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RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF MUTUAL PRODRUG OF PROPYPHENAZONE AND FLURBIPROFEN

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ABSTRACT

Present study involves development of a liquid chromatography method for the estimation of Mutual Prodrug of Propyphenazone and Flurbiprofen. RP-HPLC method was developed using Column C18 (Shimadzu Shim-pack Gist (250* 4.6 mm, 5 µm) as stationary phase and Acetonitrile:Water (90:10) as mobile phase. Flow rate was kept at 1 mL/min and detection was performed using UV detector at 245 nm. The retention Mutual Prodrug was observed to be 4.5 min. The linear regression analysis data for the calibration plot showed a good linear relationship for Mutual Prodrug over a concentration range of 10–50 µg/mL. The proposed method was validated with respect to linearity, accuracy, precision and robustness. The developed method can be used for routing quality control evaluation of Mutual Prodrug in bulk.

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

INTRODUCTION:

The widely used medications for the treatment of pain, fever, and inflammation are nonsteroidal anti-inflammatory drugs (NSAIDs) **[1]**. The utility of this class of medications, however, is limited due to a higher prevalence of gastrointestinal adverse

effects such as stomach ulcers, perforation, bleeding, and other related issues such as cardiovascular, hepatic, and renal toxicity **[2]**. Many of these consequences can be fatal, and some people have died as a result of them **[3]**. Prostaglandins, which are major physiological and pathological mediators in inflammation, pain, pyrexia, cancer, and neurological diseases, are secreted via the COX pathway **[4]**. Phospholipase A2 produce free arachidonic acid from biomembrane bound arachidonate **[4]**. The two known COX isoforms are found in this COX pathway: Arachidonic acid is converted by COX-1 and COX-2 into prostaglandin G2, which is then reduced by peroxidase to form PGH2 **[4]**. Thromboxane A2 and PGD2, PGE2, PGF2, and this PGH2 are formed. The prostanoids produced by this isoform of COX-1 mediate functions such as the regulation of renal blood flow, cytoprotection of the gastric mucosa, and platelet aggregation. COX-1 is expressed in the majority of tissues **[5]**. The kidneys, brain, and spinal cord all express COX-2 **[5]**. It is an immediate early response gene highly restricted under basal conditions but highly inducible in response to inflammatory stimuli, including endotoxin, cytokines, hormones and tumour promoters **[6]**.

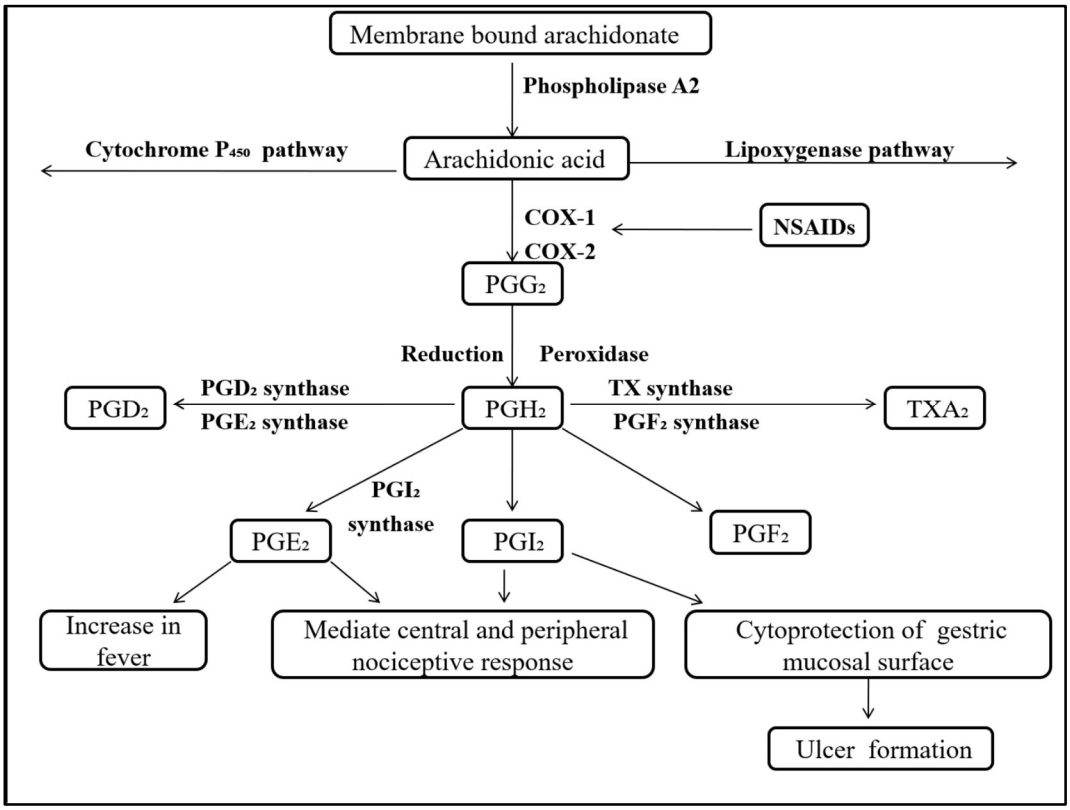

Figure 1: Mechanism of Nsaids Drugs

The synthesis of prostaglandins was reduced by blocking the COX enzyme, which leads to decrease in inflammation, pain and fever **[7]**. Reduced prostaglandin secretion causes a variety of adverse effects, including GI discomfort, cardiovascular consequences, renal toxicity, hypertension aggravation, and fluid retention **[7]**. Because NSAIDs cause GI mucosal injury via two pathways, non-selective NSAIDs inhibit not only COX-2 but also COX-1, causing GI ulceration and potentially upper GI perforation and haemorrhage **[8]**. The first technique includes direct contact, which results in regional suppression of prostaglandin formation in the GI tract as well as localised irritation from the carboxylic group of NSAIDs. COX-2 selective drugs gave the same efficacy without gastrointestinal symptoms, but increased the risk of elevated serum potassium levels and possible liver damage **[5]**.

It has been well documented in recent years that the creation of reactive oxygen species (ROS) plays a substantial role in the establishment of gastric mucosal lesions associated with NSAID therapy **[9]**. These investigations suggest that the production of mutual prodrugs may reduce the likelihood of NSAID-induced GI ulcerogenicity **[9]**. As previously stated, NSAIDs have poor physicochemical features that can be modified by using a prodrug method **[6]**. The mutual prodrugs have better physicochemical and pharmacological properties and are designed to release the parent drugs at the site of action **[10]**. NSAIDs were transformed to different ester or amide prodrugs that prevent the parent drug from coming into direct touch with the gastric mucosal lining in the GI tract, resulting in improved physicochemical characteristics and increased bioavailability **[6]**.

The present study aimed for development and validation of analytical method for the estimation of mutual prodrug of Propyphenazone and flurbiprofen.

Literature review revealed that no any liquid chromatographic method has been reported for the estimation of Prodrug of Propyphenazone and flurbiprofen. So, the present study aimed at development and validation of analytical method for the estimation of mutual prodrug of Propyphenazone and flurbiprofen.

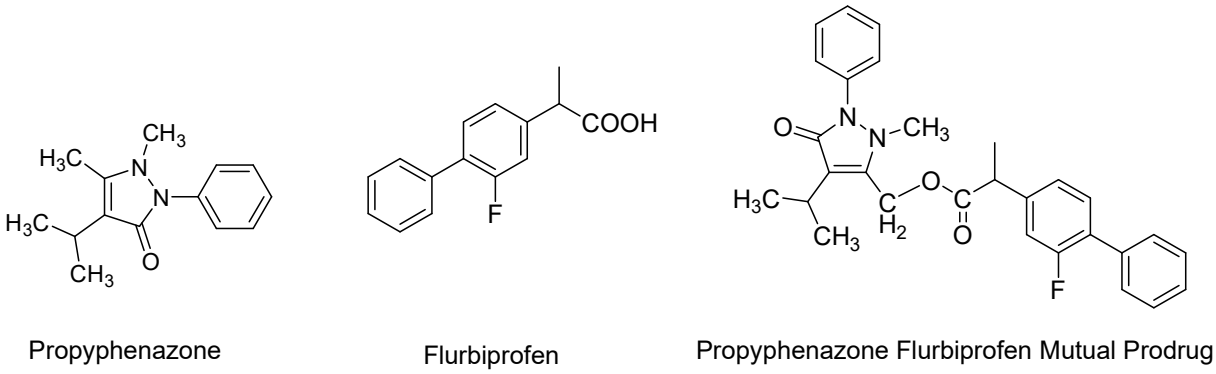

Figure 2: Chemical Structure of Drugs and Prodrug

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 was 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**.

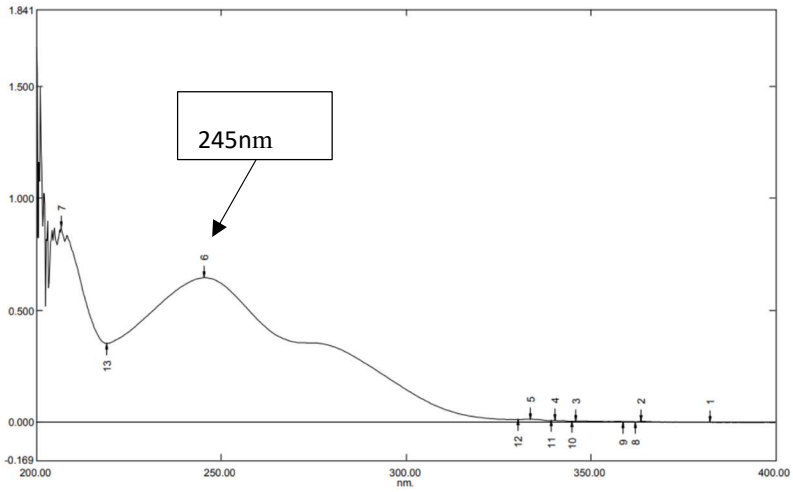

Figure 3: UV Spectrum of Prodrug

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 up 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.

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

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

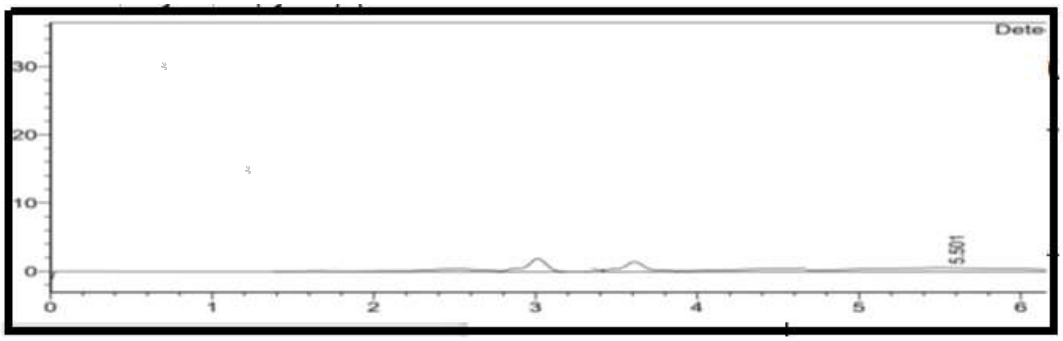

Figure 4: Chromatogram of Blank

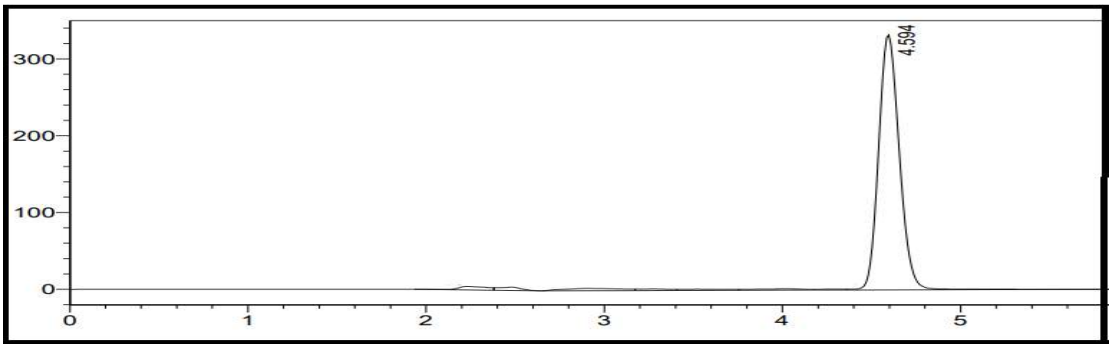

Figure 5: Chromatogram of Prodrug

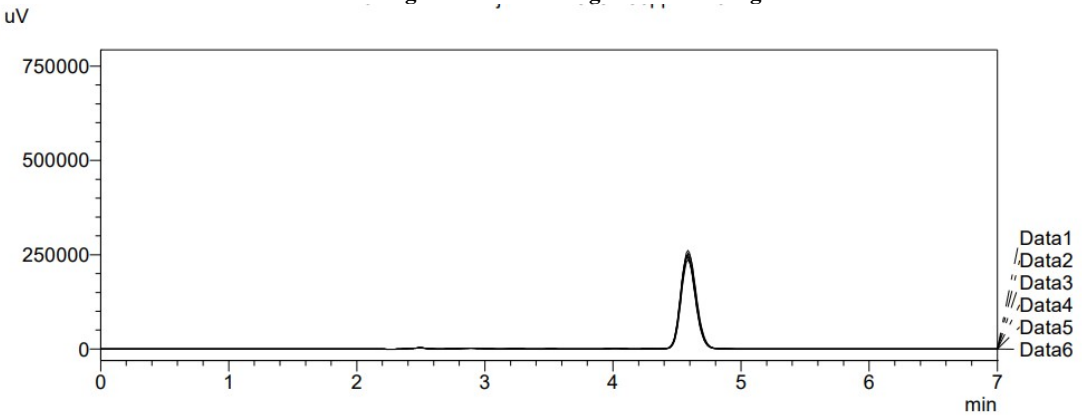

Figure 6: Chromatogram of System Suitability

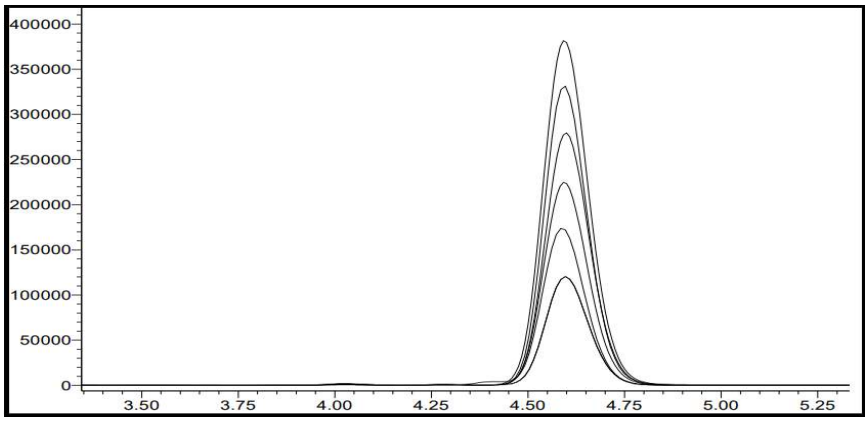

Figure 7: Chromatogram of linearity

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

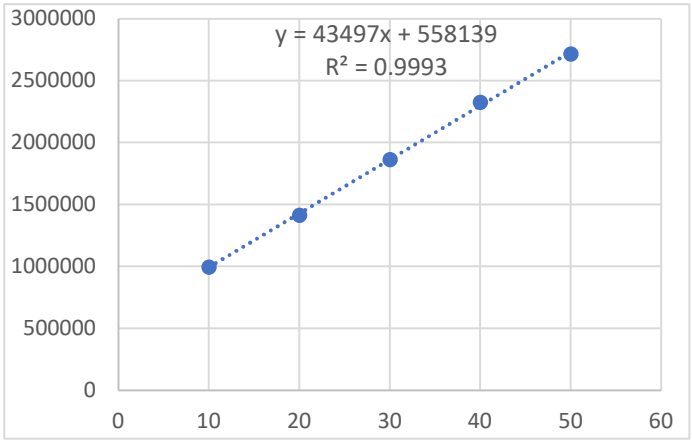

Figure 8: Calibration Cure of Prodrug

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 inter-day 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

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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