Chapter 1

Introduction
1.1 INTRODUCTION OF ANALYTICAL METHODS

The aim of this doctoral thesis is to study validation processes development of new method validation in pharmaceutical analysis. The advent of novel and highly potent drugs may even give rise to equally potent impurities with severe deleterious effects. The scenario becomes worse if the drug is to be administered for prolonged periods leading to unknown reactions and side effects. Under such a scenario, the rule to characterize and quantify impurities exceeding 0.1 per cent may not hold well, and may demand for further lower levels of quantitation and characterization. These impurities might have derived from diverse origins, for example process related compounds, by products, reaction intermediates, degradation products etc. Analytical chemistry refers to any kind of assessment that provides qualitative and/or quantitative information on the chemicals present and persists in a sample. The sample in question may originate from any kind of material in any kind of location or matrix, e.g. food, an environmental compartment or living organism. Hence, the analysis is often challenging since most matrices contain not only the compounds of interest but also a wide range of other substances. Furthermore, the target compounds are often present at concentrations lower than their detection limits and require a preliminary concentration step. Therefore, the first step of an analysis is usually some kind of sample pre-treatment to improve both the selectivity and sensitivity of the subsequent detection. Many techniques are available for this purpose and the suitability of which depends primarily on the physical state of the sample, e.g. solid-phase extraction (SPE) for liquid and pressurized-liquid extraction for solid samples.

Validation is an another important feature in any method of measurement because it is closely related to the quality of the results. A method of analysis is characterised by its performance parameters, which have to be assessed if they are to provide the correct performance values. These performance values must be in accordance with previously defined requirements the method of analysis should satisfy. But above all, the performance parameters primarily depend on the type of method and its intrinsic characteristics. So depending on what is needed, the user must choose which method of analysis will best solve the analytical problem. Qualitative methods of analysis provide basic information about the composition
of a sample and perform quite simple chemical reactions to identify the analyst it contains [1, 2]. Quantitative methods of analysis provide information not only about the composition but also about the concentration of the analyst present in the sample and, generally speaking, they often require more complex analytical techniques to obtain more accurate and reliable information about the sample. Semi-quantitative methods of analysis lie between the qualitative and the quantitative methods because they assign samples to different classes which delimitate specific ranges after measuring the corresponding property. These different categories are defined by a particular criterion: concentration of a compound, index value, etc. [3, 4]. One example of this sort of method is how the acid-base character of a sample is determined by means of the pH measurement: different colours mean different pH values. These are the semi-quantitative methods of analysis [5].

1.2 CHROMATOGRAPHY

Chromatography is a technique (Fig.1.1) used to separate and analyze complex mixtures. The components to be separated are distributed between two phases: a stationary phase and a mobile phase, which percolates through the stationary bed. The phases are chosen such that components of the sample having different solubilities in each phase. A component, which is quite soluble in the stationary phase, will take longer to travel through it than a component, which is not highly soluble in the stationary phase but soluble in the mobile phase. As a result of these differences in motilities, sample components separate from each other as they travel through the stationary phase. The separated molecules leave the column and get detected by one or more on-line electrical devices with signals proportional to the concentration of the analyst [6 – 8].
1.2.1 High performance liquid chromatography (HPLC)

Among all separation tools, high performance liquid chromatography (HPLC) takes a central role in pharmaceutical analysis. Liquid chromatography (LC) is a separation method of the great importance to the chemical, pharmaceutical and biotechnological industry. The principle is that a sample of a solution of the substances is injected into a column of a porous material (stationary phase) and a liquid (mobile phase) is pumped through the column. The separation of substances is based on differences in rates of migration through the column arising from different partition of the substances between the stationary and the mobile phase. Depending on the partition behaviour of the different types of substances, these will elute at different times from the column outlet. The technique was originally developed by the Russian Botanist M.S. Tswett in 1903 [9–10] and since then three has been an enormous development of this technique. The definite break through for liquid chromatography of low molecular weight compounds was the introduction of chemically modified small diameter particles (3 to 10μm), e.g. octadecyl groups bound to silica in the late 1960s [11]. The new technique became rapidly a powerful separation technique and is today called high performance liquid chromatography during this time period were summarized by Giddings [12]. For biological samples where the analyst often are non-volatile and/or occur in an aqueous matrix, the reversed-phase mode, using a hydrophobic stationary phase and an aqueous mobile phase is extremely useful. The usefulness and popularity of HPLC was further increased by the possibility to automate and computerize the systems providing unattended operations and high sample capacities. Many Nobel Prize awards have been based upon work in which
chromatography played an important role [13]. Most recently, the 2002 Nobel Prize in chemistry was awarded to "the development of methods for identification and structure analyses of biological macromolecules" in which HPLC and Mass spectrometry (MS) were used [14].

In analytical chromatography the aim of the separation is to obtain quantitative and qualitative information about the compounds of interest (analyst) in a sample. Analytical chemists have to analyze a variety of complex samples often originating in difficult matrices to answer questions about the quality and quantity of different analyst. A complex sample often contains a wide range of components with varying solubilities. Therefore the sample preparation and the separation methods must be highly selective and sensitive. These requirements are satisfied with HPLC especially if combined with an advanced detection technique such as diode – array detection (DAD) or mass spectrometry (MS). A very large number of reports have been published in the analytical and bio analytical (analysis of drugs in biological fluids) areas using these techniques.

1.2.2 Instrumentation HPLC system

HPLC instrumentation includes a pump, injector, column, detector and data system. The heart of the system is the column where separation occurs. Since the stationary phase is composed of micrometre size porous particles, a high pressure pump is required to move the mobile phase through the column. The chromatographic process begins by injecting the solute onto the top of the column. Separation of components occurs as the analyst and mobile phase are pumped through the column. Eventually, each component elutes from the column as a narrow band (or peak) on the recorder.

Detection of the eluting components is important, and this can be either selective or universal, depending upon the detector used. The response of the detector to each component is displayed on a chart recorder or computer screen and is known as a chromatogram. To collect, store and analyse the chromatographic data, computer, integrator and other data processing equipment are frequently used.
Fig.1.2 depicts the main components of a modern HPLC system and their interrelationships. In HPLC, a solution containing the compound(s) of interest is injected into a loop which has been calibrated to contain a specified volume (a 20 μL loop injector is a commonly used size). The valve switch is then rotated, allowing a sample stream of mobile phase (the eluent) to sweep the sample from the loop onto a column, packed with a suitable stationary phase, where the separation occurs. The eluent is delivered from a pump at a constant rate, (on the order of 1 mL/min) at a pressure sufficiently high to overcome the backpressure of the column. Pressures of 600-2000 psi are commonly necessary. An upper limit of 5000 psi is normally set on the instrument. The separation efficiency is inversely proportional to the particle size of the column packing material. High pressures are required to force a liquid through a tightly-packed column filled with small particle material, and the availability of high pressure solvent delivery systems is directly responsible for the "high performance". Assuming that a suitable column has been chosen for the separation of interest, all components should pass through the column and "elute" at different times (differential migration). This time differential is due to the differences in the distribution (partitioning) of the various components between the mobile phase (eluent) and stationary phase (column packing), which arise from the physical/chemical differences among the components of the mixture. Thus, each component will pass through the detector and be identified separately. The time for a substance to pass through the column, termed the retention time, is therefore, related to the identity of the compound and is the basis for qualitative analysis. Quantitative information is obtained from the area or height of the peak produced by the detector.
Several different approaches to HPLC detection exist. Perhaps most common is a detector based on the absorbance of ultraviolet or visible light, a UV/VIS absorbance detector. These detectors are, in reality, miniature UV/VIS spectrophotometers, equipped with a flow-through cell, allowing continuous monitoring of the eluent. The wavelength selected corresponds to the region of the electromagnetic spectrum where the compounds of interest and/or their associated chromophores absorb light. In the linear dynamic range of the calibration curve the absorbance is proportional to the concentration of the compound of interest. The data is recorded and presented using a chromatographic integrator. The absorbance measured by the detector produces a peak with a characteristic retention time; each peak has an area which is listed as well. Measurement of a series of standards, along with the unknown, allows the use of the "standard series" method, which produce the calibration curve to determine the concentration of the unknown compound.

Fig.1.2 Diagram of a Typical HPLC
1.2.3 Functional description of the instrument

The instrument consists of the following parts which have their own characteristic functions.

Mobile phase reservoir filtering
Pump
Column
Injector
Detector
Data system

1.2.3.1 Mobile phase reservoir filtering

The most common type of solvent reservoir is a glass bottle. Most of the manufacturers supply these bottles with special caps, Teflon tubing and filters to connect to the pump inlet and to the purge gas (helium) used to remove dissolved air. Helium purging and storage of the solvent under helium is not sufficient for degassing aqueous solvents. It is useful to apply a vacuum for 5-10 min. and then keep the solvent under a helium atmosphere.

1.2.3.2 Pump

High pressure pumps are needed to force solvents through packed stationary phase beds. Smaller bed particles require higher pressures. There are many advantages to using smaller particles, but they may not be essential for all separations. The most important advantages are: higher resolution, faster analyses and increased sample load capacity. However, only the most demanding separations require these advances in significant amounts. Many separation problems can be resolved with larger particle packing that requires less pressure. Flow rate stability is another important pump feature that distinguishes pumps. Very stable flow rates are usually not essential for analytical chromatography. However, if the user plans to use a system in size exclusion mode, then there must be a pump which provides an extremely stable flow rate. An additional feature found on the more elaborate pumps is external electronic control. Although it adds to the expense of the pump, external electronic control is a very desirable feature.
when automation or electronically controlled gradients are to be run. Alternatively, this becomes an undesirable feature (since it is an unnecessary expense) when using isocratic methods. The degree of flow control also varies with pump expense. More expensive pumps include such state-of-the-art technology as electronic feedback and multi headed configurations.

Modern pumps have the following parameters:

- Flow rate range: 0.01 to 5 mL/min
- Flow rate stability: not more than 1%
- For SEC flow rate stability should be less than 0.2%
- Maximum pressure: up to 300 h Pa.

It is desirable to have an integrated degassing system, either helium purging or membrane filtering.

1.2.3.3 Column

Typical HPLC columns are 5, 10, 15 and 25 cm in length and are filled with small diameter (3, 5 or 10 µm) particles. The internal diameter of the columns is usually 4.6 mm and is considered the best compromise for sample capacity, mobile phase consumption, speed and resolution. However, larger diameter columns may be needed if pure substances are to be collected (preparative scale). Packing the column tubing with small diameter particles requires high skill and specialized equipment. For this reason, it is generally recommended that all but the most experienced chromatographers purchase repacked columns, since it is difficult to match the high performance of professionally packed LC columns without a large investment in time and equipment. In general, LC columns are fairly durable and one can expect a long service life unless they are used in some manner which is intrinsically destructive, as for example, with highly acidic or basic eluents, or with continual injections of 'dirty' biological or crude samples. It is wise to inject some test mixture (under fixed conditions) into a column when new and to retain the chromatogram. If questionable results are obtained later, the test mixture can be injected again under specified conditions. The two chromatograms may be compared to establish whether or not the column is still useful.
1.2.3.4 Injector

Sample introduction can be accomplished in various ways. The simplest method is to use an injection valve. In more sophisticated LC systems, automatic sampling devices are incorporated where the sample is introduced with the help of auto-samplers and microprocessors. In liquid chromatography, liquid samples may be injected directly and solid samples need only be dissolved in an appropriate solvent. The solvent need not be the mobile phase, but frequently it is judiciously chosen to avoid detector interference, column/component interference, and loss in efficiency or all of these. It is always best to remove particles from the sample by filtering over a 5 μm filter, or centrifuging, since continuous injections of particulate material will eventually cause blockages in injection devices or columns. Sample sizes may vary widely. The availability of highly sensitive detectors frequently allows use of the small samples which yield the highest column performance. Typical sample mass with 4.6 mm ID columns ranging from the nanogram level up to about 2 mg was diluted in 20 ml of solvent. In general, it will be noted that much less sample preparation is required in LC than in GC since unwanted or interfering compounds or both, may often be extracted, or eliminated, by selective detection. Examples of injectors are presented in the fig.1.3.
Today, optical detectors are used most frequently in liquid chromatographic systems. These detectors pass a beam of light through the flowing column effluent as it passes through a low volume (~10 μl) flow cell. The variations in light intensity caused by UV absorption, fluorescence emission or change in refractive index, from the sample components passing through the cell, are monitored as changes in the output voltage. These voltage changes are recorded on a strip chart recorder and frequently are fed into a computer to provide retention time and peak area data. The most commonly used detector in LC is the ultraviolet absorption detector (fig.1.4). A variable wavelength detector of this type, capable of monitoring from 190 to 400 nm, will be found suitable for the detection of the majority samples. Other detectors in common use include: Photo Diode Array UV detector (PDA), refractive index (RI), fluorescence (FLU) and electrochemical (EC). The RI detector is not only universal but also the less sensitive one. FLU and EC detectors are quite sensitive (up to 10-15 per mole) but quite selective.
Since the detector signal is electronic, using modern data collection techniques can aid the signal analysis. In addition, some systems can store data in a retrievable form for highly sophisticated computer analysis at a later time. The main goal in using electronic data systems is to increase analysis accuracy and precision, while reducing operator attention. There are several types of data systems, each differing in terms of available features. In routine analysis, where no automation (in terms of data management or process control) is needed, a pre-programmed computing integrator may be sufficient. If higher control levels are desired, a more intelligent device is necessary, such as a data station or minicomputer. The advantages of intelligent processors in chromatographs are found in several areas. First, additional automation options become easier to implement. Secondly, complex data analysis becomes more feasible. These analysis options include such features as run parameter optimisation and deconvolution (i.e. resolution) of overlapping peaks.
1.3 METHOD VALIDATION

During method development, analysts establish the most suitable steps of the analytical process that will lead to the information required: sample pre-treatment, when necessary, separation technique and the detection system, among others. The best analytical conditions for obtaining good results are also considered. The information gathered after the analysis may have several goals, viz. to take decisions involving the control of the manufacturing process of a product, to assess whether a product complies with regulatory limits, to take decisions about legal affairs, international trade, health problems or the environment, etc. Therefore, the analytical information must be of sufficient quality, which means that it must be reliable and match the purposes of the analysis. To meet these premises, analysts must define the purposes of the analysis and the requirements that the method should fulfil. Therefore, the validation of the method of analysis will provide, according to the ISO definition [15] the “confirmation by examination and provision of evidences that the particular requirements for a specified intended use are fulfilled”. Another definition given in the Handbook for the Quality Assurance of Metrological Measurements [16] states that “method validation consists of documenting the quality of an analytical procedure, by establishing adequate requirements for performance criteria, such as accuracy, precision, detection limit, etc. and by measuring the values of these criteria”. In general terms, then, the requirements and performance parameters must first be defined for every analytical method and purpose of analysis and second, the value for these parameters must be estimated and checked to see if they really meet the criteria. This is an essential condition if the results provided are to be used. The process of assessing the performance criteria is closely related to the concept of ‘fitness-for-purpose’, which is defined by IUPAC in the Orange Book [17] as the “degree to which data produced by a measurement process enables a user to make technically and administratively correct decisions for a stated purpose”. Hence, it is important, first, to consider the necessary conditions related to the problem at hand, second to choose the method of analysis that best fits the necessities and finally to validate it as is shown in Fig. 1.5.
Fig. 1.5 Fitness for purpose concept (Adapted from the Eurachem the Fitness for Purpose of Analytical Methods)

The Eurachem Guide, the Fitness for Purpose of Analytical Methods [18], also describes how important it is for the analytical performance and the analytical problem to be suited. It also describes the importance of method validation and indicates when, how and who should perform the validation, among other equally relevant statements. Fitness for purpose also involves practicability and suitability criteria [19], which entail evaluating operational and time constraints, as well as such other parameters as reusability or possibilities of automation.

Although the users of the method of analysis will focus the validation process on their own needs, there are some common features that all validation procedures must have. The validation process must satisfy three requirements [4].

1) The whole method must be validated. It is quite usual to focus on the detection technique or the instrumental measurement, which often means that just this stage is validated. However, the previous steps of sample pre-treatment, extraction or pre-concentration also belong to the method of analysis and are of utmost importance. So they must all be validated.
2) The whole range of concentrations must be validated. It is difficult to comply with this condition because a method may work very well in one particular concentration range but not in others.

3) The whole range of matrices must be validated. It is well known that the matrix can have a decisive effect on the analysis. Therefore for the sake of representativeness, several matrices must be submitted to method validation.

In addition to the conditions mentioned above, it should also be pointed out that the method developed, before it is validated, should include the various types of equipment and the locations where it will be run. That is to say, if the analysis is always to be performed with the same equipment and in the same laboratory, then other equipment and other laboratories need not be taken into account. Before the equipment is used, its performance must be checked with generic standards.

The analytical requirements that the analyst has defined are translated to the performance criteria of the method of analysis. So one of the stages of method validation is to estimate and assess the values of the quality parameters. In general terms, performance criteria can be divided into two main categories [20] although some authors may suggest other classifications. The basic parameters usually refer to the reliability of the method and are commonly derived with statistical procedures. Some examples are trueness, precision, selectivity, sensitivity, limit of detection and quantification. Criteria such as cost, ease of use, rapidity etc. are considered to be complements of these.

In the Handbook of Chemometrics and Qualimetrics [21], Massart states that there are two types of performance criteria: primary and secondary. Precision, bias, accuracy, trueness and the detection limit belong to the first group while the other parameters that can influence these primary criteria belong to the second (e.g. linearity, the range of linearity, the quantification limit, selectivity and sensitivity or ruggedness).
1.3.1 Types of method validation

Because methods of analysis are designed for different uses, not all validation procedures are equal. Some examples of factors that can influence the definition of these procedures and which must be carefully considered are the quality of the final results, the consequences in terms of economy and time, whether the method has been developed recently or whether it is an adaptation of a previously adapted one. Depending on these factors, different methods of validation will be carried out in a different manner.

A validation procedure cannot be performed if the validation level required is not taken into account. The validation level is the degree of effort invested in the validation process, so a high validation level requires greater effort. On the other hand, if the validation level is low, the effort investment will also be low. In both cases, the quality of the results obtained by the validated method of analysis will be rather different. Fig.1.6 shows the different levels of method validation. So establishing the most suitable validation level is fundamental because the definition of the process depends on it and the results after the validation will also be of a different quality. To correctly choose the most appropriate validation level, operational, economic and material resources or the requirements the method must fulfil must be considered. The analyst can then choose to perform either an internal method validation or an inter laboratory validation [22, 23]. A recently accepted alternative is for a third laboratory to make an assessment of the properties claimed.

![Validation Diagram]

Fig.1.6 The types of method validation that involve different validation levels
Internal method validation is the lowest validation level [24]. The laboratory that incorporates a new method of analysis that has been developed internally or externally tests the quality of both the method and the results. Internal method validation is mainly carried out in three cases: to assess new methods developed in-house, to assess methods transferred from other laboratories and for instance, to estimate long-term precision. Routine internal quality control is also considered as internal method validation.

Each of the above mentioned situations requires a particular validation scheme because the requirements of every individual case are different. As a general philosophy, fitness-for-purpose is also applicable here. The main types of internal method validation are briefly described below. A full validation process is undertaken when the laboratory develops a new method and has to be used in routine control. Again, before carrying out the full validation process, the most appropriate performance parameters must be considered. If there is no information about the method's performance characteristics, it is recommended first to check if it is suitable for the intended purpose with several samples: for example if the method is selective enough, if the sensitivity is tolerable or if the matrix will not interfere excessively. If the results are favourable, then the subsequent quality parameters are determined. If not, the method itself, the equipment, the analysis technique or the acceptance limits should be changed. Method development and validation, then, is an iterative process. This is so-called prospective validation.

Transferring analytical methods from one laboratory to another is quite a common situation. Because the transferred method must be fully validated in the source laboratory, the receiving laboratory does not need to undergo another complete validation process. However, it must assess whether the methods of analysis perform correctly under the corresponding conditions. This is called suitability checks. Retrospective validation is performed on validated methods that are already being used. It may be necessary to examine accumulated results to assess whether the method keeps on performing appropriately. Likewise, long-term precision can also be assessed by collecting data over a long period of time. Once the method is in normal use, a quality control program should be run. Control charts [25] are a very useful tool for this purpose.
On the other hand, inter laboratory trials provide the highest validation level because several laboratories assess one property of a sample, usually the concentration of one or more analysts. Depending on the aim, any one of three main trial types can be used. Method performance or collaborative studies are performed on analytical methods that will be extensively used and which must provide high quality results. In these cases, several laboratories participate in validating the analytical method. The participating laboratories have been inspected, they are known to perform well and it is assumed that their results are highly reliable. They follow the same analytical procedure, which is described in detail and they analyse the same samples to establish the performance criteria. After all the results have been reviewed, the final values of the quality parameters defined are calculated.

To perform a collaborator trial, either the ISO guideline 5725-2 or the IUPAC technical report [26] are good starting points because they define all necessary terms, they specify the optimum number of participating laboratories and samples analysed, and they describe how the study must be performed and how the data must be treated if the method is to be validated. A laboratory proficiency study tests the performance of the laboratory itself. Though it is not always possible, it is advisable to analyse a material, whose true concentration is known, by using the method of analysis that each laboratory considers most suitable for the problem at hand. When the results are compared, appropriate conclusions about the individual performance of each laboratory can be inferred. The ISO/IEC Guide 43-1: 1997 [27] reports a procedure for performing proficiency tests. The last objective when performing an inter laboratory trial is to certify a material. The group of participating laboratories have been proven to be good and reliable, so they analyse a material containing one or more analyst using several methods of analysis to determine the most probable concentration value/s with the minimum uncertainty. Although these studies are not the most commonly used ones, there is an ISO guideline that describes the suitable protocol [28].

Inter laboratory trials are not easy whatever their purpose may be. Collaborative studies need to find enough laboratories that have been proven to perform well. Economical investment is also important so that samples and
materials can be shipped. And the samples themselves can be problematical despite having the ideal composition they are often not stable. And finally, the trials are time-consuming for the organizing laboratory. Because of these drawbacks in inter laboratory trials the alternative of a third laboratory to test method performance is an interesting one. To be more precise, the laboratory which verifies the quality parameters of the method under examination belongs to an institution or has the competence to assess the quality of other laboratories. This option consists of providing the examining laboratory with the quality parameters claimed by the method developer. Then, the examining laboratory must verify if the values provided are correct or if, on the contrary, they must be estimated again. The best example of this in operation is the Peer Verified Methods [29] program of the AOAC International. The International Seed Testing Association (ISTA) [30] also provides a program called Performance Validated Method, which has similar characteristics. Reporting method validation correctly is also an important issue. After the validation procedure, all the actions taken must be clearly and documented. In the same way, the values of the performance criteria must be documented so that any change or variation due to different laboratory conditions can be easily avoided. As is usual in these cases, the ISO has a guideline [31] that describes how standards should be laid out. Written documents also need to be revised all copies must be up-to-date and any uncontrolled copy must be withdrawn.

1.4 IMPURITY PROFILING OF PHARMACEUTICAL COMPOUNDS

Impurity profiling is the common name of a group of analytical activities, the aim of which is the detection, identification/structure elucidation and quantitative determination of organic and inorganic impurities, as well as residual solvents in bulk drugs and pharmaceutical formulations. Since this is the best way to evaluate and standardize the quality and stability of bulk drugs and pharmaceutical formulations, this becomes the core activity in modern drug analysis [32]. In the pharmaceutical field, an impurity is considered as any other organic or inorganic material, besides the drug substance, or ingredients, arise out of synthesis or unwanted chemicals that remains with API. Either the impurity may originate during the synthesis of the drug substance or making of a
formulation or upon aging of both API and formulated API in medicines. Regulatory authorities' world wide like ICH, USFDA, Canadian Drug and Health Agency are emphasizing on the purity requirements and the identification of impurities in Active Pharmaceutical Ingredient (API). The process called qualification of the impurities is acquiring and evaluating data that establishes biological safety of an individual impurity; thus establishes biological safety of an individual impurity; thus, emphasizing the need and scope of impurity profiling of drugs in pharmaceutical research [33 – 36].

1.4.1 Stability

Many solutes readily decompose prior to chromatographic investigations, for example, during the preparation of the sample solutions, extraction, cleanup, phase transfer or storage of prepared vials (in refrigerators or in an automatic sampler). Under these circumstances, method development should investigate the stability of the analyst and standards.

The term system stability has been defined as the stability of the samples being analyzed in a sample solution. It is a measure of the bias in assay results generated during a pre selected time interval, for example, every hour up to 46 hours, using a single solution. System stability should be determined by replicate analysis of the sample solution. System stability is considered appropriate when the RSD, calculated on the assay results obtained at different time intervals, does not exceed more than 20 per cent of the corresponding value of the system precision. If, on plotting the assay results as a function of time, the value is higher, the maximum duration of the usability of the sample solution can be calculated.

1.4.2 Forced Degradation Studies of Pharmaceutical Compounds

Forced degradation studies are conducted to gain better understanding of active pharmaceutical ingredient (API) and drug product (DP) stability and to provide information about degradation pathways, which in turn will facilitate the development of analytical methodology.

A part from its intrinsic stability characteristics every drug molecule has its own characteristic tendency of sensitivity and stability towards its
surroundings, both physical and chemical. Under these external conditions drug molecule may undergo transformation giving rise to degradation products. These susceptibilities have to be ascertained by conducting controlled experiments called “forced degradation studies”. Thus forced degradation study is a process of subjecting drug substance or drug product to extreme chemical and environmental (physical) conditions for a stipulated period of time to determine drug product breakdown levels, preliminary degradation kinetics and to identify degrading species. Forced degradation studies are used for multiple purposes, including demonstration of the specificity of separation methods, gaining insight into degradation pathways, and discernment of degradation products in formulations that are related to drug substances versus those that are related to other ingredients of a formulation. Reliable chemical stability testing data can show how a drug product changes over time with the influence of environmental factors.

The forced degradation study practices that different companies use may vary significantly and can have a serious impact on the analytical methodologies, which are being used. A recent survey revealed that a wide variety of conditions are used for forced degradation studies in the pharmaceutical industry [37-40]. Although forced degradation studies are a regulatory requirement and scientific necessity during development, they are not considered as apart of the formal stability program.

The drug from its initial discovery stage as a new chemical entity (NCE) undergoes various stages of testing called clinical trials namely Phase1, Phase2 and Phase3. To begin these trails and also throughout these studies various regulatory applications and approvals are involved with regulatory authorities. The guidance gives recommendations for conducting studies at various phases of development [41 – 46]. However, the overall questions that were expected to be answered from forced degradation studies will remain same and they are the following:

- What is the chemical stability of the API?
- What physical form should be selected for development?
- Which salt form should be used?
- What are the chemical degradation pathways of the API?
- Do the major degradation products match the metabolites of the API?
- Which formulation should be selected?
- What are appropriate storage conditions for the API and formulations?

The general process of forced degradation study will include various conditions and may require multiple analytical technologies and instrumentation [47-49]. Forced degradation study will begin with designing the experimental protocol of incubating sample under various chemical and temperature conditions. A typical generalized procedure is shown in Fig.1.7.

Certain laboratories were known to use prediction software’s before beginning the studies. When no prediction can be made a more generalized condition, a diverse or a stretched condition can be chosen for the study. Stress studies will be carried out in both liquid and solid conditions. Samples should be analyzed and compared at regular intervals along with a blank and standard drug. Following are the general conditions, considered for forced degradation study of a drug substance.
Design the study protocol
Define samples and conditions
Use predicting software if available

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Experiments
Perform sampling at reasonable intervals

↓

Analytical Methodology
Choose an established method for screening samples

↓

Estimate purity of degradation samples
Study extent of degradation and optimize HPLC conditions

↓

Track and Identify major degradants
Use of MS along with PDA detection recommended

↓

Isolate and Characterize degradants
By Prep HPLC, NMR, HRMS

↓

Conclude degradation by establishing degradant structures and pathways
Documentation

Fig.1.7 General Flow of conducting forced degradation study
1.4.3 Sample preparations/concentrations

Samples can be studied at a concentration of 1 mg/ml, 2mg/ml or 20mg/ml, probably adjusted with strength of the reagent or condition.

1.4.4 Acid conditions

Generally used acid is the 0.1M hydrochloric acid. However, other acids such as sulphuric acid can also be used. Reactions can be carried out at 25°C (RT), 40°C, and 80°C.

1.4.5 Basic conditions

Most vastly used basic condition is 0.1 N NaOH. However, LiOH or KOH can also be used. Reactions can be carried out at 25°C (RT), 40°C, and 80°C. Sampling intervals can be hourly to begin with and can be prolonged up to 8 days if no degradation is observed.

1.4.6 Oxidative conditions

Oxidative stress study can be done at oxygen rich environment or with 3% or 30% hydrogen peroxide. Reactions can be further accelerated by heating at 40°C or 80°C. To enhance reactions a free radical initiator can be used.

1.4.7 Thermal/Humidity

Thermal stability is carried out in solid state at more than 50°C and 75% relative humidity. Samples are incubated for 10 days.

1.4.8 Stability in water

Hydrolysis in water is studied at room temperature (25°C) incubated for 10 days and above 50°C. Samples are incubated for 10 days.

1.4.9 Photo stability

Photo stability is carried out at a wavelength range of 254 nm-800 nm in solution state or solid state with over all illumination not is less than 1.2 million lux hours. In solid state the solid is exposed at a thickness of 1mm spread on a watch glass.
1.4.10 Timelines and intervals of degradation profiling

Standard drug and blank should be analyzed before analyzing actual sample. Initial analysis should probably begin with hourly analysis, however the degradation profiling can continue up to 8 or 10 days. The profiling study can be terminated depending on extent of degradation. Certain compounds can be highly stable till the end of the study. For such compounds, it is not necessary to extend the study or it is not necessary to apply harsh conditions than the normal. Sampling intervals can be hourly to begin with and can be prolonged up to 8 to 10 days. The initial samples for analysis should be diluted and/or processed to suit the analytical methodology.

1.5 VALIDATION OF STABILITY INDICATING HPLC METHODS

The HPLC method developed may have some intrinsic problems which cannot be taken in pharmaceutical industry as the products assessed by faulty to the market and were administered to already ill patients. And also the methods need to be transferred to different laboratories once they are developed, where a less skilled analyst may be executing the methods to analyse the samples. Hence the methods need to be tested and proven for their accuracy, precision, rugged and robustness before they are implemented or transferred to other laboratories. The whole validation process is done by an expert supervisor. Thus, “validation is the process by which it is established by laboratory studies, that the performance characteristics of the method meet the requirements for the intended application”.

There are various guidelines and general protocols available for various types of method validation. Among various parameters that are evaluated during validation, certain tests may not require all the parameters to be validated (Table 1.1). Following are the types of tests:

Type-I-assay and identification of the main component they are (active ingredient, preservative, key excipient etc.)

Type-II-assay and identification of impurities

Type-III-Limit tests
### Table 1.1

Various tests and the corresponding validation parameters

<table>
<thead>
<tr>
<th>Validation parameters</th>
<th>Type-I</th>
<th>Type-II</th>
<th>Type-III</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mandatory Validation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Accuracy/Precision</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>System repeatability</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Analysis Repeatability</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Intermediate precision</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Reproducibility</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Detection limit</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Quantitation limit</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Linearity</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Range</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Robustness</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Additional Validation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stability of solutions</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>System suitability</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

### 1.5.1 Accuracy

The accuracy of an analytical method expresses the closeness of agreement between the obtained values or as an accepted reference values. Accuracy is expressed as the percentage recovery. Generally it is performed to test three replicates per level at a minimum of three concentration levels across the specified experimental range.
1.5.2 Precision

The precision of an analytical method expresses the closeness of agreement of the same homogeneous sample under the prescribed conditions in the method.

1.5.3 Intermediate precision

Intermediate precision expresses within laboratory variations for e.g. different days, different analysts, different equipment etc.

1.6 ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (UPLC)

Ultra high performance liquid chromatography (UPLC), with its shorter analysis times, narrow peak widths and enhanced peak heights, has not been widely accepted in our drug metabolism laboratories due to the requirement for better performing radioactivity flow detectors. In drug metabolism studies, radio labelled compounds are commonly used to provide a complete metabolite profile. In this study we present an alternative to radioactivity flow detection to screen samples for the presence of metabolites to identify by mass spectrometry. Isotope pattern directed mass chromatograms were generated by software in development by Cerno Bioscience. The narrow chromatographic peak widths (3-6 s) from UPLC analysis were preserved in the isotope pattern directed chromatogram that was diminished by the radioactivity flow detector. Isotope ratios more distinguishable from those of non-related sample components provided the best results.

In recent years, significant technological advances have been made in particle chemistry performance, system optimisation, detector design, and data processing and control. When brought together, the individual achievements in each discipline have created a step-function improvement in chromatographic performance. Ultra performance liquid chromatography (UPLC) is a new category of analytical separation science that retains the practicality and principles of HPLC, while increasing the overall interlaced attributes of speed, sensitivity and resolution. During the past 30 years or so, high performance liquid
chromatography (HPLC) has proven to be the predominant technology used in laboratories worldwide. One of the primary drivers for the growth of this technique has been the evolution of the packing materials used to effect the separation. The underlying principles of this evolution are governed by the Van Deemter equation, which any student of chromatography is familiar with [50].

Increasing demand for greater pharmaceutical analysis throughput prompted the testing of the waters ACQUITY Ultra Performance LC (UPLC™). This system claims to provide faster analyses through the use of a novel separation material of very fine particle size (1.7 μm) and unique core chemistry [51-55]. To effect fast separations on this material, the column hardware and instrument have significant design modifications from typical HPLC. The UPLC operates at higher pressures (up to 15,000 psi.), injects samples into a smaller system dwell volume, and captures detector signals at high data rates for fast eluting peaks. A new needle design has been claimed to substantially reduce carryover which can aid in the lowering of limits of quantisation (LOQ).

1.7 AIMS AND OBJECTIVES

The present study has proposed the following aims and objectives.

1) A new HPLC method was developed for selective and simultaneous determination of Rilpivirine. The developed method is also applicable for the related substances determination in bulk drugs.

2) The forced degradation and solid state stability indicating study and Validation of method for the determination of Assay of Artesunate by HPLC.

3) A Novel method developed ilaprazole by Ultra Performance Liquid Chromatography (UPLC). It is used to treat drug for in the treatment of duodenal ulcers and provide some characteristics of the dose-response relationship for later studies.
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