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Understanding the Dynamic Process of Dissolution Using In-situ FT-IR Spectroscopy

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Understanding the Dynamic Process of Dissolution
Using In-situ FT-IR Spectroscopy

By

Vrushali M. Bhawtankar

Ph.D. Dissertation

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Chemistry and Biochemistry of Seton Hall University

May, 2017

South Orange, New Jersey
Dissertation Committee Approvals

We certify that we have read this thesis and that in our opinion it is sufficient in scientific scope and quality as a dissertation for the degree of Doctor of Philosophy

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Dedicated to my dear husband Mangesh for his encouragement and support to pursue higher education.
Abstract

Dissolution studies provide valuable and critical drug release information (in vitro) that are important for quality control drug development. Using in-situ FT-IR spectroscopy methods has been developed for analyzing and monitoring dissolutions of pharmaceutical APIs. The accuracy of this technique was found to be ± 3% relative to HPLC and UV/Vis Spectroscopy. A dynamic analysis of the dissolution and subsequent hydrolysis of aspirin has been determined by in-situ FT-IR. This technique allows real-time analysis of the behavior of aspirin under simulated physiological conditions (pH 1.2, 4.5, 6.8) as aspirin (1205 cm⁻¹) and salicylic acid (1388 cm⁻¹) are detected as separate and distinct peaks in the in-situ FT-IR.

For this study, 325 mg Bayer aspirin tablet was used. The data shows dissolution of aspirin with the hydrolysis profile and the formation of salicylic acid. Comparison of the dissolution behavior of generic brands of aspirin has been performed. The formulations contain the same amount of drug substance as present in Bayer aspirin. Dissolution and hydrolysis of enteric coated aspirin tablets has been performed, and compared with the Bayer aspirin tablets. The dissolution was carried out as per the USP procedure.

This technique indicates a future potential for real-time studies of dissolution and hydrolysis of other prodrugs such as Loratadine. The in-situ FT-IR system is extremely capable of measuring low concentrations (0.03 mg/ml) and for distinguishing separate components of a multiple component systems without requiring manual sampling. Specifically, the unique fingerprint region of the in-situ FT-IR spectra provides detailed information about the release profile of the drug.

One more demonstration of the in-situ FT-IR novel technique is the dissolution study of Loratadine.
(hydrolysis of Loratadine), a pro-drug that has a tendency to hydrolyze to an active compound desloratadine.

In addition, research on the acetaminophen dissolution observed on extra strength Tylenol and on Tylenol Arthritis has been validated by UV/Vis spectroscopy and HPLC. In this study the non-linear behavior of Beer-Lamberts’s law was observed. The hypothesis has been established that the acetaminophen forms dimer at higher concentrations. The investigation was carried out by NMR and FTIR Spectroscopy and by measuring colligative properties such as vapor pressure osmometer and freezing point depression.

In-situ FT-IR spectroscopy is a novel technique used for monitoring the dissolution testing. The fingerprint region of the in-situ FT-IR has great potential for studying dissolution kinetics as well as hydrolysis kinetics for aspirin. A separate peak for aspirin as well as salicylic acid has been observed. This technique has excellent potential for the dissolution studies of tablets without disturbing the dissolution media.
Acknowledgement

During my graduate career at Seton Hall University, I have had the privilege and opportunity to develop myself as a research scientist. First and foremost, I would like to thank Dr. Nicholas Snow for adopting me into his research group. His constant support, motivation, and enthusiasm were irreplaceable and greatly appreciated. I would like to express my sincere gratitude to my advisor for his patient guidance, encouragement and generous advice he has provided and inspiring me to become better.

I would like to give special thanks to my former research advisor Dr. John R. Sowa, Jr. for the continuous support, valuable guidance, expertise, encouragement of my Ph.D. study and research. I will be forever grateful for his motivation, enthusiasm, and assistance. I would like to acknowledge Wes Walker and Jane Riley from Mettler-Toledo for technical support for the iC10IR Instrument.

Specifically, I would like to give a special appreciation of my thesis committee to Dr. Wyatt Murphy and Dr. Stephen Kelty for their scientific advice, knowledge and many insightful suggestions with encouraging, motivating and supporting my research ambitions. I would also like to acknowledge Dr. Cecilia Marzabadi, Dr. Yufeng Wei, Dr. Yuri Kazakevich Dr. James Hanson, Dr. Alexander Fadeev, Dr. Rosario Lobutto. Dr. David Sabatino and Dr. Sergiu Gorun for their guidance and mentoring throughout my studies. I will forever be indebted to those who have supported me through the years in this endeavor. It is with great appreciation that I offer my gratitude to all.
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<td>Attenuated Total Reflection</td>
</tr>
<tr>
<td>In-situ FT-IR</td>
<td>Attenuated Total Reflection-Infrared Spectroscopy</td>
</tr>
<tr>
<td>API</td>
<td>Active Pharmaceutical Ingredient</td>
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<tr>
<td>Disso</td>
<td>Dissolution</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
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<tr>
<td>UV</td>
<td>Ultra-Violet</td>
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<tr>
<td>Vis</td>
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CHAPTER 1

INTRODUCTION

1.1 Introduction

Dissolution of solids has been studied for many decades and applied to many industrial fields for various chemical or physical applications, for example, food and pharmaceutical products. Dissolution is the process by which a solid substance enters the solvent phase yielding a solution.

[1] In the past few years, the importance of dissolution testing has increased substantially, as seen by the high level of regulations imposed on the pharmaceutical industry by various health agencies around the world. Dissolution testing is critical for measuring the performance of a pharmaceutical formulation. For a solid dosage form, it will be disintegrated into small granules then dissolution process of the granules can be studied and its outcomes such as solubility can be obtained.

Dissolution testing plays several important roles in the pharmaceutical industry. First, the dissolution test is a quality control tool that measures the change in stability of the formulation. Some of the relevant changes that dissolution testing is able to detect include changes caused by temperature, humidity, and photosensitivity. Second, the dissolution testing is also important for formulation development. During development of a drug product, the formulators use dissolution testing to distinguish between different variations of the drug product. The physical characteristics of particles applied in dissolution studies are normally pointed as its objective properties. The physical properties of particles are related to its size, shape, surface and structure. In the pharmaceutical industry, it is important to develop with several variations of the drug product since these are needed to access the drug’s performance in clinical trials. From the clinical trials, the
efficacy of the variants is distinguished and obtained. Third, once in vivo (clinical trials) data has been established for the drug product, a correlation between in vivo (human blood data)/ in vitro (lab dissolution results) is attempted. [2] [3] Fourth, dissolution testing is used for the development of the quality control specifications for the manufacturing process, such as compression and binding agents in tablets and other parameters for other dosage forms. [4] With this, dissolution plays a very important role for measuring the stability of the investigational product. In the manufacturing industry, another application of dissolution testing is to assess the batch-to-batch consistency preferably in solid dosage forms. Finally, the Food and Drug Administration (FDA) requires dissolution testing in regulatory filings for all oral dosage forms and many other dosage forms as well.

Dissolution is the process by which a solid dosage form enters in the dissolution media to yield a solution. It is simply a process by which a solid dosage form dissolves. It is controlled by the affinity between the molecules comprising the solid substance and the solvent. Moreover, the dissolution rate plays an important role in the understanding of dissolution chemistry. The dissolution rate is defined as the amount of drug substance that goes into solution per unit time under standardized conditions of liquid/solid interface, temperature and solvent composition.

Dissolution testing can be performed using different types of testing apparatus for pharmaceutical dosage forms. Even though there is increased research interest in this area, the techniques used for studying dissolution rates remain constant. In fact, there are only a few instruments used to analyze and understand the dissolution rates of drugs. This chapter discusses the current techniques used for dissolution testing. Additionally, a detailed literature review into the use of in-situ FT-IR spectroscopy in general and as a dissolution technique has been made and is discussed in this chapter. [5]
1.2 Dissolution Models and Theories

In the dissolution research, Higuchi provided fundamental contributions to the development of dissolution theory. [6] According to Higuchi’s research, there are three basic models used alone or in combination to describe the dissolution process and mechanisms. These three models are; diffusion layer, interfacial barrier and Danckwerts.

The diffusion layer model was simply used for a crystal solid to describe the dissolution process in the pure solvent without any reactive actions. This model was once expressed by Nernst. [7] According to the diffusion layer model as shown in Figure 1, it is assumed that there is a saturated layer of solvent $C_s$ around the solid surface and equilibrium conditions occur on the surface. Then the dissolution process is driven by the diffusion movement of molecules in the diffusion layer, $\delta$ cm in thickness. Therefore the dissolution process can be expressed such that the first step of solid forming an interface in the solvent is very rapid. This leads to the formation of a saturated stagnant layer around the surface. Then slow diffusion occurs from the surface into the bulk solution through the diffusion layer. The rate of dissolution is entirely based on the diffusion of the solid molecule from the diffusion layer to bulk liquid.
Figure 1: The diffusion layer model

(All figures in this dissertation were created by Vrushali M. Bhawtankar)
The interfacial barrier model assumes that there is a high activation energy to promote the interfacial transport process. [8] According to this model as shown in Figure 2, a free energy barrier surmounts before the solid can dissolve. The surface of the solid is continually exposed to the solvent and equilibrium is assumed at the solute-solvent interface $C_s$. The solute then diffuses from free energy barrier and is carried into the solvent under the agitation. This leads to the formation of a saturated stagnant layer around the surface. Then slow diffusion occurs from the surface into the bulk solution through the diffusion layer. Therefore there is no layer around the surface and the surface is continually replaced by the solvent medium.

The Danckwerts model assumes that the macroscopic packets of solvent reach the solute-solvent interface first by eddy diffusion in some random fashion [9] as explained in Figure 3. At the interface, the packet is able to absorb solute according to the laws of diffusion and then replaced by new packets. This process continually occurs and the surface is continually replaced by the new packets of solvent. Therefore the surface renewal process can be related to the solute transport process.

These three models explain the mechanism of the drug dissolution testing, where solid drug reacts with the fluid of dissolution medium. This reaction takes place at the solid-liquid interface. Therefore dissolution kinetics are dependent on three things-the flow rate of the dissolution medium towards the solid-liquid interface, the reaction rate at the interface and the diffusion of the dissolved drug molecules from the interface towards a bulk solution. The most commonly used model is diffusion layer model.
Figure 2: The interfacial barrier model
Figure 3: The Danckwert's model
1.3 Mathematical Models of Dissolution

The in vitro dissolution has been recognized as an important testing in the drug development. There are several mathematical and kinetic models for dissolution testing are greatly developed and explained. These models describe drug dissolution from immediate and sustained release dosage forms. There are several models represents the drug dissolution profile, which is a function of time-related to the amount of drug released from the pharmaceutical dosage form. [10] This section covers a brief information about each model with the final derived equation.

There are many fundamental mathematical models based on the Noyes-Whitney equation and on Nernst-Brunner film theory on dissolution kinetics which can be expressed as statistical methods. These include the exploratory data analysis method, repeated measures design and multivariate approach. Model dependent methods including the zero-order model, first-order model, Higuchi model, Hixson-Crowell model, Baker-Lonsdale model, Korsmeyer-Peppas model and Weibull model have been developed. Model independent methods including difference factor and similarity factor also have been used.

The first equation for dissolution kinetics was described by Noyes and Whitney in 1897 with the equation. [11]

\[
\frac{dM}{dt} = kS( C_S - C_t) \tag{1}
\]

Where \( M \) is the dissolved mass, \( t \) is the dissolution time, \( S \) is the surface area of particles, \( C_S \) is the equilibrium solubility at the temperature, \( C_t \) is the concentration in solution at the time and \( k \) is the dissolution rate constant.
This Noyes-Whitney law was developed from observing the dissolution of two different materials dissolving in water. With this equation, the dissolution could be assumed to be driven by the difference between the concentrations at the particle surface, which can be regarded as the equilibrium solubility with the concentration in the bulk. Therefore the dissolution process was controlled by the mass transfer from the surface of the particle to bulk. 

Then, the dissolution mathematical model was greatly developed by Brunner and Nernst. In their studies, a relationship between diffusion coefficient and the concentration in bulk was explained. The equation to describe this theory is expressed by Nernst and Brunner as

\[ k = \frac{DS}{v \cdot h} \]  

(2)

Where D is the diffusion coefficient, h is the diffusion layer thickness, v is the volume of solution, S is the surface area of particles and k is constant. With this model, Nernst and Brunner stated that the dissolution process could be proposed in two steps. They assumed that the fluid in the diffusion layer was stagnant. Furthermore, this theory also assumed that the dissolution process at the surface of the particle is much faster than the mass transfer process and a linear concentration gradient happens in the particle surface layer. However, this ideal condition assumption may never occur because the surface area of the particle changes permanently with the dissolution process.

**Exploratory data analysis**

This method is used to understand and compare the dissolution data with a controlled dissolution process. [12] The comparison can be achieved with dissolution data in graphical and numerical methods. The dissolution data can be plotted for every formulation with one or two error bars at each dissolution time point. Therefore the dissolution data can be summarized numerically and the differences between every dissolution data profile can be compared at each dissolution time point.
**Multivariate approach**

The multivariate analysis of variance (MANOVA) is a statistical method, based on the repeated measurements designs where the percent dissolved material is depended with the repeated factor of time. With the repeated measurements, the factors are measured repeatedly with more than two levels. [13] The data were collected with repeated measurements over time on the same experimental instrument. These are compound symmetry assumptions and the assumptions of sphericity, it refers to the condition where the variances of the differences between all possible pairs of within subject conditions are equal. Because of these assumptions, MANOVA approach to repeated measures has gained popularity in recent years.

**Zero-order model**

The zero-order model assumes that dissolution is independent of the concentration in bulk, and is only changing with dissolution time. In this way, the application of this model is limited with the only consideration of dissolution proportional to time. This model states that dissolution from particle will not disaggregate and the process is slow. [14] This model is expressed as

\[ Q_t = Q_0 + k_0 t \]  \hspace{1cm} (3)

where \( Q_t \) is the amount of particles that has dissolved at time \( t \), \( Q_0 \) is the initial amount of particle and \( k_0 \) is the zero-order dissolution constant.

**First-order model**

This method is usually used to describe the absorption or elimination of solid particles. [15] However, the mechanism of dissolution process with this model is difficult to be conceptualized from a theoretical context, as after saturation point with the first-order dissolution rate shifts to
zero order. Because of this scenario, it is also called mixed-order kinetics. The first order kinetics can be represented as follows, where $dC$ is the change in concentration, $dt$ is the change in time, $C$ is the concentration and $k_f$ is the first-order dissolution rate constant.

$$\frac{-dC}{dt} = k_f C \quad (4)$$

**Higuchi model**

This model was firstly expressed by Higuchi to describe drug release from a matrix system. The assumption of this model can be described that the initial concentration should be higher than solubility, and the diffusion occurs only in one dimension, the drug particles are much smaller than diffusion layer thickness. [16] The model can be expressed as

$$Q = A \sqrt{D(2C_0 - C_s)C_s t} \quad (5)$$

where $Q$ is amount of dissolved particles in area A at time $t$, $C_0$ is the initial concentration, $C_s$ is the solubility of particles and $D$ is the diffusion coefficient of particle molecules. The common application of this equation is the simplified equation where $k_H$ is the Higuchi dissolution rate constant.

$$Q = k_H t^{1/2} \quad (6)$$

The application of the Higuchi model is for the drug release from the dispersed matrices to particles of general shape using the pseudo-steady-state approximation of Higuchi. [17]

**Hixson-Crowell model**

This mathematical model was first expressed by Hixson and Crowell as the particle regular area is proportional to the cube root of the volume. [18] The equation can be described as
\[ W_0^{1/3} - W_t^{1/3} = k_h t \]  \hspace{1cm} (7)

where \( W_0 \) is the initial concentration of drug in pharmaceutical dosage from, \( W_t \) is the remaining concentration of drug in pharmaceutical dosage from at time \( t \) and \( k_h \) is the Hixson-Crowell dissolution rate constant. This model assumes that the surface of the tablet is allowed to change with time and its geometrical shape diminished proportionally with time. Hixson and Crowell expressed that the dissolution rate was controlled by the particles dissolution rate rather than the diffusion speed through the diffusion layer.

**Korsmeyer-Peppas model**

This model was expressed by Korsmeyer, in 1983 as a simple relationship to describe the drug dissolution from a polymeric system. [19]

\[ \frac{M_t}{M_\infty} = k t^n \]  \hspace{1cm} (8)

Where \( \frac{M_t}{M_\infty} \) is the ratio of dissolved drug at time \( t \), \( k \) is the dissolution rate constant and \( n \) is the exponent. It is noticed that the value of \( n \) is used to define different dissolution types for cylindrically shaped matrices.

The review of the kinetic modeling on the drug release show that these models have been established to describe the relationship between drug dissolution and geometry on the drug release patterns mathematically. The drug transport inside pharmaceutical systems involve multiple steps provoked by different physical and chemical phenomenon making it difficult to formulate a mathematical model describing it in the correct way. The release models with the major application and the commonly used to study drug release phenomenon are the Higuchi model, zero order model, first order model and Korsmeyer-Peppas model.
1.4 USP Dissolution Information

In the history of dissolution the results revealed in late 1800’s to the mid-1900’s, the first official dissolution testing method appeared in United States of Pharmacopeia XVIII in 1970. The United States Pharmacopeia or USP is a non-government, official public standards–setting authority for prescription and over-the-counter medicines and other healthcare products manufactured or sold in the United States. [20] The USP also sets widely recognized standards for food ingredients and dietary supplements. The USP sets standards for the quality, purity, strength, and consistency of these products which are critical to the public health. Increased interest in dissolution regulations continued to grow well into the 1970’s.

In 1978 the Food and Drug Administration (FDA) published the document entitled, “Guidelines for Dissolution Testing.” [21] The intention behind this publication was to harmonize and streamline the systems and processes of different laboratories. This was due to the fact that dissolution results were observed to have high variability. There are minor changes in the equipment parameters which causes higher variability. The FDA realized that more controls on the tolerances of the dissolution equipment were needed so that results would be more reproducible. Additionally, the FDA along with USP introduced the idea of calibrator tablets.

In 1978 the USP launched three calibrator tablets; prednisone, salicylic Acid and nitrofurantoin. These calibrator tablets were used during the calibration of the instrument to validate the dissolution testing system. The calibrator tablets have known specification limits and the calibrations of the instruments have to be within those limits. To have an appreciation of the
complexity of the dissolution system and equipment parameters, an overview of the technology will be given in the next section.

In 1995, the USP assigned unique numbers to the different dissolution apparatus that were available to the scientific community. All dissolution testing apparatus are listed in Table 1. There are seven different types of dissolution equipment that are available to the dissolution chemist. The most widely used dissolution apparatus in the pharmaceutical industry are apparatus I, II and IV. The use of apparatus IV has been developed more importance in recent years.

Apparatus 1 and 2 are most widely used in the pharmaceutical industry. As shown in Table 1, Apparatus 1 uses baskets while Apparatus 2 uses paddles. Figure 4, shown below, is a schematic and brief illustration of these two apparatuses and more precise dissolution testing vessels. The apparatuses are comprised of a covered vessel which is cylindrical with hemispherical bottom with capacity of minimal 1 liter, a metallic drive shaft which rotates smoothly that could affect the results, a motor to spin the shaft, a cylindrical basket (Apparatus 1) or a paddle (Apparatus 2) which are explained in detail in figure 5 and 6 respectively and dissolution medium.

The dissolution testing apparatus comprised a water bath or heated jacket capable of maintaining the temperature of the vessels at 37°C ± 0.5°C. The dissolution testing apparatus is connected to the auto sampler and if not then need to collect aliquots manually at the particular time point. Although the figure below appears simple in design, there are strict regulations for the specifications of each component of the apparatus and tolerances for each component are specified by USP General Chapter Dissolution <711>. [22]
Table 1: List of dissolution apparatus as per USP [20]

<table>
<thead>
<tr>
<th>APPARATUS</th>
<th>NAME</th>
<th>DRUG PRODUCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apparatus 1</td>
<td>Rotating basket</td>
<td>Tablets, Capsules</td>
</tr>
<tr>
<td>Apparatus 2</td>
<td>Paddle</td>
<td>Tablets, capsules, modified drug products</td>
</tr>
<tr>
<td>Apparatus 3</td>
<td>Reciprocating cylinder</td>
<td>Extended-release drug products, Beads</td>
</tr>
<tr>
<td>Apparatus 4</td>
<td>Flow through cell</td>
<td>Drug products containing low-water-soluble drug</td>
</tr>
<tr>
<td>Apparatus 5</td>
<td>Paddle over-disk</td>
<td>Transdermal drug products, Ointments, Gels, Emulsion</td>
</tr>
<tr>
<td>Apparatus 6</td>
<td>Rotating Cylinder</td>
<td>Transdermal drug products, Ointments, Gels, Emulsion</td>
</tr>
<tr>
<td>Apparatus 7</td>
<td>Reciprocating holder</td>
<td>Extended-release drug products, Transdermal Patches, Ointments, Gels, Emulsion</td>
</tr>
</tbody>
</table>
Figure 4: Schematic illustration of apparatus 1 and apparatus 2
Figure 5 shows the stirring basket elements of dissolution apparatus 1. The apparatus has a basket on the bottom of the shaft with 37 ±3 mm, made of stainless steel. At the bottom of the basket a mesh with 25 ±3 mm diameter. The figure shows the dimensions of the each part of the shaft and basket and the position of the each stirring elements. These are the USP requirements for the instrumentation of the dissolution apparatus 1. A dosage unit is placed in a dry basket at the beginning of the test. The distance between the bottom of vessel and bottom of the flask is 25 ± 2 mm is required.

Figure 6 shows the stirring for schematic diagrams of dissolution apparatus 2. This apparatus is similar to the apparatus 1, as the only difference is a paddle used instead of a basket. This apparatus is the most widely used to develop a dissolution method in the pharmaceutical industry. The specifications of the shaft and the paddle are mentioned in detail in the figure. The distance between the bottom of paddle and bottom of the flask is 25 ± 2 mm is required. The total length of the paddle is required to be 74-75 mm with a thickness of 4 ± 1 mm.

The specifications of other USP apparatus are specified in general chapter Drug Release<724>. [23] The general chapter <1092> the dissolution of Procedure: Development and Validation were published in 2001. The authors proposed that the chapter contains detailed information not only on the development of dissolution tests to supplement the information that was in <1088> but also on the validation procedures particular to dissolution testing. [24] As a result of these regulations of the past fifty years, the number of USP monographs has exponentially increased. In 1970 there were only twelve monographs. [25] In 2011 there were 740 dissolution USP monographs and in the recently updated version of USP has 4900 USP monographs.
Figure 5: USP apparatus 1 basket stirring elements (© 104 U.S. Pharmacopeial Convention, Used with Permission)
Figure 6: USP apparatus 2 specifications (© 104 U.S. Pharmacopeial Convention, Used with Permission)
Another most widely used dissolution apparatus in the pharmaceutical industry is USP Apparatus 4, Flow-through cell. It was first appeared in 1957 and developed by FDA. [26] The method was adapted by USP, European Pharmacopeia, and Japanese Pharmacopeia, and the flow through cell became an official apparatus as USP apparatus-4. The system consists of a reservoir containing dissolution medium, a pump that forces the medium upwards through the vertically positioned flow-cell and a water bath to control the temperature in the cell. Different types of cells are available for testing different dosage forms. Usually, bottom cone of the cell is filled with small glass beads (about 1 mm diameter) and the dosage unit is placed on top of the beads. Peristaltic or pulsating pistons are used in the pumps.

Dissolution testing using apparatus 4 continues to grow in popularity. Many pharmaceutical companies in the United States and throughout the world are currently developing new methods utilizing apparatus 4 and become more widespread. In the dissolution method development different variations such as the size of the cell, flow rate, filter size and media with pH change can be varied with difficulty in apparatus 1 and 2. This technique is useful for the low solubility and rapidly degrading drugs. [27] Two different types of configurations can be used: an open loop or closed loop where dissolution media is circulating through drug sample. In the open loop method, the dissolution media flows through a cell containing the drug sample and goes into the waste after sampling as it flows in one direction only. [28]

Figure 7 shows all parts of the dissolution apparatus 4. The dissolution medium is placed in a covered flask from where the medium passes to the cell with the help of a pump. The flow is in the upward direction, so a pump is required. The cell contains the dosage unit, through which dissolution media flows and passes through the filtration unit which is placed on the top of the cell and finally collected in a waste flask or circulates again for a closed system.
Figure 7: Schematic diagram of flow-through cell apparatus-4
1.5 Infrared Spectroscopy

Infrared spectroscopy (IR) is performed using wavelengths, 4000 cm\(^{-1}\) to 400 cm\(^{-1}\), of the electromagnetic spectrum. Additionally, the IR portion of the electromagnetic spectrum is divided into three regions; near-infrared, mid-infrared and far-infrared. The near-infrared energy is approximately in the region between 14000 to 4000 cm\(^{-1}\) and can excite overtone or harmonic vibrations. The mid-infrared energy is approximately in the region between 4000 (2500 nm) to 400 cm\(^{-1}\) (2500 nm) and can be used to study the fundamental vibrations of structures. The far-infrared region is approximately in the region between 400 to 10 cm\(^{-1}\) and can be used to study to rotations of structures. [29] [30] With IR spectroscopy, different functional groups absorb at different IR bands or regions. Thus, this technique can help identify and even quantify organic and inorganic molecules.

Figure 8 shows the infrared spectrum of a sample can be obtained by passing a beam of infrared light through the sample. The light passes through a beam splitter, which sends the light in two directions at right angles. One beam goes to a stationary mirror then back to the beam splitter. The other goes to a moving mirror. The motion of the mirror makes the total path length variable versus that taken by the stationary mirror beam. When the two meet up again at the beam splitter, they recombine, but the difference in path lengths creates constructive and destructive interference: an interferogram.
Figure 8: FT-IR schematic diagram
A Fourier transform instrument can be used to measure how much energy was absorbed by the sample over the entire wavelength range. [31] The interferogram represents the light output as a function of mirror position. The FT-IR raw data is processed to give the actual spectrum of light output as a function of wavenumber. The FT-IR system can produce both transmittance and absorbance spectrum. Molecules absorb IR radiation and excite to higher energy states. The IR frequency matches with the natural vibrational frequencies of the molecule. Only those bonds that have a dipole moment are capable of absorbing IR radiation. Symmetric bond such as those of H$_2$ or Cl$_2$ does not absorb IR radiation. [32]

1.5.1 Attenuated Total Reflectance-Infrared (ATR-IR) spectroscopy

Attenuated Total Reflectance generally allows qualitative or quantitative analysis of solid and liquid samples with little or no sample preparation, greatly improves the rate of analysis. The main benefit of ATR sampling comes from the very thin sampling path length or depth of penetration of the IR beam into the sample. This is in contrast to traditional FT-IR sampling by transmission where most of the time the sample must be diluted with an IR transparent salt (e.g. KBr), pressed into a pellet or pressed to a thin film, prior to analysis to minimize absorption and reflection by the sample. [33]

In ATR a beam of infrared light is allowed to interact with the sample and the beam gets internally reflected as it bounces through a crystal, changes relative to a background signal are recorded by the instrument. An optically dense crystal (e.g. Diamond, Zinc selenide or Germanium) with a high refractive index (typically refractive index values between 2.38 and 4.01) is used and the infrared beam is allowed to impact at a certain angle. The most frequently used small crystal in ATR is diamond, because it has the best durability and chemical inertness.
Figure 9 shows that a beam of infrared light is directed onto a crystal with a high refractive index at a certain angle. An evanescent wave is formed due to the internal reflectance which penetrates out of the crystal and passes through the sample which is in contact with the crystal. However, the evanescent wave moves only 0.5 µ to 5 µ out of the crystal making it mandatory for the sample to be in very good contact with the crystal. The evanescent wave is attenuated in the regions where the sample absorbs energy and the altered wave is reflected back to the IR beam, which then exits the opposite end of the crystal and is sent to the detector of the spectrometer which ultimately gives an IR spectrum after processing.

The following experimental factors that affect the quality of final spectrum are the refractive indices of the ATR crystal, the angle of incidence of the IR beam, the depth of penetration, the wavelength of the IR beam, the number of reflections and ATR crystal characteristics. ATR provides excellent quality data in conjunction with the best possible reproducibility of any IR sampling technique. ATR allows faster sampling, improved sample to sample reproducibility and minimizing user to user spectral variation.

Figure 10 shows the in-situ FT-IR instrument on which the research has been done. The FT-IR instrument is the ReactIR™ iC10, from the Mettler Toledo. The FiberConduit™ is comprised of flexible IR transparent silver chloride/silver bromide optical fibers. The fiber optic probe interface (AgX 9.5 mm x 1.5 m fiber (Silver Halide)) contains a diamond tip-DiComp ATR crystal. The enlarged view of the tip of the probe, works with ATR technique.
Figure 9: A multiple reflection ATR system
Figure 10: A representation of ATR with \textit{in-situ} FT-IR (Adopted from Mettler Toledo)
Recent use and application of the ATR-IR spectroscopy has increased in pharmaceutical industry, especially in the region of the Mid-IR and the Near-IR. [34] Near-IR spectroscopy has gained acceptance in the pharmaceutical industry for analysis of incoming raw materials and finished dosage forms in the last century. [35] The determination of the multiple parameters from a single near-IR spectrum is the most valuable attribute. One recent application of near-IR is in the prediction of dissolution behavior of pharmaceutical tablets with near-IR imaging. [36] Wendy and Zhuangji described detection and quantitation of volatile organic contaminants in water using ATR-IR technique. [37]

The FT-IR Imaging technique was first introduced in 1997. The technique has been applied to study many different systems from all facets of science, from areas such as polymer diffusion and dissolution. [38] Van der Weerd and his colleagues described the design and implementation of a new cell, which allows the study of drug release from tablets by macro-FT-IR ATR imaging with a diamond ATR accessory. Tablet formulation can be impacted directly on the ATR crystal. The authors explain that various components in the tablet can be determined FT-IR imaging. [39] Wray and his colleagues explored the novel application of FT-IR imaging to study the dissolution of delayed release and pH resistant compressed coating pharmaceutical tablets. It was used to accurately determine the swelling of the hydroxypropyl cellulose core and its relation the rate of water ingress.
In the pharmaceutical industry, there are two main solid oral dosage forms: tablets and capsules and others as well. The tablets or capsules are immersed in an aqueous solution for dissolution testing, and the concentration of the active ingredient is monitored as a function of time. Dissolution testing alone provides limited information on chemical processes that take place within a dissolution vessel, because of the limited capabilities of the techniques that are used during dissolution. Most dissolution analysis is carried out using ultra-violet and visible spectroscopy which only gives concentration as a function of time. High-performance liquid chromatography is used to separate multi-component drugs but still utilizes UV/Vis detection. [40]

One of the challenges in the industry is faced with is to increase an understanding of the mechanisms governing dissolution. The current approach relies heavily on a data-driven approach. To have a better understanding of dissolution, dissolution chemists need to explore other applications that could give insight into what is happening inside of the vessel. This research focuses on using ATR mid-infrared spectroscopy as an \textit{in situ} technique to monitor and study the dissolution of pharmaceutical tablets. The fiber-optic probes for reaction monitoring and spectroscopy have been used as an important device in research. The flexible fiber optical probes and spectroscopic software have enhanced the development of real-time applications. However, recently, on-line methodologies have been used for obtaining accurate data during the dissolution process.
1.6 Drugs used in this Research

As discussed earlier, this research is conducted using in situ FT-IR technique to monitor the dissolution process. For this research, solid dosage form such as tablets were selected which were available over the counter and easily available, these are acetaminophen, aspirin, salicylic acid and Loratadine which are shown in Figure 11.

1.6.1 Acetaminophen

Acetaminophen (paracetamol) has been widely used for nearly a century and is currently one of the most commonly used medications in the United States. Acetaminophen is an effective and well-tolerated analgesic and antipyretic agent when used as indicated. [41] Acetaminophen is a synthetic, nonopiate, centrally acting analgesic derived from p-aminophenol and it is also a degradation product. It is reported to have significant nephrotoxic and teratogenic effects, therefore its dosage should be strictly controlled. [42] [43] Drug metabolism plays a key role in acetaminophen-induced hepatotoxicity. Acetaminophen metabolism occurs primarily in the liver, where the drug undergoes glucuronidation sulfation by UDP-glucuronosyltransferases and sulfotransferases, respectively.

Figure 12 shows the FT-IR spectra of a standard solution of acetaminophen and phosphate buffer. The blue line is for the buffer and the yellow line is for acetaminophen which shows peak at 1246 cm\(^{-1}\) for the analysis, corresponding to C-N stretch. For the research, the standard IR spectra of acetaminophen collected via FT-IR were confirmed and compared with standard spectra, which is collected from SDBS database for acetaminophen shown in Figure 13. [44] One more confirmation test has been performed by studying calibration of acetaminophen using different concentrations.
Figure 11: Structures of drugs used in this research
Figure 12: In-situ FT-IR spectra of acetaminophen
Figure 13: IR spectra of acetaminophen from SDBS database (Adopted from reference 45)
1.6.2 Aspirin and Salicylic Acid

Aspirin is unique in its history and has many important roles in drug therapy. Aspirin is a pro-drug and known to decrease pain. It fights pain and inflammation by blocking the action of an enzyme called cyclooxygenase which inhibits the formation of prostaglandins, [45] which signal an injury and trigger pain. Aspirin also inhibits the formation of prostaglandins. This results in the inhibition of blood clots which could cause a heart attack or stroke. Aspirin was first synthesized by the German chemist Felix Hoffmann (1868-1946) in the laboratories of Farbenfabriken Bayer, Elberfeld, Germany in 1897. [46] Salicylic acid was used prior to aspirin, has several bad flaws as a medicine. It has a bad taste, cause stomach irritation and presents other side effects spurred researchers to look for other derivatives. The intent was to keep salicylic acid efficacy without the disadvantages it posed. Acetylation of the hydroxyl group was one of the logical modifications. This eventually led to the synthesis and discovery of aspirin.

Felix Hoffman used acetic anhydride for preparation of aspirin. Figure 14 shows the formation of aspirin from salicylic acid and acetic anhydride. Salicylic acid can react with ethenone and acetyl chloride to form aspirin. This is the general synthesis pathways for the formation of salicylic acid that were investigated back in the late 1800’s. [47]

A chemical reaction of particular relevance to aspirin’s stability in biological fluids is hydrolysis. There are two types of hydrolysis which are based on the pH. Figure 15 shows the acid catalyzed mechanism, if the proton source is hydronium (H₃O⁺), the catalysis is termed specifically acid catalysis. The source of the proton is a dissociated acid and the substrate (the ester) is already protonated in the rate-limiting (slow) step of the reaction.
Figure 14: Synthesis of aspirin in 1800’s [48]
Figure 16 shows the base catalyzed aspirin hydrolysis mechanism. Any undissociated acid (if present) does not appear in the rate-limiting step. [48] In base catalysis the base is hydroxide (HO\(^-\)) and the substrate is attacked by hydroxide in the rate-limiting step of the reaction. There are no other bases (such as the conjugate base of an acid) in the rate-limiting step. The spontaneous process shows water acting as the nucleophile attacking a neutral substrate. [49]

Figure 17 is the FT-IR spectra of a standard solution of aspirin, salicylic acid and buffer. The blue line is for salicylic acid, the green one is for buffer solution and the pink line is for aspirin. Aspirin shows peak at 1205 cm\(^{-1}\) for the analysis, corresponds to C-O stretch and salicylic acid shows peak at 1388 cm\(^{-1}\) for the analysis, corresponds to C-C stretch in the aromatic ring, selected for the further analysis. The most interesting part of this study is that that, these picks are not interfering with each other that make analysis very clear.

For the research, the standard IR spectra of aspirin and salicylic acid collected on FT-IR were confirmed and compared with standard spectra, which is collected from SDBS database for aspirin shown in Figure 18 and for salicylic acid shown in Figure 19. [50] Additional confirmation test perform was done by performing calibration of aspirin and salicylic acid at different concentrations.
Figure 15: Acid-catalyzed aspirin hydrolysis
Figure 16: Base-catalyzed aspirin hydrolysis
Figure 17: *In-situ* FT-IR spectra of aspirin and salicylic acid
Figure 18: IR spectra of aspirin from SDBS database (Adopted from reference 51)
Figure 19: IR spectra of salicylic acid from SDBS database (Adopted from reference 51)
CHAPTER 2

DISSOLUTION AND NONLINEAR BEHAVIOR OF ACETAMINOPHEN

2.1 Introduction

Dissolution testing can give an *in-vitro* snapshot of how the drug product may behave *in-vivo*. As a result, the number of dissolution methods in the United States Pharmacopeia has grown substantially. [51] The potential impact of a new analytical technique that permits *in-situ* analysis of multiple active ingredients during dissolution testing is profound.

This section focuses on the dissolution of over-the-counter acetaminophen tablets. The study of the dissolution of acetaminophen was done earlier by conventional methods. [52] These conventional methods need manual sampling which disturbs the dissolution process during analysis by the traditional way using UV or HPLC. [53] The main purpose of this study is to perform dissolution and analysis of acetaminophen tablet by a novel technique using *in-situ* FT-IR which is compared to UV and HPLC.

2.2 Experimental Section

2.2.1 Chemicals and materials

Acetaminophen reference material used in this study (batch no. 104K0154) was purchased from Sigma-Aldrich (St. Louis, MO). Acetaminophen tablets (Tylenol extra strength batch no. SLA175) were purchased from a local pharmacy. Acetaminophen extended release tablets (Tylenol Arthritis Pain batch no. 09FMC085) were purchased from a local pharmacy. Methanol, acetone and acetonitrile (HPLC grades) were purchased from Pharmaco-Aaper (Brookfield, CT). Sodium
hydroxide (batch no. 064214BH), used to prepare the pH buffered solutions, was purchased from Sigma-Aldrich (St. Louis, MO). Potassium phosphate monobasic (batch no. 103K0060), used to prepare the pH buffered solutions, was purchased from Sigma-Aldrich (St. Louis, MO). Sodium chloride crystals (batch no. J39602) was purchased from Mallinckrodt Chemicals (England, UK). All solutions were prepared using water treated by a Milli-Q plus Millipore purification system (Milford, MA). All purified water aliquots have a resistivity of not less than 18 MΩm-cm⁻¹.

2.2.2 Instrumentation

All chemicals were weighed on a Mettler Toledo balance (Washington Crossing, PA) PB303 Delta-Range® and AG204 Delta-Range®. The pH of the buffer was measured on pH meter by VWR SympHony (Radnor, PA) SB70P. Dissolutions were carried out on the Easy Max™ 102 by Mettler Toledo. HPLC analysis was performed on a Hewlett-Packard 1050 HPLC, with ChemStation software. All UV measurements were carried out using a Hewlett-Packard UV instrument (model no. 8452A diode array). The UV instrument was operated using Olis Spectralworks. All manual dissolutions were tested using a Van-kel Dissolution Bath (model no. 700 and serial no. 31-214-1296).

In-situ FT-IR analysis was performed on a Mettler Toledo iC 10 ReactIR, with iCIR versions 3.0 and 4.0 software. The ReactIR™ iC10 FTIR instrument is composed of a Mercury Cadmium Telluride detector (liquid nitrogen cooled) and a FiberConduit™. The FiberConduit™ is comprised of flexible IR transparent silver chloride/silver bromide optical fibers. The fiber optic probe interface (AgX 9.5 mm x 1.5 m Fiber (Silver Halide)) contains a diamond tip-DiComp ATR crystal. The resolution was set to 8 wavenumbers. The optical range used by the system is: 1900 cm⁻¹ to 650 cm⁻¹. The gain adjustment was set to normal (1x) and the apodization method was set
to Happ-Genzel. The system uses compressed air (house air, filtered and dehumidified) to purge the optics.

2.2.3 Potassium phosphate buffer (pH 5.8)

Potassium phosphate buffer (0.2 M) was prepared as per the USP procedures of buffer solutions. [54] For 200 mL of buffer 50 mL of 0.2 M potassium phosphate monobasic solution and 3.6 mL of 0.2 M sodium hydroxide solution were used. The volume was made up with deionized water to 200 mL and then the pH was measured.

2.2.4 Acetaminophen solutions for calibration

For the calibration study, 137.5 mg of acetaminophen (99 %) was weighed and dissolved in 100 mL of phosphate buffer pH 5.8 and used as stock solution. Then 14 dilutions were made using the stock solution. For dilution added 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 mL stock solution by using volumetric pipettes in each 25 mL volumetric flask and made volume with phosphate buffer pH 5.8, then concentration of each flask was 0.0275, 0.055, 0.0825, 0.110, 0.165, 0.220, 0.275, 0.330, 0.385, 0.440, 0.495, 0.550, 0.605 and 0.660 mg/mL respectively analyzed samples on UV spectroscopy and in-situ FT-IR.

2.2.5 Standard solutions

The standard solution of acetaminophen was prepared by weighing 55 mg of acetaminophen and dissolved in 100 mL of phosphate buffer pH 5.8 resulting concentration 0.55 mg/mL.

2.2.6 Dissolution of acetaminophen (Extra Strength Tylenol)

Tylenol tablets 500 mg of acetaminophen (Label Claim) were tested in pH 5.8 phosphate buffer as per acetaminophen USP monograph. [55] Dissolutions were conducted using vessel volumes of
900 mL and at 37.0 °C on Van-kel dissolution bath. All dissolutions were conducted using USP Apparatus Type II (paddles) with an agitation speed of 50 rpm.

**2.2.7 Dissolution of acetaminophen (Arthritis Tylenol)**

Tylenol (Arthritis Pain) tablets 650 mg of acetaminophen (Label Claim) were tested in pH 5.8 phosphate buffer 64 mins. Dissolutions were conducted using vessel volumes of 900 mL and at 37.0 °C on Van-kel dissolution bath. All dissolutions were conducted using USP Apparatus Type II (paddles) with an agitation speed of 50 rpm.

**2.2.8 In-situ FT-IR analysis**

For the calibration as well as the dissolution of acetaminophen experiments, data treatment was carried out using the following methodology: The data was first subjected to baseline correction. The absorption band at 1246 cm\(^{-1}\) (corresponding to the C-N stretch) was selected for acetaminophen. The peak area was calculated using a two-point baseline correction set at 1265 cm\(^{-1}\) and 1230 cm\(^{-1}\). The ATR-IR system was configured to collect spectra every two minutes at room temperature.

**2.2.9 HPLC analysis**

1.0 mL aliquots were collected during dissolution testing after a time interval (2, 4, 8, 16, 32 and 64 mins) and filtered using a syringe filter (0.45 µm). The column used was Phenomenex Luna 5µm Phenyl-Hexyl column (4.6 x 150 mm), with mobile phase 70/30/1 (methanol/water/trifluoroacetic acid). The flow rate was set 0.5 mL/min, isocratic mode, with an injection volume 10 µL and the absorbance set at 296 nm.
2.3 Results and Discussion

2.3.1 Calibration of acetaminophen

The USP monograph specifies the pH 5.8 phosphate buffer as the medium of choice for acetaminophen dissolution. Hence all standard diluted solutions were prepared in phosphate buffer pH 5.8. Based on the in-situ FT-IR spectra and UV/Vis data obtained for acetaminophen, at low concentrations (≤ 0.11 mg/ml), the relationship between absorbance and concentration is effectively linear ($r^2 = 0.9967$). However, non-linear behavior was observed for both IR ($r^2 = 0.9985$) and UV-Vis ($r^2 = 0.9981$) methods at higher concentrations.

Figure 20 shows the calibration data of acetaminophen standard solutions. The calibration was run from 0.05 to 0.66 mg/mL concentration of acetaminophen. 2nd order polynomial curve with $r^2 = 0.999$ was observed for both FT-IR as well as UV/Vis spectroscopy.

Figure 21 shows the calibration data of acetaminophen low standard solutions. The calibration data was from 0.05 to 0.11 mg/mL concentration of acetaminophen. The calibration is a linear, which ran on the UV/Vis spectroscopy with $r^2 = 0.997$.

Both methods can be modeled using second-order polynomial equations and has been confirmed after multiple replications. [56] The hypothesis has established that it is because of the dimerization of the acetaminophen. The proposed statement is that in the higher concentrations the acetaminophen molecules form dimers. The detailed experimentation is discussed in the later part of this section.
Figure 20: Non-linear graph for different levels of acetaminophen reference standards
Figure 21: Linear calibration graph of acetaminophen at low concentrations

Acetaminophen reference standards in 5.8 phosphate buffer, UV/Vis absorbance at 296 nm, 5-20% dilutions

\[ y = 2.1924x + 0.0122 \]

\[ R^2 = 0.9967 \]
2.3.2 Dissolution of Extra Strength Tylenol

At the initial stage of the research, dissolution of extra strength Tylenol was performed using UV/Vis spectroscopy to understand the dissolution procedure and analysis. As per the procedure which is discussed in the experimental section, dissolution was performed three times using 5 vessels with a Van-Kel dissolution bath. Aliquots were collected for UV/Vis analysis after 2, 4, 8, 16, 32 and 64 mins respectively. The percent dissolution was calculated by using following formula.

\[
\% \text{ Dissolution} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \times \frac{\text{Standard Mass}}{\text{Standard Volume}} \times \frac{\text{Vessel Volume}}{\text{Potency}} \times \text{Standard Purity} \times 100
\]  

Figure 22 shows the dissolution of extra strength Tylenol. The tablets were dissolved in 15-20 mins and all vessel data overlapped as per expectations. Maximum drug dissolved after 60 mins were 91%.

It is noticed that percent dissolution observed during this study is slightly lower than percent dissolution observed during other experiments carried out with Extra strength Tylenol in last few decades. This observation could be due to the hypothesis of the calibration of acetaminophen which showed polynomial curve at increased concentration.

Figure 23 shows that recalculated percent dissolution of acetaminophen by using polynomial equation from the calibration graph and observed that the percentage of dissolution has increased to 96%. Based on these calculations and results, it confirmed the hypothesis at higher concentrations acetaminophen molecules form dimers.
Figure 22: Dissolution of acetaminophen

Dissolution data 5 vessels of Acetaminophen in 5.8 phosphate buffer, UV/Vis absorbance at 296 nm
Figure 23: Dissolution of acetaminophen after polynomial calculations
2.3.3 Dissolution of Tylenol (Arthritis Pain)

After studying the UV/Vis analysis and dissolution procedures, the research was extended towards the main goal of the thesis which was the in-situ FT-IR dissolution and its comparison with HPLC. Tylenol has a long lasting effect on the arthritis pain and hence the dissolution was performed for 8 Hrs. Aliquots were taken throughout the experiment and analyzed to compare the in situ FT-IR method with HPLC and UV/Vis methods.

The results from the in situ FT-IR, UV/Vis and HPLC systems are overlaid as shown in Figure 24. An excellent correlation was observed between these three methods. The amount of released acetaminophen by HPLC (yellow Line) and UV/Vis (green Line) is little higher than the in-situ FT-IR (Orange line) in the early part of the dissolution from 30 mins to 2 hr. This might be because of the dissolution-in-dissolution concept which happens in the HPLC and UV/Vis vials; fine particles of acetaminophen can appear after filtration in the vial was dissolved again in the dissolution media upon storage of sample for the analysis and showed higher concentration than the in-situ FT-IR. This shows the advantage of the novel technique in-situ FT-IR, which avoids further dissolution of the drug in the sample vial upon wait time for analysis.
Figure 24: Dissolution data of acetaminophen (Arthritis pain)
2.4 Non-Linear behavior of Acetaminophen

In acetaminophen research, it has been observed from the calibration data that acetaminophen has a non-linear behavior. The calibration study was done by UV-vis spectroscopy and \textit{in-situ} FT-IR from concentration ranges from 0.005 mg/mL to 0.66 mg/mL. At the lower concentration the calibration showed a linear graph in Figure 21, but at higher concentrations, it is non-linear as shown in Figure 20. On searching for literature explaining this behavior specifically for acetaminophen, it was found that acetaminophen has a tendency to form dimer with respect to pH of the solution. [57] [58] The polymorphism study of acetaminophen showed that the interplay of molecular flexibility and hydrogen bonding manifested in the monoclinic and orthorhombic polymorphs of acetaminophen and with the X-ray crystallographic study has confirmed. [59] [60] There is evidence about the formation of acetaminophen hemi adducts due to the formation of chains for acetaminophen molecule linked via OH…O=C interaction or NH….O=C interaction. [61]

In this research, the acetaminophen was in solution form, where it was dissolved in phosphate buffer pH 5.8. There is no evidence which claiming the formation of a dimer in these conditions. Because of the importance of acetaminophen as medicine, there is a need to better understand this unusual behavior of acetaminophen. Based on the observed results, the hypothesis was proposed that the non-linear behavior is because of the dimerization showed in reaction 1 or aggregation of the acetaminophen molecules showed in reaction 2.
The main focus is to study the non-linear behavior of acetaminophen by different instrumental techniques and by studying colligative properties to calculate the number of moles of acetaminophen in the buffer solution.

Investigation of the dimerization of acetaminophen has been performed by different techniques, Infrared spectroscopy (IR), Nuclear Magnetic Resonance spectroscopy (NMR) and colligative properties in that by vapor pressure osmometry and freezing point depression.

Investigation of dimerization of acetaminophen is possible by IR spectroscopy. It can identify interactions that occur as a result of hydrogen bonding which leads to dimeric and oligomeric structures shown in Reaction 1 and Reaction 2. The carbonyl group present is around 1600-1700 cm\(^{-1}\) wave numbers. There is literature, which describes the use of IR spectroscopy to detect
dimerization of carboxylic acid structures. This suggests that this technique can be used to study acetaminophen in nonpolar solvents. [62] [63] [64] Literature gave evidence that, because of formation of dimer, which creates a new hydrogen bond and shows a new broad peak for the dimer in the region of 3300 cm\(^{-1}\)-3700 cm\(^{-1}\). [65] IR Spectroscopy has been used to identify the phosphoric acid dimerization a well-known example of dimerization. [66] [67]

Another common example for the study of the nonlinear behavior is benzoic acid which is shown in reaction 3.

**Reaction 3**

![Reaction 3](image)

The investigation of the benzoic acid dimer was done by IR spectroscopy. They observed a broadening and shift of the carbonyl stretching frequency. [68] Literature studies provide evidence about the benzoic acid dimer study. [69] [70] [71] Literature study of dimerization shows the IR spectroscopy study will be useful for investigation and confirmation.

The non-linear behavior of acetaminophen which is because of the aggregation or dimerization of molecules can be investigated by NMR. That gives the information about the chemical shift of hydrogen and carbon peak which moves to downfield. [72] The proton transfer in the benzoic acid dimer was measured by the NMR. [73] The literature showed that the study of dimerization and the oligomerization can be analyzed by NMR, even quantitative analysis can be done by NMR.
Literature suggested another way to determine the dimer formation of acetaminophen in phosphate buffer by NMR analysis.

The presence of one or more solutes alters the ability of the solvent molecules to interact, reducing their freedom of movement, thus the ability to move from liquid to solid is altered. These changes, which collectively are referred to as colligative properties, are dependent on the total number of particles present in the solution. One of the colligative property, osmotic pressure a direct measurement of the molar concentration of total solutes in an aqueous solution. Osmotic pressure is defined as the force which would be required to resist movement of solvent across a membrane which is permeable to the solvent but not to the solute molecules. A solute particle can be a molecule or an ion or an aggregated species such as dimer that can exist discretely in solution. Since osmotic pressure is directly related to the total molar concentration of solute, there is a direct and linear relationship between osmotic pressure.

Another excellent way to determine the molecular weight of dissolved acetaminophen is by measuring the vapor pressure of the solution. By osmometry, one can measure the vapor pressure of the acetaminophen solution and can calculate the molecular weight and then moles of the solution.

Thus, if acetaminophen dimerizes or oligomerizes, the measured molar mass will be larger than the molar mass of the monomer of 151.16 g/mol. However, it is expected that the measured molar mass will be a weighted average of monomer, dimer and oligomer species that will be present. Thus \( M = x \) (monomer) + \( y \) (dimer) + \( z \) (trimer) + \( zz \) (tetramer +…). Robert performed some
forensic laboratory experiments to calculate the weights of drugs and diluents by osmometry, which gave the motivation of performing the experiments to calculate the moles of acetaminophen.

A solution freezes at a lower temperature than does the pure solvent. This phenomenon is called freezing point depression. The freezing point depression of a solution is a colligative property of the solution which is dependent upon the number of dissolved particles in the solution. The higher the solute concentration, the greater the freezing point depression of the solution. [81] The freezing point values are determined from the cooling curves obtained by recording the temperature as a function of time. [82] Another application of this method is mainly in the food industry to study the thermodynamic properties of frozen food and fruits. [83] [84] Freezing point depression is used to determine the molecular mass of polyols which avoids the interference by water, by using acetophenone as a solvent having freezing point depression 20°C. [85]

After reviewing all this literature performed the different experiments on IR, NMR and for colligative properties such as vapor pressure osmometer and Freezing point depression as well. The results from these experiments could not support the hypothesis that at higher concentrations acetaminophen molecules form dimer. There is a lack of any evidence to explain or confirm the dimerization of acetaminophen as per the proposed hypothesis.
2.5 Conclusion

The *in-situ* infrared spectroscopy is a viable alternative for measuring dissolution profiles of pharmaceutical tablets. The *in-situ* FT-IR system was found to be impressive and validated by comparing dissolution profile using UV/Vis and HPLC. The method was calibrated with acetaminophen standard solutions ranging from 0.05 mg/mL to 0.66 mg/mL, with $r^2=0.998$. The percent dissolution of acetaminophen was found in the range of 95-97 % by all three techniques.

The *in-situ* FT-IR has great advantage of minimizing the manual efforts required for manual sampling of dissolution. On the contrary, UV/Vis and HPLC methods require laborious manual sampling. With the current configuration of the *in-situ* FT-IR instrument, this analysis is limited by the sensitivity and wavelength range of the *in-situ* fiber optic probe. However, since this chapter successfully demonstrates the versatility of this novel application of *in-situ* FT-IR spectroscopy.

Unsatisfactory results were observed for the hypothesis of the dimerization of acetaminophen. As part of this study, multiple experiments were designed and performed on the respective instruments (IR, NMR and using colligative properties such as vapor pressure osmometer and freezing point depression) for the dimerization study of acetaminophen. But still, there is a lack of evidence to support the proposed dimerization hypothesis.
CHAPTER - 3
DISSOLUTION AND HYDROLYSIS STUDY OF ASPIRIN

3.1 Introduction

This section focuses on monitoring the dissolution and hydrolysis of over the counter tablet acetylsalicylic acid (aspirin) under acidic conditions by In-situ FT-IR spectroscopy using attenuated total reflectance infrared probe (ReactIR). The acetylsalicylic acid undergoes hydrolysis to form salicylic acid and acetic acid. [86] The equation of aspirin hydrolysis is written as mentioned below.

Reaction 4

\[
\text{Aspirin} + \text{H}_2\text{O} \rightarrow \text{Salicylic Acid} + \text{Acetic Acid}
\]

The majority of the methods for monitoring dissolution utilize UV/Vis spectroscopy. For example, aliquots are manually withdrawn and analyzed by UV/Vis spectroscopy or HPLC with ultraviolet detection. However, the sampling process is disruptive to the dissolution profile since the removal of aliquots from the vessel disturbs the solution. In addition, there are instruments that allow real-time analysis using in-situ UV/Vis probes. For example, fiber optic dissolution testing is used in the pharmaceutical industry to monitor drug product release. Fiber optic dissolution is also used for formulation development. [87] Formulators are using these in-situ UV/Vis systems to profile
and develop drugs faster, instead of conventional techniques where dissolutions are conducted manually and analyzed offline. The advantage of the fiber optic system is that it allows real-time data analysis. But the major disadvantage of UV/Vis system is a small range of the analysis that is 200-400 nm, in the case of aspirin and salicylic acid study both peaks overlap of the main maxima and limits the analysis which needs additional determination for methods. [88]

There is an interest to develop new methods that do not require manual sampling. The use of in-situ FT-IR for analyzing aqueous samples is limited by the relatively high concentration of analyte required for detection. [89] Hence this research investigated the use of in-situ FT-IR spectroscopy as a potential technique for monitoring and understanding dissolution. The research showed that dissolution using in-situ FT-IR spectroscopy for the single component as well as multi-component formulations is useful for monitoring the release profile of each active pharmaceutical ingredient (API). [90] Thus, there is also an interest in the development of hydrolysis of a prodrug aspirin while performing dissolution and study kinetics of that process.

3.2 Experimental Section

3.2.1 Chemicals and materials

Acetylsalicylic acid (aspirin) reference material (batch no. 016K0131) was purchased from Sigma-Aldrich (St. Louis, MO). Salicylic acid reference material (batch no. 04708HE) was purchased from Sigma-Aldrich (St. Louis, MO). Acetylsalicylic acid 325 mg tablets (Genuine Bayer batch no. 219050N) were purchased from a local pharmacy. Methanol, acetone and acetonitrile (HPLC grades) were purchased from Pharmaco-Aaper (Brookfield, CT). Sodium chloride crystals (batch no. J39602) was purchased from Mallinckrodt Chemicals (England, UK). Hydrochloric acid (batch no. H44032) was purchased from J.T. Baker (Central Valley, PA). Sodium acetate crystals (batch
no.05816HD, potassium phosphate monobasic (batch no. 103K0060) and sodium hydroxide (batch no. 06414BH) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Glacial acetic acid (batch no. 971666) was purchased from Fisher Chemicals (Waltham, MA). All solutions were prepared using water treated by a Milli-Q plus Millipore purification system (Milford, MA). All purified water aliquots have a resistivity of not less than 18 MOhm-cm\textsuperscript{-1}.

### 3.2.2 Instrumentation

All chemicals were weighed on a Mettler Toledo balance (Washington Crossing, PA) PB303 Delta-Range\textsuperscript{®} and AG204 Delta-Range\textsuperscript{®}. The pH of the buffer was measured on a VWR SympHony (Radnor, PA) SB70P pH meter. Dissolutions were carried out on the Easy Max\textsuperscript{TM} 102 by Mettler Toledo. HPLC analysis was performed on a Hewlett-Packard 1050 HPLC, with ChemStation software. In-situ FT-IR analysis was carried on a Mettler Toledo iC 10 ReactIR, with iCIR versions 3.0 and 4.0 software.

### 3.2.3 Simulated gastric fluid

Simulated gastric fluid was prepared with hydrochloric acid and sodium chloride as per USP buffer procedure. [91] 2 g of sodium chloride was dissolved in 100 mL of deionized water, in that 7 mL HCl was added and then volume made up to 1000 mL by adding deionized water, and then measured pH of that solution and was adjusted to 1.2.

### 3.2.4 Sodium acetate buffer

Sodium acetate buffer was prepared with glacial acetic acid and sodium acetate anhydrous as per the USP buffer procedures. [92] 2.99 g of sodium acetate was dissolved in 100 mL of deionized water, in that 1.66 mL glacial acetic acid was added and then volume made up to 1000 mL by adding deionized water and then measured pH of that solution and was adjusted to 4.5.
3.2.5 Potassium phosphate buffer

Potassium phosphate buffer (0.2 M) was prepared with monobasic potassium phosphate and sodium hydroxide as per the USP buffer procedures. [93] First potassium phosphate solution was prepared by weighing 27.22 g of monobasic potassium phosphate and dissolved in 100 mL of deionized water and diluted with up to 1000 mL. Next sodium hydroxide solution was prepared by weighing 8 g of sodium hydroxide dissolved in water and made volume up to 100 mL. For phosphate buffer added 50 mL of monobasic potassium phosphate solution in a 200 mL volumetric flask then added 22.4 mL of sodium hydroxide solution and then added water to make a volume. Finally measured the pH of this solution and was adjusted to 6.8.

3.2.6 Aspirin solution for calibration

Aspirin stock solution was prepared by dissolving 500 mg of aspirin in 100 mL of simulated gastric fluid (pH 1.2), sodium acetate buffer (pH 4.5) and phosphate buffer (pH 6.8). To prepare a calibration curve, nine dilutions were made by using this stock solution by adding 1.00, 2.00, 3.00, 4.00, 5.00, 6.00, 7.00, 8.00 and 9.00 mL to 10.00 mL volumetric flasks respectively and diluting to volume with respective buffer solution. The concentration of aspirin in each flask was 0.500, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00 and 4.50 mg/mL as well as 3.62, 7.24, 10.9, 14.5, 18.1, 21.7, 25.3, 28.9 and 32.6 mM respectively. Aspirin starts hydrolyzing as it goes into solution, to avoid or to slow down that process; solutions were placed into an ice bath immediately after the preparation. As soon as solutions were ready, they were scanned by in-situ FT-IR for the calibration study at room temperature.
3.2.7 Salicylic acid solution for calibration

A stock solution of salicylic acid was made by dissolving 500 mg of salicylic acid in 100.00 mL of simulated gastric fluid (pH 1.2), sodium acetate buffer (pH 4.5) and phosphate buffer (pH 6.8). To prepare a calibration curve, nine dilutions were made by using this stock solution by adding 1.00, 2.00, 3.00, 4.00, 5.00, 6.00, 7.00, 8.00 and 9.00 mL to 10.00 mL volumetric flasks respectively and diluting to volume with respective buffer solution. The concentration of salicylic acid in each flask was 0.500, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00 and 4.50 mg/mL as well as 2.78, 5.55, 8.33, 11.1, 13.9, 16.7, 19.4, 22.2 and 25.0 mM respectively.

3.2.8 Hydrolysis of aspirin to salicylic acid in test tubes

Prior to the hydrolysis experiments, a qualitative measurement was investigated for determining the presence of salicylic acid in solution. Acetylsalicylic acid hydrolyzes to salicylic acid in the of acidic medium. Ferric chloride test was found to be a good qualitative test for phenols. Ferric chloride in solution gives colors with a number of organic derivatives. The common ones are phenols, enols, oximes, hydroxamic acids, and some carboxylic acids. It will, however, react with salicylic acid, which is used to synthesize aspirin, forming an iron-phenol complex. [94] [95] Thus, adding an aqueous ferric chloride solution to an aspirin solution is a good way to see if there is any salicylic acid present. The ferric chloride color test is shown below. A small experiment was performed in that used three vials containing simulated gastric fluid (vial C) as control, aspirin solution (vial A) and salicylic acid solution (vial S).
3.2.9 Standard solutions

The standard solutions were prepared by weighing 325 mg of aspirin and 325 mg salicylic acid and dissolved in 100 mL of simulated gastric fluid, sodium acetate buffer and potassium phosphate respectively and obtained concentration 3.25 mg/mL.

3.2.10 Dissolution and hydrolysis of aspirin

Bayer Aspirin tablets 325 mg of aspirin (Label Claim) were measured in pH 1.2 simulated gastric fluid solutions for 1.5 hr., pH 4.5 sodium acetate buffer for 24 hr and potassium phosphate buffer for 24 hr. Dissolutions were conducted using vessel volumes of 100 mL at 37.0 °C. All dissolutions were conducted using USP Apparatus Type II (paddles) with an agitation speed of 100 rpm as per the USP procedure and collected samples for HPLC as well.

3.2.11 In-situ FT-IR Analysis

3.2.11.1 Aspirin analysis

A distinct peak for aspirin without interference was observed. Calibration of aspirin with standard diluted solutions as discussed in experimental section. For the calibration of aspirin experiments, data treatment was carried out using the following methodology: The data was first subjected to baseline correction. The absorption band at 1205 cm⁻¹ (corresponding to the C-O stretch) was selected for acetylsalicylic acid. The study was done by using three different body pHs such as simulated gastric fluid (pH 1.2), sodium acetate buffer (pH 4.5) and phosphate buffer (pH 6.8) which depicts stomach, duodenum and small intestine pHs respectively.
3.2.11.2 Salicylic acid analysis

A distinct peak for salicylic acid without interference was observed which is shown in Figure 17, then performed calibration of salicylic acid with standard diluted solutions as discussed in experimental section. For the calibration of salicylic acid experiments, data treatment was carried out using the following methodology: The data was first subjected to baseline correction. The absorption band at 1488 cm\(^{-1}\) (corresponding to the C-C stretch in the aromatic ring) was selected for salicylic acid. Although a unique absorption at 1161 cm\(^{-1}\) (corresponding to the C-O stretch) was observed for salicylic acid, 1488 cm\(^{-1}\) was chosen for further analysis because of its higher intensity. The study was done by using three different body pHs such as, simulated gastric fluid (pH 1.2), sodium acetate buffer (pH 4.5) and phosphate buffer (pH 6.8) which depicts stomach, duodenum and small intestine pHs respectively.

3.2.11.3 Dissolution and hydrolysis of aspirin tablet

The dissolution testing was carried out as discussed in the experiment section. The in-situ FT-IR fiber optic probe collected baseline spectra (blank medium) for the first 30 minutes. A single 325 mg Bayer aspirin tablet was dropped into the vessel containing 100 mL of buffer solution (simulated gastric fluid pH 1.2, sodium acetate buffer pH 4.5 and potassium phosphate buffer pH 6.8) and the dissolution profiles were collected using in-situ FT-IR spectroscopy. Standard spectra of pure aspirin and salicylic acid were collected prior to running the dissolutions. These standards were used to identify the unique peaks of interests for these active drugs. For the aspirin tablet analysis, the same steps as discussed in calibration analysis were followed.
For this experiment, data processing is critical to obtain well-resolved spectra. For all \textit{in-situ} FT-IR experiments, 256 scans were collected and co-added for each spectral point and each data point took about two minutes to complete. The peak of interest was identified (1205 cm\(^{-1}\)) and the area was calculated using a two-point baseline correction. A trend analysis was performed using the iCIR software on this peak. This provided a plot of absorbance vs. time. This plot was subjected to smoothing using a 25 point smoothing parameter. After smoothing, and while still in the trend analysis, the baseline correct routine was performed. Then, the data were exported to Excel. Further data processing in Excel involved the subtraction of solvent spectra background. A 30 min set of spectra at the beginning of the experiment were obtained before aspirin pill was added. These spectra were subtracted from the spectra taken after the pill was dropped to give the final set of data. Within the final set of data, the area of the 1205 cm\(^{-1}\) peak was calculated at each time point. Then the plot of area vs. time was performed to give rate information. In addition, using a calibration curve, peak areas were converted to concentration to provide concentration vs. time plots.

\subsection*{3.2.12 HPLC analysis}

The dissolution aliquots (1.0 mL) were collected after a certain time interval and filtered using syringe filter (0.45 µm) transferred to an ice bath to prevent further hydrolysis. The column was used is Phenomenex C18 5 µm Luna column (4.6 x 150 mm), with mobile phase 60/40/1 (methanol/water/trifluoroacetic acid) with 13 min run time. The flow rate was set to 0.5 mL/min, isocratic mode, with an injection volume 10 µL and absorbance was measured at 300 nm.
3.3 Results and Discussion

3.3.1 Calibration of aspirin

Based on the IR spectra obtained, aspirin has unique IR bands at 1205 cm$^{-1}$. Linear regression for aspirin was calculated by using the area under curve (AUC) calculations of the IR absorption band at 1205 cm$^{-1}$. The analysis was performed as discussed in the experimental section and by plotting the concentration vs Intensity of AUC of the peak, it was determined that aspirin had a linear correlation.

Figure 25 shows the calibration data of aspirin with different concentrations of standard aspirin solution. The concentrations range from 3 mM to 33 mM with $r^2 = 0.9922$ of AUC of aspirin IR peak vs. concentration in simulated gastric fluid (pH 1.2).

Figure 26 shows the calibration data of aspirin with different concentrations of standard aspirin solution. The concentrations range from 3 mM to 33 mM with $r^2 = 0.9979$ of AUC of aspirin IR peak vs. concentration in sodium acetate buffer (pH 4.5).

Figure 27 shows the calibration data of aspirin with different concentrations of standard aspirin solution. The concentrations range from 3 mM to 33 mM with $r^2 = 0.9926$ of AUC of aspirin IR peak vs. concentration in potassium phosphate buffer (pH 6.8).
3.3.2 Calibration of salicylic acid

Based on the IR spectra obtained, salicylic acid has an IR frequency of interest at 1488 cm\(^{-1}\) (for simulated gastric fluid study) and 1388 cm\(^{-1}\) (for duodenum and intestine study). Linear regression for salicylic acid was calculated by using the area under curve calculations of the IR absorption band at 1388 and 1488 cm\(^{-1}\). The analysis was performed as discussed in the experimental section and by plotting the concentration vs intensity of AUC of the peak, it was determined that salicylic acid had a linear correlation.

Figure 28 shows the calibration data of salicylic acid with different concentrations of standards with concentrations range from 3 mM to 33 mM with \(r^2=0.9908\) of AUC of aspirin IR peak vs. concentration in simulated gastric fluid pH (1.2).

Figure 29 shows the calibration data of salicylic acid with different concentrations of standards concentrations from 3 mM to 33 mM with \(r^2=0.9961\) of AUC of aspirin IR peak vs. concentration in sodium acetate buffer (pH 4.5).

Figure 30 shows the calibration data of salicylic acid with different concentrations of standards concentrations from 3 mM to 33 mM with \(r^2=0.9954\) of AUC of aspirin IR peak vs. concentration in potassium phosphate buffer (pH 6.8).
Figure 25: Calibration graph of aspirin in simulated gastric fluid (pH 1.2)

\[ y = 0.027x + 0.3284 \]

\[ R^2 = 0.9922 \]
Figure 26: Calibration of aspirin in sodium acetate buffer (pH 4.5)

\[ y = 0.0153x - 0.0088 \]

\[ R^2 = 0.9926 \]
Figure 27: Calibration of aspirin in potassium phosphate buffer (pH 6.8)

\[ y = 0.0088x + 0.0296 \]
\[ R^2 = 0.9979 \]
Figure 28: Calibration graph of salicylic acid in simulated gastric fluid (pH 1.2)
Figure 29: Calibration graph of salicylic acid sodium acetate buffer (pH 4.5)

\[ y = 0.0092x + 0.0231 \]

\[ R^2 = 0.9961 \]
Figure 30: Calibration graph of salicylic acid potassium phosphate buffer (pH 6.8) in [mM]

\[ y = 0.0086x + 0.0373 \]

\[ R^2 = 0.9954 \]
3.3.3 **Hydrolysis of aspirin to salicylic acid in test tube**

A qualitative measurement was investigated to determine the presence of salicylic acid in solution. This was carried out for the confirmation of the hydrolysis of the aspirin. Figure 31 shows that after adding ferric chloride solution at 0 min, a purple color has formed immediately in a vial containing salicylic acid (Vial-S), due to the formation of the iron-phenol complex. A sample of pure aspirin (vial-A) should not exhibit any color change at time 0 min. Formation of iron phenol complex gives definite color ranging from red to violet, depending on the amount of salicylic acid present and pH of the buffer solution. After 60 min aspirin (vial A) showed a color change from colorless to violet indicates that formation of salicylic acid after hydrolysis. The same test was performed after the dissolution of aspirin tablet in simulated gastric fluid. The color of the dissolution solution was changed from clear to purple after adding few drops of ferric chloride solution, which confirmed the hydrolysis of aspirin to salicylic acid.
Figure 31: Ferric chloride color test on standard solutions and on samples in the flask.
3.3.4 Dissolution and hydrolysis of aspirin tablet in simulated gastric fluid pH 1.2

This study was focused on performing dissolution, simultaneously observing hydrolysis of aspirin by in-situ FT-IR in simulated gastric fluid. Figure 32 shows the dissolution and hydrolysis of aspirin and formation of salicylic acid with time. The dissolution profile was monitored by in-situ FT-IR and confirmed by HPLC analysis. The graph for both methods showed overlapping data which gives the authentication of the in-situ FT-IR method. The graph has two lines, a brown line depicts the dissolution of aspirin and a blue line depicts the formation of salicylic acid. A brown line with steep upward trend indicates dissolution of aspirin in first 20 mins followed by downward trend indicating hydrolysis of aspirin after 20 min. The blue line indicates the formation of salicylic acid during this process. At initial stage of dissolution, small amount of salicylic acid was detected. This might be because of the salicylic acid produced by aspirin in the process of manufacturing or hydrolysis of aspirin can occurred upon storage. It is observed that the rate of dissolution is faster at pH 1.2 compared to other pHs. The rate of dissolution is 1.706 mM/min in simulated gastric fluid. Almost 90% of the aspirin was dissolved in first 15-20 min in simulated gastric fluid pH 1.2, which indicates that stomach is the best place for the dissolution of the aspirin tablet. It was observed that the rate of the dissolution rate of hydrolysis also faster than other pHs.
After the dissolution and hydrolysis study was confirmed, a replicate study with the same experimental conditions was performed as shown in Figure 33. During the replicate study, the result of dissolution and hydrolysis of aspirin and formation of salicylic acid overlapped with the initial study which confirmed the method.

Figure 34 shows the combined study of all dissolutions and hydrolysis of aspirin from all the experiments. Data from all these experiments were combined to calculate standard deviation, which is shown in the form of error bars within the range of 4-5%.

Table 2 shows the amount of aspirin and salicylic acid by in-situ FT-IR and HPLC methods. In this table, the quantities are mentioned in the mM concentration instead of percent dissolution. The Bayer aspirin tablet contains 325 mg of aspirin, was converted into millimolar concentration of 18.03 mM. The percent dissolution and hydrolysis of aspirin and formation of salicylic acid was also converted to mM.
Figure 32: Dissolution and hydrolysis of data in simulated gastric fluid (pH 1.2): *in-situ* FT-IR vs. HPLC
Figure 33: Dissolution and hydrolysis of data in simulated gastric fluid (pH 1.2): in-situ FT-IR, Overlay
Figure 34: Dissolution and hydrolysis of data in simulated gastric fluid (pH 1.2): *in-situ* FT-IR, Combined study
Table 2: Dissolution and hydrolysis data of aspirin tablet in simulated gastric fluid

<table>
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<th>Time in mins</th>
<th>Salicylic Acid by IR [mM]</th>
<th>Aspirin by IR [mM]</th>
<th>Salicylic Acid by HPLC [mM]</th>
<th>Aspirin by HPLC [mM]</th>
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<td>0.9</td>
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</tr>
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3.3.5 Dissolution and hydrolysis of aspirin tablet in sodium acetate buffer pH 4.5

In the method development of dissolution, it is a good practice to perform the dissolutions in different body pHs and study the dissolution pattern. After gastric simulated fluid tablet moves to the duodenum, another place where a tablet can dissolve in the body. This set of experiments performed dissolution and simultaneously observed hydrolysis of aspirin by *in-situ* FT-IR in sodium acetate buffer having pH 4.5 depicts duodenum pH and compared with HPLC.

Figure 35 shows the dissolution and hydrolysis of aspirin and formation of salicylic acid with time. The dissolution profile was monitored by *in-situ* FT-IR and confirmed by HPLC analysis. The graph for both methods showed overlapping data which gives the authentication of the *in-situ* FT-IR method. The graph has two lines, a brown line depicts the dissolution of aspirin and a blue line depicts the formation of salicylic acid. A brown line with steep upward trend indicates dissolution of aspirin in first 3-4 hrs followed by downward trend indicating hydrolysis of aspirin after 3-4 hrs. The blue line indicates the formation of salicylic acid during this process. Thus the dissolution and the hydrolysis of aspirin and at the same time formation of the salicylic acid was observed. At initial stage of dissolution, small amount of salicylic acid was detected. This might be because of the salicylic acid produced by aspirin in the process of manufacturing or hydrolysis of aspirin can occurred upon storage. It was observed that the rate of dissolution is slower in pH 4.5 sodium acetate buffer compared to simulated gastric fluid pH 1.2. The rate is affected on the basis of acidity of dissolution medium that is 0.545 mM/min. The dissolution and hydrolysis of aspirin
with the comparison of HPLC data and confirmed by this method. It was observed that the rate of the dissolution rate of hydrolysis was slower than simulated gastric fluid pH 1.2.

After the dissolution and hydrolysis study was confirmed, a replicate study with the same experimental conditions was performed as shown in Figure 36. During the replicate study, the result of dissolution and hydrolysis of aspirin and formation of salicylic acid are overlapped with the initial study which confirmed the method.

Figure 37 shows the combined study of all dissolutions and hydrolysis of aspirin from all the experiments. Data from all these experiments were combined to calculate standard deviation, which is shown in the form of error bars within the range of 3-4%.

Table 3 shows the amount of aspirin and salicylic acid by in-situ FT-IR and HPLC methods. In this table, the quantities are mentioned in the mM concentration instead of percent dissolution. The Bayer aspirin tablet contains 325 mg of aspirin, was converted into millimolar concentration of 18.03 mM. The percent dissolution and hydrolysis of aspirin and formation of salicylic acid was also converted to mM
Figure 35: Dissolution and hydrolysis of data in sodium acetate buffer (pH 4.5): *in-situ* FT-IR vs. HPLC
Figure 36: Dissolution and hydrolysis of data in sodium acetate buffer (pH 4.5): *in-situ* FT-IR, overlay
Figure 37: Dissolution and hydrolysis of data in sodium acetate buffer (pH 4.5): in-situ FT-IR, combined study
Table 3: Dissolution and hydrolysis data of aspirin tablet in sodium acetate buffer

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<th>Time in hrs.</th>
<th>Salicylic Acid by IR [mM]</th>
<th>Aspirin by IR [mM]</th>
<th>Salicylic Acid by HPLC [mM]</th>
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3.3.6 Dissolution and hydrolysis of aspirin tablet in potassium phosphate buffer pH 6.8

As per the physiological conditions, finally tablet reaches to the small intestine another place for dissolution of pharmaceutical dosage forms. In this set of experiments were performed dissolution and simultaneously observed hydrolysis of aspirin by in-situ FT-IR in phosphate buffer having pH 6.8 depicts small intestine pH and compared with HPLC.

Figure 38 shows the dissolution and hydrolysis of aspirin and formation of salicylic acid with time. The dissolution profile was monitored by in-situ FT-IR and confirmed by HPLC analysis. The graph for both methods showed overlapping data which gives the authentication of the in-situ FT-IR method. The graph has two lines, a brown line depicts the dissolution of aspirin and a blue line depicts the formation of salicylic acid. A brown line with steep upward trend indicates dissolution of aspirin in first 7-8 hrs followed by downward trend indicating hydrolysis of aspirin after 7-8 hrs. The blue line indicates the formation of salicylic acid during this process. Thus the dissolution and the hydrolysis of aspirin and at the same time formation of the salicylic acid was observed. At initial stage of dissolution, small amount of salicylic acid was detected. This might be because of the salicylic acid produced by aspirin in the process of manufacturing or hydrolysis of aspirin can occurred upon storage. It was observed that the rate of dissolution is slowest in potassium phosphate buffer pH 6.8 compared to other two pHs is 0.085mM/min. The dissolution and hydrolysis of aspirin with the comparison of HPLC data and confirmed by this method. It was observed that the rate of dissolution and hydrolysis was slowest than other pHs.
After the dissolution and hydrolysis study was confirmed, replicate study with the same experimental conditions were performed as shown in Figure 39. During the replicate study, the result of dissolution and hydrolysis of aspirin and formation of salicylic acid overlapped with the initial study which confirmed the method.

Figure 40 shows the combined study of all dissolutions and hydrolysis of aspirin from all the experiments. Data from all these experiments were combined to calculate standard deviation, which is shown in the form of error bars within the range of 3-4%.

Table 4 shows the amount of aspirin and salicylic acid by in-situ FT-IR and HPLC methods. In this table the quantities are mentioned in the mM concentration instead of percent dissolution. The Bayer aspirin tablet contains 325 mg of aspirin, was converted into millimolar concentration of 18.03 mM. The percent dissolution and hydrolysis of aspirin and formation of salicylic acid was also converted to mM.
Figure 38: Dissolution and hydrolysis of data in potassium phosphate buffer (pH 6.8): *in-situ* FT-IR vs. HPLC
Figure 39: Dissolution and hydrolysis of data in potassium phosphate buffer (pH 6.8): in situ, overlay
Figure 40: Dissolution and hydrolysis of data in potassium phosphate buffer (pH 6.8): *in-situ* FT-IR, combined study
Table 4: Dissolution and hydrolysis data of aspirin tablet in potassium phosphate buffer (pH 6.8)

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3.3.7 Kinetic study

The kinetic rates were also determined for the experiments discussed earlier. The kinetics of the dissolution and hydrolysis is based on the concentration of aspirin, so it is a first order reaction. Kinetic study was determined by using the slope of the line and by using following formula to study rate of dissolution and rate of hydrolysis.

\[
\ln[\text{A}] = -kt + \ln[\text{A}]_0
\]  

(10)

The rates are compared to literature research [97] [98] [99] and were found dissolution rate to be equivalent. The literature data shows the rate of hydrolysis of aspirin has a specific pH rate profile. [100] The experimental values showed comparable rate profile. In this study, the rate of hydrolysis of aspirin after dissolution is completely depends on the rate of dissolution. The main goal of this is to monitor dissolution and hydrolysis of aspirin tablet. For the hydrolysis reaction, there must be free aspirin present in the solution, which will be available after the dissolution of the tablet. So as the rate of dissolution is slow with a change in the pH, hydrolysis is also slow.

The rates of the reactions were determined from the dissolution plots. In order to calculate the rates using the plot, the following procedure was implemented. First, a plot log of percent dissolved vs. time is obtained which was shown in the initial part of result section. Next, a straight line is drawn over the area of the dissolution curve where dissolution starts to occur (middle area in this case). Furthermore, the rate of hydrolysis is also calculated with the same method using the graph where the hydrolysis starts, showed in the hydrolysis that is disappearance curve.
Table 5 shows the rates of the dissolution and hydrolysis of aspirin in simulated gastric fluid pH 1.2. The rate was calculated using the equation 10. The table shows the rate of dissolution and hydrolysis by both methods by calculation and the slope of the line. Rates of all the experiments are shown in the table with an average of all data with standard deviation. The rate of dissolution is 1.706 which is higher than the other buffer solutions. In this study, the rate of hydrolysis is totally based on the rate of the dissolution, as the hydrolysis needs the free aspirin which generates after dissolution.

Table 6 shows the rates of the dissolution and hydrolysis of aspirin in sodium acetate buffer pH 4.5. The table shows the rate of dissolution and hydrolysis by both methods by calculation and the slope of the line. Rates of all the experiments are shown in the table with an average of all data with standard deviation. The rate of dissolution and rate of hydrolysis slowed compare to simulated gastric fluid is 0.545. As the dissolution slows the rate of hydrolysis is also slow.

Table 7 shows the rates of the dissolution and hydrolysis of aspirin in potassium phosphate buffer pH 5.8. The table shows the rate of dissolution and hydrolysis by both methods by calculation and the slope of the line. Rates of all the experiments are shown in the table with an average of all data with standard deviation. The rate of dissolution and rate of hydrolysis slowest compare to other two buffers is 0.085. As the dissolution slowest, the rate of hydrolysis is also slowest in this buffer.
Table 5: Rate of dissolution and hydrolysis in simulated gastric fluid (pH 1.2)

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<th>Exp</th>
<th>Rate of Dissolution (mM/min)</th>
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Table 6: Rate of dissolution and hydrolysis in sodium acetate buffer (pH 4.5)

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Table 7: Rate of dissolution and hydrolysis study in potassium phosphate buffer (pH 6.8)

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3.4 Conclusion

The USP monograph of dissolution of aspirin explains about the conventional methods that are UV/Vis and HPLC analysis, but the importance of this study is to perform the dissolution by FT-IR in real time. The in-situ FT-IR measures the vibrations of a molecule in the fingerprint region, which is important for the analyst, because each molecule has its own specific spectrum. Aspirin has the tendency to hydrolyze and to form salicylic acid and acetic acid when it dissolves. So the advantage of the in-situ FT-IR is to monitor dissolution as well as the hydrolysis of aspirin, which is very difficult by UV/Vis and HPLC, because of the overlapping of these two peaks.

When the aspirin tablet was dropped into the dissolution medium, it starts to dissolve and slowly hydrolyze into salicylic acid and acetic acid. The dissolution was performed on three different body pHs and compared with HPLC. The results of the dissolution and hydrolysis study by both methods are comparable, and this gives the validation of the new in-situ FT-IR technique. The dissolution and hydrolysis of aspirin was monitored, in which two trends of the data was observed. First trend explains about the dissolution of aspirin and followed by hydrolysis, so intensity if the peak decreases gradually. The second trend is the formation of salicylic acid where the gradual increase in the peak intensity was observed, based on the rate of hydrolysis. The rate of hydrolysis and rate of formation of salicylic acid is based on the dissolution of the tablet. Once the tablet dissolves, then the free aspirin will be available for the hydrolysis and as hydrolysis proceeds formation of salicylic acid. So the rate of the reaction depends on the concentration as well as the dissolution. This research was possible because of the versatility of the in-situ FT-IR system.
This study demonstrated that *in situ* infrared spectroscopy is a viable technique for detecting and measuring the hydrolysis of aspirin at different body pH. The *in-situ* FT-IR system was found to be impressive in its capability of measuring low concentrations and was able to distinguish separate components of a multiple component hydrolysis systems without requiring manual sampling.

This versatility is demonstrated by observing the simultaneous dissolution and then elimination of the acetylsalicylic acid component while monitoring the formation of the salicylic acid component as acetylsalicylic acid undergoes hydrolysis. Specifically, the unique fingerprint region of the IR spectra gave detailed information about the release profile of the drug. Conventional methods would only show the release profile of one component. Moreover, the hydrolysis and transformation of the drug from one form to another is missed with conventional techniques. However, *in-situ* FT-IR captures those minor details and displays them in the fingerprint region, as shown earlier. Furthermore, since this chapter successfully demonstrates the versatility of this novel application of *in-situ* FT-IR spectroscopy, it suggests that the method has excellent potential for the study of the dissolution and hydrolysis kinetics of pro-drug formulations.
CHAPTER – 4

HYDROLYSIS STUDY OF ASPIRIN

4.1 Introduction

This chapter focuses on performing the dissolution and hydrolysis of different generic brands of over the counter aspirin tablets with brand Bayer aspirin tablet by in situ FT-IR. As discussed earlier aspirin undergoes hydrolysis to form salicylic acid and acetic acid. To perform dissolution and hydrolysis of the different generic aspirin tablets were bought from Rite-Aid, Walgreen, CVS pharmacies and Bayer aspirin with two different lot numbers. Two different brands of enteric coated aspirin tablets are bought from India. The enteric coating prevents the dissolution of tablet in acidic medium such as stomach and duodenum, tablet should dissolve in small intestine and slowly release drug from pharmaceutical dosage form.

The comparison study of dissolution helps in the drug development of pharmaceutical dosage forms. Fawzia and his group performed the comparison study of in vitro dissolution testing for commercial available aspirin tablets, they observed similar dissolution profile for all brands of aspirin. Literature showed that comparison study of the dissolution with different brands has been carried out. Author compared the dissolution study with 23 generic tablets of Levofloxacin with one branded tablet in Japan. Another study was on the design of the sustained release tablets with different polymers and theirs quality control test to check the polymer effect on the quality. Clement and his group have compared the dissolution profile of 7 immediate-release metformin 500 mg tablets. This study compared other characteristics such as weight variation and drug content. The dissolution was carried on USP-II and they observed only one formulation showed <85 % drug release in 15 min. Issa and group have performed the
comparative study of chlorpheniramine maleate orally disintegrating tablets. They have prepared the different formulations by using different diluents and different disintegrating agents by direct compression method. The formulation contains glucose crospovidone showed faster dissolution as it disintegrates at faster rate compare to other formulations. [105] Ozkan and his group have performed the comparison of dissolution on different designed and formulated salbutamol tablets. This study was carried out using USP-I and USP-II. They observed the commercial formulation showed the faster release of salbutamol. [106]

Literature study explains about the importance of the comparison study of dissolution and developed motivation for this study. Thus interest has created to perform the dissolution of different generic brands of aspirin tablets and compare with Bayer aspirin tablet. The different generic brands of aspirin tablets were compared with Bayer aspirin tablet with same strength by using in-situ FT-IR spectroscopy. The comparison of Bayer aspirin has performed with the enteric coated tablets as well. The enteric coated tablets has a coating so that these tablets could not dissolve in the stomach and duodenum.

### 4.2 Experimental Section

#### 4.2.1 Chemicals and materials

Acetylsalicylic acid (aspirin) reference material (batch no. 016K0131) was purchased from Sigma-Aldrich. Salicylic acid reference material (batch no. 04708HE) was purchased from Sigma-Aldrich. Acetylsalicylic acid 325 mg tablets (Genuine Bayer batch no. 219050N) were purchased from a local pharmacy. Another acetylsalicylic acid 325 mg tablets (Genuine Bayer batch no. NAA01N9), Rite-Aid Pain relief Aspirin 325 mg tablets (batch no. 4125), CVS Genuine Aspirin
325 mg tablets (batch no. 4200), Walgreen Regular strength Aspirin 325 mg tablets (batch no. 4148) and Bayer Advanced aspirin 500 mg tablets (batch no. 231051E) were purchased from local Pharmacy store. Ecosprin 150 mg (batch no. 04005074) and Delisprin 150 mg (batch no. M109M032) tablets were purchased from a local pharmacy from India. Methanol, acetone and acetonitrile (HPLC grades) were purchased from Pharmaco-Aaper (Brookfield, CT). Sodium chloride crystals (batch no. J39602) was purchased from Mallinckrodt Chemicals (England, UK). Hydrochloric acid (batch no. H44032) was purchased from J.T. Baker (Central Valley, PA). Sodium acetate crystals (batch no.05816HD, potassium phosphate monobasic (batch no. 103K0060) and sodium hydroxide (batch no. 06414BH) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Glacial acetic acid (batch no. 971666) was purchased from Fisher Chemicals (Waltham, MA). All solutions were prepared using water treated by a Milli-Q plus Millipore purification system (Milford, MA). All purified water aliquots have resistivity of not less than 18 MOhm-cm\(^{-1}\).

### 4.2.2 Instrumentation

All chemicals were weighed on a Mettler Toledo balance (Washington Crossing, PA) PB303 Delta-Range\(^{\circledR}\) and AG204 Delta-Range\(^{\circledR}\). The pH of the buffer was measured on a VWR SympHony (Radnor, PA) SB70P pH meter. Dissolutions were carried out on the Easy Max\(^{\text{TM}}\) 102 by Mettler Toledo. HPLC analysis was performed on a Hewlett-Packard 1050 HPLC, with Chem Station software. In-situ FT-IR analysis was carried on a Mettler Toledo iC 10 ReactIR, with iCIR versions 3.0 and 4.0 software.
4.2.3 Simulated gastric fluid

Simulated gastric fluid was prepared with hydrochloric acid and sodium chloride as per USP procedure for buffers. 2 g of sodium chloride was dissolved in 100 mL of deionized amount of water, in that 7 mL HCl was added and then volume made up to 1000 mL by adding deionized water, and then measured pH of that solution and adjusted to 1.2.

4.2.4 Sodium acetate buffer

Sodium acetate buffer was prepared with glacial acetic acid and sodium acetate anhydrous as per the USP procedures for buffers. 2.99 g of sodium acetate was dissolved in 100 mL of deionized of water, in that 1.66 mL glacial acetic acid was added and then volume made up to 1000 mL by adding deionized water and then measured pH of that solution and adjusted to 4.5.

4.2.5 Potassium phosphate buffer

Potassium phosphate buffer (0.2 M) was prepared with monobasic potassium phosphate and sodium hydroxide as per the USP buffer procedures. First potassium phosphate solution was prepared by weighing 27.22 g of monobasic potassium phosphate and dissolved in 100 mL of deionized water and diluted with up to 1000 mL. Next sodium hydroxide solution was prepared by weighing 8 g of sodium hydroxide dissolved in water and made volume up to 100 mL. For phosphate buffer added 50 mL of monobasic potassium phosphate solution in a 200 mL volumetric flask then added 22.4 mL of sodium hydroxide solution and then added water to make a volume. Finally measured the pH of this solution and was adjusted to 6.8.
4.2.6 Standard solutions

For standard solutions weighed 325 mg of aspirin and salicylic acid and dissolved in 100 mL of simulated gastric fluid, sodium acetate buffer and potassium phosphate and made concentration 3.25 mg/mL.

4.2.7 Dissolution and hydrolysis of all brands of aspirin tablets

All aspirin tablets were measured in pH 1.2 simulated gastric fluid solutions for 1.5 hr., pH 4.5 sodium acetate buffer for 24 hr and potassium phosphate buffer for 24 hr. Dissolutions were conducted using vessel volumes of 100 mL and at 37.0 °C. All dissolutions were conducted using USP Apparatus Type II (paddles) with an agitation speed of 100 rpm as per USP specifications.

4.2.8 In-situ FT-IR analysis

The set for dissolution testing was carried out as discussed in the experiment section. The in-situ FT-IR fiber optic probe has collected baseline spectra (blank medium) for the first 30 minutes. A single 325 mg Bayer aspirin tablet was dropped into the vessel containing 100 mL of buffer solution (simulated gastric fluid pH 1.2, sodium acetate buffer pH 4.5 and potassium phosphate buffer pH 6.8) and the dissolution profiles were collected using in-situ FT-IR spectroscopy. Standard spectra of pure aspirin and salicylic acid were collected prior to running the dissolutions. These standards were used to identify the unique peaks of interests for these active drugs. For the aspirin tablet analysis, the same steps as discussed in calibration analysis were followed. The data processing was carried out as mentioned in the previous chapter.
4.3 Results and Discussion

This study was focused to perform dissolution and simultaneously observation of hydrolysis of different generic brands of aspirin with Bayer aspirin tablet by in-situ FT-IR in simulated gastric fluid pH 1.2, sodium acetate buffer pH 4.5 and phosphate buffer pH 5.8.

4.3.1 Comparison between Bayer aspirin and Bayer advanced aspirin tablets

Two separate dissolutionss were conducted by the procedure as mentioned in experimental section on Bayer aspirin and Bayer Advanced aspirin tablet. This study was focused on performing dissolution and simultaneously observe hydrolysis of aspirin by in-situ FT-IR in simulated gastric fluid which depicts stomach environment having pH 1.2.

Figure 41 shows the dissolution profile of aspirin as per time with simultaneous hydrolysis of aspirin and formation of salicylic acid of both the tablets. The dissolution profile was monitored by in-situ FT-IR. The graph showed that the Bayer Advanced tablet showed the higher dissolution than the Bayer aspirin tablet, as that tablet is immediate release tablet. In pH 1.2 gastric fluid the rate of the dissolution is faster with Advanced Bayer aspirin tablet. Almost 90% dissolution of the aspirin was observed in first 15-20 mins. which indicates that stomach is the best place for the dissolution of the aspirin tablet. The Advanced Bayer aspirin tablet is an immediate release tablet so it dissolves in the stomach at a high rate. The brown line in the graph represents Advanced Bayer aspirin tablet and blue line represents Bayer aspirin tablet.
Figure 41: Comparison of dissolution and hydrolysis of Bayer aspirin tablet and Bayer advanced aspirin tablet by In-situ FT-IR in SGF
4.3.2 Comparison between Bayer aspirin and generic aspirin tablets

This set of experiments performed dissolution and simultaneously observed hydrolysis of aspirin by \textit{in-situ} FT-IR in simulated gastric acid pH 1.2, sodium acetate buffer having pH 4.5 and phosphate buffer pH 6.8 on Bayer aspirin tablets and other generic aspirin tablets. Each dissolution was performed separately with same dissolution conditions and gone through the data treatment on \textit{in-situ} FT-IR.

Figure 42 shows the overlay plot of the dissolution profile of aspirin as per time, simultaneous hydrolysis of aspirin and formation of salicylic acid in simulated gastric fluid pH 1.2. The plot showed the dissolution and the hydrolysis data of 5 types of aspirin tablets which are mentioned in the experimental section. All these tablets showed a similar trend for dissolution plot, then aspirin peak intensity has decreased and at the same time formation of salicylic trend starts increasing. The aspirin trend line has decreased shows the hydrolysis of aspirin. The dissolution and hydrolysis all brands of aspirin tablets are similar to each other and they overlapped.

Figure 43 shows the overlay plot of the dissolution profile of aspirin as per time, simultaneous hydrolysis of aspirin and formation of salicylic acid in sodium acetate buffer pH 4.5. The plot showed the dissolution and the hydrolysis data of 5 types of aspirin tablets which are mentioned in the experimental section. All these tablets showed a similar trend for dissolution plot, then aspirin peak intensity has decreased and at the same time formation of salicylic trend starts increasing. The aspirin trend line has decreased shows the hydrolysis of aspirin. The dissolution and hydrolysis all brands of aspirin tablets are similar to each other and overlapped.
Figure 42: Comparison of dissolution and hydrolysis of all brands of aspirin tablets by \textit{in-situ} FT-IR in simulated gastric fluid
Figure 43: Comparison of dissolution and hydrolysis of all brands of aspirin tablets by *in-situ* FT-IR in sodium acetate buffer
Figure 44: Comparison of dissolution and hydrolysis of all brands of aspirin tablets by *in-situ* FT-IR in phosphate buffer
Figure 44 shows the overlay plot of the dissolution profile of aspirin as per time, simultaneous hydrolysis of aspirin and formation of salicylic acid in potassium phosphate buffer pH 5.8. The plot showed the dissolution and the hydrolysis data of 5 types of aspirin tablets which are mentioned in the experimental section. All these tablets showed a similar trend for dissolution plot, then aspirin peak intensity has decreased and at the same time formation of salicylic trend starts increasing. The aspirin trend line has decreased shows the hydrolysis of aspirin. The dissolution and hydrolysis all brands of aspirin tablets are similar to each other and overlapped on.

4.3.3 Dissolution of enteric-coated tablets

The next step of the comparison study of dissolution and hydrolysis of aspirin is to perform the dissolution of enteric coated aspirin tablets. The enteric coated means, that the tablet is coated with a material to prevent the dissolution process in the stomach and in duodenum but allow dissolution in the small intestine. The advantage of this coating is to avoid dissolution of those compounds which creates stomach irritations. The Ecospirin-150 and Delisiprin-150 tablets were selected for this study. The dissolution of these tablets was carried out in simulated gastric fluid pH 1.2 and sodium acetate buffer pH 4.5 up to 24 hrs. for the confirmation of the enteric coating and observed that both tablets showed no dissolution in both buffers. At the end of 24 hrs. tablets were present in the dissolution media in its initial form. However, the dissolution of these tablets were performed in phosphate buffer pH 6.8 and observed the dissolution of aspirin followed by hydrolysis.
The dissolution of Ecospirin-150 was carried as per the procedure mentioned in the experimental section. Figure 45 shows the comparison of dissolution and hydrolysis data of aspirin. The similar profile was observed as discussed earlier. The dissolution of aspirin plot was increased as per the time and after dissolution, simultaneous hydrolysis of aspirin was observed. With this formation of salicylic acid was also observed in potassium phosphate buffer pH 6.8. The dissolution was compared with Bayer aspirin tablet. The data was overlapped.

The dissolution of Delisprin-150 was carried as per the procedure mentioned in the experimental section. Figure 46 shows the comparison of dissolution and hydrolysis data of aspirin. The similar profile was observed as discussed earlier. The dissolution of aspirin plot was increased as per the time and after dissolution, simultaneous hydrolysis of aspirin was observed. With this formation of salicylic acid was also observed in potassium phosphate buffer pH 6.8. The dissolution was compared with Bayer aspirin tablet. The data was overlapped.
Figure 45: Dissolution and hydrolysis of Ecospirin-150 tablet by *in-situ* compared with Bayer aspirin in Phosphate buffer.
Figure 46: Dissolution and hydrolysis of Delisprin-150 tablet by *in-situ* FT-IR compared with Bayer aspirin tablet in Phosphate buffer
4.3.4 Kinetic study

The kinetic study was determined by using the slope of the line and by using following formula calculated the rate of dissolution and rate of hydrolysis.

\[
\ln[A] = -kt + \ln[A]_0
\]  

(11)

The rates of the reactions were determined from the dissolution plots. In order to calculate the rates using the plot, the following procedure was implemented. First, a plot log of percent dissolved vs. time is obtained which was shown in the initial part of result section. Next, a straight line is drawn over the area of the dissolution curve where dissolution starts to occur (middle area in this case). Furthermore, the rate of hydrolysis is also calculated with the same method using the graph where the hydrolysis starts, showed in the hydrolysis that is disappearance curve.

As the main goal is to study the dissolution of an aspirin tablet and with that monitoring hydrolysis of aspirin. For the hydrolysis reaction, there must be free aspirin present in the solution, which will come after the dissolution of the tablet. So as the rate of dissolution is slow with a change in the pH, hydrolysis is also slow.

Table 8 shows the rate of dissolution and rate of hydrolysis of all dissolutions data. The list of brands of aspirin tablets and their dissolution and hydrolysis rates has shown in the table. The kinetic data is similar with Bayer aspirin tablet.
Table 8: Kinetic data of all brands of aspirin tablets

<table>
<thead>
<tr>
<th>Name of Aspirin Tablet</th>
<th>Simulated Gastric Fluid</th>
<th>Sodium Acetate Buffer</th>
<th>Potassium Phosphate Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rate of Dissolution (mM/min)</td>
<td>Rate of Hydrolysis (mM/min)</td>
<td>Rate of Dissolution (mM/min)</td>
</tr>
<tr>
<td>Bayer</td>
<td>1.706</td>
<td>0.610</td>
<td>0.545</td>
</tr>
<tr>
<td>New Bayer</td>
<td>1.679</td>
<td>0.723</td>
<td>0.523</td>
</tr>
<tr>
<td>Rite-Aid</td>
<td>1.721</td>
<td>0.654</td>
<td>0.567</td>
</tr>
<tr>
<td>Walgreens</td>
<td>1.680</td>
<td>0.741</td>
<td>0.601</td>
</tr>
<tr>
<td>CVS</td>
<td>1.694</td>
<td>0.634</td>
<td>0.569</td>
</tr>
<tr>
<td>Ecospirin</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Delisprin</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
4.4 Conclusion

The *in-situ* FT-IR records vibrations in the fingerprint region, which is important for the analyst, because each molecule has its own specific spectrum. Aspirin has the tendency to hydrolyze and to form salicylic acid and acetic acid when it dissolves. So the advantage of the *in-situ* FT-IR is to monitor dissolution as well as the hydrolysis of aspirin, which is very difficult by UV/Vis and HPLC, because of the overlapping of these two peaks. As the aspirin tablet dropped into the dissolution medium, it starts to dissolve and slowly hydrolyzes into salicylic acid and acetic acid. The dissolution was performed on three different body pHs and compared with HPLC in the previous chapter and validated.

The dissolution and hydrolysis of Bayer aspirin tablets were monitored and compared with the generic aspirin tablet and enteric coated aspirin tablet. The profile showed the two trends of the data has been seen in all the tablets. The first trend explains about the dissolution of aspirin and followed by hydrolysis so intensity if the peak decreases gradually. The second trend is the formation of salicylic acid where the gradual increase in the peak intensity can has seen, based on the rate of hydrolysis. The enteric coated tablets dissolution and hydrolysis has compared with Bayer aspirin tablet. This research was possible because of the versatility of the *in-situ* FT-IR system.
CHAPTER - 5

DISSOLUTION OF LORATADINE

5.1 Introduction

In the previous sections, the versatility of *in-situ* FT-IR which is dissolution and hydrolysis study of aspirin and dissolution of acetaminophen were discussed in detail. Another over-the-counter antihistaminic drug, loratadine has been chosen for the further confirmation of the new *in-situ* analysis technique to monitor dissolution. [107]

Loratadine is a second-generation antihistamine antagonist drug used to treat allergies. The FDA approved Loratadine in 1993. Loratadine is effective for both nasal and eye symptoms: sneezing, runny nose, and itchy or burning eyes. [108] Loratadine could be also used to treat mild to moderate pain from headaches. The mechanism of action of loratadine is a tricyclic antihistamine which acts as a selective inverse agonist of peripheral histamine H1-receptors. Histamine is responsible for many features of allergic reactions. Loratadine is metabolized by cytochrome P450, primarily by CYP3A4 and CYP2D6 isozymes. [109] Loratadine is a prodrug which hydrolyzes into descarboethoxy loratadine also known as desloratadine. [110] [111] There are recent studies of dissolution of loratadine and the formulation with different dosage forms by different drug delivery system are found in the literature. [112]

Mondal and Islam have prepared loratadine formulations based on Self Emulsifying Drug Delivery System (SEDDS). They used oleic acid and cremophore EL surfactant with different concentrations and performed the dissolution study by using USP-II. They observed the rate of drug release has increased with increased amount of cremophor EL surfactant in the formulation.
Borgaonkar and group have studied the dissolution of loratadine buccal tablets to enhance the bioavailability and to minimize the first pass metabolism, they prepared loratadine tablets using different polymers. They finalized that the hydroxypropylmethylcellulose is good for the dissolution study as they found 99% in-vitro drug release in 8 hr. with satisfactory mucoadhesion strength. [114]

Soni and Sagar designed the quick dissolving loratadine tablets by compression method. The dissolution of the formulation is used for the stability study. [115] Qadir and group have formulated fast dissolving films of loratadine for sublingual use. They performed the dissolution of loratadine films by USP-II with the observation that with increased content of PVP, the rate and extent of drug release was faster. [116] Another study was carried out to enhance the solubility if loratadine by using cyclodextrin binary system. [117]

The novelty of this study is to perform the dissolution by in-situ FT-IR, without manual sampling and disturbing dissolution process. The dissolution data

5.2 Experimental Section

5.2.1 Chemicals and materials

Loratadine reference material used in this study is CAS # 79794-75-5. Claritin 10 mg tablets which contains loratadine was purchased with (lot number 2RXF523) from a Rit-Aid pharmacy. Methanol, acetone and acetonitrile (HPLC grades) were purchased from Pharmaco-Aaper (Brookfield, CT). Hydrochloric acid (batch no. H44032) was purchased from J.T. Baker (Central Valley, PA). Solutions were prepared using water treated by a Milli-Q Millipore purification system (Milford, MA). All purified water aliquots have resistivity of not less than 18 MOhm-cm.
5.2.2 Instrumentation

All chemicals were weighed on a Mettler Toledo balance (Washington Crossing, PA) PB303 Delta-Range® and AG204 Delta-Range®. The pH of the buffer was measured on a VWR SympHony (Radnor, PA) SB70P pH meter. Dissolutions were carried out on the Easy Max™ 102 by Mettler Toledo. In-situ FT-IR analysis was carried on a Mettler Toledo iC 10 ReactIR, with iCIR versions 3.0 and 4.0 software.

5.2.3 0.1 N hydrochloric acid

0.1 N HCl was prepared as per the USP procedure for buffers. [118] 8.5 mL of concentrated HCl was diluted with 1000 mL of deionized water for the analysis.

5.2.4 Loratadine solution for calibration

Loratadine stock solution of concentration 10 mg/mL was prepared by dissolving 500 mg of loratadine in 50 mL of 0.1 N HCl. Nine dilutions were prepared by adding 1.00, 2.00, 3.00, 4.00, 5.00, 6.00, 7.00, 8.00 and 9.00 mL of this stock solution respectively to 10.00 mL volumetric flasks respectively and diluting to volume with 0.1 N HCl for the calibration curve. The concentration of loratadine in each flask was 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50 and 5.00 mg/mL respectively. Loratadine has a tendency to hydrolyze as it goes into solution. Solutions were placed into an ice bath immediately after the preparation to slow down the hydrolysis process. As soon as solutions were ready, they were scanned by in-situ FT-IR for the calibration study at room temperature.
5.2.5 Standard solutions

The standard solutions of concentration 0.1 mg/mL was prepared using 10 mg of loratadine was dissolved in 100 mL of 0.1 N HCl.

5.2.6 Dissolution of loratadine

Claritin tablet 10 mg of loratadine (label claim) were tested in pH 0.1N HCl solutions for 2 hr. Dissolutions were conducted using vessel volumes of 100 mL and at 37.0 °C. All dissolutions were conducted using USP-II (paddles) with an agitation speed of 50 rpm as per the USP monograph of loratadine. [119]

5.2.7 In-situ FT-IR analysis

For the calibration of aspirin experiments, data treatment was carried out using the following methodology: The data was first subjected to baseline correction. The absorption band at 1246 cm\(^{-1}\) (corresponding to the C-N stretch) was selected for loratadine as shown in Figure 47 with the calibration study. [120] The figure is the overlay calibration data of the loratadine from the in-situ FT-IR software in 0.1 N HCl. A distinct peak for loratadine without any interfering peak was observed. The peak at 1246 cm\(^{-1}\) has increased intensity with increased concentration. The different concentration of loratadine solution also ran three times and the spectra overlapped on each other. The peak area was calculated using a two-point baseline correction set at 1265 cm\(^{-1}\) and 1230 cm\(^{-1}\). The in-situ FT-IR system was configured to collect spectra every two minutes at room temperature.
For this experiment, data processing is critical to obtain well-resolved spectra. Here is how the data is processed. For all *in-situ* FT-IR experiments, 256 scans were collected and co-added for each spectral point on average and each data point took about two minutes to complete. First, the peak of interest was identified (1246 cm$^{-1}$) and the area was calculated using a two-point baseline correction. Next, a trend analysis was performed using the iCIR software on this peak. This provided a plot of absorbance vs. time. This plot was subjected to smoothing using a 25 point smoothing parameter. After smoothing, and while still in the trend analysis, the baseline correct routine was performed. Then, the data were exported to Excel. Further data processing in Excel involved the subtraction of solvent spectra from reaction spectra. To perform this, a 30 min set of spectra at the beginning of the experiment, before claritin pill was added, was selected. These spectra were subtracted from the spectra taken after the pill was dropped to give the final set of data. Within the final set of data, the area of the 1246 cm$^{-1}$ peak was calculated at each time point. Then the plot of area vs. time was performed to give rate information. In addition, using a calibration curve, peak areas were converted to concentration to provide concentration vs. time plots.
Figure 47: Loratadine in-situ FT-IR peak at 1246 cm$^{-1}$ for calibration
5.3 Results and Discussion

5.3.1 Calibration of loratadine

Based on the in-situ FT-IR spectra obtained, loratadine has an IR frequency of interest at 1246 cm$^{-1}$. Based on the analysis, it was determined that loratadine had excellent linear correlation using IR spectroscopy with a 0.9951 in 0.1 N HCl correlation coefficient ($r^2$) and shown in Figure 48. Linear regression for loratadine was calculated by using the area under curve calculations of the IR absorption bands.

5.3.2. Dissolution of loratadine

The data was compared to reference standards measurements collected prior to the start of the linearity and dissolution experiment. Dissolution data was plotted vs. time. Figure 49 shows the dissolution profiles of loratadine by the in-situ FT-IR instrument software. Experiment and the dissolution media was equilibrated for first 30 mins and then dropped the tablet in the dissolution vessel. The dissolution performed for longer time to check the stagnant line after complete dissolution. Figure 49 showed the dissolution profile of loratadine per time, within 15-20 min 50% of loratadine dissolved. Once the dissolution study has confirmed, performed a replicate study with the same experimental conditions.
Figure 48: Calibration graph of loratadine

\[ y = 0.1373x - 0.0545 \]

\[ R^2 = 0.9951 \]
Figure 49: Dissolution of loratadine-combined (n=3)
5.4 Conclusion

The dissolution loratadine was performed successfully by using in-situ FT-IR spectroscopy. The calibration of the loratadine from 1 mg/mL to 5 mg/mL was carried out and showed liner correlation with $r^2$ value 0.9951. The dissolution was performed successfully, in first 40 mins more than 80% of loratadine has dissolved. This study demonstrated and confirmed that in situ FT-IR is a viable technique for monitoring the dissolution of the pharmaceutical dosage form. The in situ FT-IR system was found to be impressive in its capability of measuring low concentrations without requiring manual sampling.

This versatility has demonstrated by observing the simultaneous dissolution and then hydrolysis of prodrug such as aspirin. Specifically, the unique fingerprint region of the IR spectra gave detailed information about the release profile of the drug.

As future work of this study is to monitor the hydrolysis of loratadine as it is a prodrug. The active metabolite of loratadine metabolism is after hydrolysis, which has more pharmacological potencies than the parent drug. As per the conventional methods, it is very difficult to analyze these components. By this technique, these two components can find distinct separate peaks for loratadine and desloratadine as like the aspirin and salicylic acid. By this method, the dissolution of loratadine can monitor with the transformation of loratadine to desloratadine which is difficult in the conventional method.
Research Summary

Dissolution studies are critical tests for measuring the performance of a drug product. In this dissertation, a new technique, in-situ attenuated total reflection infrared spectroscopy (in-situ FT-IR), has been developed to monitor dissolutions of pharmaceutical drug formulations. The accuracy of this technique was determined to be relative to HPLC. Three over-the-counter (OTC) active drugs (i.e., acetaminophen, acetylsalicylic acid and loratadine) were tested during the research. Moreover, these drug formulations were tested and the individual components were identified by in-situ FT-IR spectroscopy.

Very first project in this research is dissolution of acetaminophen, the potential of the in-situ FT-IR spectroscopy for the quantitative analysis such as monitor the dissolution was observed. During these experimentation, the non-linear Beer-Lambert’s behavior was observed, the hypothesis behind this behavior was proposed due to the dimerization of the acetaminophen molecules. As per the literature, for the confirmation of the dimerization studies were performed on the NMR, FT-IR, Osmometry and freezing point depression. But, there was a lack of any evidence to confirm the hypothesis of dimerization of acetaminophen.

Furthermore, the system has the capability of monitoring drug transformations during dissolution. For instance, using this technique the hydrolysis of aspirin to salicylic acid was monitored. Moreover, the simultaneous dissolution and elimination of aspirin has been monitored and at the same time the formation of salicylic acid in solution has been monitored. The dissolution monitored on the three different body pHs, that is stomach pH 1.2, duodenal pH 4.5 and last intestinal pH 6.8 and the kinetics of the dissolution as well as hydrolysis were performed. However, the stomach pH is the best dissolution media where the rate of dissolution and hydrolysis is fast.
As the rate of hydrolysis is based on the dissolution of aspirin because it needs free aspirin in the media, then aspirin transforms into salicylic acid, which observed slow in higher pHs as aspirin dissolves slowly.

Dissolution and hydrolysis of aspirin study was performed using in-situ FT-IR spectroscopy and compared with HPLC on Bayer aspirin tablet. In-situ FT-IR spectroscopy was compared with different brands of generic aspirin tablets with the same strength. The rate of dissolution and hydrolysis of all these brands are similar to Bayer aspirin. Dissolution of the enteric coated aspirin tablet were performed, which dissolved in intestinal pH and used for the delayed release. Loratadine was another pro-drug selected for the dissolution and hydrolysis study. This thesis explains about the dissolution of loratadine and hydrolysis can be a study for future work.

The in-situ FT-IR system was found to have very good sensitivity for monitoring dissolution test. The in-situ FT-IR has great potential for use in studies of dissolution of drugs and monitoring competing for reactions such as hydrolysis of prodrugs in pharmaceutical development, formulation and quality assurance without disturbing dissolution system as well as manual sampling.
References


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Appendix

Standard Operating Procedure for ReactIR

This chapter explains the detail and stepwise standard operating procedure.

Starting Instrument:

1. Verify whether the instrument is on or off. (Usually, instrument is kept on. Please do an additional check).

2. Fill in the liquid nitrogen through the funnel shown in Figure 50 and wait for 45 mins for cooling the detector. To filling the liquid nitrogen, remove the nozzle and fit in the funnel, then pour the liquid nitrogen with the help of the liquid nitrogen container in the funnel. Stop pouring the liquid nitrogen in the funnel, after it starts overflowing.

3. Turn on the computer.

Figure 50: ReactIR nozzle for liquid nitrogen
Starting Software:

1. The ReactIR uses iC IR 4.0 for analysis. On the desktop, the iC IR 4.0 icon is highlighted with purple color. To start the software double-clicks the icon, which would bring up the software window shown in Figure 52.
2. On the left hand side, there is a button for the” new experiment” as shown in Figure 54. To start the new experiment click that button, and a small wizard window as shown below should pop up. To check previous experimental data, then “open experiment” button opens completed experiments.

Figure 53: Screenshot of start page of ReactIR software

Figure 54: Experimental section of iC IR software
3. Before starting any experiment, it’s a good practice to configure instrument. To configure the instrument, click the configure instrument button, which is located on the bottom left of the new experiment window.
Configuration of Instrument:

1. Once the “Configure instrument” button is selected, the following window below will appear. The specific instrument parameters can be modified using this part of the software. In most cases, the default settings, as shown in Figure 57, will be acceptable for the dissolution experiments. However, in some instances, the settings may be modified. These modified settings will be explained later in the document.
Figure 57: Configure hardware and software setting

a. The very first point in this configuration window is the name or type of the instrument.

Here, ReactIR 10 is the default option for the instrument because this is the name of the instrument. As shown in Figure 58, the next option is the detector. There are 2 kinds of detector one is MCT (Mercuric Cadmium Telluride) and another one is DTGS (deuterated triglycine sulfate). For this instrument, MCT detector is used because this detector is more sensitive than the DTGS. Usually, these options are selected, but if not, it can be modified manually by clicking the restore defaults button.

Figure 58: Detector settings
b. The next part in this window is the “Probe Hardware settings”. Since the instrument contains a 9.5 mm x 1.5 m silver halide probe with a covered diamond tip, the following parameters need to be selected as shown in Figure 59. There are numerous probe settings available in the system. These can be found by selecting the drop down arrow as shown below. So that it creates the facility to change the probe.

![Figure 59: Probe hardware settings](image1)

Figure 59: Probe hardware settings

c. This instrument software can allow other kinds of the probe as shown in Figure 26. Once the probe has changed, it’s mandatory to change the probe hardware setting in the configuration window, such as in probe interface K4 conduit and in probe tip SiComp (Silicon) option by using the drop-down arrow as shown in Figure 60. These three probes below can be used for this instrument and software also.

![Figure 60: Different type of IR Probes](image2)
Figure 61: Probe hardware settings for different types of probe
d. The last step of configuring the instrument is “Probe acquisition settings”. Resolution is the first option which is shown in Figure 62. There are three kinds of resolution options.

- Normal (every 8 wavenumbers) resolution, usually this option is the best option for dissolution experiment.
- High (every 4 wavenumbers) resolution, will slow down scan rate and application processing. It may be useful in certain reactions to help resolve overlapping peaks.
- Low (every 16 wavenumbers), this choice is only useful in reactions with broad, non-overlapped peaks that require a fast sampling rate.

For the wave number range, instrument can run from 1900 to 650 cm\(^{-1}\) is because of the diamond probe tip. By using the dropdown arrow for the scans options, will change the number of scans. By choosing an auto select option, the instrument will select 256 scans.
2. Once finished with all modifications in parameters for the configuration of the instrument, it’s time to move to the next step. Click the “Next” button. The next window will appear as shown in Figure 65, with the picture of the instrument and the probe. Click “Next” button to move further.

Figure 62: Probe acquisition Settings

Figure 63: Position of the probe
3. The pop-up window as shown in Figure 64 seen on the screen, which explains about the alignment of the probe. For the alignment of the probe, Peak Height should be more than 18,000 and less than 28,000 and Contrast should be more than 10.00. If peak height and contrast is more than these mentioned values, the two bars will change into green. If peak height and contrast is less than these values, the bars are in red color and length is also less compare to green bars. Once the bars turns to green, then press the “Next” button.

Figure 64: Align probe window
4. The window shown in Figure 65 is used to collect the background. Here is the option for the scans, from the drop-down list, the values for the number of scans can be changed. Then click the “Collect Background” button. To collect the background normally takes 1-2 min, but it also depends on the number of scans option. After collection of the background click the “Next” button.

![Figure 65: Collect background window](image)
5. Then “collect water vapor sample” window will open. This page of the wizard is used to collect a reference spectrum of water vapor. This reference spectrum is used for subtracting out water vapor from a data set. It is not intended to be a replacement for an effective dry air or nitrogen purge. The water vapor reference spectrum is simply a convenient way to remove small amounts of water vapor which may enter or leave the instrument over a long reaction period. After collection of the water vapor click the “Finish” button for completion of the configuration of the instrument.

![Figure 66: Collect water vapor sample window](image-url)
**Starting New Experiment:**

1. After completion of the configuration, the Figure 67 will appear on the screen. In the “Experimental File” the first option is for the name of the experiment. The naming of the experiment is dependence on the patron/performer. The “Experiment Folder” option gives options of where the experiment will be saved. Click the “Next” button.

![Figure 67: Name experiment file](image-url)

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2. This window gives the choice to set the time for the experiment and give the interval time for the scans. As per the mentioned experiment time and interval time, the software will show the total number of samples. Then click the “Next” button for next step.

![Experiment duration window]

**Figure 68: Experiment duration window**
3. This window is the “reference spectra needed”. There are 4 kinds of reference options that are available in this window. Usually, the first option is selected, that option is for the collections of the reference spectra. There are other three types of options, in which one is for the previously collected reference spectra; the next one is for no spectra and the last one is for the direct start of the experiment. Then click the “Next” button.

![Reference Spectra Window](image)

**Figure 69: Reference spectra window**
4. The next window again will show the picture of the instrument and the probe. The picture will appear as below. Then click the “Next” button.

![Figure 70: Position of the probe](image)

Figure 70: Position of the probe
5. The align probe window will appear as shown in Figure 64. The position of the probe and mirror should be aligned and if it is aligned then the bars will turn into the green color. Otherwise, it will remain red. Click the “Next” Button.

6. In the next window, which is for the cleaning of the probe, there are two bars, one is for the purging and one is for cleaning the probe. If purging and cleaning are proper then these bars are green as shown below. The deionized water is the best solvent, for the cleaning of the probe, also use acetone and 0.2 N hydrochloric acid (very very dilute). So that all small traces of the previous experiment will dissolve in these solvents and the probe will be clean. Click the “Next” button.

Figure 71: Clean probe window
7. The “collect background” window will open. The main purpose of this window is to collect the air background. The number of scans option can be chosen from the drop-down arrow list or the default option is 256 scans. Then click the “Collect Background” button. The collection of the background normally takes 1-2 min, but it also depends on the number of scan options. Once the background is collected, click the “Next” button.

Figure 72: Collect background window
8. In the sequence of collecting primary data, the next window is for the “collect sample”. Collect sample means the collection of spectra from the reference material, such as solvent, reagent or any other reference material which is needed for the experiment. The window contains three kinds of options, the first option is for naming the reference. The second option gives an idea about, which kind of reference (the options are given by the drop-down arrow). The last option is the functional group, which is present in the reference. (This option is also chosen by using the drop-down arrow). Once all settings are completed, insert the probe into a beaker which contains sample or reference, then click the “Collect Sample” button. (The collect button is disabled in the picture below, but after the name of the reference is written and the reference option is selected, the “Collect Sample” button will be enabled). Then Click the “Next” button.

![Collect reference window](image)

**Figure 73: Collect reference window**
9. This window gives an idea of the names of all references which are already collected. There are four different buttons at the bottom of this window.

- View Button - used to study or to check the reference spectra. The spectra will open in a new window with all references. All the names of reference spectra can be seen in the empty space under the “Component” heading as shown in the picture below.

- Delete Button - the delete button is used to delete a spectrum that is not of good quality. For deleting the spectrum, first select the name of the spectrum, (this will enable the delete button) then click the delete button.

Once finished click the “Next” button for the next step.

Figure 74: Edit reference window
10. After collecting the reference spectra, the instrument will show the clean probe window again as shown in Figure 71. It means that before starting the experiment, the probe should be clean. Whatever traces from the reference material which is present on the probe must be removed by cleaning. The clean probe window will appear as shown below. Once the probe is cleaned two green bars will appear showed as below will appear on the screen. Click the “Next” button.

11. In front of the screen should be a “Collection Background” window as shown in Figure 72. The main importance of collecting background is, the instrument can start its new experiment with a fresh setting for the better results and good quality of spectra. Then click the “Finish” button which is at the bottom of this window. After this step all the preliminary requirements are finished, it will open a new experiment window.