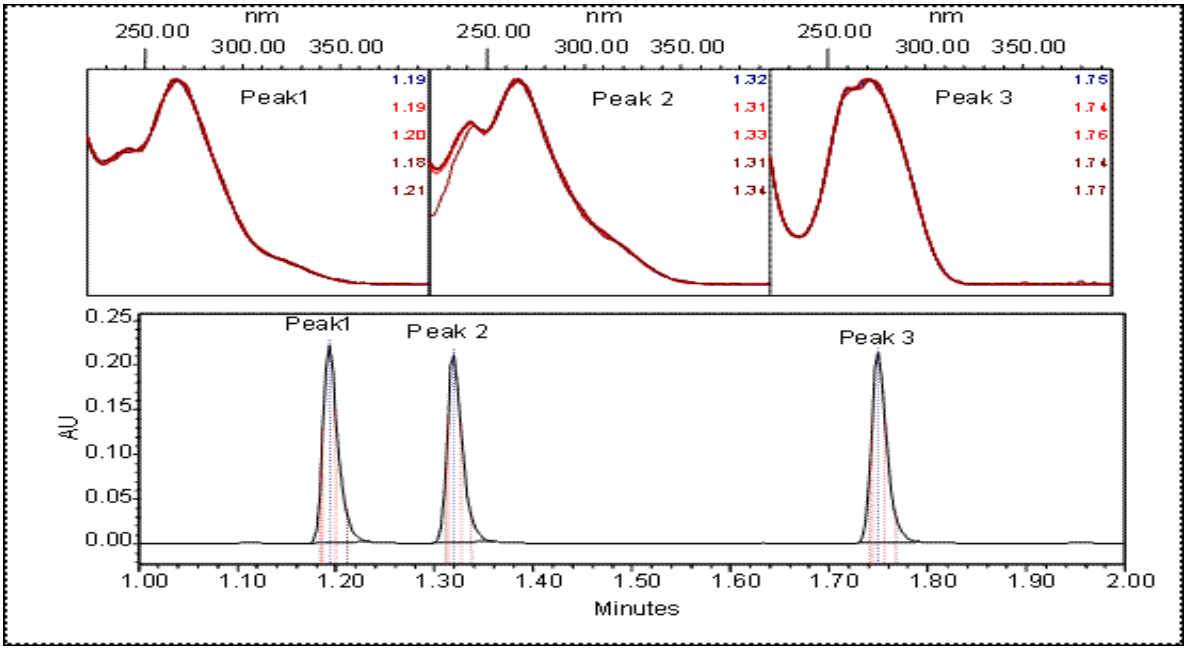

Figure 11: UV chromatogram at 270 nm of three sulfa drugs shown in Figure 9, and a plot of UV Spectra collected from each peak's Apex, Inflection and two offset points, to demonstrate comparison of spectra collected at various points across the peak.

The nature of the stress testing will depend on the individual drug substance and the type of drug product involved. It usually includes the effect of temperatures, humidity, oxidation, and photolysis on the drug substance. The testing also evaluates the vulnerability of the drug substance to hydrolysis across a wide range of pH values when in solution or suspension. Photo-stability testing is also an integral part of a stress testing .

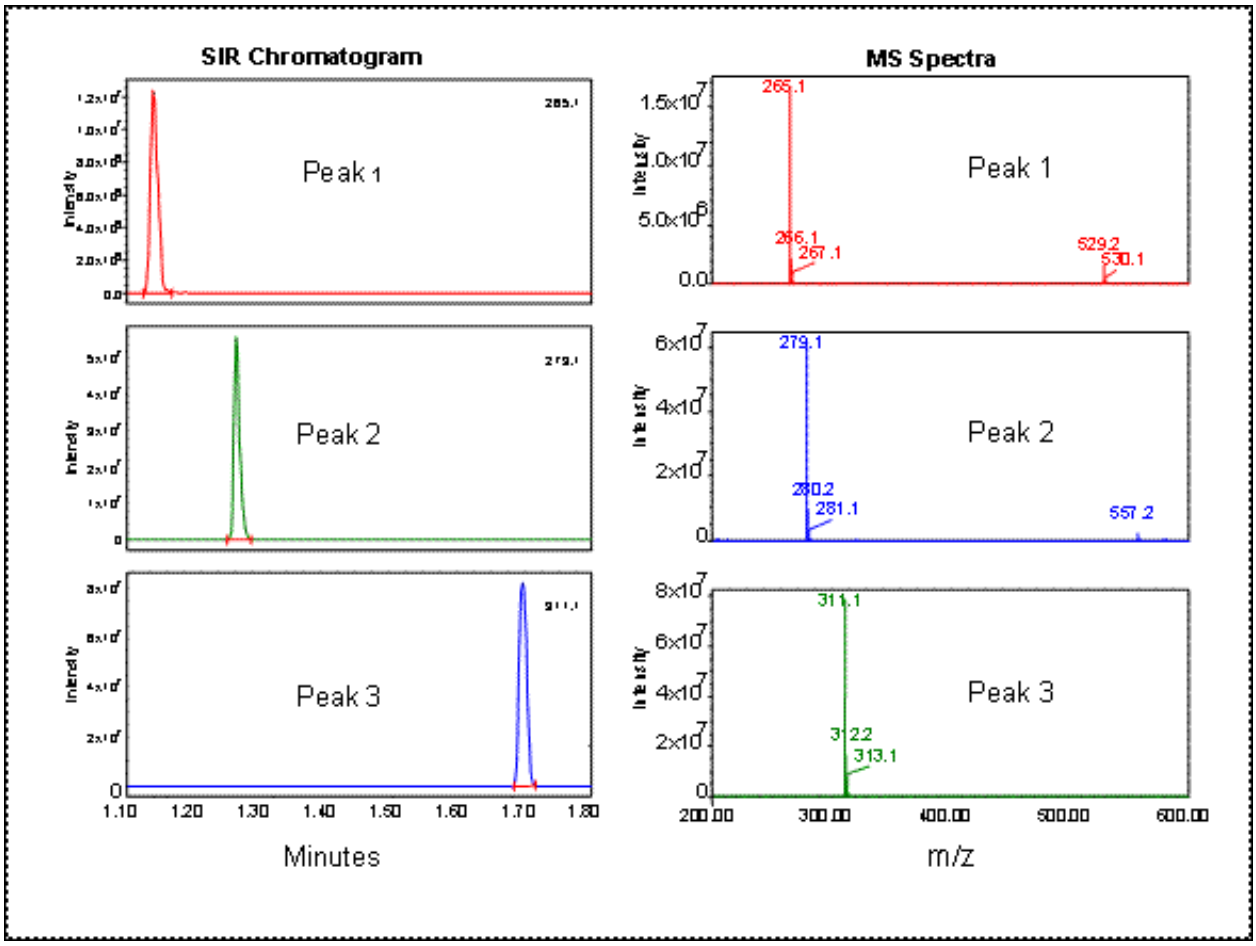

Figure 12: SIR chromatograms of the same sulfa drugs as in Figure 10 and their corresponding mass spectra, obtained by combining spectra across the entire peak.

Cleaning Validation

Cleaning validation tests are performed to assure the cleanliness of the pharmaceutical manufacturing equipment, such as blender, tablet press, etc. There are strict specifications for the maximum allowable amounts of the residues, according to their toxicology and daily dosage. The analytical methods must be extremely sensitive, to be able to track the residues of the analytes, their degradants or impurities, other contaminations or the cleaning reagents themselves.The methods must be very sensitive and short, as the entire manufacturing process depends on the approval of the cleanliness of apparatus. Due to the enhanced sensitivity as well as speed of analysis, the use of ultra performance liquid chromatography has immediately adopted in the cleaning validation tests.

In-Process Control

In-process control method monitors the progress in the manufacturing of an active pharmaceutical ingredient or its formulation. HPLC is frequently employed for the in-process assay of active pharmaceutical ingredient synthesis. The results signal the production chemist/pharmacist whether to proceed with a subsequent unit operation. The decision about whether to use an in-process control test in a manufacturing is established during process development and is based on scientific judgment. In recent years the FDA issued a recommendations for process analytical technology

(PAT). The guidance intended to describe a regulatory framework that will encourage the voluntary development and implementation of innovative pharmaceutical development, manufacturing, and quality assurance. The new LC ultra performance/rapid technologies are emerging as the technology of choice for the in-process control assay due to their extended efficiency, sensitivity and speed of the analysis.

4. Special Topics

4.1 Ultra High Performance Liquid Chromatography

In order to enhance chromatographic performances in terms of efficiency and speed, LC has recently evolved thanks to the development of short columns packed with small particles (sub-2 µm), working at high pressures (Above 1000 bar). This approach has been described 30 years ago according to the fundamental chromatographic equations. However, systems and columns compatible with such high pressures have been introduced into the market since 2004 only. Advantages of small particles working at high pressure have been vividly discussed in the literature, in terms of sensitivity, efficiency, resolution, and analysis time, showing how systems working at a maximum pressure of 1000 bar give reliable and reproducible results. The implementation of the new technology in the pharmaceutical and life sciences is soaring and it becomes the next benchmark technology for the near future.

There are important experimental parameters one should be aware of while reducing the particle size. First and foremost is the column pressure. Equation 10 shows the dependence of column head pressure on a number of experimental parameters including the particle size. As can be seen in Eqn 10, the pressure is inversely proportional to the square of the particle size.

Eqn 10: $$P = \frac{\Phi L \eta \mu}{100 \times d_p^2}$$

Where *P* is the pressure drop, *phi* is the flow resistance parameter, *heta* is viscosity (mPa/s), *L* is column length (mm), *u*- is the linear velocity (mm/s) and d_p particle size (um). So when the particle size is halved, the pressure goes up by a factor of four. However, often for fast separations, the column length is also reduced, so the pressure increase is not nearly as high as one would expect, because pressure is proportional to column length.

If longer columns such as 100 or 150 mm are required to achieve higher resolution and sensitivity, then higher pressure pumps are required. Currently, there are commercial HPLC systems with upper pressure limits as high as 15,000 psi. It should be noted that the total pressure that the HPLC system experiences is the sum of the column and the instrument backpressures. The latter results when small internal diameter capillaries are used in the flow paths to reduce extra column effects and minimize the gradient delay volume .As the flow rate increases, the back pressure due to these capillaries increases proportionally.

An experimental parameter that requires the most careful consideration when reducing the column length and especially internal diameter is the extra column band broadening. Some of the modern ultra-fast LC columns are only 1.5-cm long with an internal diameter of 2.1 mm. Such a column has a total void volume of around 33 µL. Many conventional HPLC instruments were developed for typical 15 and 25 cm x 4.6 mm analytical columns, whose void volumes are ~1.6 and ~2.6 mL respectively. A 1.5 cm x 2.1 mm column packed with 1.8-um particles often produces a few microliters peaks, which requires that extra-column band broadening will be minimized to accommodate for the true advantages of these small columns. Some modern liquid chromatographs have been designed or modified to minimize the extra column effects. For others, upgrade kits are available to modify some older HPLC instruments to work satisfactorily with sub-2-µm columns.

Some other experimental parameters that must be taken into account when running fast and ultrafast separations in LC are as follows :

1) Detector time constant: Peaks can be only a second or two wide when short narrow-bore columns run at high flow rates. A time constant that is too slow will make the peaks artificially broad because the detector cannot keep up with the rapidly changing signal.

2) Data sampling (acquisition) rate: Data system needs enough data points, minimum of 20 across a peak to define the peak as proper for integration of area, determine retention time, etc.

3) Auto sampler cycle time: Separations whose run time is less than a minute or two, throughput can be slowed down by a slow auto sampler.

4) Gradient delay volume: If the volume between the points, where the gradient forms (mixer), to the column head is too large, gradient profiles will be compromised because gradient has not reached column in time.

When transferring methods from HPLC to UPLC there is a scale-down process of the sample load, flow-rate, and gradient times . Sample load must be reduced proportionally to the columns volumes, a typical factor can be from ~1.5 mL (4.6x150 mm column) to ~0.15 mL (2.1x100 mm column). Equation 11 describes the calculations required for such a scale-down.

Eqn 11:

$$M_{Uplc} = M_{HPLC} \frac{L_{Uplc}}{L_{HPLC}} \frac{ID_{Uplc}}{ID_{HPLC}}$$

Where M is the sample load in mg, L is column's length and ID is internal column's diameter.

It should be noted that the separation mechanisms in UPLC compared to HPLC are still the same; chromatographic principles are maintained while speed, sensitivity and resolution is improved. This all supports easier method transfer from HPLC to UPLC and its revalidation. The main advantages are significant reduction of analysis time and flow-rates (which means also reduction in solvent consumption!), enhanced peak capacity and sensitivity, which means that complex analytical determination of pharmaceutical preparations can be accomplished much easier within a reasonable time. Moreover, time spent with new method development, optimization and validation is significantly saved.

A typical chromatogram obtained in the UPLC system is shown in Figure 13. A mixture of alkylphonone is chromatographed on a 2.1x50 mm Acquity BEH C18 column packed with 1.7µM particles at room temperature using a gradient of 5% MeCN to 95% Acetonitril within 3 minutes at flow rate of 0.5 mL/min. Re-equilibration time was 1.5 min and solvent consumption was 2.5 ml/run. The spectacular resolution and peak widths in such a short time is noticable.

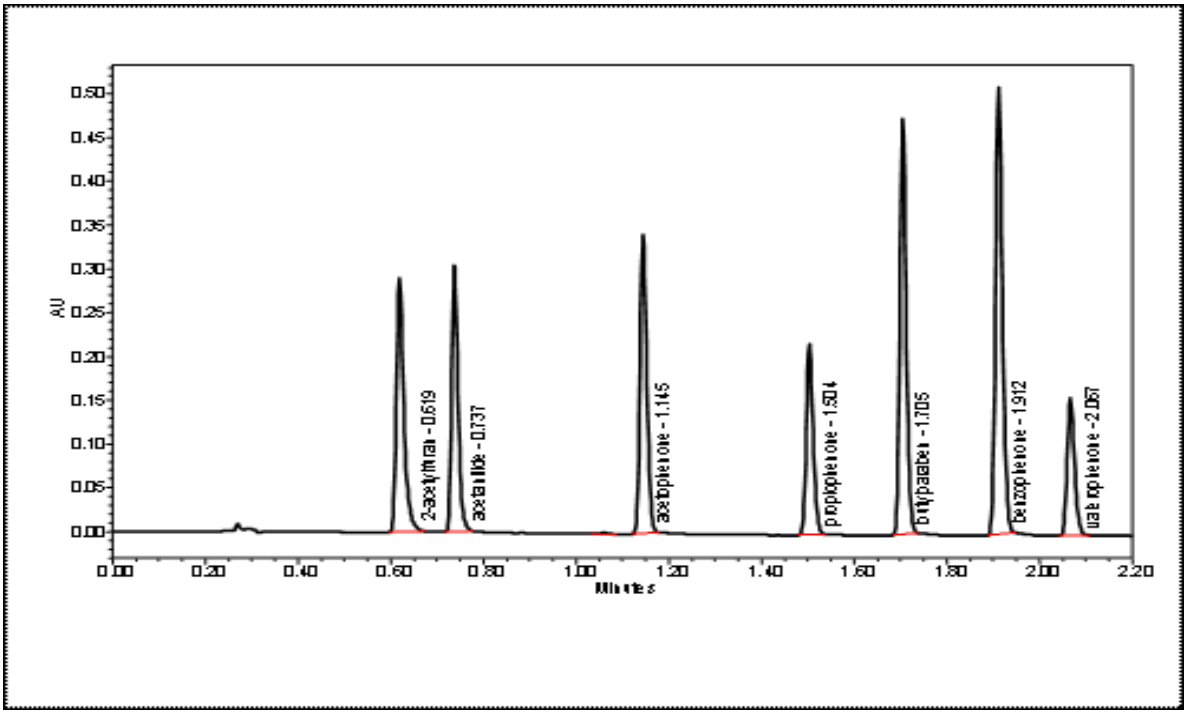

Figure 13: Mixture of alkylphenones injected into Waters' Acquity UPLC using Acquity BEH C18 2.1x50 mm 1.7 µM

4.2 Preparative Chromatography

Preparative chromatography is used to isolate and purify compounds .Typically this type of separations requires larger columns and the chromatograms generally display broader peaks, due to the increased column load. The separation is optimized for higher throughput (weight/time), then sample load is maximized on small analytical columns, and finally quantities are scaled up to the preparative column according to the desired purity and recovery.

The parameters that will govern efficiency of the preparative separation are the plate count, the capacity factor k' and the selectivity between the solutes (see section 2). As selectivity, *a*, between two adjacent peaks of interest is improved, a better separation between them at higher sample loads is obtained, getting better product purity. Mobile phase changes cause this change in selectivity, as well as selecting the appropriate stationary phase.

Peak shape is no longer a concern, only the collected product's purity. The scaling up of the mass load can be done according to the column's volume ratio as is shown in Eqn 11, but instead of ratio between UPLC and HPLC, the ratio is between the analytical column and the preparative one.

A rational approach to scaling up HPLC runs, using adsorption isotherms measurements and calculations has been described. In the pharmaceutical work preparative chromatography is used mostly to isolate drug impurities for the purpose of their identification and/or using them as working standards for impurities determination. Purification of compounds during drug development and manufacturing has been done in recent years using UV in line with mass spectrometer, to achieve a mass-directed purification, whereby the mass spectrometer is used to determine the cutoff of the purified compound and direct the pure compound into its collection vessel.

4.3 Chiral Chromatography

The biological activity of chiral substances often depends upon their stereochemistry, since the living body is a highly chiral environment. A large percentage of commercial and investigational pharmaceutical compounds are enantiomers, and many of them show significant enantioselective differences in their pharmacokinetics and pharmacodynamics. The importance of chirality of drugs has been long recognized, and the consequence of using them as racemates or as enantiomers has been frequently discussed in the pharmaceutical literature. With increasing evidence of problems related to stereoselectivity in drug action, chiral chromatography has become the major tool in

their analysis . Great efforts have been devoted to the development of better methodology for enantioselective chromatography during the past two decades, and have resulted in variety of chiral stationary phases. Chiral agents were derivatized and immobilized on the surface of the support (silica gel mostly), and served as the in situ chiral discriminators during the chromatographic process.

The chiral stationary phases can be categorized into the following classes:

- Chiral affinity by proteins (serum albumin, a1-acid glycoprotein, ovomucoid and chymotrypsin) .
- Stereoselective access to helical chiral polymers (derivatized or free polysaccharides).
- Steric interactions between p-Donor p-acceptor type of chiral aromatic amide groups (Pirkle) .
- Host-guest interactions inside chiral cavities (cyclodextrins, crown ethers, macrocyclic antibiotics and imprinted polymers).

Major developments in chiral stationary phases have led to dramatic advances in enantioselective chromatography . Chromatographic enantioseparation on chiral stationary phases results from energy differences between transient diastereomeric complexes formed by the solute with the chiral agent on the surface of the chiral stationary phase interactions.These differential interactions are directly influenced by the mobile phase environment. The solvents in the mobile phase can be either normal phase, or aqueous reversed phase's or polar organic .

During recent years the trend in the pharmaceutical manufacturing of drugs containing chiral centers is to develop them as single enantiomers rather than racemates due to the complexity of the tests and research involved in the use of racemates as API's .

Conclusion

HPLC technology has matured to the extent that almost any existing organic compound can be analyzed by an existing method that can be found in the analytical literature, such as professional journals, protocol books such as Pharmacopeia's or AOAC manuals. The most remarkable change in the pharmacopoeias in the past 25 years has been the increasing importance of HPLC technology in the analysis of all aspects of drug development and manufacturing.