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Principles of Chromatography Method Development

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Abstract

This chapter aims to explain the key parameters of analytical method development using the chromatography techniques which are used for the identification, separation, purification, and quantitative estimation of complex mixtures of organic compounds. Mainly, the versatile techniques of ultra-/high-performance liquid chromatography (UPLC/HPLC) are in use for the analysis of assay and organic impurities/related substances/degradation products of a drug substance or drug product or intermediate or raw material of pharmaceuticals. A suitable analytical method is developed only after evaluating the major and critical separation parameters of chromatography (examples for UPLC/HPLC are selection of diluent, wavelength, detector, stationary phase, column temperature, flow rate, solvent system, elution mode, and injection volume, etc.). The analytical method development is a process of proving the developed analytical method is suitable for its intended use for the quantitative estimation of the targeted analyte present in pharmaceutical drugs. And it mostly plays a vital role in the development and manufacture of pharmaceuticals drugs.

Keywords: analytical method development, ultra performance liquid chromatography (UPLC), high-performance liquid chromatography (HPLC), assay, impurities, impurity profiling study, forced degradation study

1. Introduction

It is well known that chromatography is a laboratory technique used for separation and quantification of complex organic mixtures which cannot be separated effectively by other purification techniques. The constituents of a mixture dissolved in solvent get separated gradiently according to their affinities to the stationary phase with the help of mobile phase one after another. Chromatography is invented by *Mikhail Semenovich Tswett* in 1903 during his research on plant pigments such as chlorophylls, xanthophylls, and carotenoids [1] which got extended for analyzing organic molecules of different kinds especially pharmaceutical from the year 1920 [2]. Invention of chromatography made the jobs of organic chemist and the whole industry relying on them especially pharma industry easier. Keeping in mind the various fields where this technique has been used, this chapter focuses on the use of chromatography in pharmaceuticals for separating the drug (API) mixture in particular.

Nowadays, many different kinds of chromatography techniques, such as thin-layer chromatography (TLC), paper chromatography, and liquid chromatography (e.g., HPLC, UPLC, and preparative HPLC), supercritical fluid chromatography, and gas chromatography (GC)) have been designed and utilized for the separation

and purification of pharmaceutical drugs [3]. In this chapter, the authors discuss the principles for chromatography method development using ultra/high-performance liquid chromatography (UPLC/HPLC) techniques for the analysis of assay and organic impurities/related substances/degradation products of pharmaceuticals (any drug product/drug substance/intermediate/raw material of pharmaceuticals). These techniques are developed substantially as a result of the work of *Archer John Porter Martin* and *Richard Laurence Millington Synge* during the 1940s and 1950s, for which they won the 1952 Nobel Prize in Chemistry [4]. Commonly used characterizing technique in pharma industry is liquid chromatography (e.g., HPLC, UPLC, and LC-MS). Each one varies in the stationary phase and operational conditions. HPLC and UPLC can be used as a quantitative technique if coupled with a mass detector (MS) to elucidate the structure of the molecule and quantification.

In pharma industry specific, stability-indicating HPLC/UPLC methods have to be developed to estimate the assay and to quantitatively determine the impurities of new drug substances and drug products [5]. Assay is a quantitative test of a substance to determine the amount of an individual components present in it. Impurity is an unknown component of drug substance that is not the chemical entity. Assay and impurity tests are major and critical quality attributes of the pharmaceutical dosage forms which help to check and ensure the quality, safety, and efficacy of drug substances and drug products. This chapter will discuss the various parameters that have to be chosen to run the chromatography in order to have a better separation and maximum purity. The process of changing the conditions in order to design a best method run for a particular drug mixture or compound is called the analytical method development.

2. Analytical method development

Analytical method development is a process of proving that the developed chromatography method is suitable for its intended use in the development and manufacturing of the pharmaceutical drug substance and drug product. The basic separation techniques and principles involved in the analytical method development using the HPLC and UPLC are listed as follows:

- Selection of chromatography mode
- Selection of detector
- Selection of column (stationary phase)
- Selection and optimization of mobile phase
 - Buffer and its strength
 - pH of buffer
 - Mobile-phase composition
- Selection of organic modifiers
- Selection of ion-pair reagents
- Selection of flow rate

- Selection of solvent delivery system (elution mode)
- Selection of diluent
- Methods of extraction
- Samples to be used
- Experimentation to finalize the method
- Selection of test concentration and injection volume
- Forced degradation studies (stress testing)
- Evaluation of stress testing
- Mass balance study
- Finalization of wavelengths
- Stability of solution
- System suitability
- Robustness of the method
- Relative response factor
- Quantification methods

2.1 Literature search

- i. Before starting an analytical method development, literature on some of the column characteristics as mentioned below has to be referred for the target molecules or similar molecules or precursors from open resources like articles, books, pharmacopeia reports, etc. This will give a tentative choice in designing a method for initial or test experiments, which will be further modified or updated to develop a method which fits the separation process for better results in terms of reproducibility, quantification, etc. *Solubility profile* of drug substance in different solvents at different pH conditions is useful while selecting the diluents for standard solutions and extraction solvents for test solutions.
- ii. *Analytical profile* is useful in understanding the physicochemical properties (e.g., *pKa*, melting point, degradation pathways, etc.) and absorption characteristics of drug in selecting the detector wavelength for analysis.
- iii. *Stability profile* of the drug substance with respect to storage conditions (sensitivity of the drug towards light, heat, moisture etc.) is useful as it helps in adopting the suitable/adequate precautions while handling drug and its formulated products.
- iv. *Impurity profile* collects the information of impurities and degradation profile of the drug substance during their formation pathways. This helps

a lot in developing the method for separation of all possible impurities and degradation products of targeted analyte. It should be borne in mind that impurity profile may vary depending on the manufacturing process (which uses different methods, precursors, and conditions), which makes it clear that not all manufacturing processes yield the same impurity profile.

- v. *Metabolic pathway* is a chemical reaction which occurs within a cell when the drug molecule reacts with an enzyme and forms a metabolite [6]. Metabolic pathway gives the information on oxidation, reduction, and hydrolysis products which gives critical inputs on the possible degradation products.
- vi. *Stability-indicating method* is to identify the closely related structures by collecting the structures of the molecule and its impurities and degradation products. This helps to develop a specific and stability-indication method with a good resolution between the closely related structures.
- vii. *Checking the polarity* of the drug molecule using the functional groups as elucidated from structural analysis techniques. By comparing the structures of impurities and degradation products with the structure of drug molecule, it will help in understanding the polarity based on the nature of functional groups. This makes the scientists' job easy in choosing the right solvents with either lesser or higher in polarity than the compound of interest.
- viii. *Estimation of maximum daily dose (MDD)*. Calculate the reporting, identification, and qualification thresholds of drug substance and drug product based on the maximum daily dose as per ICH Q3A guideline [7, 8]. MDD info can also be obtained from physical desk reference (PDR), innovator product information leaflet (PIL), and the website of RX-list (www.rxlist.com).

2.2 Selection of chromatography mode

Chromatography can be operated by two ways, normal mode and reverse phase modes. The choice of the mode is very important, which is dependent on the type of sample which has to be separated. In general, the usage of reversed-phase chromatography (in which the mobile phase is polar and stationary phase is nonpolar in nature) is the preferred mode for most of the molecules, except in the case of isomer (enantiomers) separation where the normal-phase chromatography (in which the mobile phase is nonpolar and stationary phase is polar in nature) is used. Reversed-phase chromatography separates the components with a good resolution based on their hydrophobicity. A compound with a greater polarity elutes earlier, and those with the least polarity elute later.

2.3 Selection of detector

Detector plays an important role in the finalization of any analytical method. Generally most of the organic/drug molecules are aromatic or unsaturated in nature, which has an absorption in the UV-vis region. This comes as an advantage in quantifying and analyzing the molecules and its associated impurities. The absorbance maxima of the compound shall be collected by analyzing the UV-vis spectrophotometer or diode array detector (DAD) of HPLC/UPLC. From the area intensity of the test compound using calibration curves, the quantification of the test compound can be done [9–10].

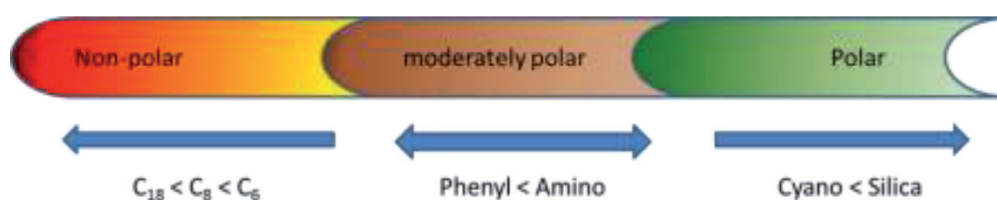
If the compounds do not absorb and if they do not have chromophores, other detectors like refractive index detector (RID) and evaporative light scattering detector (ELSD)/corona-charged aerosol detector (CAD) can be used for the quantitative determination of assay and impurities [11]. If the compounds of interest contain a part, which is non-chromophoric, which may likely be cleaved and produce a non-chromophoric impurity, then both UV and other detectors like RI/ELSD/CAD can be coupled in order not to miss any impurity.

Alternatively, non-chromophoric compounds can also be analyzed by UV after converting it into a derivative which will be active. But the usage of derivatives has to be carefully assessed keeping in view the functional group involved in the derivatization reaction [12, 13]. In case the molecule of interest is having fluorescence properties, a fluorescence detector (FLD) can be used for compounds for which structural information is available [14]. But when FLD is to be used for estimation of unknowns, it needs to be carefully assessed whether fluorescence properties are available in all possible impurities and degradation products.

2.4 Selection of column stationary phase

The choice of the right column (stationary phase) is the basis of the whole technology. Most chromatographic separations are achieved due to a wide variety of columns available in the market and due to their flexibility in changing and controlling the parameters. A widely used choice of column material is silica either as neat or modified depending on the nature of the solute mixture in normal-phase chromatography, wherein the eluent (mobile phase) is nonpolar an organic solvent. The silanol groups on the surface of the silica give it a polar character.

Though silica remains the most common support for liquid chromatography (LC) columns, other commonly used materials are cross-linked organic polymers, zirconia, etc. The silica support for columns was gradually modified for the betterment through the years by three different manufacturing technologies commonly described as “evolution through three generations.” The initial process started with type A silica where the raw material used is from inorganic sols. A slightly modified type A silica by performing a chemical treatment to remove the metal impurities is termed as a second-generation material which is called as base-deactivated silica. Third generation silica (type B) is an altogether new process which uses organic sols instead of inorganic sols. These materials are similar in properties to the second-generation silica because both have a minimum level of metal impurities. Silica-based liquid chromatography columns with a different percent of cross-linking and functionalization of silanol groups with substituted aliphatic and aromatic moieties were designed for varying polarities of the separating medium. An increasing order of functionalized silica is represented below with alkyl groups at the nonpolar end, phenyl and amino functionalized in the moderate polar region, and cyano and silica groups at the polar end.



The following are the parameters of a chromatographic column which need to be considered while choosing a column (stationary phase) for separation of assay, impurities, and degradation products:

i. Length and diameter of column

ii. Packing material

iii. Shape of particles

iv. Size of particles

v. Percent (%) of carbon loading

Column dimension: Length and internal diameter of packing bed.

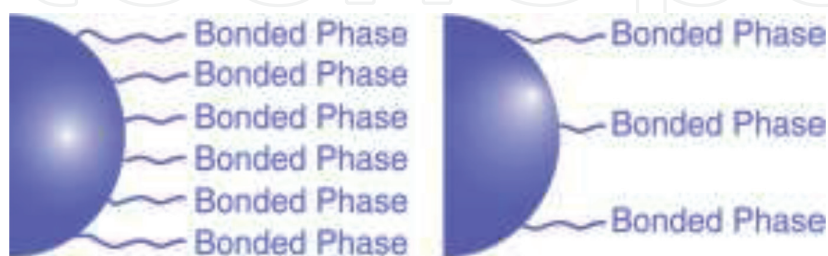
- Short (30–50 mm)—can result in short run times and low back pressure
- Long (250–300 mm)—can result in higher-resolution and long run times

A column with a diameter of 2.1 mm leads to a high resolution.

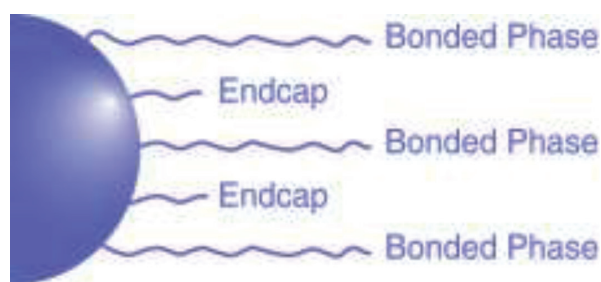
Particle size: Decrease in particle size leads to increase in resolution but with a corresponding increase in back pressure. In general smaller particles offer higher efficiency, but there is a chance to get high back pressure limiting the separation efficiency. Less (3 μm) particles are usually used for resolving complex and multicomponent samples, where the lesser surface area induces better resolution and separation characteristics.

Pore size and surface area: Larger pores allow larger solute molecules to be retained for a longer time through maximum surface area exposure. High surface area generally provides greater retention, capacity, and resolution for multicomponent samples. Low surface area materials generally equilibrate quickly and provide lesser separation efficiency but can be highly preferred and important in gradient analyses.

Carbon loading: Higher carbon loads generally offer greater resolution and longer run times. Low carbon loads shorten run times, and many show a different selectivity. A pictorial representation of difference in carbon loading is as shown below.



End capping: End capping reduces peak tailing of polar compounds that interact excessively with the otherwise exposed, mostly acidic silanols. Non-end capped packing provides a different selectivity than do end-capped packing, especially for polar compounds. A pictorial representation of difference in end capping is shown below.



2.5 Selection and optimization of mobile phase

Though adsorption is the principle behind chromatography, real separation happens only when the adsorbed compound is eluted using a mobile phase of the required polarity. The selection of mobile phase is done always in combination with the selection of column (stationary phase). The following are the parameters which shall be taken into consideration while selecting and optimizing the mobile phase.

- The right choice of buffer and its eluting efficiency
- pH of the buffer or pH of the mobile phase
- Mobile-phase composition inclusive of binary and tertiary solvent mixture

2.5.1 Buffer and its strength

Buffer and its efficiency play an important role in deciding the peak symmetries (shapes) and peak separation. Various types of organic/inorganic buffers are employed for achieving the required separation. The most commonly used buffers are:

- Phosphate buffers— KH_2PO_4 , K_2HPO_4 , NaH_2PO_4 , Na_2HPO_4 , H_3PO_4 , etc.
- Acetate buffers—Ammonium acetate, sodium acetate, etc.
- Triethylamine/diethylamine buffers
- Buffers with various ion-pair reagents like tetrabutyl ammonium hydrogen sulfate, butane sulfonic acid, hexane sulfonic acid, heptane sulfonic acids, etc.

The choice of buffer is to reduce the tailing factor for each peak separated which occurs due to varying ionic strength. The retention time of analyte(s) is delayed and got separated well when more concentrated buffer is used [15]. Better separation happens when the molarity of buffer used is in the range of 0.05 to 0.20 M. The concentration of buffer is chosen by carefully choosing the composition of organic mobile phase.

Depending on the need of the chosen mixture of separation, the strength of the buffer can be increased or decreased if necessary to achieve the required separation, and it can be varied between 10 and 20%, and the effect of variation has to be studied in detail before using. But it should be ensured that increased or decreased buffer strength should not result in precipitation or turbidity either in mobile phase during operation or during storage in refrigerator. Before using the chosen buffer of specific strength to run a column, test experiments have to be done in optimizing the separation to avoid peak tailing, better separation, and reproducibility.

2.5.2 pH of buffer

pH plays an important role in achieving the chromatographic separations as it controls the elution properties by controlling the ionization characteristics. The pH of buffer or mobile phase should be selected based on the pK_a of analyte or test mixture, which is based on the structure of the molecule. Depending on the pK_a , drug molecules change retentions, e.g., acids show an increase in retention as the pH is reduced, while the base shows a decrease. If the pK_a of the compound is high, lower pH or acidic mobile phase has to be chosen as it will stop unwanted association with the stationary phase. For basic compounds, the use of high pH or basic mobile phase and, for neutral compound, neutral mobile phase is highly preferable for better separation.

It is important to maintain the pH of the mobile phase in the range of 2.0 ~ 8.0 as most columns do not withstand the pH which is outside this range. This is due to the fact that the mostly used silica column gets deactivated at high pH (>2) and at low pH (<8) due to cleavage of siloxane linkages. If a pH outside the range of 2.0 ~ 8.0 is found to be necessary, stationary phase which can withstand the range shall be chosen [16–18].

2.5.3 Mobile-phase composition

It is well reported in literature that to achieve better efficiency, binary and tertiary solvent mixtures are used along with other components like buffer and acids or bases. The ratio of the organic versus (vs.) aqueous or polar vs. nonpolar solvents is varied accordingly to get better separation. This is due to the fact that a fairly large amount of selectivity can be achieved by choosing the qualitative and quantitative composition of aqueous and organic portions. Experiments shall be conducted with mobile phases having buffers of different pH and different organic phases to check for the best separations between the impurities. Most chromatographic separations can be achieved by choosing the optimum mobile phase composition [18].

2.6 Selection of organic modifiers

Most widely used solvents in reverse-phase chromatography are methanol and acetonitrile. Tetrahydrofuran (THF) is also used but to a lesser extent [19, 20]. In most of the systems, acetonitrile is used as the default organic modifier because of favorable UV transmittance and low viscosity. It is recommended to mix acetonitrile with 5–10% of the aqueous solution(s) to avoid the pumping problems associated with a higher percent (%) of acetonitrile usage. Methanol is also the second most widely used solvent in liquid chromatography, but it gives the back pressure to LC column. Though THF has some disadvantages like higher UV absorbance, reactivity with oxygen, and slower column equilibration, sometimes it gives very unique selectivity for closely eluting peaks. Intermediate selectivity (if needed for a particular sample) can be obtained by blending appropriate amounts of each of these solvents.

Order of polarity: methanol > acetonitrile > ethanol > THF > propanol.

Order of solvent strength: propanol > THF > ethanol > acetonitrile > methanol.

2.7 Selection of ion-pair reagents

Ion pair reagents are necessary as a mobile-phase additive when structurally or chemically or polarity wise inseparable closely related compounds are to be separated [21, 22]. For example, if a mixture of ionic and nonionic analyte(s) having the same polarity and same retention time is required to be separated, start by optimizing for one of the analytes by adding an ion pair reagent in a mobile phase which

reduces or increases the polarity of component and helps in increasing the elution time difference. Careful choice of an appropriate ion-pair reagent is required in such cases to get the necessary selectivity. A dedicated LC column is used when an ion pair reagent (0.0005 M to 0.02 M) is intended to employ for specific analysis, but an appropriate cleaning procedure has to be established to enhance the lifetime of the column material. Alkyl ammonium salts (tertiary or quaternary) and alkyl sulfonate salts are the most useful in the separation of acidic and basic compounds, respectively. Sodium perchlorate can also be used for acidic components.

2.8 Selection of flow rate

Separation of mixtures is highly influenced by the flow of mobile phase inside the column [23, 24]. The flow rate is highly crucial in having well-separated peaks with no tailing. The flow rate of the mobile phase can be optimized based on the retention time, column back pressure, and separation of closely eluting adjacent peaks or impurities and peak symmetries from the test run. Preferably the flow rate is fixed not more than 2.0 mL/minute. The flow which gives the least retention times, good peak symmetries, least back pressures, and better separation of adjacent peaks/impurities could be the chosen as an optimized flow rate for the analysis.

2.9 Selection of column temperature

Temperature is another criterion which has to be optimized for any sample, as the flow rate and the rate of adsorption vary with temperature. It is generally believed that with increasing temperature, it can help to improve the resolution between the adjacent/closely eluting peaks and peak merging. So a careful choice of the temperature is a must which might change the pressure of the column and ultimately the elution and resolution [25–28].

Choosing ambient temperature for the analysis is always preferred as it will minimize the degradation of the test sample; however, higher temperatures are also advisable under unavoidable conditions after confirming the stability of the compound. The temperature range which is usually allowed in liquid chromatography is 25 and 60°C. Higher temperatures above 60°C are preferred if the peak symmetry is not good and to increase the retention time for closely occurring peaks.

2.10 Selection of solvent delivery system (elution mode)

Chromatographic separations with a single eluent (isocratic elution: all the constituents of the mobile phase are mixed and pumped together as a single eluent) are always preferable. However, the gradient elution is a powerful tool in achieving separation between closely eluting compounds or compounds having narrow polarity difference [29–31]. An important feature of the gradient elution mode which makes it a powerful tool is that the polarity and ionic strength of the mobile phase are changed (increased or decreased) during the run. Experiments using different mobile-phase combinations and different gradient programs have to be performed prior to achieving better separation.

In a gradient run, two mobile phases which have different compositions of polar and nonpolar solvents are premixed using a single pump before introducing to the column which is called as *low pressure gradient (LPG)*, and when the mobile phases are pumped at different flow rate and mixed in a chamber, then introduced into the column is known as *high pressure gradient (HPG)*. It is better to select the gradient run, whether *LPG* or *HPG*, while optimizing the chromatography method. *HPG* can be only preferred for use when more than 80% organic

phase is pumped. To avoid the pumping problems due to the low viscous solvents like acetonitrile in mobile phase, at least 10% aqueous portion could be added to the organic phase.

While optimizing the gradient program, it is important to monitor the following. Pressure graph is needed to be monitored so as to ensure that the overall system pressure will not cross 400 bar or 6000 psi at any point during the run. Flow rate has to be physically cross-checked by collecting the output from the detector during the run at different time intervals, especially when the gradient is running with higher organic-phase composition so as to ensure that there were no pumping problems during the run when mobile phases of different compositions are pumped. It is also important to optimize the program for initialization after each run and before going for the next injection. The program for initialization shall be optimized such that there shall be no carry-over to the next run and the system stabilizes with initial composition before the next injection.

One standard program which can be used for optimizing is discussed below. For starting a method development, a solvent gradient system is always preferred. Initially, start with a gradient of 50:50 buffer and mobile phase, and change the program linearly up to 5:95, and retain the ratio for at least 30 minutes. Then try with a gradient of 95:5 and the program linearly changed up to 5: 95, and retain for at least 30 minutes. The typical gradient program is as follows:

2.11 Selection of diluent

Diluent is an aqueous solution or a solvent used to dissolve and extract the drug moiety for analysis. Select a diluent in which impurities, starting material, by-product, intermediates, degradation products, and the analyte are soluble. It is advisable to check first in the mobile phase. All the analytes should be completely soluble and the solution should be clear [32]. Diluent should be compatible with the mobile phase to obtain the good peak shape.

- Selection of diluent based on extraction efficiency and peak shapes: Select the diluent for finished dosage forms, in which the analyte should be extracted at least 95% for assay and 90% for organic impurities. Calculate the % extraction against pure standard compound in the concentration of linear range, (preferably <1 AU) by diluting the test preparation.
- The peak shapes of all compounds should be good in the selected diluent: Select an initial flow rate of 1.0 mL/min or 1.5 mL/min and select column temperature as ambient (25–30°C).

Diluent is selected initially based on solubility of the substance. However, the finalization of diluent is based on its extraction efficiency, peak symmetries, resolution of impurities, and diluent blank injection interference. Inject the diluent blank and test solution spiked with known impurities into the chromatographic system, and establish the noninterference of blank in estimation of the drug and the effect of diluent on resolution of impurities from drug peak and peak symmetry.

2.12 Methods of extraction

General methods followed for extraction are sonication, rotary shaking, or seldom both. In some cases where the analyte cannot be extracted by the above

procedures, heating can be adapted if the substance is stable and should not precipitate upon cooling to room temperature [33–34].

2.13 Samples to be used for analysis

- Use mixture of impurities, starting material, by-product, intermediates, and degradation products to establish the separations.
- Use the reaction mass/mother liquor/what if study samples for the above study if all the impurities samples are not available in the beginning for method development.
- Use forced degradation samples, if degradation products are not available in the case of drug API.
- Prepare a mixture of known impurities spiked on API at a test concentration of about 0.5 or 1.0 mg/mL.
- Prepare a placebo (mixture of excipients to be used in formulation) solution at a concentration equivalent to test concentration (of about 0.5 or 1.0 mg/mL) for the dose in which higher placebo content is expected.

2.14 Experimentation to finalize the method

Inject individual solution of standard and impurities to confirm the retention times. Check for the interference from blank. Check for the interference from placebo components in the case of the formulation.

Gradient program will provide an assessment of the elution pattern of polar and nonpolar impurities. Also, run an isocratic run with a mobile phase of a buffer with a suitable pH and acetonitrile in the ratio of acetonitrile: buffer (90:10) using a 250 × 4.6 mm, 5 µm silica column. This will help to know whether any highly nonpolar impurities are still un-eluted from the column.

2.15 Selection of test concentration and injection volume

The test concentration and injection volume are generally chosen based upon the response of API peak at the selected detector wavelength [35]. However, the test concentration shall be finalized after it is proven that drug (API) is completely extractable at the selected test concentration. After finalizing the test concentration and diluent, prepare a test solution, and keep the filtered solution in closed condition on a bench top, and check whether the solution has any precipitation or turbidity after 24 hours. Generally, the test solution must be clear and should not show any turbidity or precipitation.

2.16 Forced degradation studies (stress testing)

It's a method of subjecting the drug substance or drug product to stress with varied strengths of stressing agents to obtain the degradation. The stressed samples were analyzed using an LC system equipped with a PDA detector and monitored for the separation of degradation products formed under the stressed conditions and the peak purity of the analyte peak. The method is considered as stability-indicating for the estimation of the drug if it meets the peak purity requirement [36, 37].

Forced degradation studies are conducted basically to meet the following objectives:

- To investigate the likely degradation products; this, in turn, helps to establish the degradation pathways and the intrinsic stability of the drug molecule.
- To provide a foundation for developing a suitable stability-indicating method.
- Ensure the force degradation limit of 2–20%.

The major forced degradation studies which are to be carried out are as follows:

a. Thermolytic degradation

This stress testing method studies the degradation that is caused by exposure to temperature high enough to induce bond breakage. Solid-state reactions often proceed in an autocatalytic pathway involving an induction period (lag), followed by a period of rapidly increasing degradation and then slowing down of the degradation rate as the compound is consumed. Thus, solid-state reaction kinetics will often follow an S-shaped curve when degradation vs. time is plotted. Thus, before conducting thermolytic degradation, determine the melting point of the compounds of interest. Then, choose a temperature of 70°C for all the drugs for which melting point is <100°C, or choose a temperature which is 40°C below the melting point.

For the compounds for which melting point is >150°C, stress the samples at 105°C. Keep the samples directly exposed in the oven for 1 week or until about 2–20% degradation is achieved, whichever is earlier. Stress the drug substance, placebo, and drug product separately. In the case of the multicomponent drug products, stress testing of placebo with other actives excluding the one at a time shall be performed additionally.

b. Hydrolytic degradation

Drug degradation that involves hydrolysis reaction is called hydrolytic degradation. Hydrolysis reactions are typically acid or base catalyzed. Acidic, neutral, and basic conditions should therefore be employed in order to induce potential hydrolytic reactions. As these hydrolytic stress studies are to be conducted in aqueous solutions, solubility of the drug molecule of interest in water has to be estimated first. Many small molecule drugs are not soluble in water at the concentrations typically used for analytical evaluations (0.1 to 1 mg/mL); in those cases either a slurry or suspension must be used to examine the hydrolytic stability of a compound, or a cosolvent must be added to facilitate the dissolution under the conditions of low solubility. Two most commonly used cosolvents are acetonitrile and methanol. Methanol has the potential of participating in the degradation chemistry which has to be used with caution especially under acidic conditions when the compound being tested contains a carboxylic acid, ester, or amide.

Acetonitrile is generally regarded as inert solvent and is typically preferable to methanol in hydrolytic stress testing studies. However, acetonitrile is not completely inert and can participate in the degradation reactions, leading to artificial degradation results.

The other cosolvents that are recommended for the hydrolytic stress testing studies are shown below.

Acidic pH	Neutral pH	Basic pH
Acetonitrile	Acetonitrile	Acetonitrile
DMSO	N-methyl pyrrolidine	DMSO
Acetic acid		Diglyme
Propionic acid		p-Dioxane

The hydrolytic degradations (using water/0.1 M HCl/0.1 M NaOH with or without cosolvent) are recommended to be performed at a temperature of about 70°C with a reflux condenser installed to avoid the loss of evaporation. Reflux until about 2–20% degradation is achieved. Stress the drug substance, placebo, and drug product separately. Neutralize the stressed solutions before injection. Prepare a stressed solution at a higher concentration than that of test concentration. In the case of the multicomponent drug products, stress testing of placebo with other actives excluding the one at a time shall be performed additionally.

c. Humidity stress

Stress the samples to 90% humidity for 1 week. Stress the drug substance, placebo, and drug product separately. In the case of the multicomponent drug products, stress testing of placebo with other actives excluding the one at a time shall be performed additionally.

d.Oxidative degradation

Oxidative degradation is one of the most common mechanisms of drug degradation. Oxidative drug degradation reactions are typically autoxidative, that is, the reaction is radical initiated. Radical initiated reactions start with an initiation phase involving the formation of radicals followed by propagation phase and eventually a termination phase. Thus, the reaction kinetics will often follow S-shaped curve when the degradation vs. time is plotted and will not follow Arrhenius kinetics.

In oxidative stress study, the use of temperature > 30°C is not recommended because the reaction rate in solution may reduce at higher temp due to the decrease in oxygen content of the solvent. Thus, it is always suggested to perform the degradation with 3% hydrogen peroxide at room temperature (25–30°C) with constant stirring in the dark. Stress for 24 hours or until about 1–20% degradation is achieved or whichever is earlier. Stress the drug substance, placebo, and drug product separately.

In the case of the multicomponent drug products, stress testing of placebo with other actives excluding the one at a time shall be performed additionally.

e. Photolytic degradation

Photolytic degradation is the degradation that results from exposure to UV or visible light. Expose the samples to 3 times to 1.2 million lux-hr visible and 200 W-hr/m² UVA. Stress the drug substance, placebo, and drug product separately. In the case of the multicomponent drug products, stress testing of placebo with other actives excluding the one at a time shall be performed additionally.

2.17 Evaluation of stress testing

Peak purity can be evaluated for the main peak and the major degradants which have the peak heights less than 1 AU. Identify the degradation products by co-injection, in case of known impurities and have comparable spectra.

If any known impurity is observed to be increased in stress, it can be examined properly. If process impurity is found to be increased in stress study, it needs to be assessed whether there is any secondary pathway of formation of this impurity via some other degradant route.

After conducting these studies, verify the chromatograms, and observe any peaks merging with respect to main peak and any critical pairs. If any situations were arrived, adjust the mobile-phase compositions, column parameters, etc. and conclude the method parameters.

After method finalization, check the method using different detectors (RI/ELSD/CE/LC-MS), and compare the data with other detectors like UV, fluorescence, etc. The UV inactive components can be found with these experiments. Identify the mass of major degradant which may be formed greater than 1.0% in stress studies, and try to establish the structures.

2.18 Mass balance study

Mass balance is a process of adding together the assay value and levels of degradation products to see how closely these add up to 100% of the initial value. It is important to have methods that detect all major degradation products. This is generally done by performing the assay of forced degraded samples and assesses the mass balance. Mass balance has to be achieved at least up to 95% level. If it is less than the required criteria, investigation has to be done and justified. The following are some of the reasons for not achieving the mass balance.:

- Degradation products are:
 - Not eluted from the LC column
 - Not detected by the detector used
 - Lost from the sample matrix, due to insolubility, volatility, or adsorption losses
 - Co-eluted with the parent compound
 - Not integrated due to poor chromatography
- Parent compound may be lost from the sample matrix, due to insolubility, volatility, or adsorption losses
- Inaccurate quantification due to differences in response factors

2.19 Detector wavelengths

After separation of all impurities and degradation products, absorption spectra of all the compounds are recorded and compared by taking overlay spectra of all known impurities along with the main analyte in each stress condition and finalizing a wavelength where all impurities are detected and quantified and have the

maximum absorbance. In case this is not feasible, select different wavelengths to estimate all impurities. It is also recommended to extract the chromatograms at lower wavelengths like 210 nm–220 nm to see if there is any additional impurities found, which are found to be missing at higher wavelengths; this is likely the case when parent compound breaks into two parts during forced degradation study with one part highly UV active and second part an alkyl chain where alkyl chain will have poor UV character.

2.20 Stability of analytical solutions

The stability of analytical solutions (sample or standard) can be established on auto-injector for at least 12 hours continuously in a sequence mode to know the stability of all components and ruggedness of the method (peak shapes, column back pressure over the period of time). To get better results, choose a diluent in which a test solution is stable for at least 12 hours. If the solution is found to be unstable by its nature, then incorporate the stability of solution in test method.

2.21 System suitability

System suitability tests verify and ensure whether the system's performance is acceptable at the time of analysis in accordance with the criteria set forth in the procedure or not. System suitability parameters are chosen based on the criticality of separation. In general, resolution factor for the two adjacent peaks or closely eluting peaks is selected as a system suitability requirement. If the separation of impurities from each other and from API peak is found to be satisfactory, there is no need to keep a resolution factor as a system suitability parameter. In such a case, only a diluted standard reproducibility can be adopted as a system suitability requirement. Before finalizing the system suitability parameters, the separation needs to be studied during the robustness study to understand its behavior during the various deliberate changes in method.

System suitability checking must be performed on two different make of HPLC systems whenever the separation of any impurities is critical. For in-process-related impurity issues, the quantification limit (QL) concentration is to be injected, and signal to noise ratio (S/N) must be kept as a system suitability parameter.

2.22 Robustness of the method

Robustness by definition means the reliability of an analysis with respect to deliberate variations in method parameters. After finalizing all chromatographic conditions, robustness study with regard to mobile phase composition ($\pm 10\%$), pH (± 0.2), gradient ($\pm 0.2\%/min$), flow rate ($\pm 0.2\text{ mL/min}$), and temperature ($\pm 5^\circ\text{C}$) can be carried out to ensure that the developed method is stability-indicating. If the method of analysis is in a gradient mode, it needs to be checked on two different brands of HPLC or different HPLC to check the effect of the system volumes on separations.

2.23 Relative response factor

The relative response factor is used to correct the difference in the detector response of impurities with respect to the main analyte peak. It is mainly used to control the impurities or degradation products in a drug substance or drug product. RRF is established for all the known impurities using any of the slope methods. The standard solutions of API and all impurity can be prepared in at least five different

concentrations in the range of 0.1–1.0% (e.g., 0.1, 0.3, 0.5, 0.7, and 1.0%) and analyzed using the liquid chromatography. RRF is calculated by using the slope of the respective impurity and slope of the main drug (API) [38, 39].

2.24 Quantification methods

The following methods can be used for the quantitative determination of assay and organic impurities [40, 41]:

- a. *External standard method*: This method is used for the assay and impurity estimation in a given sample, where the impurities are estimated using the respective impurity standard and without the API standard peak. It's possible to estimate the concentration from calibration curve.
- b. *Area normalization*: If the RRF value of known impurity is close to the API (analyte), i.e., 0.9–1.1, the area normalization method is chosen for quantification. The recovery needs to be established without using the response factors.
- c. *Diluted standard method*: If the RRF values of impurities are different from the analyte, the diluted standard method can be chosen.
- d. *Internal standard method*: If the sample preparation procedure involves different extraction steps to avoid the error in the extraction procedure, internal standard procedure shall be chosen (normally for derivatization techniques and bioanalytical methods).

3. Conclusion

Principles involved in chromatography method development, especially for the analytical method development for the separation, identification, purification, and quantitative estimation of organic compounds using the liquid chromatography techniques (HPLC, UPLC, LC–MS, preparative HPLC, etc.), were emphasized in this chapter. Though many different types of chromatography techniques are currently in use, the liquid chromatographic methods HPLC, UPLC, and LC–MS are most widely utilized for the separation and quantitative determination of organic compounds. This chapter mainly focused on and explained the major and critical parameters of the liquid chromatography for the method development and optimization of a suitable stability-indicating LC method and impurity profiling studies. Each and every parameter which controls the purification of most of the organic compounds inclusive of drug, its precursors, and degraded products has been explained in detail in this chapter. The information given in this chapter will help the reader in choosing the right conditions for a particular compound to quantitatively separate from the reaction mixture or drug composition.

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References

- [1] Ettre LS. Milestones in Chromatography. LCGC North America. 2003. Available from: http://files.pharmtech.com/alfresco_images/pharma/2014/08/22/e5b0c0c2-2e1c-463d-8d4f-4993f7245f47/article-56954.pdf [Accessed: 10 July 2019]
- [2] Blomberg L. Stationary phases for capillary gas chromatography. *Trends in Analytical Chemistry*. 1987;**6**(2):41-45
- [3] Zlatkis A, editors. 75 Years of chromatography: A historical dialogue. *Journal of Chromatography Library*. Elsevier; 1979:7. ISBN 0-444-41754-0 (Vol. 17); ISBN 0-444-41616-1 (Series)
- [4] The Nobel Prize in Chemistry. 1952. Available from: nobelprize.org [Retrieved: 25 August 2016]
- [5] FDA, United States-Published in the Federal Register. Q6A Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances. 2000. Vol. 65; 83041-83063. Available from: https://database.ich.org/sites/default/files/Q6A_Guideline.pdf
- [6] Nicholson DE. An Introduction to Metabolic Pathways by S. Dagley. 59, No. 2 ed. Sigma Xi. The Scientific Research Society; 1971. 266p. Available from: <https://onlinelibrary.wiley.com/doi/pdf/10.1002/food.19710150432>
- [7] Q3A(R2) Impurities in New Drug Substances: FDA, United States-Published in the Federal Register on June 2008. Available from: https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q3A_R2/Step4/Q3A_R2_Guideline.pdf [Accessed: 10 July 2019]
- [8] Q3B(R2) Impurities in New Drug Products. FDA, United States-Published in the Federal Register. 14 November 2003; 68; 220; 64628-9. Available from: https://database.ich.org/sites/default/files/Q3B_R2_Guideline.pdf; https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q3B_R2/Step4/Q3B_R2_Guideline.pdf [Accessed: 10 July 2019]
- [9] Liljefors T, Allinger NL. Conformational analysis. 128. The Woodward-Fieser rules and α,β -unsaturated ketones. *Journal of the American Chemical Society*. 1978;**100**(4):1073. DOI: 10.1021/ja00472a008
- [10] Silverstein B. Morrill: Spectroscopic Determination of Organic Compounds. 5th ed1991
- [11] Swartz M. HPLC detectors: A brief review. *Journal of Liquid Chromatography & Related Technologies*. 2010;**33**:1130-1150. DOI: 10.1080/10826076.2010.484356
- [12] Rigas PG. Review: Liquid chromatography—post-column derivatization for amino acid analysis: Strategies, instrumentation, and applications. *Instrumentation Science & Technology*. 2012;**40**(2-3): 161-193. DOI: 10.1080/10739149.2011.651669
- [13] Santa T, Al-Dirbashi OY, Fukushima T. Derivatization reagents in liquid chromatography/electrospray ionization tandem mass spectrometry for biomedical analysis. *Drug Discoveries & Therapeutics*. 2007;**1**(2):108-118
- [14] Scott RPW. liquid chromatography detectors. Chapter 3. The fluorometric detector. *Journal of Chromatography Library*. 1977;**11**:121-130. DOI: 10.1016/S0301-4770(08)61018-0
- [15] McCalley DV. Choice of Buffer for the Analysis of Basic Peptides in Reversed-Phase HPLC. LCGC

ASIA PACIFIC VOLUME 8. 2005.
Available from: http://alfresco.ubm-us.net/alfresco_images/pharma/2014/08/22/806d27ce-71f0-4239-b9a6-f0541666d136/article-254604.pdf [Accessed: 10 July 2019]

[16] Chemical Analysis. ZORBAX HPLC Columns. Available from: <http://quimica.udea.edu.co/~carlopez/cromatogc/ph/95121tb.html> [Accessed: 10 July 2019]

[17] Zapala W. Influence of mobile phase composition on retention factors in different HPLC systems with chemically bonded stationary phases. *Journal of Chromatographic Science*. 2003;**41**. Available from: <https://pdfs.semanticscholar.org/2310/bbddcddcf462b99666f1d7cc351fcab48952.pdf> [Accessed: 10 July 2019]

[18] Janeček M, Šlais K. Design of mobile phase composition for liquid chromatography with an internal pH gradient. *Chromatographia*. 1993;**36**(1):246-250. DOI: 10.1007/BF02263872

[19] Joshi VS, Kumar V, Rathore AS. Role of organic modifier and gradient shape in RP-HPLC separation: Analysis of GCSF variants. *Journal of Chromatographic Science*. 2015;**53**(3):417-423. DOI: 10.1093/chromsci/bmu222

[20] Shimada K, Yoshida H, Komine Y. The effect of organic modifier in the mobile phase on the separation of bile acids and its fluorescent derivatives in inclusion chromatography. *Journal of Liquid Chromatography*. 1991;**14**(4):605-617. DOI: 10.1080/01483919108049274

[21] Ion-pair Reagents for HPLC. TCI/A1084E 20170303. Available from: https://www.tcichemicals.com/a-cmn/en/common/support-download/brochure/ion-pair_reagents_for_hplc.pdf [Accessed: 10 July 2019]

[22] Shibue M, Mant CT, Hodges RS. Effect of anionic ion-pairing reagent hydrophobicity on selectivity of peptide separations by reversed-phase liquid chromatography. *Journal of Chromatography A*. 2005;**1080**(1):68-75. DOI: 10.1016/j.chroma.2005.03.035

[23] 1.14.4 High-Performance Liquid Chromatography: The International Pharmacopoeia. 8th ed2018. Available from: <http://apps.who.int/phint/pdf/b/7.1.14.4.1.14.4-High-performance-liquid-chromatography.pdf> [Accessed: 10 July 2019]

[24] Du Q, CaijuanWu GQ, Wu P, Ito Y. Relationship between the flow-rate of the mobile phase and retention of the stationary phase in counter-current chromatography. *Journal of Chromatography A*. 1999;**835**(1-2):231-235

[25] Heidorn M. The Role of Temperature and Column Thermostating in Liquid Chromatography. White Paper. WP71499-EN 09/16S. Thermo Scientific. Available from: <http://tools.thermofisher.com/content/sfs/brochures/WP-71499-LC-Temperature-Column-Thermostating-WP71499-EN.pdf> [Accessed: 10 July 2019]

[26] Thermo Scientific Product Spotlight SP71195: Best in UHPLC Column Thermostating to Fit All Needs. 2014. Available from: <http://www.thermoscientific.com/content/dam/tfs/ATG/CMD/cmd-documents/bro/bro/chrom/lc/sys/SP-71195-Vanquish-Column-Thermostating-SP71195-EN.pdf> [Accessed: 10 July 2019]

[27] Thermo Scientific Poster Note PN71314. Thermostating in UHPLC: Forced Air Mode, Still Air Mode, and Method Transfer. Germering, Germany. 2014. Available from: <http://www.thermoscientific.com/content/dam/tfs/ATG/CMD/cmd-documents/sci-res/posters/chrom/lc/sys/>

- PN-71314-ISC2014-UHPLC-Thermostating-PN71314-EN.pdf [Accessed: 10 July 2019]
- [28] Dolan J. Separation Science. HPLC Solutions # 53. Temperature and Retention. Available from: <https://owl.english.purdue.edu/owl/resource/747/08/> [Accessed: 10 July 2019]
- [29] Gradient HPLC Solvent Delivery System: WPP10. Waters. Available from: <https://gimitec.com/file/wpp10.pdf> [Accessed: 10 July 2019]
- [30] Gradient Design and Development, Breaking the Bad Gradient Cycle: Agilent Technologies. Available from: <https://www.agilent.com/cs/library/slidepresentation/public/Gradient%20Design%20and%20Development.pdf> [Accessed: 10 July 2019]
- [31] Good Habits for Successful Gradient Separations: Getting the Most From Your Method. Agilent Technologies. Available from: <https://www.agilent.com/cs/library/slidepresentation/Public/Successful%20Gradient%20Separations.pdf> [Accessed: 10 July 2019]
- [32] Wrezel PW, Chion I, Pakula R. Key factors in sample diluent selection for HPLC assays of active pharmaceutical ingredients. LCGC North America. 2005;23(7):682-686
- [33] Slack GC, Snow NH. 8 HPLC sample preparation. Separation Science and Technology. 2007;8:237-268. DOI: 10.1016/S0149-6395(07)80014-6
- [34] LCGC Editors. Overview of sample preparation. 2015;33(11):46-51. Available from: <http://www.chromatographyonline.com/overview-sample-preparation> [Accessed: 10 July 2019]
- [35] John W. Dolan: How much can i inject? Part I: Injecting in mobile phase. LCGC North America. 2014;32(10):780-785. Available from: <http://www.chromatographyonline.com/how-much-can-i-inject-part-i-injecting-mobile-phase> [Accessed: 10 July 2019]
- [36] Singh S, Junwal M, Modhe G, Tiwari H, Kurmi M, Parashar N, et al. Forced degradation studies to assess the stability of drugs and products. Trends in Analytical Chemistry. 2013;49:71-88
- [37] Jain D, Basniwal PK. Forced degradation and impurity profiling: Recent trends in analytical perspectives. Journal of Pharmaceutical and Biomedical Analysis. 2013;86:11-35
- [38] Kalyana Chakravarthy VV, Kishore Babu G, Lakshmana Dasu R, Prathyusha P, Aparna Kiran G. The role of relative response factor in related substances method development by high performance liquid chromatography (HPLC). Rasayan Journal of Chemistry. 2011;4(4):919-943
- [39] Bhattacharyya L, Pappa H, Russo KA, Sheinin E, Roger L. Williams: U.S. pharmacopeia. The use of relative response factors to determine impurities. Pharmacopeial Forum. 2005;31(3). Available from: https://www.researchgate.net/publication/295423011_The_use_of_Relative_Response_Factors_to_determine_impurities
- [40] Altria KD. Essential peak area normalisation for quantitative impurity content determination by capillary electrophoresis. Chromatographia. 1993;35(3-4):177-182
- [41] de Oliveira EC, Muller EI, Abad F, Dallarosa J, Adriano C. Internal standard versus external standard calibration: an uncertainty case study of a liquid chromatography analysis. Química Nova. 2010;33:4. DOI: 10.1590/S0100-40422010000400041