International Journal of Education and Science Research Review
Volume-11, Issue-2 March-April-2024 E-ISSN 2348-6457
(Peer-Reviewed, Open Access, Fully Refereed International Journal) P-ISSN 2349-1817
www.ijesrr.org Impact Factor - 7.02 Email- editor@ijesrr.org

ANALYTICAL METHOD VALIDATION OF RP-HPLC FOR THE DETERMINATION OF A MUTUAL PRODRUG OF PROPYPHENAZONE AND FLURBIPROFEN

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ABSTRACT

The present investigation focuses on the development of a liquid chromatography method for estimating the mutual prodrug of Propyphenazone and Flurbiprofen. An RP-HPLC method was established using a C18 column (Shimadzu Shim-pack Gist, 250 × 4.6 mm, 5 µm) as the stationary phase, with Acetonitrile: Water (90:10) as the mobile phase. The flow rate was maintained at 1 mL/min, and detection was carried out using a UV detector at 245 nm. The retention time for the mutual prodrug was recorded at 4.5 minutes. Linear regression analysis of the calibration curve demonstrated a strong linear correlation within the concentration range of 10–50 µg/mL. The method was validated for linearity, accuracy, precision, and robustness. This developed method is suitable for routine quality control assessment of the mutual prodrug in bulk.

Keywords: Flurbiprofen, Validation, RP-HPLC, Prodrug, NSAIDs, Propyphenazone

1. INTRODUCTION:

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely prescribed for managing pain, fever, and inflammation. However, their therapeutic use is often restricted due to a high incidence of gastrointestinal side effects, including stomach ulcers, perforation, bleeding, and other complications such as cardiovascular, hepatic, and renal toxicity. In severe cases, these adverse effects can be life-threatening. Prostaglandins play a crucial role as physiological and pathological mediators in inflammation, pain, fever, cancer, and neurological disorders. Their secretion occurs through the COX pathway, where phospholipase A2 releases free arachidonic acid from membrane-bound arachidonate. This pathway consists of two known COX isoforms: COX-1 and COX-2. COX-1 and COX-2 convert arachidonic acid into prostaglandin G2, which is then reduced by peroxidase to form PGH2. This PGH2 serves as a precursor for thromboxane A2 and various prostaglandins, including PGD2, PGE2, and PGF2. The prostanoids synthesized via COX-1 are essential for physiological functions such as maintaining renal blood flow, protecting the gastric mucosa, and facilitating platelet aggregation. COX-1 is present in most tissues, whereas COX-2 is primarily expressed in the kidneys, brain, and spinal cord. Under normal conditions, COX-2 expression remains limited; however, it is highly inducible in response to inflammatory stimuli, including endotoxins, cytokines, hormones, and tumor promoters.

The inhibition of cyclooxygenase (COX) enzymes plays a crucial role in reducing prostaglandin synthesis, which, in turn, alleviates inflammation, pain, and fever. However, the suppression of prostaglandin production is associated with several adverse effects, including gastrointestinal (GI) discomfort, cardiovascular complications, renal toxicity, fluid retention, and exacerbation of hypertension. Nonsteroidal anti-inflammatory drugs (NSAIDs) exert their effects through dual inhibition of COX enzymes, specifically COX-1 and COX-2.

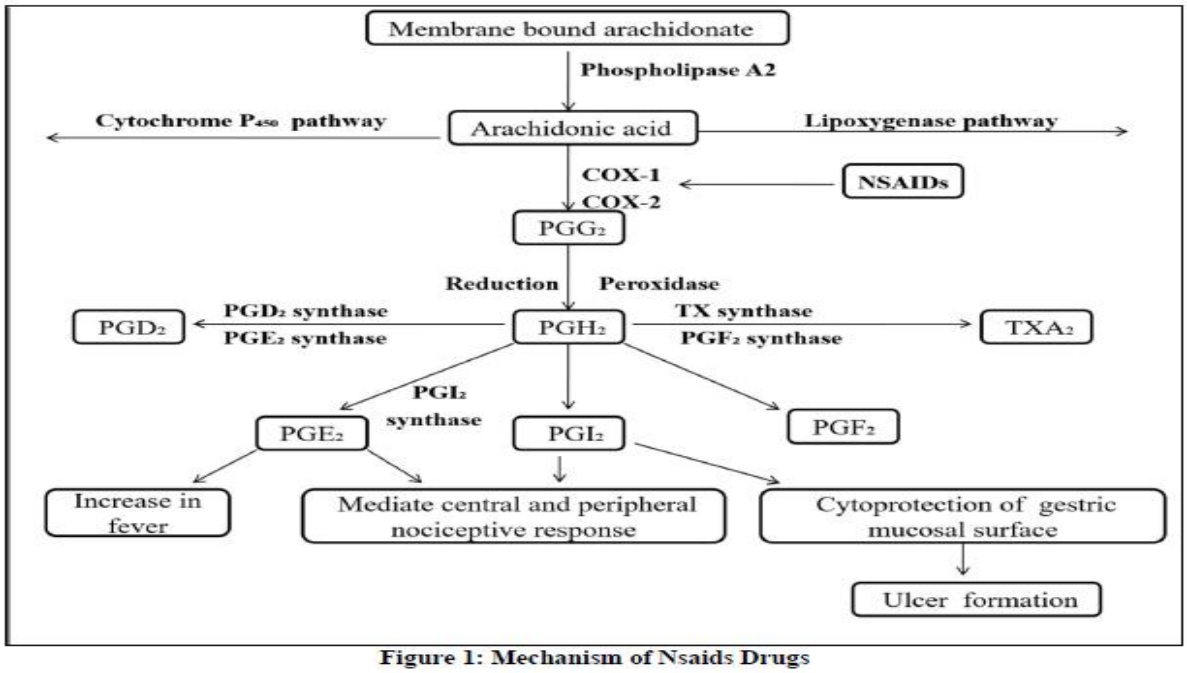

Figure 1: Mechanism of Nsaids Drugs

While COX-2 inhibition is primarily responsible for the anti-inflammatory and analgesic properties of NSAIDs, the inhibition of COX-1 contributes to GI mucosal damage, leading to complications such as ulceration, upper gastrointestinal perforation, and hemorrhage.NSAID-induced GI toxicity occurs through two primary mechanisms. The first involves direct contact between NSAIDs and the gastric mucosa, leading to localized suppression of prostaglandin synthesis in the GI tract. Additionally, the presence of the carboxyl functional group in NSAIDs contributes to local irritation and mucosal damage. While COX-2 selective inhibitors were developed to mitigate GI-related adverse effects, their use has been linked to other complications, including elevated serum potassium levels and potential hepatotoxicity.Recent studies have highlighted the role of reactive oxygen species (ROS) in the development of gastric mucosal injuries associated with NSAID therapy. The excessive generation of ROS exacerbates oxidative stress, further contributing to NSAID-induced ulcerogenicity. To overcome these challenges, the concept of mutual prodrugs has gained attention as a strategy to enhance the safety and efficacy of NSAIDs. Mutual prodrugs are designed to improve the physicochemical and pharmacological properties of parent drugs by modifying their structure to minimize direct exposure to the gastric mucosa. These prodrugs undergo enzymatic or chemical conversion at the target site, releasing the active drug in a controlled manner. Various NSAID derivatives, including ester and amide prodrugs, have been synthesized to improve solubility, bioavailability, and gastrointestinal safety. By preventing the direct interaction of the parent drug with the gastric mucosa, these prodrugs offer enhanced therapeutic benefits while reducing adverse effects. The present study is focused on the development and validation of a robust and reliable analytical method for the quantification of a mutual prodrug of Propyphenazone and Flurbiprofen. A comprehensive literature review revealed that no liquid chromatographic method has been previously reported for the estimation of this specific mutual prodrug. Therefore, this study aims to bridge this gap by establishing and validating a novel reverse-phase

high-performance liquid chromatography (RP-HPLC) method to accurately quantify the prodrug in bulk and pharmaceutical formulations.

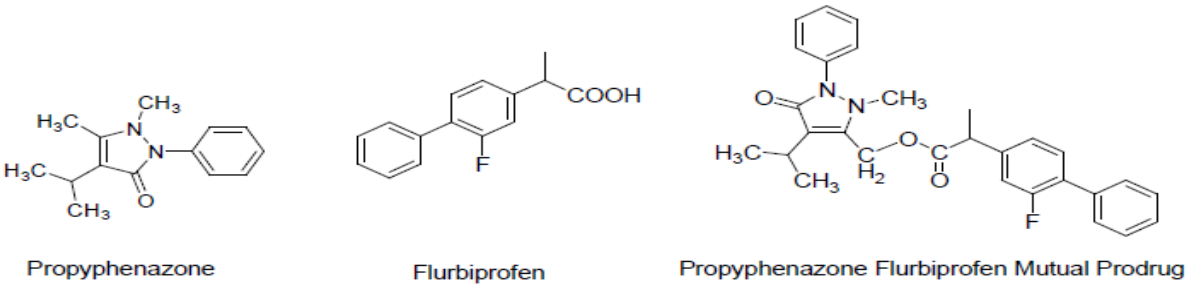

Figure 2: Chemical Structure of Drugs and Prodrug

2. MATERIALS AND METHODS

Instruments

The separation was carried out by RP-HPLC instrument (Shimadzu) Having a UV Detector. The Lab solutions Software used for data processing and as a stationary phase Shimadzu C18 Shim-pack Gist (250 × 4.6 mm, 5µm) column was used in the study. Other Instruments used were vacuum pump filtration assembly (Rocker 300), Sonicator water bath (Janki Impex), UV–Visible spectrophotometer 1900 (Shimadzu Japan), analytical weighing balance (Mettler Toledo) and pH meter. Chemicals and reagents The mutual prodrug of Propyphenazone and flurbiprofen was synthesized in the lab. And the structure of synthesized prodrug was confirmed by IR, Mass and NMR spectra. Methanol, Acetonitrile (ACN) and water of HPLC Grade were purchased S.D. Fine Chem Mumbai, India. Selection of detection wavelength 10 µg/mL solution of Prodrug was scanned under a UV spectrophotometer between the range 200nm to 400nm, and the spectra were obtained. At 245 nm good absorption was observed for Prodrug, so 245 nm was selected as the wavelength for the detection of Prodrug. The UV spectra of prodrug was shown in Figure 3.

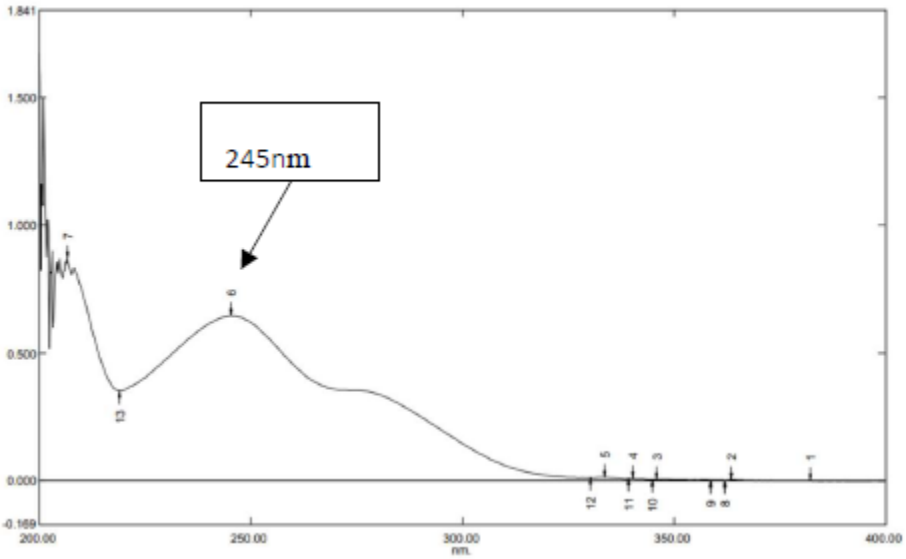

Figure 3: UV Spectrum of Prodrug

Chromatographic conditions

The separation was carried out by using C18 column and mobile phase Acetonitrile: Water (90:10) was used in the chromatographic study. The LC system was equilibrated with the mobile phase before starting the analysis. The flow rate was kept at 1 mL/min and eluent were monitored with UV detector at 245 nm. Total run time was kept at 10 min. Preparation of standard stock solutions (1000 µg/mL) and working standard solutions (100 µg/mL) Accurately 10 mg of Prodrug was weighed and transferred to 10 mL volumetric flasks. Then the drug was dissolved in few ml of solvent. The final Volume was made up to the mark by using methanol to prepared the standard stock solution of 1000 µg/Ml solution. Then again pipette out 1 mL of above solution in 10 mL volumetric flask and diluted to the mark with methanol to get working standard solution of 100 µg/ml of Prodrug.

Validation

Validation of the developed method was carried out according to the ICH Q2 (R2) guideline.

Linearity and range

Calibration curve was prepared by taking appropriate aliquot of working standard solution in different 10 mL volumetric flasks. The volume was made up to 10 mL using mobile phase as a solvent to obtain final concentration of 10-50 µg/mL for Prodrug.

The calibration curve was plotted using mean peak area versus concentration. The regression equation was computed and correlation coefficient was determined for Prodrug.

Precision

Precision was determined in terms of Repetability, intra-day and inter-day precisions. Intra-day precision was determined by analyzing sample solutions of mixture (20, 30 and 40 µg/mL) at three different concentration levels covering entire range of the calibration curve three times on the same day (n = 3). Inter-day precision was determined by analyzing sample solutions of mixture (20, 30 and 40 µg/mL) at three different concentration levels covering entire range of concentration over a period of 3 successive days (n = 3). Then from the peak areas calculate the mean, SD and relative standard deviation (% RSD) values.

Repeatability of measurement of peak area ware determined by analyzing middle concentration of 30 µg/mL for six times.

Accuracy

The accuracy of the method was determined by calculating % recovery of Prodrug by using spiking method. Known amounts of (5, 10, 15 µg/mL) standard stock solutions were added to sample. The resulting solution was analyzed by using HPLC system. The recover amount of Prodrug was calculated by using the equations of the calibration curves.

LOD and LOQ

The LOD and LOQ was calculated by using the equation given in ICH Q2 (R2) guideline.

Robustness

Flow rate, Wavelength and composition of mobile phase were changed upto ± 2% and the effects on the results were analyzed. Middle three concentration level of 20,30 and 40 µg/mL of Prodrug was used for robustness. The % RSD was calculated.

Specificity

The specificity of method was ascertained by analyzing Prodrug in presence of excipient like Starch, talc and magnesium stearate. Interference due to excipients were noted.

Solution stability

Solution stability was determined by storing the stock solution (100 µg/ml) of Prodrug at room temperature for 24 hr and analyzed at different interval of 0, 4, 8 and 24 hr.

Procedure for assay of synthetic mixture

The synthetic mixture was prepared by mixing Prodrug with common excipients used for the tablet formulation. To determine drug content 10 mg equivalent powder content taken and dissolve in methanol. Final test solution (10 µg/mL) was prepared using diluent.

3. RESULTS AND DISCUSSION

Method development

Optimization of the Mobile Phase

Different proportions of solvent like Buffers of Different pH and concentration, methanol, ACN and water were tried in different ratio but the satisfactory result was not achieved. A mobile phase composition consisting of ACN: Water (90:10 %, v/v), gave satisfactory retention time of 4.59 min for Prodrug. Moreover, it gave appropriate peak shape with satisfactory tailing factor and theoretical plate. Hence this mobile phase was selected as an optimized mobile phase. The chromatogram of Prodrug has been shown in Figure 5. Selection of detection wavelength From the UV spectrum of Prodrug in Methanol, the absorbance maxima were observed at 245 nm, so 245 nm was selected as the wavelength for the detection of Prodrug.

Validation

Specificity

The specificity was determined by comparing chromatograms of blank, Placebo and synthetic mixture which was shown in Figure 5. From this comparison of chromatograms, it is observed that all Prodrug was clearly detected, with no any interference from the sample matrix or blank. System suitability The system suitability of method was confirmed by calculating various chromatographic parameters such as Retention time, number of theoretical plates (N), Resolution (Rs) and tailing factor (Tf) from the chromatogram of standard solutions. System suitability parameters confirmed that the given chromatographic conditions were good for the method.

Linearity and range

The calibration curve was prepared by plotting the graph of concentration of Prodrug verses peak area. Standard solutions of prodrug of 10-50 µg mL-1 were prepared and 20 µL was injected into the HPLC column. The linearity was evaluated by linear regression analysis. The method was found to be linear in a concentration range of 10–50 µg/mL (n = 5) for Prodrug. The linearity data was shown in Table 2.

Table 1: Optimized Chromatographic Condition

Mobile phase	Acetonitrile:Water(90:10)v/v
Column	BDS Hypersil C18, 250*4.6, 5µ
Injection volume	20 µL
Flow rate	1.0 ml/min
Wavelength	245 nm
Detector	UV

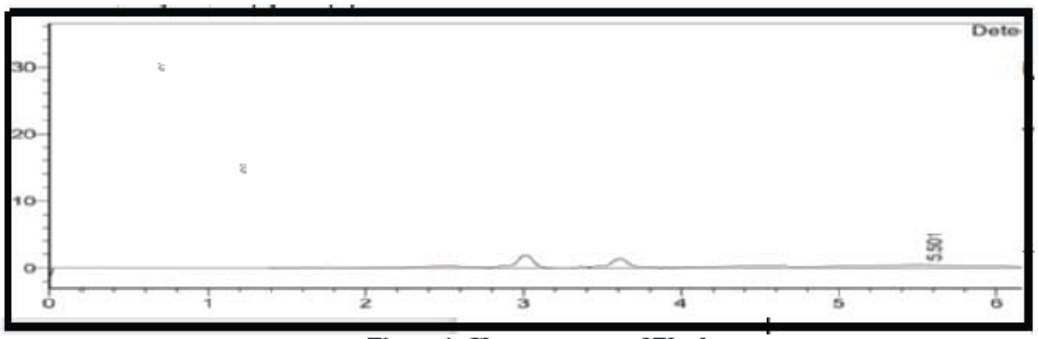

Figure 4: Chromatogram of Blank

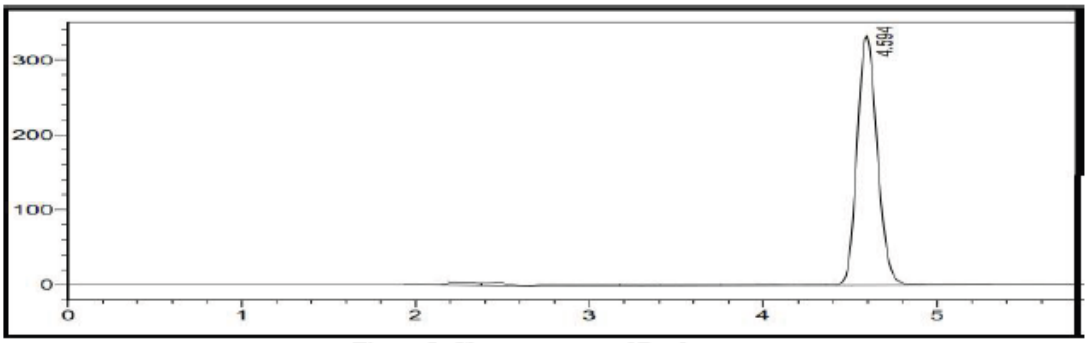

Figure 5: Chromatogram of Prodrug

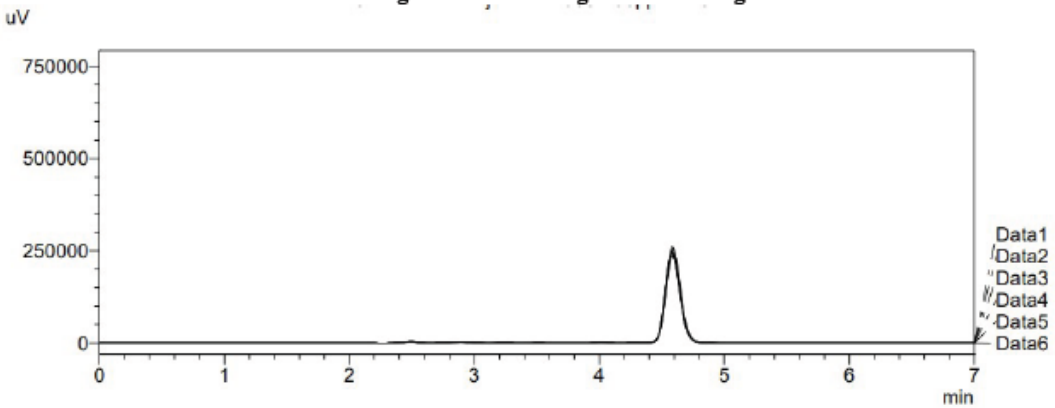

Figure 6: Chromatogram of System Suitability

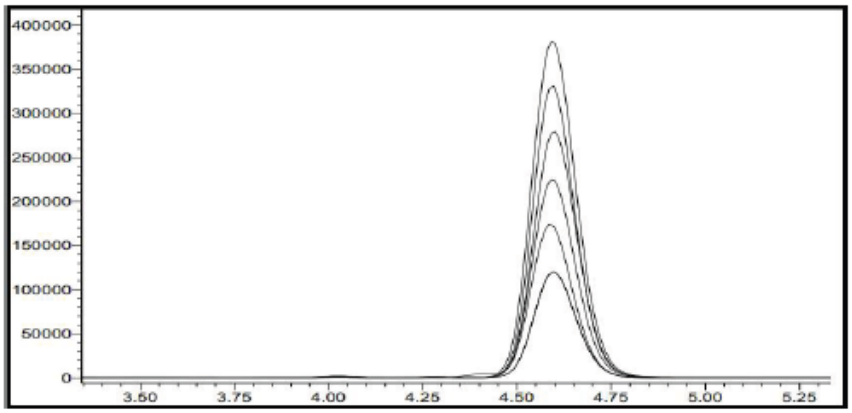

Figure 7: Chromatogram of linearity

Table 2: Data of Linearity

Sr. No	Conc. ppm	Area
1	10	995778
2	20	1415081
3	30	1863068
4	40	2326235
5	50	2715036

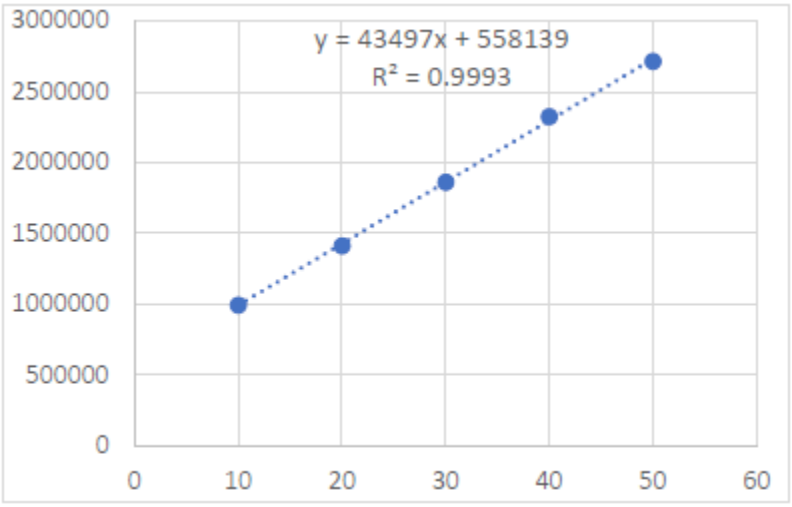

Figure 8: Calibration Cure of Prodrug

Accuracy

Accuracy of an analytical method is the closeness of Practical value to the true value (100%). A known amount of standard is spiked into pre-analyzed sample at three different concentration level (50%, 100% and 150%). The percentage (%) recovery was found to be in the range of 99.33-100.5% for Prodrug (Table 3). The values demonstrated that the method is accurate. Limit of detection and limit of quantification

The LOD and LOQ of method was calculated by using SD of Y- intercept of calibration curve and Mean slope of calibration curve.

The lowest amounts of drug that could be detected (LOD) for Prodrug was found to be 0.6 µg/mL and the limit of quantification (LOQ) for Prodrug was found to be 1.9 µg/mL.

Precision

Repeatability of method was performed by injecting a 30 µg/mL solution The average, standard deviation (SD) and % RSD of the area was calculated and reported. Intra-day precision was performed by injecting middle three concentration of an analytical procedure within a day over a short period of time by the same experimental conditions, whereas interday precision involves estimation of variations in analysis when a method is used within a laboratory on different days. The percentage (%) RSD values of the response were less than 2% for intra-day and inter-day precision which indicated that the method is precise (Table 5).

Robustness

The Prodrug was analyzed at three different wavelength and three different flow rate .The % RSD was calculated and found to be less than 2% which demonstrate that the proposed method was robust. Summary of Validation parameters are shown in Table 5.

Assay

The overlay chromatogram of placebo and synthetic mixture clearly indicates no interference of formulation excipients in analysis. The assay was found to be 103.6%, for the prodrug.

Table 3: Data of Accuracy

Standard	Spiking Level	Actual Amount (mg)	amount of Added (mg)	Total Amount (mg)	Recovered amount (mg)	% Recovery	Mean
Prodrug	50%	10	5	15	14.9	99.33	99.81
	100%	10	10	20	20.1	100.5	
	150%	10	15	25	24.9	99.6	

Table 4: Data of Linearity, LOD and LOQ

Name of Drug	Conc. Range(µg/ml)	Equation	Regression coefficient	LOD	LOQ
Mutual Prodrug	10 – 50	y = 43497x + 558139	R^2 = 0.9993	0.6	1.9

Table 5: Data of Precision and Robustness

		Prodrug
Precision	%RSD (Repeatability)	1.672119
	%RSD (Interday)	0.133109
	%RSD (Intraday)	0.095868
Robustness	Change in wavelength	0.102614
	Change in Flowrate	0.091297

4. CONCLUSION

From the validation data it was concluded that a specific, accurate and precise HPLC analytical method has been developed for the estimation of Prodrug in bulk. The method was validated and found to be sensitive, accurate and precise. Statistical analysis proved that method was repeatable and selective for the analysis of Prodrug without any interference from the excipients. The method was successfully used for the determination of drug in their Bulk form. Also, the above results indicated the suitability of the method for hydrolysis study of prodrug. As the method separates the parent drugs from its prodrug, so it can be used for drug release study of Prodrug.

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