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Radio stability Assessment of 2-acetoxybenzoic acid via HPLC and HRMS

Dr. A.S. Pratapwar[1*], Mr. G.S. Bothra[2]

[1*,2]Department of pharmaceutical chemistry Sudhakarrao naik institute of pharmacy, pusad

Abstract
The stability of pharmaceutical formulations is a critical aspect of ensuring their safety, efficacy, and quality throughout their intended shelf life. This study presents a comprehensive approach to evaluating the stability of aspirin, a widely used pharmaceutical agent, by developing and validating a stability-indicating high-performance liquid chromatography (HPLC) method. The research investigates the effects of ionizing radiation on aspirin, focusing on impurity profiling through advanced high-resolution mass spectrometry (HRMS).

Aspirin was subjected to forced degradation under hydrolysis, oxidation, photolysis, thermal stress, and ionizing radiation, including gamma and neutron exposures. The HPLC method was optimized using a reverse-phase C18 column and a tailored mobile phase to ensure the effective separation and quantification of aspirin and its degradation products. HRMS enabled precise identification and characterization of impurities, providing insights into degradation pathways.

The study highlights the significant impact of ionizing radiation on the chemical stability of aspirin, leading to the formation of unique degradation products. These findings have critical implications for Pharmaceuticals exposed to extreme environments, such as during space missions or sterilization processes. The research underscores the importance of integrating stability-indicating methods and impurity profiling in pharmaceutical quality control to ensure drug safety and efficacy under diverse conditions. This work contributes to the broader understanding of drug stability and supports the development of robust analytical methods for regulatory compliance.

Keywords: stability-indicating Methods, Aspirin, HPLC Method Development, Forced Degradation Studies, Ionizing Radiation, Impurity Profiling, High-Resolution Mass Spectrometry (HRMS), Regulatory guidelines (ICH Q1 (R2)).

Introduction

1.1 Drug Stability and the Importance of Stability Testing
Drug stability refers to the ability of pharmaceutical products to maintain their physical, chemical, microbiological, and therapeutic properties over time under specified storage conditions. Stability testing is critical for determining shelf life, storage requirements, and degradation pathways, thereby ensuring patient safety and drug efficacy. Regulatory guidelines, including those from the International Conference on Harmonization (ICH), emphasize the necessity of stability-indicating methods for assessing drug quality.
1.2 Importance of Aspirin as a Pharmaceutical Agent
Aspirin is one of the most widely used pharmaceutical agents, recognized for its analgesic, anti-inflammatory, and antipyretic properties. Additionally, its role as an antiplatelet agent in preventing cardiovascular events underscores its clinical significance. The widespread use of aspirin necessitates rigorous quality and stability studies to ensure its safety and efficacy.
1.3 Need for Stability Studies
Stability studies are essential to confirm that pharmaceutical formulations maintain their intended efficacy and safety profiles throughout their shelf life. These studies help identify degradation pathways, assess the impact of environmental factors, and ensure compliance with regulatory standards.
1.4 Overview of Ionizing Radiation Effects on Pharmaceuticals
Ionizing radiation, such as gamma rays and neutrons, can induce chemical and physical changes in pharmaceutical compounds, leading to degradation and the formation of impurities. Understanding these effects is particularly important for drugs intended for extreme environments, such as space missions, where radiation exposure is inevitable.
1.5 Role of Stability-Indicating Methods in Pharmaceutical Quality Control
Stability-indicating methods are analytical techniques designed to detect changes in drug composition over time. These methods are essential for distinguishing between active pharmaceutical ingredients (APIs), degradation products, and impurities, ensuring the reliability of stability assessments.
1.6 Relevance of Impurity Profiling for Safety Assessments

Impurity profiling involves identifying and quantifying degradation products and impurities in pharmaceutical formulations. This process is critical for assessing the safety of drug products, as some impurities may pose

significant health risks. Regulatory agencies mandate impurity profiling as part of stability testing to guarantee the safety of pharmaceutical products.

Overview of Stability Studies

1. Definition and Purpose: Stability studies assess the quality of a drug substance product, which changes over time under the influence of various environmental factors such as temperature, humidity, and light.

- Define storage conditions.
- Establish shelf life and expiration dates.
- Ensure the safety, efficacy, and quality of pharmaceutical products.

2. Types of Stability Studaies:

- **Real-time Stability Testing:** Long-term studies conducted under recommended storage conditions to evaluate degradation over time.
- **Accelerated Stability Testing:** Carried out at elevated stress conditions to predict shelf life quickly. This helps in early drug development.
- **Retained Sample Stability Study:** Regular testing of samples stored in inventory to monitor ongoing stability.
- **Cyclic Temperature Stress Testing:** Simulates fluctuating environmental conditions, especially useful for products stored or transported under variable climates.

3. Forced Degradation Studies: These are conducted under severe conditions (e.g., extreme pH, light, temperature, and oxidative stress) to:

- Identify degradation pathways and products.
- Develop stability-indicating analytical methods.

Key Degradation Condition:

- **Hydrolysis:** Acidic or basic conditions.
- **Oxidation:** Using agents like hydrogen peroxide.
- **Photolysis:** Exposure to UV or visible light.
- **Thermal:** Elevated temperatures, often coupled with humidity.

4. Factors Affecting Stability:

- **Temperature:** Increases reaction rates, especially hydrolysis and oxidation.
- **Moisture:** Affects solid dosage forms, leading to physical and chemical changes.
- **pH:** Influences hydrolysis rates in liquid formulations.
- **Oxygen and Light:** Cause oxidation or photolytic degradation.
- **Excipients and Packaging:** Interactions with excipients and inadequate packaging can reduce stability.

5. Regulatory Guidelines: Guidelines by ICH, WHO, FDA, and EMA outline protocols for stability testing:

- ICH Q1A (R2): General stability testing requirements.
- ICH Q1B: Photostability testing.
- ICH Q1C to Q1F: Stability considerations for various dosage forms and climatic zones.

6. Analytical Methods: Stability studies employ validated methods to ensure precise and accurate measurements of active ingredients and degradation products:

- **HPLC (high-performance Liquid Chromatography):** Commonly used for separation and quantification.
- **Mass Spectrometry (e.g., LC-MS):** Used for degradation product identification.
- **Spectroscopic Techniques (e.g., UV, IR):** For purity assessment.

7. Importance:

- Ensure product safety and efficacy.
- Prevents the formation of toxic degradation products.
- Assists in regulatory compliance and product approval.

HPLC Method Development for Aspirin

1. Principles of HPLC: High-performance liquid Chromatography (HPLC) is an analytical technique used for separating, identifying, and quantifying components in a mixture. It operates on the principle of separation of compounds based on their interactions with a stationary phase (column) and a mobile phase (solvent).

- **Stationary Phases:** Typically, these are solid materials that fill the column. Common types include silica-based materials, C18 (octadecylsilane), and C8 columns. The stationary phase determines the nature of the interactions with the samples, thus influencing the separation.
- **Mobile Phase:** These are solvents or mixtures of solvents that carry the sample through the column. The composition of the mobile phase (water, acetonitrile, methanol, etc.) is crucial for optimizing separation. In reversed-phase chromatography, water is commonly used in combination with organic solvents.

- **Detectors:** The most common detectors used in HPLC are UV- Vis detectors, refractive index detectors (RI), and mass spectrometrics (MS) detectors. For aspirin, UV detectors are most commonly used, typically set at a wavelength of 230nm or 254nm.

2. Parameters for Method Development:

- **Selection of Column and Mobile Phase:**

Column Selection: The column's choice depends on the aspirin's polarity. Reversed-phase C18 columns are commonly used, as aspirin is relatively non-polar.
Mobile Phase Selection: The mobile phase should be selected to ensure proper resolution of aspirin from other components. A mixture of water and acetonitrile or methanol at different ratios (e.g., 60:40 or 70:30) is often used. The pH of the mobile phase should be adjusted to optimize aspirin retention time.

- **Flow Rate, Temperature, and Detection Wavelength:**

Flow Rate: The flow rate typically ranges from 0.5 to 1.0 mL/min. A higher flow rate reduces retention time but may affect resolution.
Temperature: Columns are usually operated at temperatures between 25°C and 40°C. Temperature affects viscosity and solubility, influencing retention time and separation efficiency.
Detection Wavelength: Aspirin has a strong absorption at 230nm or 254nm in the UV-Vis range, making it ideal for detection using a UV detector.

3. Commonly Used Methods for Assay of Aspirin:

- **Methods 1: HPLC Methods for Assay of Aspirin and Its Impurities.**

Column: C18 (250mm x 4.6mm, 5µm)
Mobile Phase: 60% acetonitrile, 40% water (adjusted to pH 3.0 using phosphoric acid)
Flow Rate: 1.0mL/min Wavelength:230nm
Application: Quantification of aspirin and its degradation products.

- **Method 2: Determination of Aspirin in Pharmaceutical Formulations**

Column: C18 (150mm x 4.6mm, 3µm)
Mobile Phase: 70% methanol, 30% water with 0.1% acetic acid (adjusted pH to 3.5)
Flow Rate: 0.9mL/min
Wavelength: 254nm
Application: Used for analyzing aspirin content in tablet formulations with a focus on stability.

- **Method 3: Reverse-Phase HPLC for Aspirin in Biological Samples** Column: C18 (100mm x 2.1mm, 3µm)

Mobile Phase: 85% acetonitrile, 15% phosphate buffer (pH to 3.0)
Flow Rate: 0.8mL/min
Wavelength: 230nm
Application: This method is used for the determination of aspirin in plasma or serum samples.

Method Validation as per ICH Q2 (R1)

Method validation is a crucial process in pharmaceutical analysis that ensures the reliability, accuracy, and precision of analytical methods used to assess drug quality. The International Council for Harmonisation (ICH) Q2(R1) guidelines define validation as establishing documented evidence that a method is suitable for its intended purpose.
ICH Q2 (R1) guidelines provide a framework for validating analytical methods, ensuring accuracy, precision, sensitivity, and reproducibility. For a **stability-indicating HPLC method**, the validation parameters include:
Specificity

1. **Linearity and Range**
2. **Accuracy (Recovery Studies)**
3. **Precision (Repeatability and Intermediate Precision)**
4. **Limit of Detection (LOD) and Limit of Quantitation (LOQ)**
5. **Robustness**
6. **System Suitability Tests (SST)**

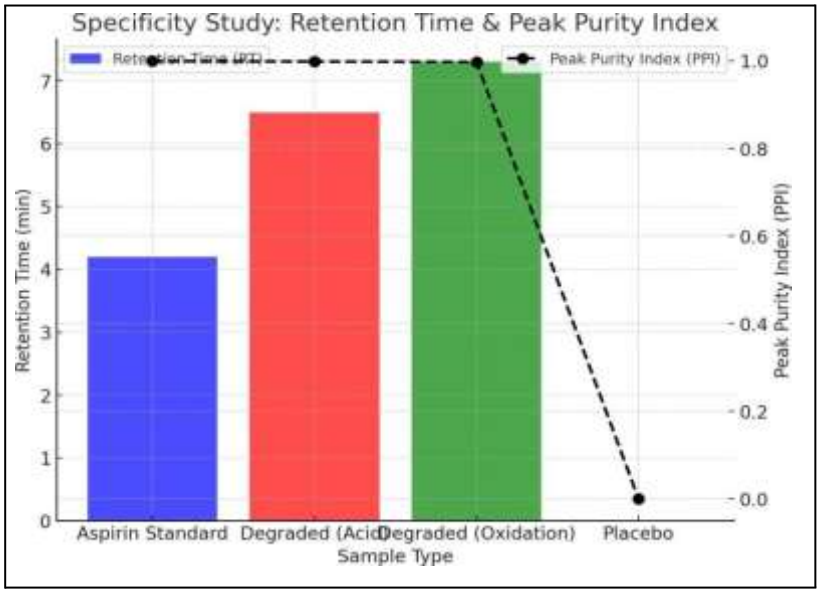
1. Specificity

Definition: The Ability to distinguish between the active pharmaceutical ingredient (API), its impurities, degradation products, and excipients.
Procedure:

- Conduct **forced degradation studies** (acid/base hydrolysis, oxidation, photolysis, thermal stress).
- Compare chromatograms of **placebo, aspirin standard, and degraded samples**.
- Ensure **no interference at the retention time (RT) of aspirin** from degradation products or excipients.

Results:

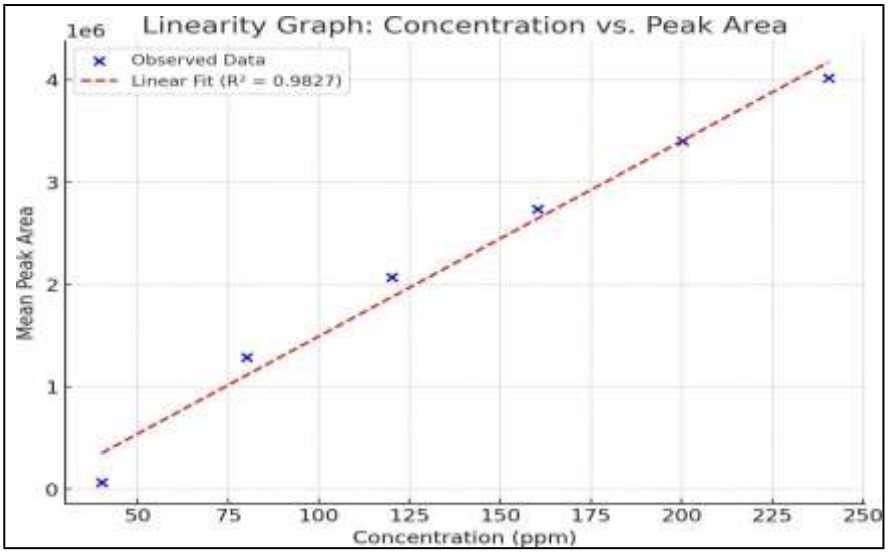
Sample	Retention Time	Peak Purity Index (PPI)	Interference Observed
Aspirin Standard	4.2 min	0.9998	No
Degraded Sample (Acid)	4.2 min, 6.5 min (Impurity 1)	0.9987	No
Degraded Sample (Oxidation)	4.2 min, 7.3 min (Impurity 2)	0.9979	No
Placebo	No peak at 4.2 min	-	No

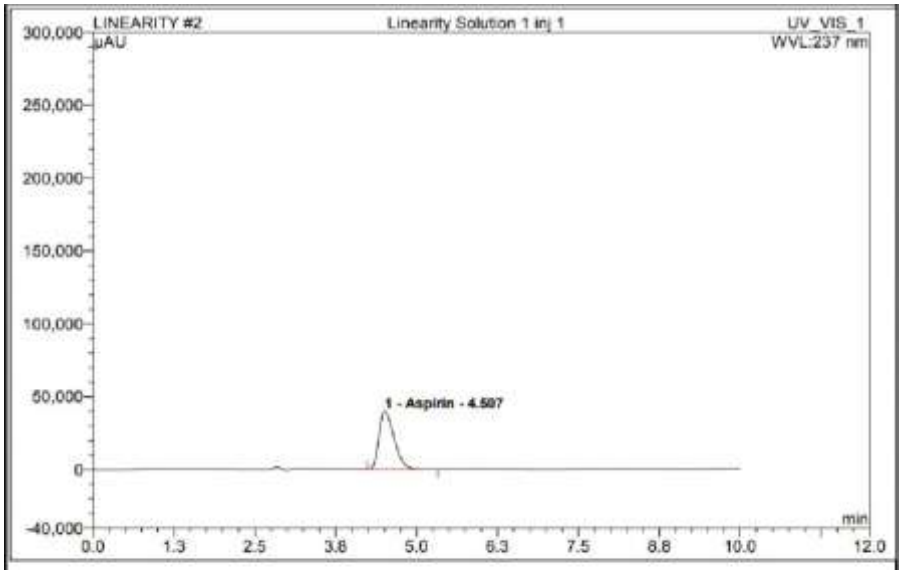


2. Linearity

It was found that Beer-Lambert's law was followed in the concentration ranges of 40-240µg/ml (40.0.8, 80.16,120.24, 160.32, 200.40,240.48 µg/ml) for aspirin API. The correlation coefficient for the calibration curve of Aspirin was found to be 0.9989. The equation of the line Aspirin is obtained as y 17018x + 29411. The linearity data is given in the table and figure below.

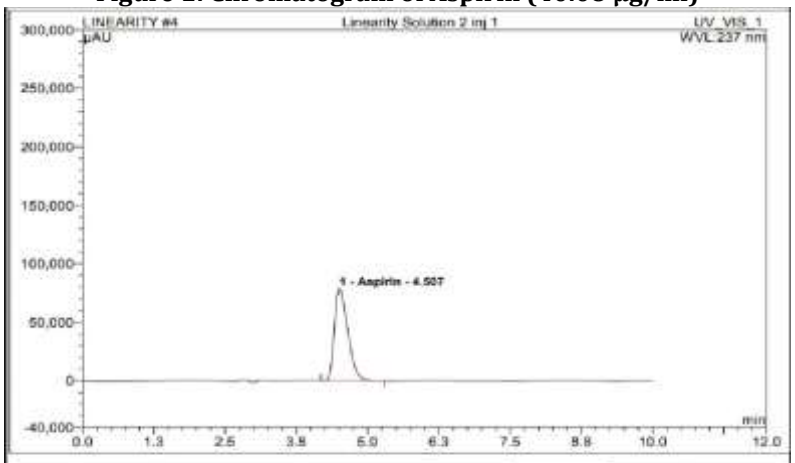
Linearity Dilution	D1	D2
Concentration (ppm)	40.08	80.16
Peak Area in Injection 1	638822	1280115
Peak Area in Injection 2	644683	1294190
Mean Peak Area	641753	1287153





Peak No.	Ret.Time min	Peak Name	Area µAU*sec	Area %	Asymmetry	Plates	Resolution
1	4.507	Aspirin	638822	100.00	1.16	8895	n.a.
Total:			638822	100.00	1.16	8895	

Figure 1: Chromatogram of Aspirin (40.08 µg/ml)



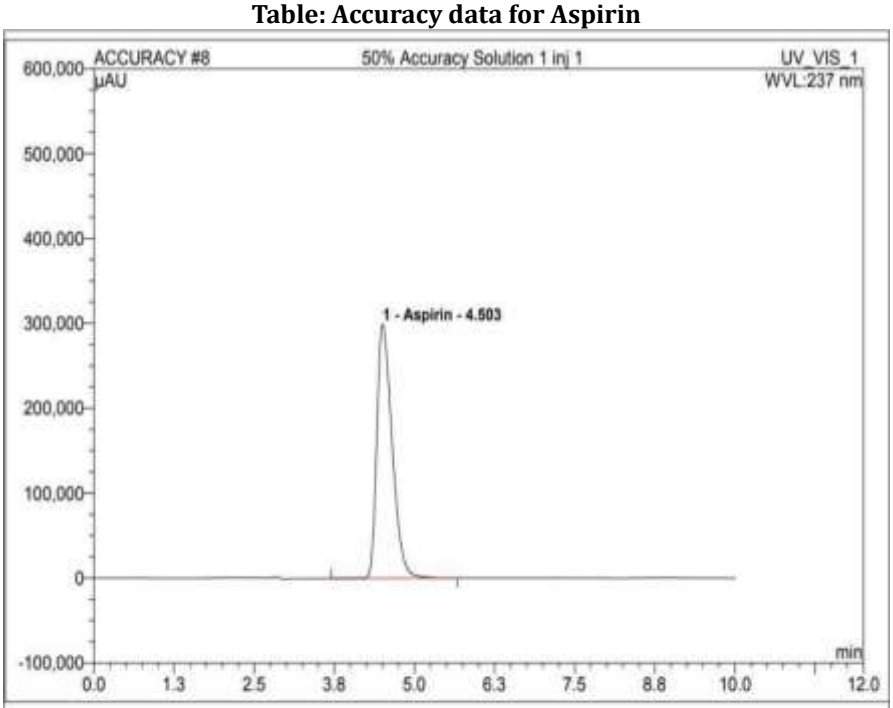
Peak No.	Ret.Time min	Peak Name	Area µAU*sec	Area %	Asymmetry	Plates	Resolution
1	4.507	Aspirin	1280115	100.00	1.17	8745	n.a.
Total:			1280115	100.00	1.17	8745	

Figure 2: Chromatogram of Aspirin (80.16µg/ml)

3. Accuracy: The results of the accuracy study for Aspirin are shown in the figures and table below. Individual recovery at each level meets the established acceptance criteria. Hence, the developed method is accurate.

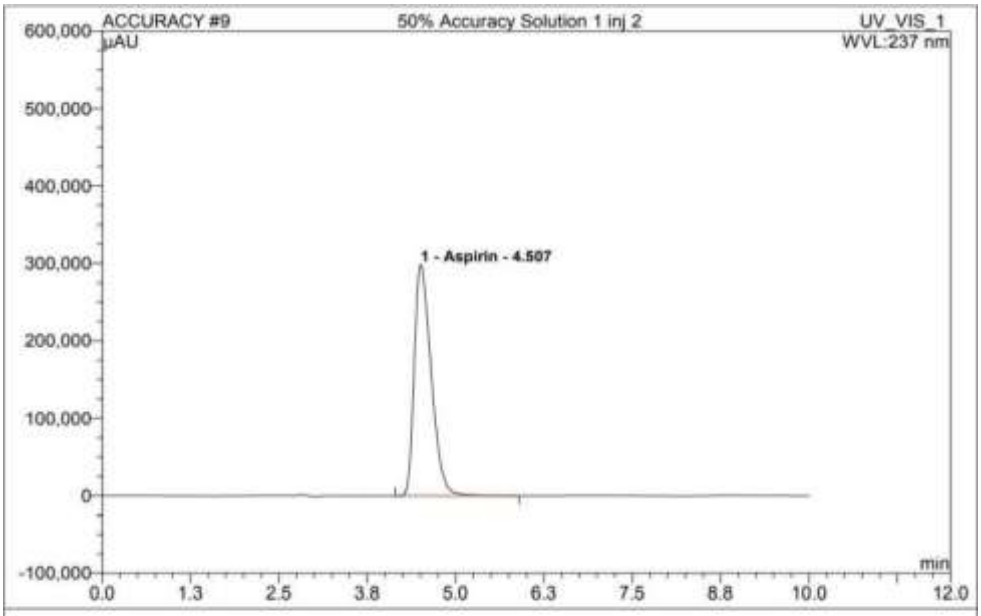
Sr. No.	Conc. Level (%)	Sample amount (µg/ml)	Amount added (µg/ml)	Peak Area	% Recovery	Average	Average of % RSD
1	50%	200	100	4977418	99.41	99.46	0.06
2		200	100	4988011	99.50		
3	100%	200	200	6681329	100.16	100.23	0.11
4		200	200	6684971	100.31		
5	150%	200	300	8389054	100.65	100.66	0.02
6		200	300	8397960	100.68		

Table: Accuracy data for Aspirin



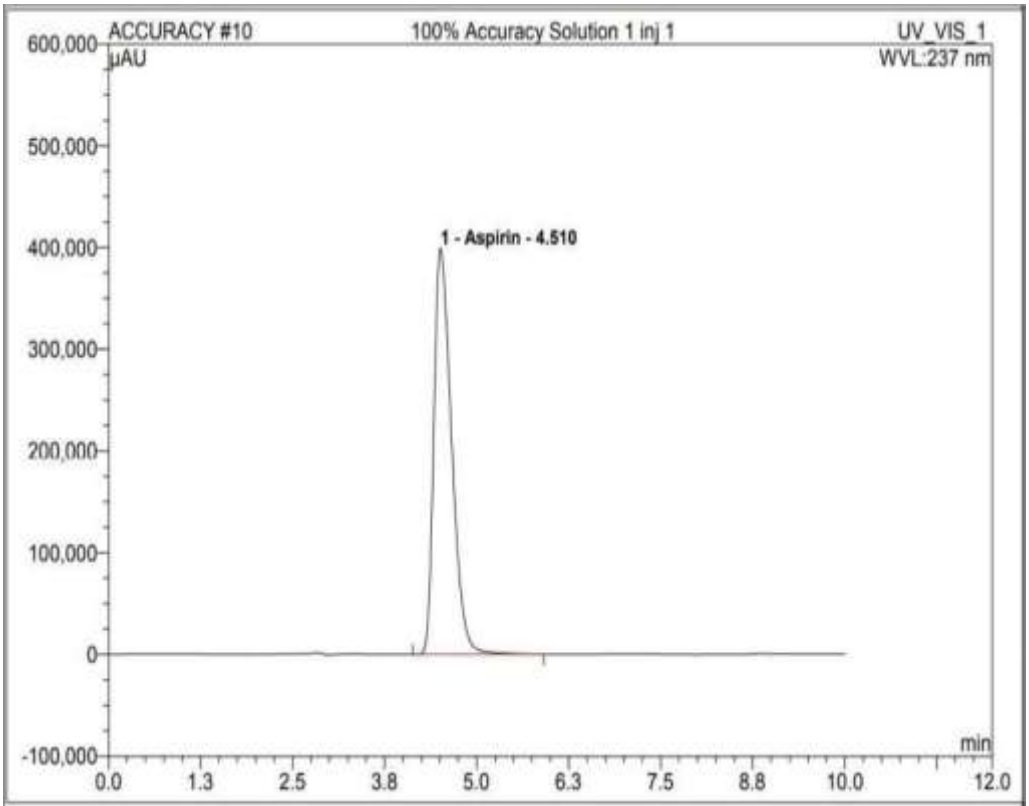
Peak No.	Ret.Time min	Peak Name	Area µAU*sec	Area %	Asymmetry	Plates	Resolution
1	4.503	Aspirin	4977418	100.00	1.18	8585	n.a.
Total:			4977418	100.00	1.18	8585	

Figure 1: Chromatogram of Aspirin for 50% accuracy



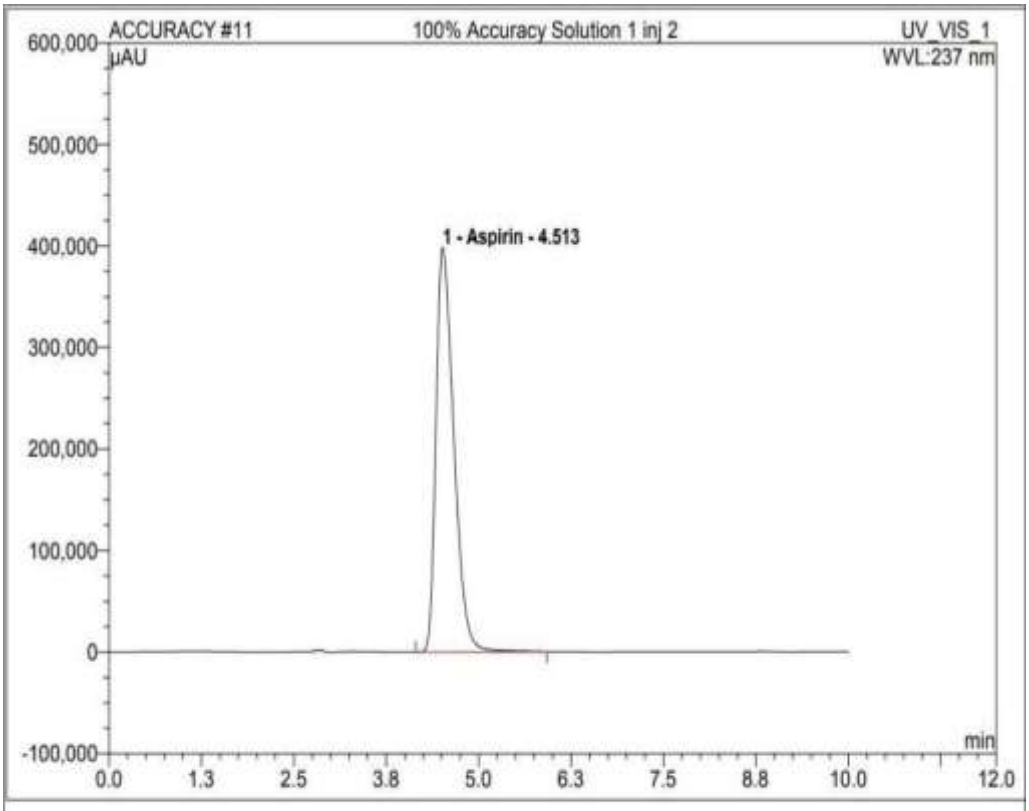
Peak No.	Ret.Time min	Peak Name	Area µAU*sec	Area %	Asymmetry	Plates	Resolution
1	4.507	Aspirin	4988011	100.00	1.17	8530	n.a.
Total:			4988011	100.00	1.17	8530	

Figure 2: Chromatogram of Aspirin for 50% accuracy



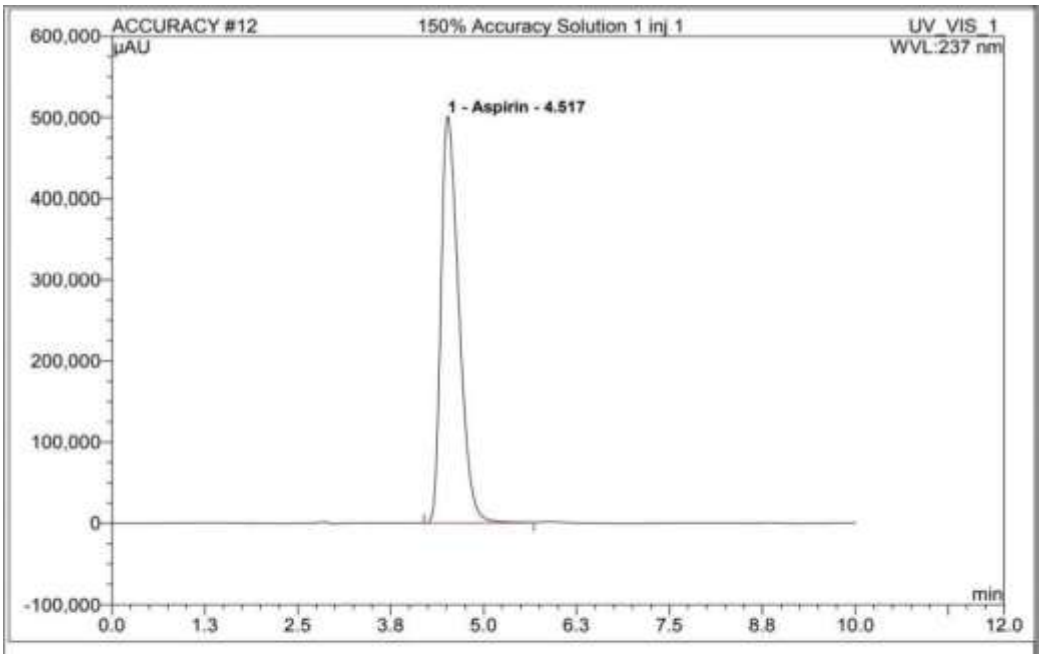
Peak No.	Ret.Time min	Peak Name	Area µAU*sec	Area %	Asymmetry	Plates	Resolution
1	4.510	Aspirin	6681329	100.00	1.18	8495	n.a.
Total:			6681329	100.00	1.18	8495	

Figure 3: Chromatogram of Aspirin for 100% accuracy



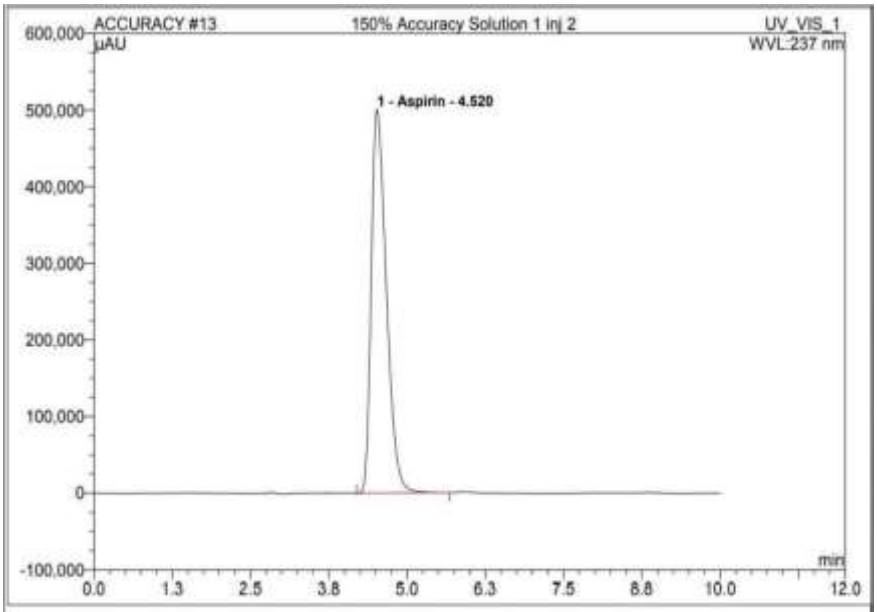
Peak No.	Ret.Time min	Peak Name	Area µAU*sec	Area %	Asymmetry	Plates	Resolution
1	4.513	Aspirin	6684971	100.00	1.19	8490	n.a.
Total:			6684971	100.00	1.19	8490	

Figure 4: Chromatogram of Aspirin for 100% accuracy



Peak No.	Ret.Time min	Peak Name	Area µAU*sec	Area %	Asymmetry	Plates	Resolution
1	4.517	Aspirin	8389054	100.00	1.18	8445	n.a.
Total:			8389054	100.00	1.18	8445	

Figure 5: Chromatogram of Aspirin for 150% accuracy



Peak No.	Ret.Time min	Peak Name	Area µAU*sec	Area %	Asymmetry	Plates	Resolution
1	4.520	Aspirin	8397960	100.00	1.17	8425	n.a.
Total:			8397960	100.00	1.17	8425	

Figure 6: Chromatogram of Aspirin for 150% accuracy

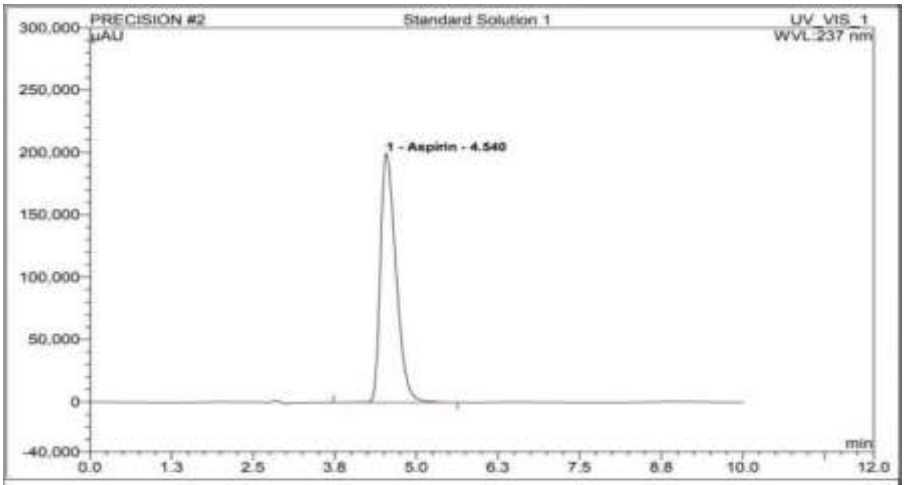
Discussion: The accuracy of all the obtained results was observed to be satisfactory. The % mean accuracy lies between 90-110%. Hence, a good accuracy was observed by this analytical method.

4. Precision:

Method precision and Intermediate precision were carried out in this study.

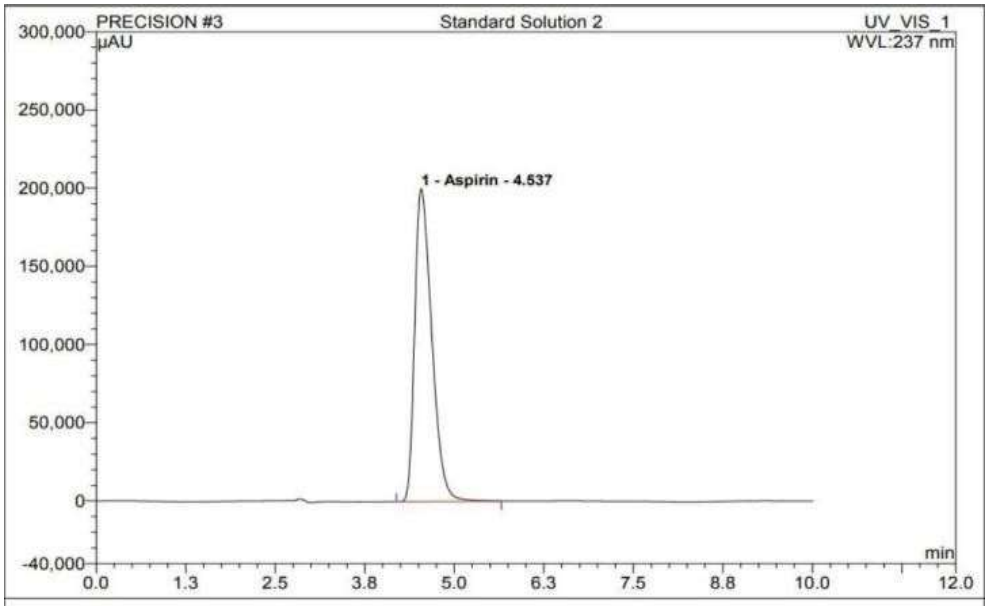
(a) Method precision

Serial No.	Peak Area
1	3323909
2	3314551
3	3343074
Mean	3327178
SD	14539.78
%RSD	0.437%



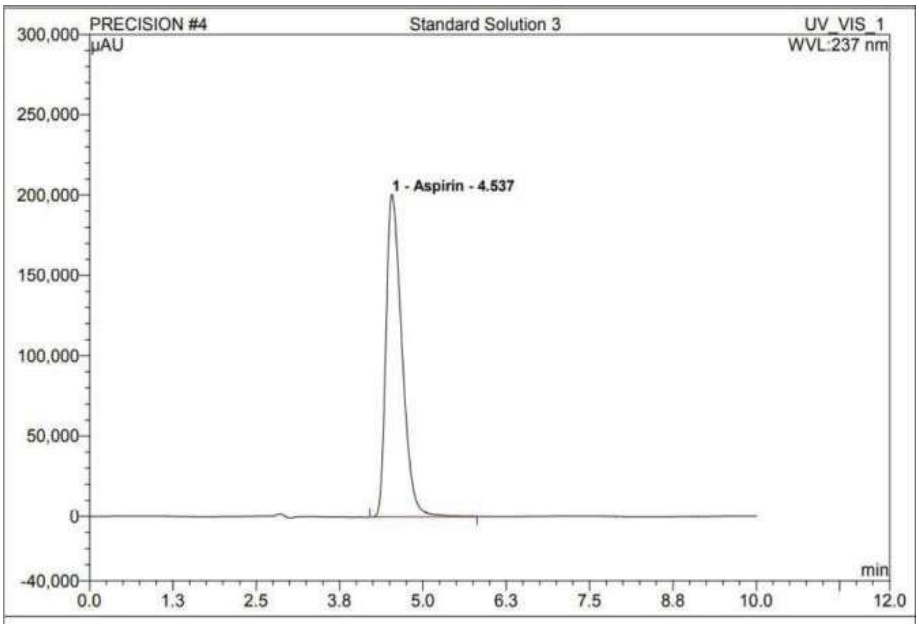
Peak No.	Ret.Time min	Peak Name	Area µAU*sec	Area %	Asymmetry	Plates	Resolution
1	4.540	Aspirin	3323909	100.00	1.17	8710	n.a.
Total:			3323909	100.00	1.17	8710	

Figure: Method Precision Chromatogram 1



Peak No.	Ret.Time min	Peak Name	Area µAU*sec	Area %	Asymmetry	Plates	Resolution
1	4.537	Aspirin	3314551	100.00	1.18	8745	n.a.
Total:			3314551	100.00	1.18	8745	

Figure: Method Precision Chromatogram 2

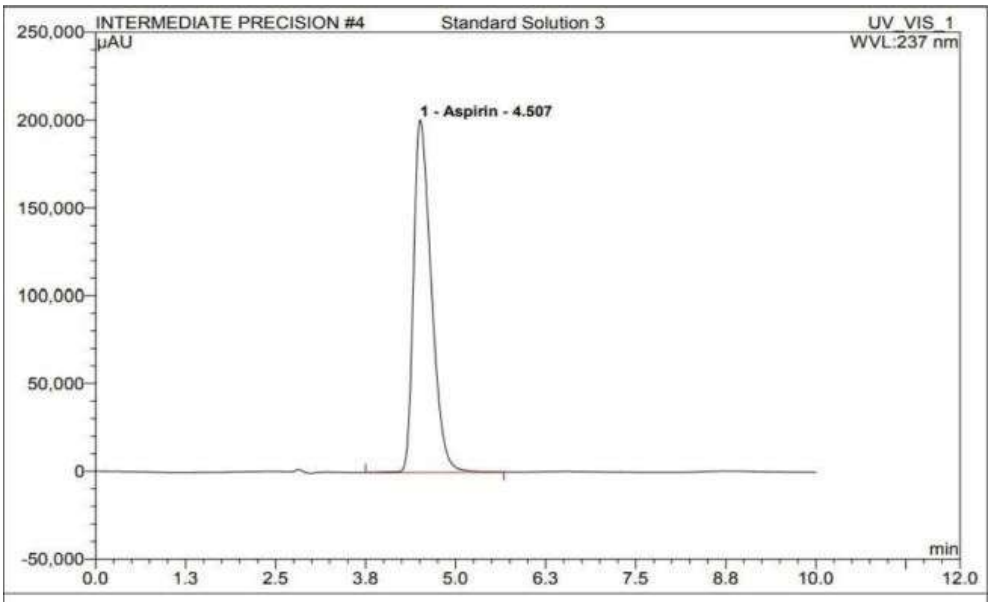


Peak No.	Ret.Time min	Peak Name	Area µAU*sec	Area %	Asymmetry	Plates	Resolution
1	4.537	Aspirin	3343074	100.00	1.18	8745	n.a.
Total:			3343074	100.00	1.18	8745	

Figure: Method Precision Chromatogram 3

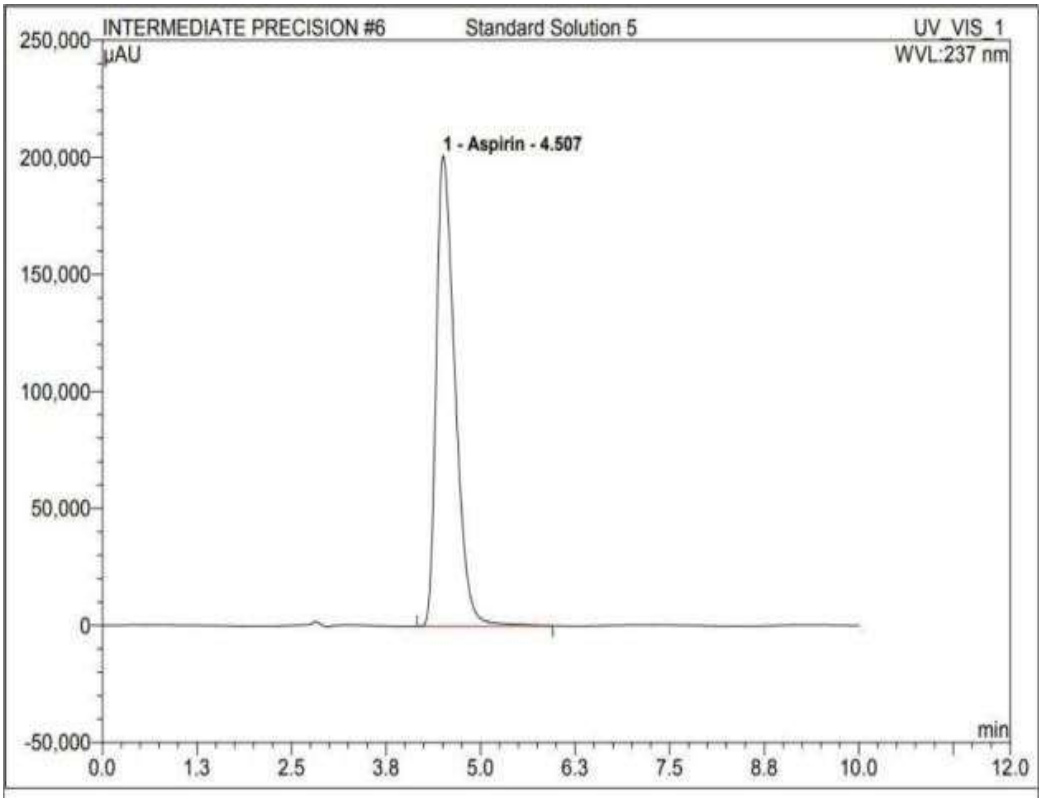
(b) Intermediate Precision

Sreial No.	Peak Area
1	3406464
2	3427010
3	3444999
Mean	3426157
SD	19,281.63
%RSD	0.56%



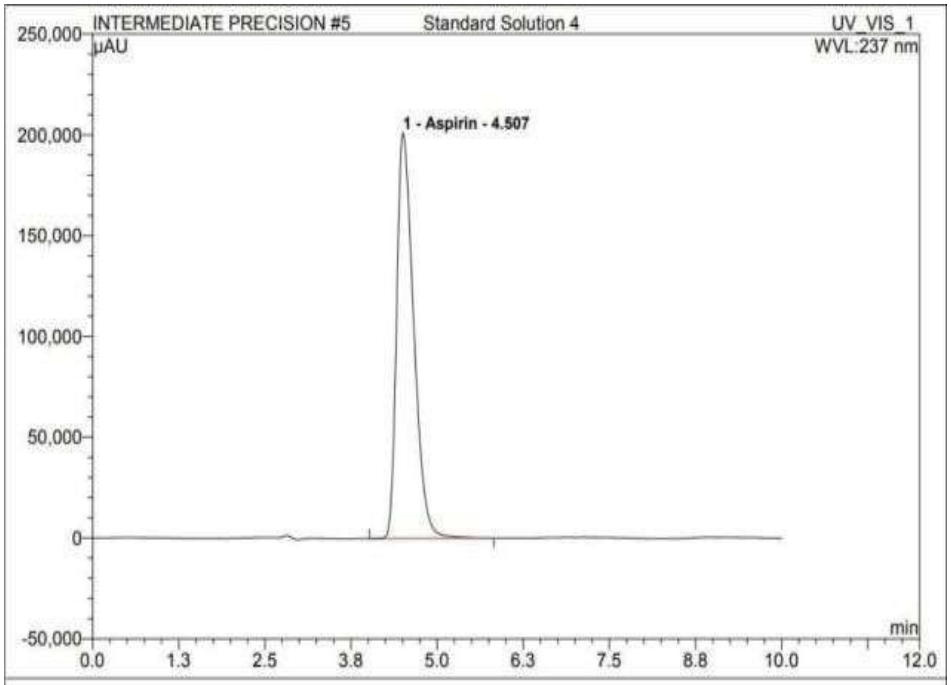
Peak No.	Ret.Time min	Peak Name	Area µAU*sec	Area %	Asymmetry	Plates	Resolution
1	4.507	Aspirin	3444999	100.00	1.18	8090	n.a.
Total:			3444999	100.00	1.18	8090	

Figure: Intermediate Precision Chromatogram of Aspirin 1



Peak No.	Ret.Time min	Peak Name	Area µAU*sec	Area %	Asymmetry	Plates	Resolution
1	4.507	Aspirin	3469731	100.00	1.19	8115	n.a.
Total:			3469731	100.00	1.19	8115	

Figure: Intermediate Precision Chromatogram of Aspirin 2



Peak No.	Ret.Time min	Peak Name	Area µAU*sec	Area %	Asymmetry	Plates	Resolution
1	4.507	Aspirin	3451916	100.00	1.17	8115	n.a.
Total:			3451916	100.00	1.17	8115	

Figure: Intermediate Precision Chromatogram of Aspirin 3

Discussion: It was found that the precision and intermediate precision results were satisfactory concerning %RSD for all levels, which were within the limit. The developed method was precise for the determination of Aspirin.

5. LOD and LOQ

LOD and LOQ were calculated as follows:

a) LOD

LOD = 3.3

LOD=3.3×SDσLOD =3.3 \times \frac{SD}{\sigma}LOD=3.3×σSD
LOD=3.3×17512.93829411LOD = 3.3 \times \frac{17512.938}{29411}LOD=3.3×2941117512.938 LOD=1.78 µg/mlLOD = 1.78 \, \mu g/mlLOD=1.78µg/ml

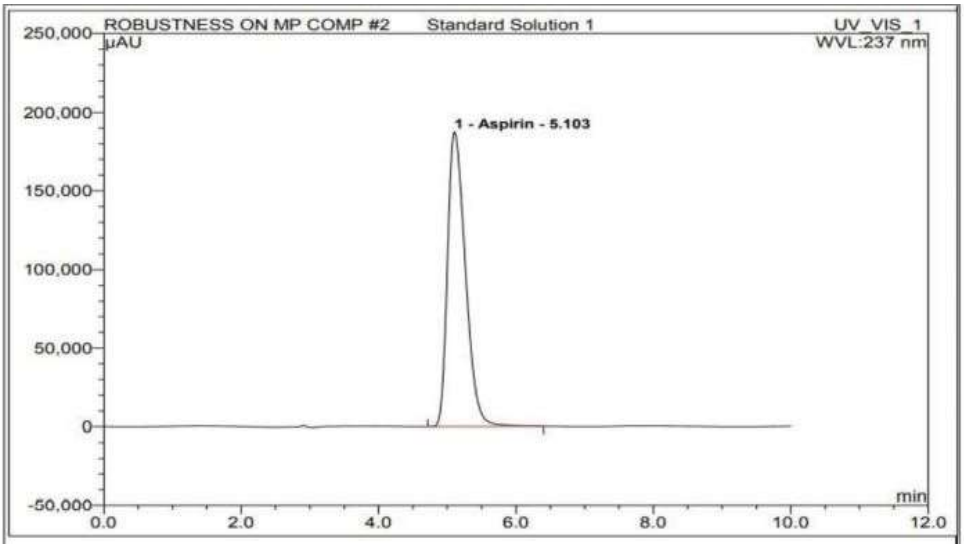
ii. LOQ

LOQ=10×SDσLOQ = 10 \times \frac{SD}{\sigma}LOQ=10×σSD
LOQ=10×17512.93829411LOQ = 10 \times \frac{17512.938}{29411}LOQ=10×2941117512.938 LOQ=5.95 µg/mlLOQ = 5.95 \, \mu g/mlLOQ=5.95µg/m

6. Robustness

Robustness is the measure of a method that remains unaffected by small, deliberate changes in method parameters like pH and detection wavelength on the assay of the analyte of interest. Here, the mobile phase composition varied ± 5, and the flow rate was varied ± 0.1 ml.

Mobile phase composition 64:35:1 v/v/v



Peak No.	Ret.Time min	Peak Name	Area µAU*sec	Area %	Asymmetry	Plates	Resolution
1	5.103	Aspirin	3396564	100.00	1.14	9215	n.a.
Total:			3396564	100.00	1.14	9215	

Figure: Typical chromatogram of Aspirin

Impurity Profiling by HRMS.

In the forced degradation and radiation studies, some degradant products were found, and the percentage of degradation was calculated by HPLC. Following are some mass fragmentation patterns in which impurity was found.

A. Forced Degradation Study: A major percentage degradation of aspirin was found in the HPLC chromatogram in acidic conditions, so the mass fragmentation pattern of acidic conditions is as follows.

1. Aspirin Standard Mass Fragmentation Pattern

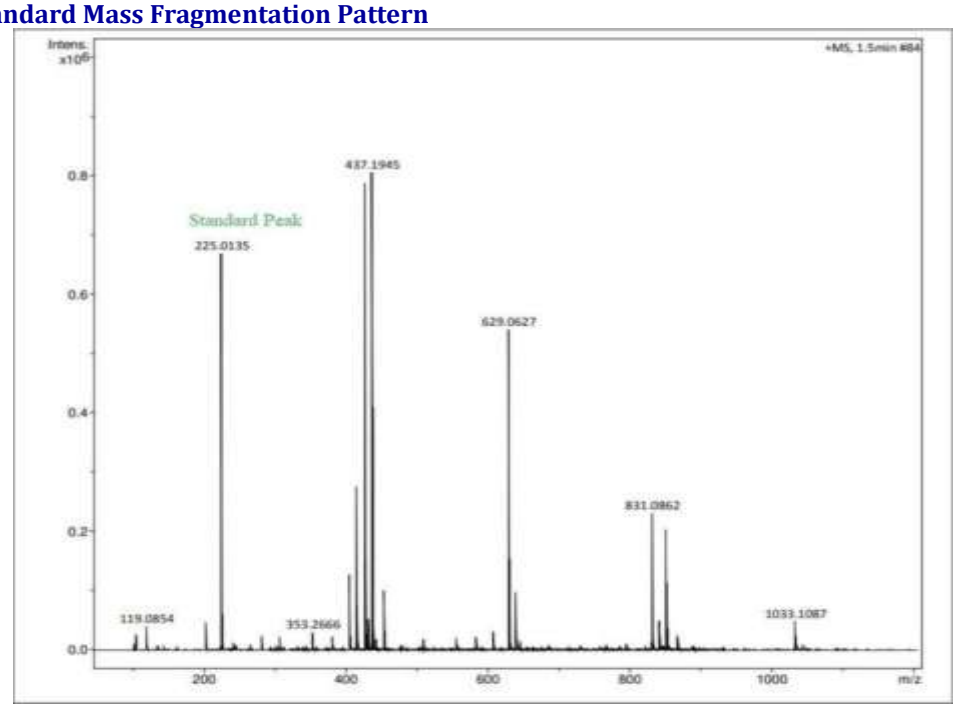


Figure: Aspirin Standard Fragmentation Pattern

2. Acidic Condition: Three fragments are found other standard and bane as impurities 1,2, and 3.1

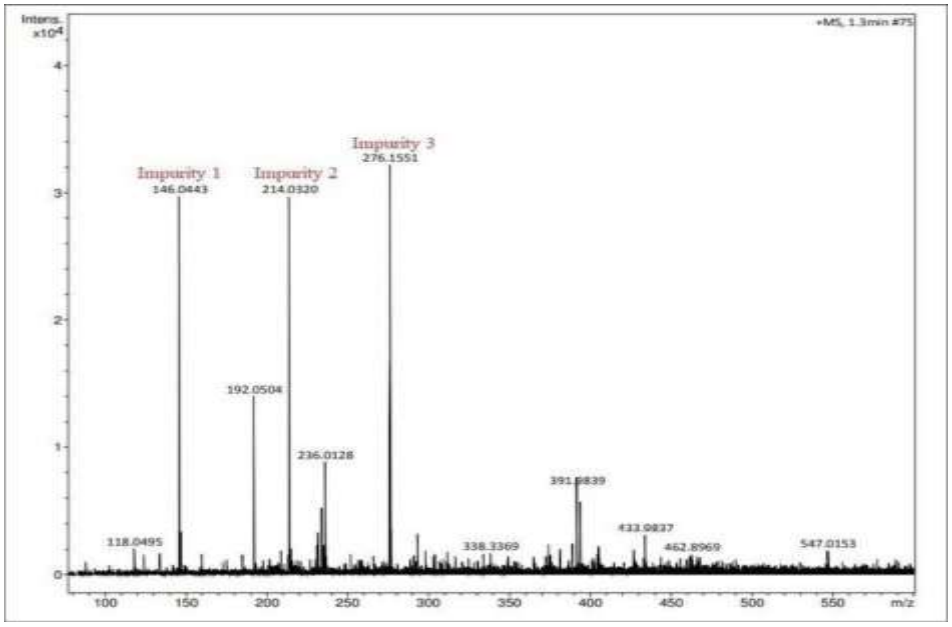


Figure: Mass fragmentation pattern of Aspirin in acidic condition

B. Irradiation Study: Among all the radiation sources like thermal neutron, fast neutron, and gamma-ray radiation, percentage degradation was found in various radiation sources in API and tablet dosage form, and mass fragmentation patterns with observed impurities are mentioned as follows.

1. Thermal Neutron API Low Dose

In Thermal Neutron API low dose, three impurities are found i.e., impurities 4, 5, and 6 at m/z ratio 274.27, 318.30, and 365.063

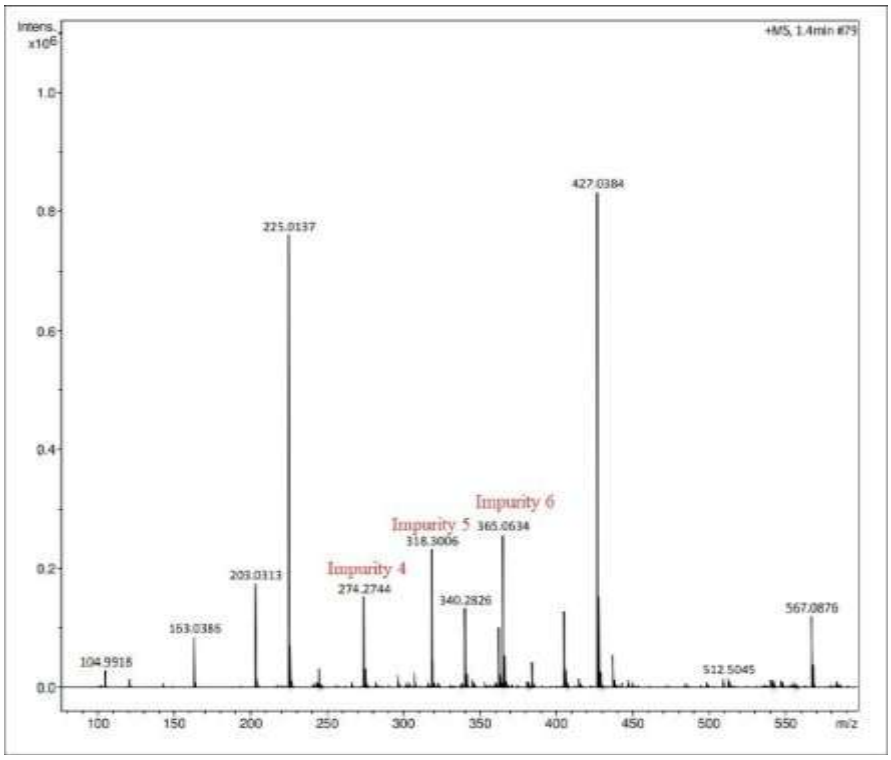


Figure: Mass fragmentation Pattern of Aspirin in Thermal Neutron API Low Dose

2. Fast Neutron API High dose

In fast neutron API high dose, two impurities, Impurity 4 and 5, are found, and a new impurity, Impurity 7, was found at an m/z ratio of 548.16

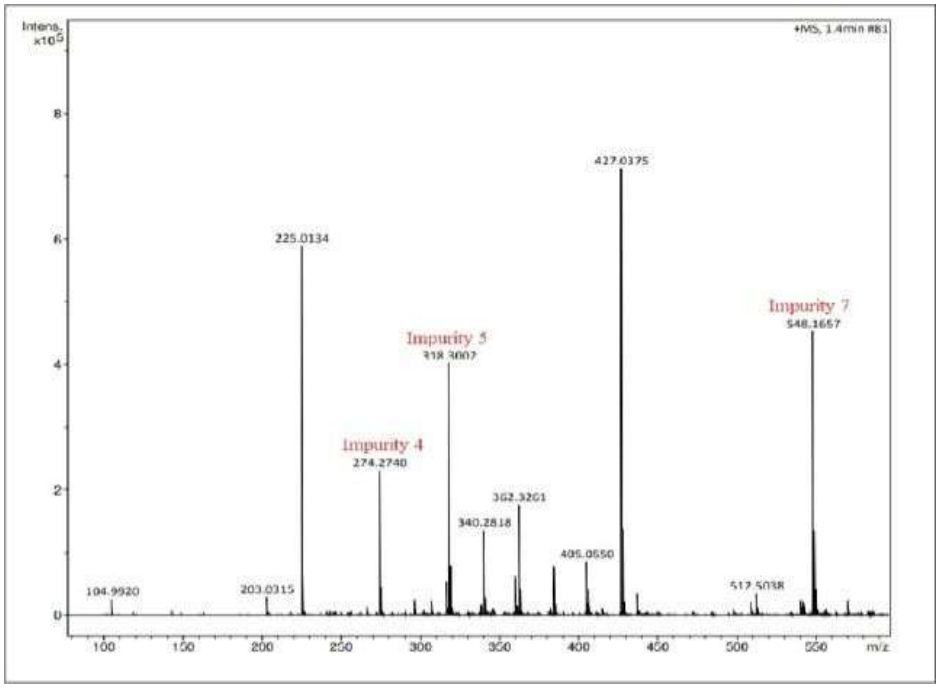


Figure: Mass fragmentation Pattern of Aspirin in Fast Neutron API High dose.

3. Fast Neutron API Low Dose:

In fast neutron API low dose, two impurities were found, i.e., impurities 4 and 5 at m/z ratios 274.27 and 318.30.

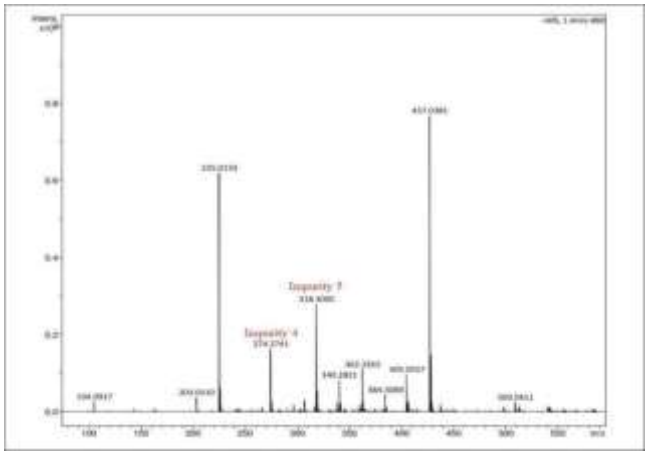


Figure: Mass fragmentation Pattern of Aspirin in Fast Neutron API Low dose.

4. Fast Neutron Tablet Low dose:

In the fast neutron tablet low dose, 3 impurities were found, i.e., impurities 4, 5, and 7 at m/z ratios 274.27, 318.30, and 548.16

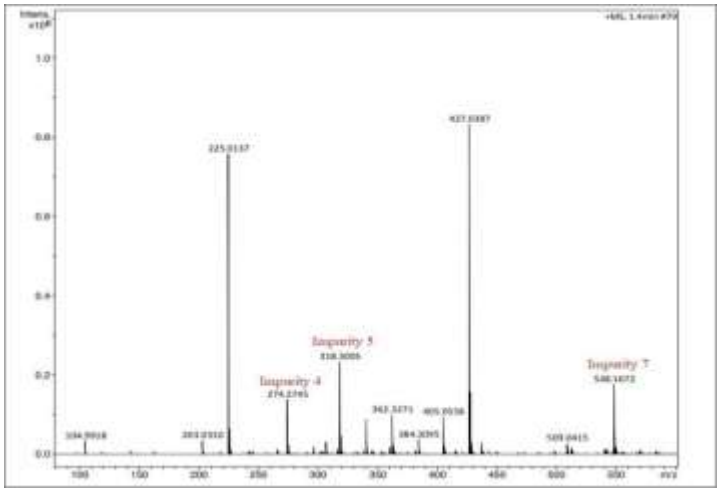


Figure: Mass fragmentation Pattern of Aspirin in Fast Neutron Tablet Low dose.

5. Gamma-ray Tablet Low-dose:

In the low-dose gamma-ray tablet, a new impurity was found at m/z ratio 851.39 called impurity 8.

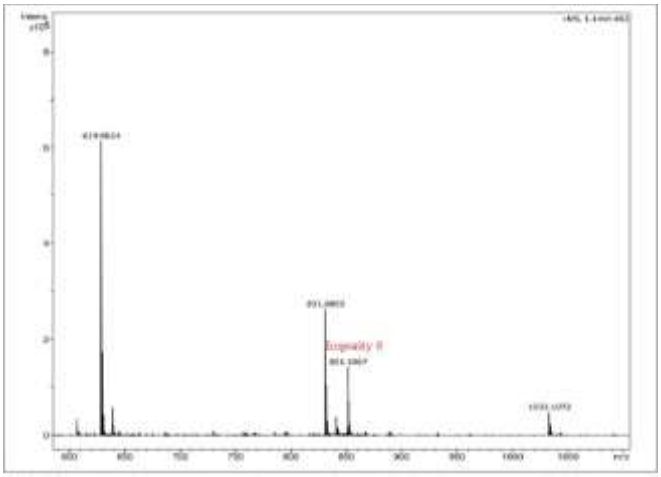


Figure: Mass fragmentation Pattern of Aspirin in Gamma-ray Tablet Low dose.

Effects of Ionizing Radiation on Aspirin and Pharmaceuticals

Ionizing radiation can significantly impact pharmaceuticals, leading to degradation, impurity formation, and reduced efficacy. Below is a detailed breakdown of its effects, specifically focusing on aspirin.

1. Radiation Impact on Pharmaceuticals

1.1 How Ionizing Radiation Causes Degradation or Impurity Formation

Ionizing radiation, including **gamma rays, X-rays, and neutron radiation**, can cause structural and chemical changes in pharmaceutical compounds. The key mechanisms include:

1. **Radiolysis**

○ The interaction of high-energy radiation with pharmaceutical molecules leads to **bond breakage** and **radical formation**.

○ For aspirin (acetylsalicylic acid), radiolysis can cause **hydrolysis of the ester bond**, leading to degradation into **salicylic acid and acetic acid**.

2. **Oxidation Reactions**

○ Ionizing radiation generates **reactive oxygen species (ROS)** such as hydroxyl radicals (•OH), superoxide radicals ($O_2•^-$), and hydrogen peroxide (H_2O_2).

○ These radicals can oxidize aspirin, forming new **degradation products**.

3. **Cross-Linking and Polymerization**

○ In some cases, ionizing radiation can induce the **polymerization** of pharmaceutical compounds, altering their **solubility and bioavailability**.

4. **Structural Modifications**

○ Radiation exposure can break down aromatic rings, **alter molecular weight**, or **introduce new functional groups** into the drug.

5. **Formation of New Impurities**

○ The degradation of aspirin due to radiation can lead to the formation of new, potentially **toxic impurities**.

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