"DEGRADATION BEHAVIOR AND KINETIC STUDY OF FEBUXOSTAT"

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FOR THE DEGREE OF

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

BY

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(12MPH308) B.PHARM.

UNDER THE GUIDANCE OF

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May 2014

CERTIFICATE

This is to certify that the dissertation work entitled ***"DEGRADATION BEHAVIOR AND KINETIC STUDY OF FEBUXOSTAT"*** *submitted by Mr. Maharshi Natavabhai Raval with Regn. No. (12MPH308) in partial fulfillment for the award of Master of Pharmacy in "Pharmaceutical Analysis" is a bonafide research work carried out by the candidate at the Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University under our guidance. This work is original and has not been submitted in part or full for any other degree or diploma to this, or any other university or institution.*

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DECLARATION

I hereby declare that the dissertation entitled ***"DEGRADATION BEHAVIOR AND KINETIC STUDY OF FEBUXOSTAT"*** *is based on the original work carried out by me under the guidance of Dr. Priti J Mehta, Head, Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University. I also affirm that this work is original and has not been submitted in part or full for any other degree or diploma to this, or any other university or institution.*

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List of Abbreviations

ABBREVIATION	FULL FORM
°C	Degree centigrade
±	Plus or Minus
<	Less than
Λ	Lambda
%	Percentage
Mg	Microgram
Ml	Micro liter
Abs.	Absorbance
API	Active Pharmaceutical Ingredient
AR	Analytical Reagent
BP	British Pharmacopoeia
CAS No.	Chemical Abstract Service Number
Cm	Centimeter
C_{max}	Maximum Plasma Concentration
T_{max}	Maximum Plasma Concentration Time
$T_{1/2}$	Half Life
Conc.	Concentration
FDA	Food and Drug Administration
CDER	Central Drug Evaluation and Research
USP	United States Pharmacopoeia
FT-IR	Fourier Transform Infrared spectrometry
GC	Gas Chromatography
Hr	Hour
HPLC	High Performance Liquid Chromatography
SFC	Super critical fluid Chromatography
i.d.	Internal Diameter
IP	Indian Pharmacopoeia

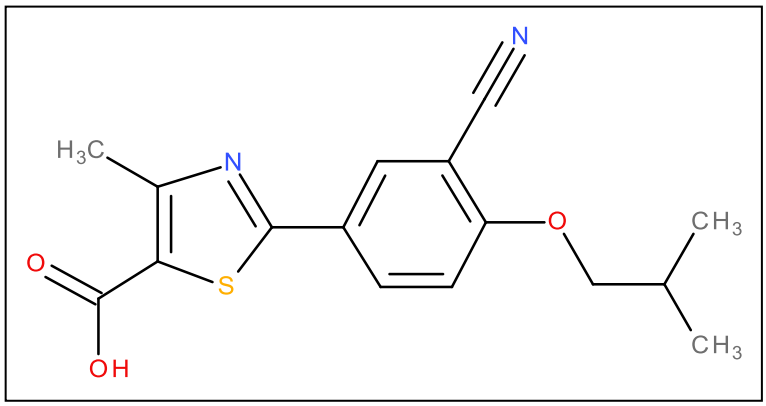
ICH	International Conference on Harmonization
IUPAC	International Union of Pure and Applied Chemistry
L	Liter
LC	Liquid Chromatography
LOQ	Limit of Quantification
LOD	Limit of Detection
FR	Flow Rate
Max	Maximum
Min	Minimum
mL	Mililiter
Min	Minute
Mm	Milimeter
MS	Mass Spectrometry
N	Number
No.	Number
Ng	Nanogram
Pg.No.	Page number
Ppm	Part Per Million
Ref.No.	Reference number
R_f	Retention Factor
RP	Reverse Phase
R.S.D.	Relative Standard Deviation
S.D.	Standard Deviation
Sec.	Second
Sr.No.	Serial number
Std.	Standard
UV/Vis	Ultra violet/Visible
UV	Ultra violet
TLC	Thin Layer Chromatography

A SFC method for Stability indicating assay was developed and validated. The separation was accomplished on column Cyno SB-CN (250 mm X 4.6 mm), 5µM, column under isocratic mode. The optimized mobile phase was CO_2 1.2 ml/min with Methanol (12.5%) as modifier. The detection wavelength was 315 nm. The method is validated as per ICH Guideline Q2 (R1) for specificity, linearity, accuracy, precision, robustness, system suitability and solution stability. The linear regression analysis data for the calibration plots showed good linear relationship with concentration range of 50-250 µg/ml for assay determination. The developed method was simple, specific, accurate and precise. Thus the developed method can also be applicable to the routine analysis of febuxostat in marketed formulations. Degradation kinetic study was carried out on HPLC as per ICH Q1A (R2). The Separation was accomplished on column Phenomenex Gemini (250 mm X 4.6 mm), 5µM. The optimized Mobile phase was Acetonitrile:Water (0.1% GAA and 0.1%NH_3) with pH 4.35. The developed method was Able to separate drug from degradation product and can be used for kinetic analysis of febuxostat.

1.1 Introduction of Drug and disease

Febuxostat is a thiazolecarboxylic acid derivative, selective for inhibition of both the oxidized and reduced forms of xanthine oxidase, and does not resemble a purine or pyrimidin. Its full chemical name is 2-(3-cyano-4-[2-methylpropoxyl] phenyl)-4-methylthiazole-5-carboxylic acid. It has a molecular weight of 316.38 (*1*).

Structure:



Common name: Febuxostat

Chemical name: 2-(3-cyano-4-[2-methylpropoxyl] phenyl)-4- methylthiazole-5-carboxylic acid

CAS No: 144060-53-7

Approval status: It was approved in INDIA on 14th November 2009

Official status: Not official. Pharmacopoeial status is pending.

Molecular mass: Molecular weight of febuxostat is 316.38

Melting Point: 205-208 °C

pKa values: 3.42

Log P value: 3.52

Solubility: freely soluble in dimethylformamide; soluble in dimethylsulfoxide; sparingly soluble in ethanol; slightly soluble in methanol and acetonitrile; and practically insoluble in water.

Appearance: Non-hygroscopic, white crystalline powder.

Mechanism of Action

Xanthine oxidase is the enzyme responsible for catalyzing the final steps, specifically the conversion of hypoxanthine to xanthine and xanthine to urate, in the degradation of purines into the end product of uric acid. Febuxostat has selective affinity for both the oxidized and reduced forms of xanthine oxidase.

Indications: Febuxostat is indicated to lower serum uric acid level in patients with gout (sUA of >6.8 mg/dl) (*2*)

Pharmacological class: Febuxostat is a non-purin selective inhibitor of xanthin oxidase.

Dosage form available: Tablet

Dose and Administration: Febuxostat is administered orally. Dose available is 40mg and 80mg.

Introduction to Disease

Gout is a monoarthritic disorder. In patient suffering with gout; crystallization of monosodium urate from supersaturated body fluids into tissues is observed. Shift in lifestyle is mainly associated with the gout and its onset. Treatment of gout includes use of NSAIDs, colchicines, or corticosteroids. (*2*)

Patient suffering from gout has hyperuricemia, in which the level of serum uric acid (sUA) reaches to >6.8 mg/dL, which approaches the limit of solubility for monosodium urate in extracellular fluids.

Purines are metabolised and degraded in final product of uric acid by enzyme xanthin oxidase. Various factors, such as age, body weight, diet, temperature, and pH, are known to influence both the concentration and solubility of monosodium urate.

During homeostasis body maintains this level below the super saturation level and prevent attack of gout by controlling hyperuricemia. Acute gouty arthritis is the most common initial consequence of hyperuricemia.

1.2 Pharmacokinetics of Febuxostat

Absorption

Febuxostat is readily absorbed (~85%) after administration of multiple oral doses. Absorption is also rapid, with Tmax of ~1 hour. In the 10- to 120-mg/d dose range, febuxostat has dose-proportional increases in Cmax and AUC. After 120 mg/d, febuxostat has a greater than dose proportional increase in AUC, may be due to decreased renal clearance of the conjugated form and an increase in biliary excretion and enterohepatic recirculation. Single daily doses of 120 mg for 13 days led to mean Tmax of 1.0 hours, mean Cmax of 4.47 μg/mL, and mean AUC24 of 11.31 μg/h/mL.

Distribution

The volume of distribution of febuxostat at steady state is 0.7 L/kg.14 Febuxostat is highly bound (99.2%) to circulating plasma proteins, it primarily bounds to albumin.

Metabolism

Febuxostat undergoes hepatic metabolism in the cytochrome P450 (CYP) enzyme system into acylglucuronide metabolites. This metabolism occurs mainly through conjugation via uridine diphosphateglucuronosyltransferase (UGT) enzymes (UGT1A1, UGT1A3, UGT1A9, and UGT2B7). The relative contribution of each isoform of enzyme is not clear. Drug undergoes oxidation of the isobutyl side chain leads to the formation of four pharmacologically active hydroxyl metabolites.

Elimination

Febuxostat is eliminated renally, with a mean $t_{1/2}$ of 9.1 hours for a 120-mg dose. Less than 5% is excreted as intact drug, with most (25%–45%) excreted as the conjugate and a small portion (2%–8%) as oxidative metabolites. it eliminates by hepatic and renal mechanisms. The mean terminal half life of febuxostat was approximately 5 to 8 hours.

1.3 Pharmacodynamics of Febuxostat

Febuxostat is non purin selective inhibitor of the xanthin oxidase. It inhibits xanthin oxidase with Ki values in range of 0.6-0.10 Nm. The compound potently inhibits xanthin oxidase in both oxidized and reduced form. It doesn't affect any other enzyme involved in purin or pyrimidin metabolism. In vivo animal studies using normal and hyperuricemic mice and rats that febuxostat exhibits hypouricemic activity.

1.4 Effects of Product Instability

A. Change in bioavailability
B. Loss of potency
C. Generation of toxic products
D. Loss of elegance

1.5 Regulatory aspects of stability study

Following are the various guidlines available for the stability studies of pharmaceuticals
ICH Q1A (R2) Stability studies of new drug substance and product
ICH Q1B photostabililty testing of new drug substance and product
ICHQ3A(R) Impurities in new drug substance
ICHQ3B (R2) impurities in new drug products
WHO stability studies of active pharmaceutical ingredients and finished pharmaceutical products

1.6 Stability indicating assay method

It is a quantitative analytical method which is based on the characteristic structural, chemical or biological properties of each active ingredient of drug product and that will distinguish each active ingredient from its degradation product. It should be sensitive enough to quantify the drug and degraded product.

1.7 Introduction to forced degradation studies

Chemical stability of pharmaceutical molecules is very crucial as it finally affects safety and efficacy of the drug product. The ICH guideline emphasises on requirement of stability testing data to understand change of stability with environmental factors. Stability data of molecule helps in selecting proper formulation and package as well as providing proper storage conditions and shelf life. This data is important to fulfill the regulatory requirements.

Forced degradation studies involve harsh conditions than that of the accelerated stability studies. Such conditions determined based on the various methods and chemical characteristics of the molecule. To validate the stability indicating method and characterise the degradation products; ICH guidelines provide valuable guidance. These guidelines are general procedures and do not provide any specific conditions to evaluate the stability. They follow general principles. It is necessary to develop and study forced degradation of new drug molecule as it is also a regulatory requirement.

The stability studies include long term studies (12 months) and accelerated stability studies (6 months). But intermediate studies (6 months) can be performed at conditions milder than that used in accelerated studies. To isolate and quantify the degradation products from accelerated stability study take more time so it is important to perform forced degradation and characterise the product to hasten the process of approval.

These studies need to be carried out as per the guidelines issued by ICH, WHO, FDA and other agencies.

It is crucial to know when to perform forced degradation studies for the development of new drug substance and product. FDA guidance says stress testing should be performed in phase III of regulatory submission process. Stress studies can be performed at different pH solutions, in the presence of oxygen and light, elevated temperatures and humidity levels to determine the stability of the drug substance. The results should be summarized and submitted in an annual report. Studies should be conducted on drug substance to obtain sufficient time for identifying degradation products and structure elucidation as well as optimizing the stress conditions. An early stress study also gives opportunities in improvements of the manufacturing process and proper selection of stability-indicating analytical procedures (*3*) (*4*).

1.7.1 Objective of forced degradation studies

Forced degradation studies are carried out to achieve the following.

- To establish degradation pathways of drug substances and drug products.
- To differentiate degradation products that is related to drug products from those that are generated from non-drug product in a formulation.
- To elucidate the structure of degradation products.
- To determine the intrinsic stability of a drug substance in formulation
- To reveal the degradation mechanisms such as hydrolysis, oxidation, thermolysis or photolysis of the drug substance and drug product. (*5*) (*6*)
- To establish stability indicating nature of a developed method.
- To understand the chemical properties of drug molecules.
- To generate more stable formulations.
- To produce a degradation profile similar to that of what would be observed in a formal stability study under ICH conditions.
- To solve stability-related problems (*7*).

1.7.2 Limits for degradation

Limit of degradation has always been deciding factor in forced degradation studies. Degradation of drug substances between 5% and 20% has been accepted as valid argument (*8*).

Some pharmaceutical scientists think 10% degradation is optimal for use in analytical validation for small pharmaceutical molecules for which acceptable stability limits of 90% of label claim is common. Others suggested that drug substance with a mixture of known degradation products can be used to check the methods employed for monitoring stability of drug product.

It is not mandatory that forced degradation would result in a degradation product. The study can be terminated if no degradation is seen after drug substance or drug product has been exposed to stress conditions than those conditions mentioned in an accelerated stability protocol (*9*). Over-stressing a sample may forms secondary degradation product that would not be seen in formal shelf-life stability studies and under-stressing may not generate sufficient degradation products (*10*).

Protocols for generation of product-related degradation may differ for drug substance and drug product due to differences in matrices and concentrations. It is recommended that maximum of 14 days for stress testing in solution (a maximum of 24 h for oxidative tests) to provide stressed samples for methods development.

1.7.3 Choice of solvent

One problem that is usually faced in designing hydrolytic stress tests is compound solubility. Many small molecule drugs are not soluble in water at the concentrations typically used for analytical evaluation (i.e., 0.1–1 mg/mL) across the entire pH range. cosolvent must be added to facilitate dissolution under the conditions of low solubility. The two most commonly used cosolvents are acetonitrile and methanol.

Methanol has the potential of participating in the degradation chemistry (e.g., acting as a nucleophile to react with electrophilic sites or intermediates in the degradation pathways), it should be used with caution. if the compound contains a carboxylic acid, ester, or amide as these groups may react with methanol. Acetonitrile is generally regarded as an inert solvent and is typically preferable to methanol in hydrolytic stress-testing studies. But it should be recognized that acetonitrile is not completely inert and can participate in the degradation reactions leading to false degradation results. For example, acetonitrile is known to contribute to base-catalyzed epoxidation reactions in the presence of peroxides Acetonitrile will also degrade, in the presence of bases (e.g., pH 13) and/or acids under elevated temperatures, to detectable levels of acetamide and/or acetic acid. It should be considered that the presence of a cosolvent may speed up or slow down the hydrolysis. There is also a possibility that degradation pathways could also change in the presence of a cosolvent.

Co solvent	Advantages	disadvantages
Methanol	Completely miscible in water. UV transparent. Good solubilizer for many compounds. Good scavenger of hydroxyl radicals at concentrations of 10% or higher.	Reactive toward some functional groups (esp. esters or carboxylic acids),especially in acid. May contain trace Formaldehyde.
Acetonitrile	Completely miscible in water. UV transparent. Cosolvent of choice for photochemistry. Good solubilizer for many compounds. Inert toward most Active pharmaceutical ingredients. Can lyophilize aqueous solutions containing ACN.	Volatile. May lose solvent at higher temperatures unless well sealed. Hydrolyzes in acid/base to form acetic acid or acetamide. Above neutral pH in oxidizing conditions can produce reactive Per-acid like compounds. Can be oxidized by alkoxyl radicals resulting in formylation of amines.

Selection of cosolvent

1.7.4 Degradation conditions

Hydrolytic conditions

Hydrolysis is one of the most common degradation chemical reactions over a wide range of pH. Hydrolysis is a degradation process due to effect of water. Hydrolytic study under acidic and basic condition involves catalysis of ionizable functional groups present in the molecule. Here drug exposed to various conditions of acid and base for evalution of their degradation. Primary degradation product and generation of secondary product gives exact prediction of the degradation pathway. Strength of acidic and basic conditions largely depends on the chemical characteristics of the molecule but it is advisable to start with the 0.1 N of HCL or NaOH. If drug is not soluble in the water, acid or base than the co-solvant can be used. Hydrochloric acid or sulphuric acids (0.1‑1 M) for acid hydrolysis and sodium hydroxide or potassium hydroxide (0.1‑1 M) for base hydrolysis are suggested as suitable reagents for hydrolysis (*11*).

The selection of co-solvent is based on the drug substance structure. Methanol is generally avoided as co-solvent, as its positive ion is strong and easily participates in the

reaction which leads to false interpretation. Selection of temperature is initiated with room temperature and gradually the temperature is increased until the satisfactory degradation is not observed. Equal amount of acid or alkali should be added to neutralise the sample which also hinders further degradation. These results will indicate whether or not the drug molecule has a particular instability in aqueous conditions as a function of pH. An approximate pH-rate profile can sometimes be constructed from the data, but caution should be exercised because it is not uncommon for a drug compound to be relatively insoluble in some pH ranges, and some samples may require co-solvents in order to achieve dissolution. Critical evaluation of the pH 1–2 degradation rate data can help assess the potential need for enteric coating of drugs to be administered orally. Evaluation of solution stability under neutral to moderately basic (e.g., pH 6–9) can help in assessing the potential for non enzymatic breakdown of the compound under physiologically relevant conditions (e.g., pH 7.5). Such information can be useful not only for metabolism studies, but also for pharmacokinetic concerns (*2*).

Oxidation conditions (*12*)

For oxidative stress degradation study hydrogen peroxide is used. Concentration of oxidising agent and its duration also depends on the chemical nature of the drug molecule. 20% degradation achieved using various concentration of oxidising agent. The oxidative degradation of drug substance involves an electron transfer mechanism to form reactive anions and cations. Amines, sulfides and phenols are susceptible to electron transfer oxidation to give N-oxides, hydroxylamine, sulfones and sulfoxide. The functional group with labile hydrogen like benzylic carbon, allylic carbon, and tertiary carbon or α-positions with respect to hetro atom is susceptible to oxidation to form hydro peroxides, hydroxide or ketone.

Photolytic conditions

Photo stability shows effect of free radical on the drug substance. UV or Fluorescent light exposure is used in studies. ICH states various degradation conditions. Light stress

conditions can induce photo oxidation by free radical mechanism. Functional groups like carbonyls, nitro aromatic, N-oxide, alkenes, aryl chlorides, weak C–H and O–H bonds, sulfides and polyenes are likely to introduce drug photosensitivity. Product should be exposed to 1.2 million lux hours and 200 watt hours/square meter of light.

Thermal conditions

Thermal degradation (e.g., dry heat and wet heat) should be carried out at more strenuous conditions than recommended ICH Q1A accelerated testing conditions. Samples of solid-state drug substances and drug products should be exposed to dry and wet heat while liquid drug products should be exposed to dry heat. Studies carried at higher temperatures for a shorter period. Effect of temperature on substance is studied through the Arrhenius equation

1.8 Degradation kinetic study (*13*)

1.8.1 Importance of degradation kinetics

A critical part of evaluation of intrinsic stability is the apparent rate of degradation under various conditions that lead to degradation.

A rigorous evaluation of the kinetics of gives useful information can be gained by kinetic evaluation. For example, relative rates of degradation at different pH conditions allows an assessment of which pH conditions will provide the most stable environment for a compound. Such information is useful for evaluation of analytical sample preparation conditions (e.g., the sample solvent for analytical assay) and is critical to some solution formulations where stability must be maximized in order to achieve acceptable shelf life.

It can also be applied to design stable solid-state formulations. if a compound is unstable under basic conditions, basic excipients that may cause an alkaline environment in formulation may be avoided.

Kinetics of degradation obtained from stress testing may also reveal the order of a reaction, the dependence of a solid state degradation on humidity, or if the reaction is autocatalytic. Such information can be very useful in designing stability studies and interpreting early time point results.

Degradation rate kinetic study is also important in prediction of the degradation pathways to follow the rates of formation of the different degradation products. Examination of the degradation profile from early time points (e.g., 0–5% degradation) can reveal which products are the primary or first formed products and which are secondary. This is important, as it is not uncommon for the degradation profiles observed during real-time stability studies to contain a mixture of primary, secondary, and even tertiary degradation products in specific cases where the initial products are particularly unstable.

Without kinetic time point analysis, it is not possible to observe primary unstable degradation product and one can lead to determination of secondary product. For example, in the reaction schematic A → B → C, at room temperature, one could observe degradant B but at elevated temperatures, one could miss B and only observe C. This would complicate predictive analysis if kinetic time points are not taken along the course of the reaction.

1.8.2 Kinetic model/Arrhenius study

Various kinetic models have been proposed for solid-state degradation Degradation. Most of these models display approximately linear or zero-order behaviour in the pharmaceutically relevant range of about 10% degradation. For most purposes, a zero-order model provides a simple and convenient means of data comparison for stress studies. Also, accurate kinetic modeling is usually not the purpose of stress studies and becomes even more tenuous for solid dosage forms containing mixtures of compounds.

If nonlinear degradation data are obtained, other models can be used if fitting data to obtain a rate constant is desired. For some studies, only initial and final time point measurements may be adequate. The number of time points should be increased for more accurate determinations of rate constants if desired or needed. For greater confidence that stress conditions are predictive of relative stability under normal conditions, an Arrhenius study of degradation rate at different temperatures can be performed and a room temperature rate calculated.

1.9 Isolation of degradation product

Degradation product is a molecule resulting from a change in the drug substance (bulk material) brought about over time. For the purpose of stability testing of the products in this guidance, such changes could occur as a result of processing or storage (e.g., deamidation, oxidation, aggregation, and proteolysis).

1.9.1 Importance of Isolation and Characterization of Impurities

Impurities are generally assumed to be inferior to API because they might not have the same level of pharmacologic activity. The drug substance and purity is compromised in even if it contains another material with superior pharmacologic or toxicologic properties. Any extra material present in the drug substance or active ingredient must be termed as an impurity even if it is totally inert or has superior pharmacologic properties, so that an appropriate evaluation of its content in the drug product can be made.

1.9.2 Isolation Methods

The following methods can be used for isolation of impurities and degradation products:

- Solid-phase extraction methods
- Liquid‑liquid extraction methods
- Accelerated solvent extraction methods
- Supercritical fluid extraction
- Column chromatography
- Flash chromatography
- Thin-layer chromatography (TLC)
- Gas chromatography (GC)
- High-pressure liquid chromatography (HPLC)
- Capillary electrophoresis (CE)
- Supercritical fluid chromatography (SFC)

1.9.3 Thin-Layer Chromatography (TLC)

Thin-layer chromatography is one of the widely used and most popular separation techniques because of its ease of use, cost-effectiveness, , speed of separation and high sensitivity.

It can also be utilized on a preparative scale to isolate a specific component. In TLC, the sample is applied as a small spot or band to the marked origin of stationary phase supported on a glass or metal plate. The sample solvent is allowed to evaporate from the plate. Then plate is placed in a closed chamber containing mobile phase at the bottom. The mobile phase moves through the stationary phase by capillary action. The components of the mixture migrate at different rates due to their different affinity y for mobile and stationary phase. When the mobile phase has moved an appropriate distance, the plate is removed from the chamber and the solvent front is marked. Mobile phase is evaporated from the plate by drying at room temperature, by heating with forced air flow. If the components of the mixture are not naturally colored or fluorescent, a detection reagent is applied to visualize the bands. Sometimes more than one detection technique is used to ensure the detection of all components in the mixture. A variety of sorbents have been used as the stationary phase in TLC, including silica gel, cellulose, alumina, polyamides, ion exchangers, chemically modified silica gel, and mixed layers of two or more materials, coated on a suitable support.

1.9.4 Preparative TLC (*14*)

To isolate the degradation product, band of the drug substance, typically at 25.0 mg/ml concentration, is applied to a 10×10 cm silica plate, usually at a volume of 200 ml, in an effort to overload the silica plate such that more of the impurity can be isolated. This process is repeated for 10 HPTLC plates or so. The plates are then developed according to the method and the impurity marked with a pencil under UV. Once the 10 plates have dried, the product band in question is scraped from the silica plate using a metal spatula and collected in separate containers. Approximately 10 ml of extraction solvent (usually the sample solvent in the relevant TLC method) is added to the container. The solution is stirred, sonicated about 10 min. The resulting clear solution is transferred to another suitable container and concentrated. This concentrated solution is then reapplied to a new TLC plate to confirm the TLC isolation of the impurities.

The process is tedious for extraction from the silica plates, and timing must be coordinated such that the sample is immediately analyzed by the HPLC system. Thi s reduces chances of any potential degradation. Sometimes, the band in question is very close to the drug substance or another band, making it difficult to extract for TLC.

1.9.5 Liquid-Liquid Extraction (LLE)

LLE initially involves pH adjustment of the original (aqueous) sample with an appropriate buffer. This pH adjustment is intended to neutralize the analyte molecules.

The next step in the process is the addition of an immiscible organic extraction solvent, followed by agitation to facilitate equilibrium partitioning of analyte molecules between two phases.

The phases are physically separated and the aqueous component is subjected to an additional extraction or discarded. If several extracts have been produced, the organic phases are combined, evaporated to dryness and, possibly, re suspended with a clean solvent.

LLE is a preparative technique, performed manually and employing separatory Funnels. When performed in the pharmaceutical analysis laboratory, LLE can be either preparative-scale using a separatory funnel, or analytical-scale (microliters to a few milliliters) and performed using test tubes, vials, and glass or polypropylene pipets. Method development for LLE is usually eased, as the approach can have a high probability success on the first attempt. The technique can be laborious and it does not provide good recoveries for highly polar molecules.

1.10 Analytical Method development and validation (*15*)

Stability indicating method and development of analytical method for it is very important. Method should serve the purpose of frequent and routine analysis. Various degradation products that have been generated during the various conditions should be easily separated and quantifiable.

Analytical method validation is the estimation of the reproducibility and reliability. Validation of method provides confidence in the data generated by the analysis.
Validation also used to evaluate the consistency and quality of method. It also shows that the method is functioning purposefully and data generated are trustworthy.
Validation as in ICH Q2 (R1) is a regulatory requirement.

1.10.1 Method Validation Parameters

The objective of the analytical procedure should be clearly understood. Since this will govern the validation characteristics which need to be evaluated. Following are the typical validation parameters

1. Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometime called trueness.

Acceptance criteria:

1. Recovery between 98-102%
2. %RSD of %Recovery should NMT 2.0%

2. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

A. Repeatability

RSD should not be more than 2.0%

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

B. Intermediate Precision

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

RSD Should not be more than 2.0%

3. Reproducibility

It expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

4. Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present.

Acceptance criteria:

There should be no interference from placebo with main peak and known impurities.

There should be separation of impurities if any.

5. Detection Limit

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

Acceptance criteria:

S/N ratio should be greater than 3

6. Quantitation Limit

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and

accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

Acceptance criteria:
S/N ratio should be greater than 10:1

7. Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

Acceptance criteria:
Correlation coefficient should be more than 0.995

8. Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

The following minimum specified ranges should be considered:
- For the assay of a drug substance or a finished (drug) product: normally from 80 to 120 percent of the test concentration;
- for content uniformity, covering a minimum of 70 to 130 percent of the test concentration, unless a wider more appropriate range, based on the nature of the dosage form (e.g., metered dose inhalers), is justified;
- For dissolution testing: +/-20 % over the specified range

9. Robustness

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

1.11 Introduction to super critical fluid chromatography

Super critical fluid chromatography has its place among rapidly growing analytical techniques. It differs from various conventional techniques in terms of its novel mobile phase and replacement of organic solvents by super critical fluid. When a mobile phase is below its critical temperature and above its critical pressure it acts as liquid and above critical temperature and below critical pressure exists as gas.

This property provides unique ability of gas to penetrate and liquid to dissolve the samples.SFC is also compatible with LC and GC detectors which make it even more useful. It can be employed to analyse thermo labile and volatile materials.

In SFC carbon dioxide is maintained at super critical state above its critical pressure and temperature. Chromatography can be carried out using open tubular capillary columns or packed silica columns. LC detectors are widely used due to their compatibility. Selection of modifiers, pressure and temperature is essential in developing analytical method using super critical fluid chromatography.

1.11.1 Principle

The concept of supercritical fluid can be understood by a phase diagram. A temperature above which a substance can no longer exist as a liquid, even after increasing the temperature is called supercritical temperature and a pressure above which the substance can no longer exist as a gas even after that the pressure is raised is called supercritical pressure.

SFC is based on the principle of density of the supercritical fluid which corresponds to solvating power. As the pressure in the system is increased, the supercritical fluid density increases and its solvating power increases. Hence, as the density of the supercritical fluid mobile phase is increased, retained components get eluted.

1.11.2 Instrumentation

1) First apparatus is like HPLC apparatus which is consists of two reciprocating pumps and a packed analytical column with oven and detector system

Pumps: Two pumps continuously flows carbon dioxide and modifier at desired rate into the column. Carbon dioxide attains its critical temperature through cool circulator system.

Column oven: Temperature has direct effect on density. Due to change in density of mobile phase properties of carbon dioxide also varies. For gaining stable extraction efficiency and reproducible peak retention time use of an oven is important. Oven controls temperature of column very precisely. They are as accurate as temperature control with a precision of ±0.1ºC.

Back pressure regulator: Changes in pressure also changes characteristics of SFC. To achieve extraction and chromatograms having high reproducibility on a supercritical fluid system requires a device which can perform pressure control in a stable manner.

2) Second apparatus which consists of a syringe pump, a capillary column, oven, a restrictor and a flame ionization detector.

Before the supercritical fluid enters the analytical column, it is brought into the supercritical region by heating it above its supercritical temperature. After that it is passed through an injection valve. The sample is applied here for analysis into the supercritical fluid. The fluid must be maintained supercritical throughout the column and up to the detector by a restrictor. To maintain temperature at working level, thermostat is used.

1.12 Introduction to high performance liquid chromatography (*16*) (*17*)

Most of the can be analyzed by HPLC method because of the several advantages like:

- Speed
- Greater sensitivity
- Improved resolution
- Reusable columns
- Easy sample recovery, handling and maintenance
- Instrumentation tends itself to automation and quantitation
- Precise and reproducible results

Different modes of separation in HPLC are available. Normal phase mode and reversed phase mode.

In the normal phase mode, the stationary phase is polar and the mobile phase is nonpolar in nature. In this technique, nonpolar compounds travel faster and are eluted first. This is because of the lower affinity between the nonpolar compounds and the stationary phase.

Polar compounds are retained for longer times because of their higher affinity with the stationary phase. These compounds, therefore take more times to elute. Normal phase mode of separation is therefore, not generally used for pharmaceutical applications because most of the drug molecules are polar in nature and hence take longer time to elute.

Various components of HPLC are:

- A solvent delivery system, including pump
- Sample injection system
- A chromatographic column
- A detector
- A strip chart recorder
- Data handling device and microprocessor control.

(a) Solvent delivery system:

A mobile phase is pumped under pressure from one or several reservoir and flows through the column at a constant rate. For normal phase separation eluting power

increases with increasing polarity of the solvent but for reversed phase separation, eluting power decreases with increasing polarity.

Pumps:

The pump is one of the most important components of HPLC, since its performance directly affects retention time, reproducibility and detector sensitivity.

Three main types of pumps are used in the HPLC system.

1. Displacement pump
2. Reciprocating pump
3. Pneumatic or constant pressure pump

(b) Sample injection system:

There are three important ways of introducing the sample into injection port.

1. Loop injection
2. Valve injection
3. On column injection

(c) Chromatographic column:

The column is usually made up of heavy glass or stainless steel tubing to withstand high pressure. The columns are usually 10-30 cm long and 4-10 mm inside diameter containing stationary phase at particle diameter of 25 µm or less.

Columns with an internal diameter of 5 mm give good results because of compromise between efficiency, sample capacity, and the amount of packing and solvent required.

Column packing:

The packing used in modern HPLC consist of small, rigid particles having a narrow particle size distribution. There are three main types of column packing in HPLC.

1. Porous polymeric beds
2. Porous layer beds
3. Totally Porous silica particles (dia. <10 µm)

d) Detectors:

The function of the detector in HPLC is to monitor the mobile phase as it merges from the column. Detectors are usually of two types:

1. **Bulk property detectors**: It compares overall changes in a physical property of the mobile phase with and without an eluting solute. e.g. refractive index, dielectric constant or density.

2. Solute property detectors: It responds to a physical property of the solute which is not exhibited by the pure mobile phase. E.g. UV absorbance, fluorescence or diffusion current. Such detectors are about 1000 times more sensitive giving a detectable signal for a few nanograms of sample.

2.1 Literature Review

Following methods are available for analysis of febuxostat.

Analytical Methods			
UV-spectrophotometric Method			
Matrix	**Title**	**Method**	**Reference No.**
Tablets	Development and application of difference spectrophotometric method for the determination of febuxostat in tablets.	Spectrophotometric method 0.1N HCL and 0.1 N NaOH Wavelength: 315 nm and 260 nm Linearity: 5-25 µg/ml	(*1*)
Bulk and Tablet	A simple UV spectrophotometric method for the determination of febuxostat in bulk and Pharmaceutical formulations	Solvent: Methanol Wavelength: 315 nm Linearity: 0.2-15 µg/ml	(*2*)
Tablet	Development and validation of new Spectrophotometric method for determination of febuxostat in tablet dosage forms	Solvent: Methanol Wavelength: 316 nm Linearity: 2-20 µg/ml	(*3*)
RP-HPLC Methods			
Tablet	Stress Degradation Studies and Validation Method for Quantification of Febuxostat in Formulations by Using RP-HPLC	Column: C8 (4.6 × 150 mm id, particle size 3 µm) Acetonitrile and 20 mM sodium dihydrogen phosphate in water (PH 2.5 with orthophosphoric acid) 50:50 Wavelength 315 nm Retention time 2.35 min	(*4*)
Tablet	Development and validation of RP-HPLC method For estimation of febuxostat in bulk and Tablet dosage form	Column YMC C8 Column C8 (4.6 × 150 mm id, particle size 3 um) Mobile phase ACN:BUFFER (60:40) phosphate buffer Wavelength 320nm Retention time 3.145	(*5*)

Bulk	Study of impurity carryover and impurity profile in Febuxostat drug substance by LC–MS/MS technique	Column : Kromasil C18 (4.6 × 150 mm id, particle size 5 µm) Mobile phase: 0.01 M aqueous ammonium acetate and pH Adjusted to 3.5 with trifluroacetic acid Acetonitrile gradient mode Wavelength 315 nm	(*6*)
Tablet	ICH Guidance In Practice: Development Of A Validated Stability-Indicating High-Performance Liquid Chromatographic Assay Method For Febuxostat And Degradation Kinetic Study In Acid Hydrolytic Condition	column C-18 Mobile phase: Methanol-water (with 0.02%v/v TFA) in the ratio of 95:5 wavelength 315 nm retention time 3.98	(*7*)
Tablet and bulk	Estimation of related substances of Febuxostat in bulk & 40/80/120mg tablets by RP-HPLC	Column : poroshell 120 E18C18 column (500 mm × 4.6 mm; 2.7 µm) Mobile phase: Phosphate buffer pH 3.0: Methanol Gradient Mode Wavelength: 318nm Retention time: 3.6 min	(*8*)
Tablet and bulk	An improved stability-indicating HPLC method for Febuxostat in bulk and pharmaceutical dosage Forms	Gracesmart C18 (4.6 × 100 mm id, particle size 5 µm) Mobile phase: methanol:Water (80:20 v/v) Wavelength: 315 nm 2.254	(*9*)
Bulk and tablet	Developed And Validated Reverse Phase Hplc Method For The Determination Of Febuxostat In Bulk And Formulations	Column: C18 250 × 4.6 mm i.d, 5µm Mobile phase: methanol:OPA,(90:10) v/v Wavelength: 316 nm Retention time: 5.28 min	(*11*)

Tablets	Reverse phase high performance liquid chromatographic Estimation of anti-gout in pharmaceutical dosage form	Column: phenomenex luna C18 250 × 4.6 mm i.d, 5µm Mobile phase: water:acetonitrile,(70:30) v/v Wavelength: 314 nm Retention time: 2.49 min	(*12*)
Bulk	Establishment of inherent stability of Febuxostat and development of a validated stability-indicating method by UPLC according to ICH requirement	Column: waters Acquity BEH 150 × 4.6 mm i.d, 1.7 µm Mobile phase: acetonitrile: Ammonium acetate buffer pH 4.5 (70:30) v/v Wavelength: 315 nm Retention time: 2.04 min	(*13*)

3.1 Rationale for selection of Drug

Febuxostat drug was approved by USFDA in february 2009 and it was approved in India in November 2009.

Sahu et al (*1*) published stability indicating assay method by UPLC recently in 2012 and reported degradation of febuxostat in alkaline condition.

Mukthinuthalapati et al (*2*) also reported stability indicating assay method for febuxostat using RP-HPLC. They reported that the febuxostat was stable under all degradation conditions.

From the structure it is evident that fabuxostat contains nitrile and caroboxylic acid functional groups. These functional groups are prone to degradation which is considered to be affected during the degradation studies.

Pandya et al (*3*) published stability indicating assay method with kinetic study of febuxostat and reported degradation in acidic condition.

Literature shows that use of methanol in forced degradation of the drug should be avoided due to its high reactivity with carboxylic acid. Degradation studies performed with methanol generates ester of drug which is not in true sense a degradation product and may not form during shelf life of the drug.

This conflict of results and uncertainty over the degradation of drug it was decided to study degradation of drug along with isolation of degradation product.

With increasing trends to reduce use of organic solvents in pharmaceutical practises it was also decided to develop competent Super Critical Fluid Chromatographic stability indicating assay method.

3.2 Objectives of study

Present work has following objectives to perform stability indicating assay method

- Development of stability indicating assay method for febuxostat in its bulk and individual dosage form using super critical fluid chromatography.
- Degradation Kinetic study of febuxostat using High performance liquid chromatography.
- Calculation of degradation rate constant and order of reaction.
- To determine the intrinsic stability of a drug substance in formulation.
- To isolate the degradation products in pure form.

4.1 Instrumentation and Equipments

Following instrument were used during the study

Analytical balance: CX220, Citizen USA

Ultrasonicator: D-compact, EIE instruments Ahmedabad, INDIA

UV VIS spectrophotometer: UV-2450, double beam, shimadzu, JAPAN

Melting point apparatus: T0603160; EIE instruments, Ahmedabad, INDIA

Fourier Transform infrared spectrophotometer (FT-IR): JASCO FT/IR 6100, JAPAN

Water bath: EIE Instruments, Ahmedabad, INDIA

Digital pH meter: PH-MV-TEMP, PH-206, LTLUTRON, Taiwan

Hot air oven: EIE 108, EIE instruments, Ahmedabad, INDIA

HPLC System: JASCO PU-2080 solvent mixing module JASCO-MX2080. Rheodyne loop injector with 20µl loop, PDA detector system MD-2015 PLUS, software BORWIN

All equipments were calibrated and operated as per the standard operating procedures.

SFC System: JASCO PU980 Intelligent HPLC pump, JASCO CO 150 intelligent HPLS Thermostat, JASCO UV 975 UV/VIS Detector, JASCO 880-81 Back pressure Regulator. CH-201 Cool circulator, SCINICS Instrument, Rheodyne 20µl loop injector.

5.1 Identification of drug

Following methods were used to identify the drug febuxostat.

5.1.1 Identification by Melting point determination

Drug	Melting point(Reported) (*1*)	Melting point (Observed)
Febuxostat	205-208 °C	205-208 °C

5.1.2 Identification using FT-IR Spectroscopy (*2*)

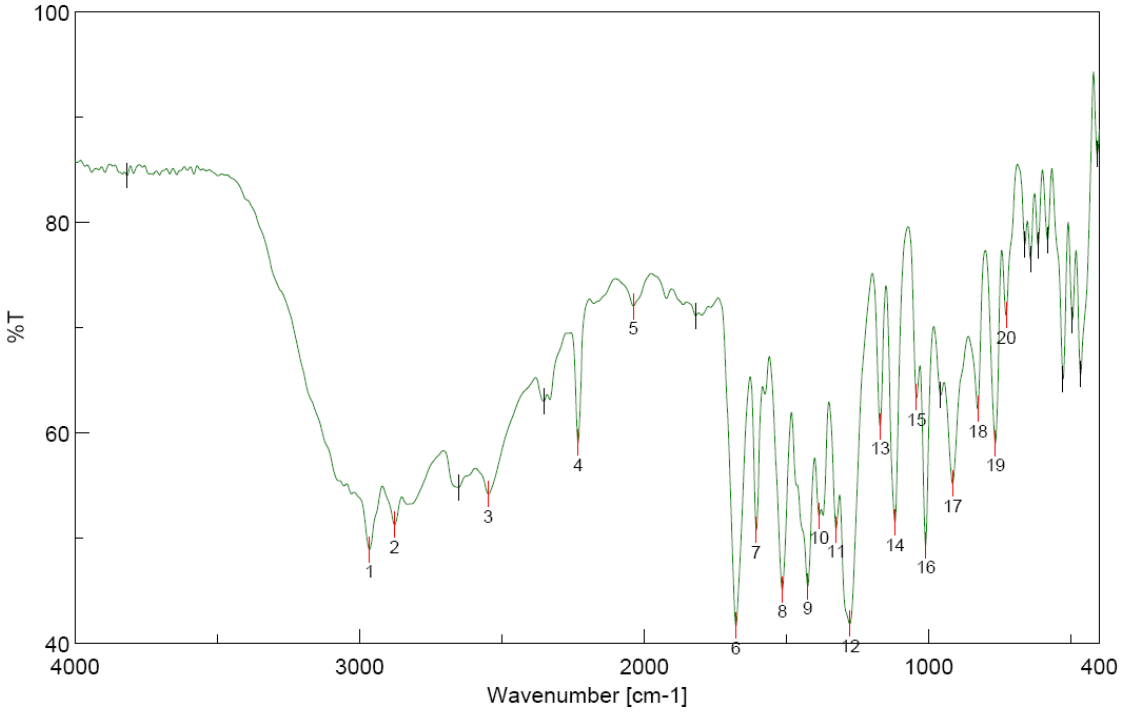


Fig 5.1 IR spectra of febuxostat

Table 5.1 Observations for IR spectra

No	Characteristic group	Theoretical frequency	Observed frequency
1	O-H stretching	2700-2500	2552
2	Aryl nitrile stretching	2240-2220	2230
3	Carboxylic acid stretching	1700-1680	1674
4	Ether stretching	1275-1200	1274
5	Ring C=C stretching	1600	1605
6	Alkane C-H stretching	2962-2853	2880

5.1.3 Identification using UV-spectroscopy

An absorption maxima was determined using standard solution of febuxostat 20µg/ml in the range of 200-400 nm against ACN as blank.

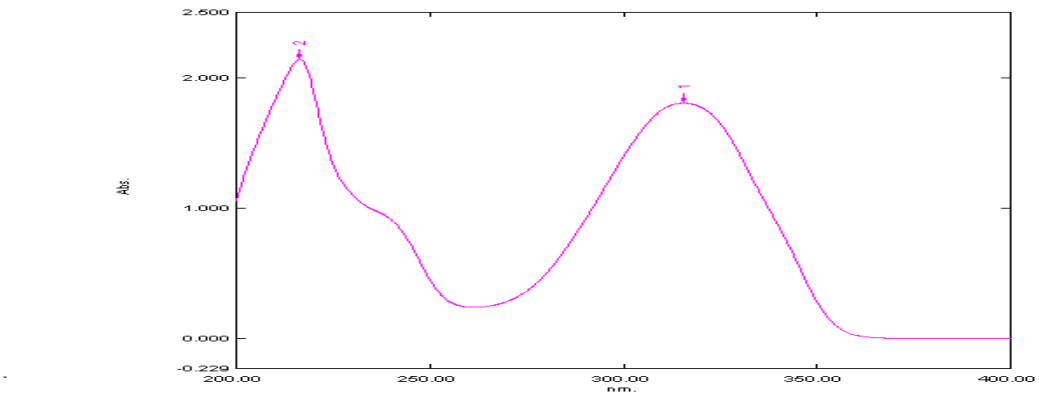


Fig 5.2 UV spectra of Febuxostat

Table 5.2 Absorption Maxima of Febuxostat

Name	Reported absorption maxima	Observed absorption maxima
Febuxostat	315 nm (Bagga *et al.* 2655-59)	315 nm

6.1 Experimental work

6.1.1 Preparation of standard stock solution

Standard stock solution of febuxostat was prepared by dissolving accurately weighed 100mg of FBX in acetonitrile using 100ml volumetric flask. Further dilution of standard was carried out by diluting appropriate aliquots of solution to required quantity of the solution.

6.1.2 Preparation of samples for forced degradation studies

Febuxosatat was directly soluble in 0.1 N NaOH. Sample was prepared by directly dissolving accurately weighed quantity of febuxostat in 0.1 N Sodium Hydroxide to prepare 1000µg/ml of solution. For alkaline degradation sample was kept at 70˚C in water bath.

6.1.3 Optimized chromatographic conditions for HPLC

Following conditions were finalised for the HPLC analysis and kept constant throughout the study (omkar et al)

Column: C18 Phenomenex (250×4.6 mm, 5 µm)

Mobile phase: Acetonitrile and water (0.1 % GAA and 0.1% Ammonia) 43:57 %v/v

Temperature: 25˚C

Detection wavelength: 315 nm

Injection volume: 20µl

6.1.4 Preparation of degradation samples for base degradation kinetic analysis

Samples were collected at the interval of one hour. Equal amount of acid (0.1 N HCl) was added for neutralisation. All samples were diluted to get final concentration of 20µg/ml. Samples were diluted using ACN:Water (50:50% v/v). Samples were filtered using syringe filter of 0.45µm prior to analysis.

7.1.6 Application of spots

A pencil line was drawn at the bottom of the plate. Solution was applied using micro pipette. Sample was applied repetitively two times. A pencil line was drawn at the bottom of the plate and a small drop of a solution was placed on it. The spot of mixture was allowed to become dry.

7.1.7 Preparation of degradation sample for Liquid-Liquid extraction

Accurately weighted 100 mg of Febuxostat was transferred to 200 ml round bottom flask. To this flask 100 ml of 0.1 N NaOH was added. This solution was sonicated until clear solution was obtained. Flask was kept in water bath for 2 hours at 70˚C.

7.1.8 Procedure 1 for Liquid-Liquid extraction

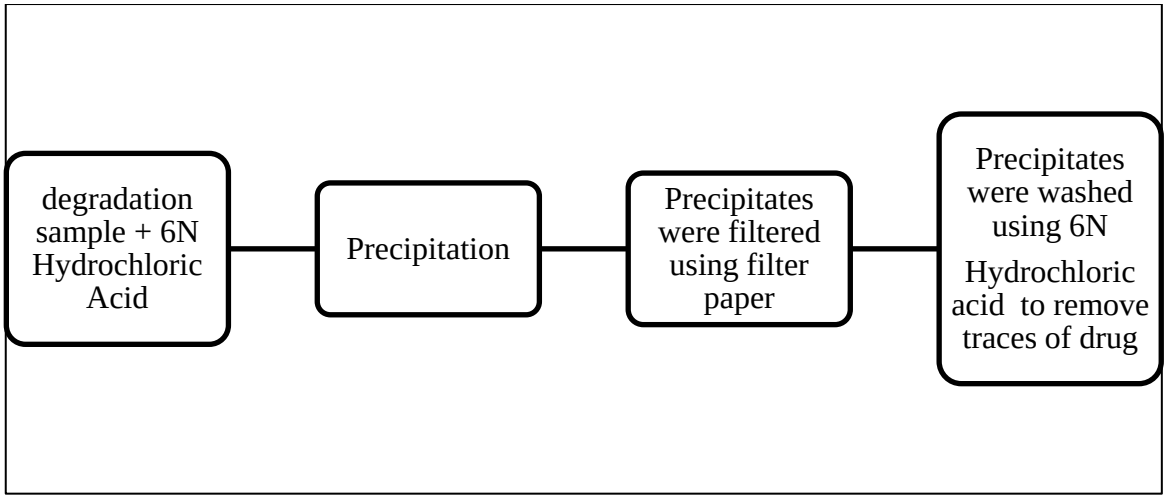


Fig 7.1 Isolation Procedure 1

7.1.9 Procedure 2 for Liquid-Liquid extraction

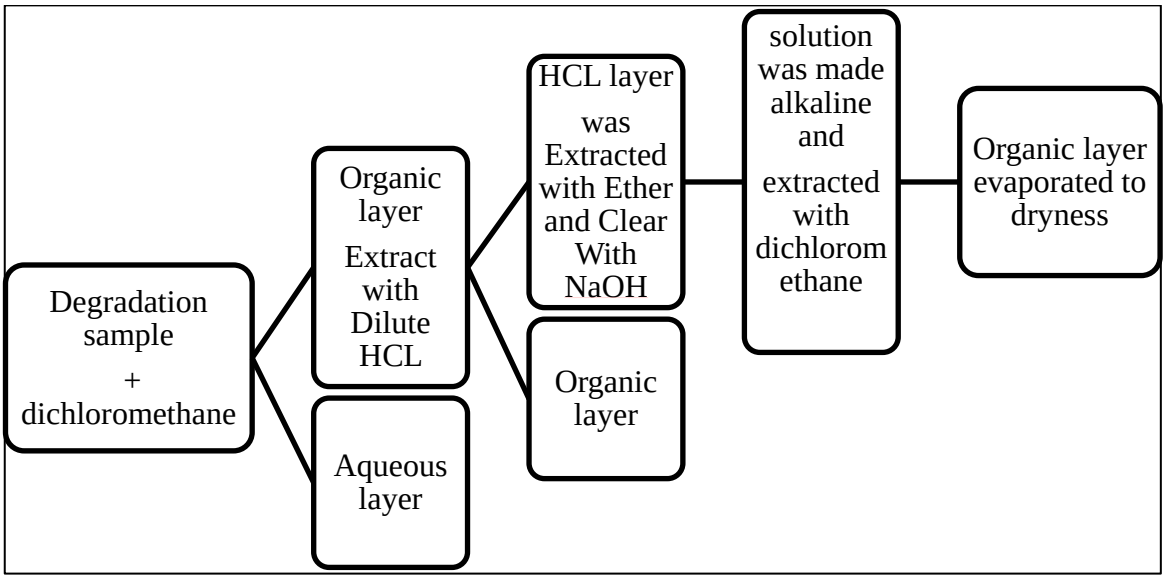


Fig 7.2 Isolation procedure 2

7.1.10 Procedure 3 for Liquid-Liquid extraction

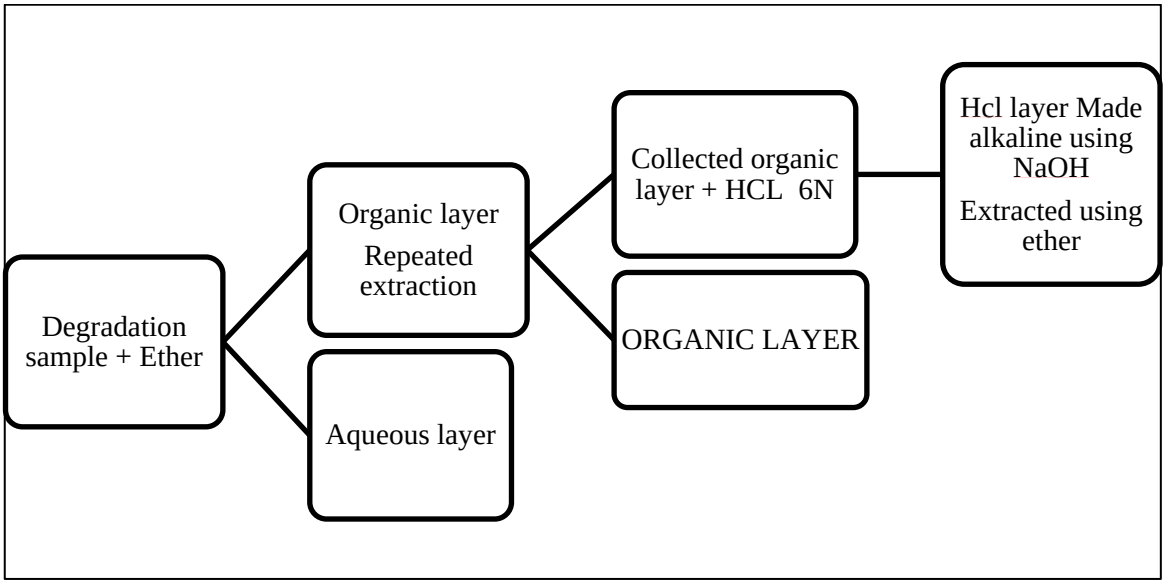


Fig 7.3 Isolation procedure 3

Liquid-liquid extraction was closely monitored at each step by simple TLC analysis

7.1.11 Preparation of solution for TLC analysis (Monitoring extraction procedure)

From degradation sample of 1000 µg/ml, 1 ml sample was taken in 10 ml volumetric flask. This solution was neutralised with 1 ml 0.1 M HCl. Solution was diluted to get final concentration of 100µg/ml.