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APPLICATION OF BIOANALYTICAL METHOD IN PHARMACOKINETIC STUDIES OF DIKAMALI GUM BASED MICROSPHERES OF FLURBIPROFEN FROM RABBIT BLOOD SAMPLES

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ABSTRACT: Preparation and evaluation of dikamali gum-based microspheres of Flurbiprofen, needed a suitable bioanalytical method. In this direction, we report the development of a simple RP-HPLC method for the determination of Flurbiprofen in rabbit plasma samples by using the standard internal method. The method was carried out on RP C18 column, with a mixture of the buffer: acetonitrile (60:40) as the mobile phase. The linearity was found to be in the range of 83.90-99.45%. The half-life of drug was 4.7-5.7 h. The developed method was validated as per USFDA guidelines and used in the estimation of Flurbiprofen microspheres on oral administration in rabbits. It was observed that the T_{max} and AUC of Flurbiprofen was 3 h, 114.24 mcg.hr/ml for the marketed formulation, and 9 h and 213.03 mcg.hr/ml for test formulation, respectively. The pharmacokinetic parameters of the test formulation showed delayed T_{max}, decreased C_{max}, and increased area under the curve (AUC) as compared to the reference formulation. This indicated that Flurbiprofen was not released significantly in the stomach and small intestine but was delivered to the colon resulting in slow absorption of the drug and making it available for local action in the colon.

INTRODUCTION: Flurbiprofen is a member of the phenyl alkanoic acid derivative family of non-steroidal anti-inflammatory drugs [1]. It is primarily indicated as a pre-operative anti-miotic as well as orally for arthritis or dental pain. In patients with rheumatoid arthritis, the anti-inflammatory action of Flurbiprofen has been evidenced by relief of pain, increase in grip strength, and reductions in joint swelling, duration of morning stiffness, and disease activity [2].

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In patients with osteoarthritis, the anti-inflammatory and analgesic effects of Flurbiprofen have been demonstrated by a reduction in tenderness as a response to pressure and reductions in night pain, stiffness, swelling, and overall disease activity.

The aim of this project is to develop and validate a bioanalytical method [4, 5] using HPLC for formulated sustained-release & colon targeted microspheres by a novel natural polymer *i.e.*, Dikamali gum obtained from a plant, *Gardenia gummifera*, [7, 8, 9] belonging to the family Rubiaceae. The novelty in the present study lies in using a new sustained-release agent in the preparation of microspheres and to evaluate these microspheres [6] by using a bioanalytical method for determination of various pharmacokinetic parameters [11, 12].

Fenoprofen was used in irritable bowel syndrome, also known as irritable colon syndrome, so, colon targeted microsphere was suitable. The $t_{1/2}$ of this drug is approximately 3 h. The drug also used in treating mild-to-moderate pain in chronic arthritis so a sustained release formulation was suitable for these drugs for which no marketed sustained release formulations were available [13].

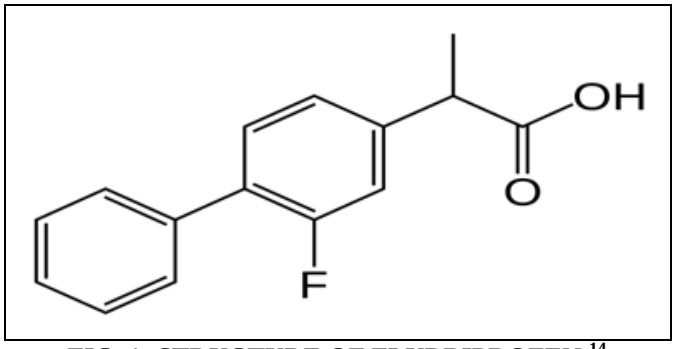

FIG. 1: STRUCTURE OF FLURBIPROFEN [14]

MATERIALS AND METHODS:

Chemicals and Reagents: All chemicals were of analytical grade. All solutions and dilutions were prepared with ultrapure water. Acetonitryle was purchased from Merck (Darmstadt, Germany) company. Working solutions of flurbiprofen and internal standard ibuprofen were daily prepared by appropriate dilutions. Rabbit blood samples were collected from healthy New Zealand albino rabbits.

TABLE 1: THE FOLLOWING TABLE GIVES THE OPTIMIZED CONDITIONS FOR BIOANALYTICAL METHOD DEVELOPED FOR ESTIMATION OF FENO-PROFEN IN DIKAMALI GUM BASED MICROSPHERES

S. no.	Parameters	Method
1	Column Specification	KROMASIL, C8, 250 x 4.6 mm, 5µ.
2	Mobile Phase	Buffer and acetonitrile (67:33)
3	pH	No pH Adjustment
4	Flow Rate (ml/min)	1
5	Run Time (min)	10
6	Volume of Injection Loop (µl)	30
7	Detection Wavelength (nm)	250
8	Detector	UV Detector
10	Diluent	WATER: ACN (50:50)
11	Retention time	10 min

Instrumentation and Chromatographic Conditions: Analysis was performed on HPLC system, which consisted of a pump (model Jasco PU-2080 plus PU- 2087 plus intelligent LC pumps) with autosampler injector. The detector used was UV (Jasco UV-2075 plus). The column was the DISCOVER C18 column (250 × 4.6 mm, i.d., 5µm). Before analysis, the mobile phase was filtered through a 0.22 µm filter and then degassed ultrasonically for 30 min. The analyses were done at a flow rate of 1.0 mL/min, and the detection wavelength was 279 nm. The run time was about 10 min. Chromatographic conditions of fenoprofen are given in **Table 1**.

Pharmacokinetic Studies for Fenoprofen Microspheres:

Animals Housing and Preparation for Study: Healthy rabbits (New Zealand Albino) of either sex weighing 2.5-3.0kgs were selected and housed with CPCSEA guidelines in Srivenkateswara College of Pharmacy, Etcherla. During the experiment, rabbits were housed in standard housing conditions like the temperature of 25 ± 1 °C, the relative humidity of 45%-55% and 12 h light: 1 2h dark cycle. The animal facilities of Department of Pharmacology at Sri Venkateswara College of Pharmacy, Etcherla are approved by the committee for Control and Supervision of Experiment Animals.

The pharmacokinetic study protocols were approved by the Institutional Animal Ethics Committee (IAEC) CPCSEA/SVCP/IAEC/2018/ Ph.D-002). Prior to initiating the pharmacokinetic study, animals were fasted overnight. Animals were separated into two experimental groups, each group consisting of three animals (n=3). The test formulation of batch FPS15 was compared with the marketed formulation (reference) with the following treatment schedule under fasted conditions.

Group I: Flurbiprofen marketed formulation used as a reference.

Group II: Flurbiprofen optimized formulation (Flurbiprofen microspheres prepared using dikamali gum as a sustained-release agent) used as a test.

The optimized formulation containing micro-spheres was administrated via oral gauge at a dose of 10.2 mg/kg for Fenoprofen [15]. Blood samples (each of about 1-2 ml from each animal) were withdrawn from marginal ear vein at regular time intervals after administration. The collected blood samples were immediately centrifuged at 5000 rpm in an ultra cooling centrifuge for 10 min at 4 °C.

The supernatant plasma sample was separated and stored in clean screw-capped 5 ml polypropylene plasma tubes at -20 °C in a deep freezer until further analysis.

Estimation of Drug from Rabbit Plasma: The stored plasma samples were processed at room temperature, 250 µl of plasma was added to 500 µl of Acetonitrile to precipitate the proteins. The samples were vortexed on a vortex mixer for 15 min, followed by centrifugation at 10000 rpm for 15 min. The respective samples were injected into the HPLC column 0.2 µ syringe filter. A 20 µl of the filtered solution was injected onto the HPLC column.

Preparation of Fenoprofen Stock Solution (1mg/mL): Taken 30 mg of Fenoprofen in 10 ml volumetric flask and made the volume with diluent to produce 3 mg/ml (3000 µg/mL).

Preparation of Fenoprofen Working Standard Solutions (6 µg/mL to 150 µg/mL): From the above Fenoprofen stock solution 0.01 ml, 0.02 ml, 0.03 ml, 0.1 ml, 0.25 ml, 0.3 ml, 0.4 ml and 0.5 ml was pipette and transferred to 8 individual 10 ml volumetric flask and make up the volume up to the mark with diluent to produce 3 µg/mL, 6 µg/mL, 9 µg/mL, 30 µg/mL, 75 µg/mL, 90 µg/mL, 120 µg/mL and 150 µg/mL.

Preparation of Internal Standard Solution (1 µg/mL): Take 100 mg of Ibuprofen in 100 ml volumetric flask and make up the volume with diluent to produce 1000 µg/mL. From the above solution, take 2.5 ml of the solution into 10 ml volumetric flask and make up the volume with diluent to produce 250 µg/mL solutions. The chromatograms of blank plasma and sample were included in **Fig. 3** and **4**.

Method Validation: Method validation was carried out according to ICH guidelines in rabbit plasma in order to evaluate the method for selectivity, the linearity of response, accuracy, precision, recovery, and stability of analytes during processing and storage.

Selectivity: Selectivity was checked by injecting blank plasma samples from six different rabbits to confirm no interfering peaks around the retention time of both Flurbiprofen and IS.

RESULTS AND DISCUSSION:

Calibration, Linearity and Quality Control Samples: Calibration curves were obtained daily for 3 days using standards containing eight different concentrations [15]. Curves were constructed by calculating the peak area ratio of Flurbiprofen to that of IS. For the preparation of calibration standards, working solutions of Flurbiprofen (0.5mL) and IS (0.5mL) were added to blank plasma (0.5ml) to obtain final concentrations of 1.2 µg/mL, 2.4 µg/mL, 3.6 µg/mL, 12 µg/mL, 30 µg/mL, 36 µg/mL, 48 µg/mL and 60 µg/mL. The quality control (QC) samples were prepared in a similar manner as the calibration standards at three different levels: 3.6 µg/mL low-quality control (LQC), 30 µg/mL medium quality control (MQC), and 48 µg/mL high-quality control (HQC). The calibration curve was obtained in **Fig. 1,** with a correlation coefficient value of 0.998.

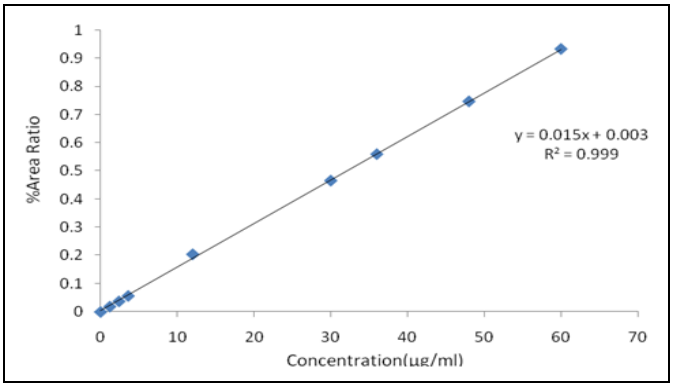

FIG. 2: CALIBRATION CURVE FOR FENOPROFEN

Precision and Accuracy: The precision and accuracy of the assay were determined using QC samples of known Flurbiprofen concentrations (*i.e.*, 3.6, 30 and 48 µg/mL), which were processed each validation day freshly as described for calibration curve standards. Six replicates of each QC were analyzed on 3 days, and the intra- and inter-assay means, standard deviation (SD) and CV were calculated. The recovery of Flurbiprofen from plasma samples was carried out at three concentration levels (LQC, MQC, and HQC) by analysis of replicate (n = 6) samples. The peak area of QC samples in plasma was compared with peak area of actual analyte (in mobile phase) at the same final concentrations. The recovery was expressed as a percentage value, and the extent of recovery of Flurbiprofen and of the IS should be consistent, precise, and reproducible.

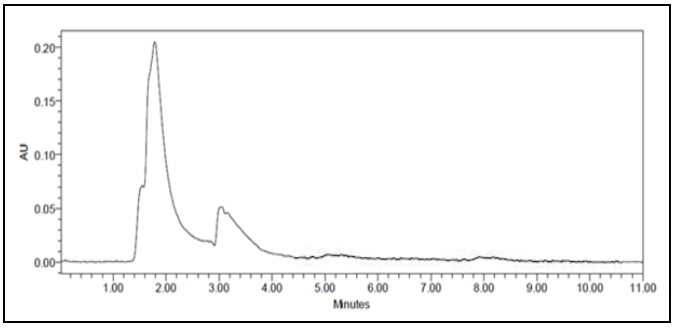

FIG. 3: CHROMATOGRAM OF THE BLANK PLASMA

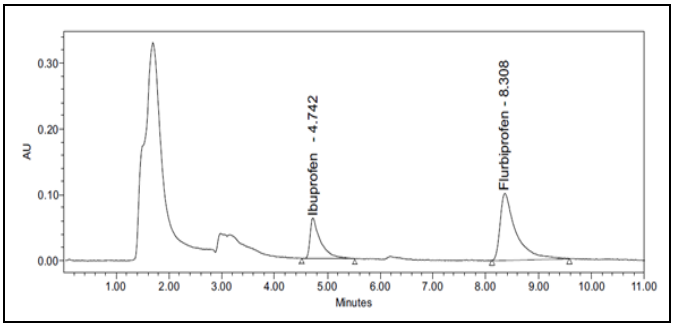

FIG. 4: CHROMATOGRAM OF OPTIMIZED FORMULATION WITH INTERNAL STANDARD IN PLASMA

Data Analysis: The total area under plasma concentration-time curve ($AUC_{0-\infty}$), the maximum plasma concentration (C_{max}), and the time to reach the maximum plasma concentration (T_{max}) were selected as parameters for pharmacokinetic evaluation. The C_{max} and T_{max} were obtained directly from the experimental data of plasma concentration versus time profile **Fig. 5** $AUC_{0-\infty}$ was obtained by adding the AUC_{0-24h}, which was calculated by the trapezoidal rule. The differences in average of data were compared by sample analysis of variance (one-way analysis of variance) or independent sample t-test. The significance of the difference was determined at the 95% confident limit (P=0.05). All these pharmacokinetic parameters were calculated and included in **Table 3**.

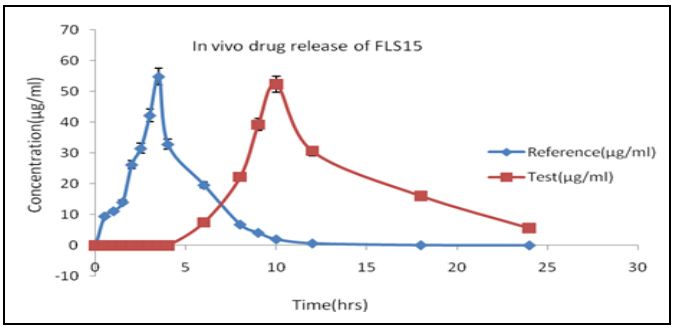

FIG. 5: PLASMA CONCENTRATION PROFILE OF FLURBIPROFEN OPTIMIZED FORMULATION (TEST) AND REFERENCE (MARKETED FORMULATION) IN HEALTHY RABBITS

TABLE 2: PLASMA CONCENTRATION PROFILE OF FENOPROFEN OPTIMIZED FORMULATION (TEST) AND REFERENCE (MARKETED FORMULATION) IN HEALTHY RABBITS

Time (h)	Reference (µg/ml)	Test (µg/ml)
0	0	0
0.5	9.5±10.84	0±1.20
1	11.16±3.67	0±0.45
1.5	14.06±5.48	0±6.87
2	26.21±2.68	0±5.29
2.5	31.5±1.75	0±6.74
3	42.21±2.46	0±3.45
3.5	54.82±2.02	0±6.87
4	32.84±1.86	0±5.98
6	19.62±2.48	7.52±2.87
8	6.84±6.84	22.22±3.68
9	4.16±3.57	39.16±1.28
10	2.04±6.54	52.26±4.30
12	0.72±2.86	30.64±2.33
18	0.14±1.58	16.12±1.28
24	0.08±2.46	5.68±2.04

TABLE 3: PHARMACOKINETIC PARAMETERS OF FLURBIPROFEN MICROSPHERES (FLS 15) AND REFERENCE FORMULATIONS

S. no.	Parameters	Marketed formulation (Reference)	Optimized formulation (Test)
1	$AUC_{(0-\infty)}$ (µg.h/ml)	194.39±11.28	448.18±13.02
2	C_{max} (µg/ml)	54.82 ±12.02	52.26±18.30
3	T_{max} (h)	3.5	10
4	Relative bioavailability	(AUC_{0-t} Test/AUC_{0-t} std)100	190.57%

CONCLUSION: This bioanalytical method was developed for estimation of flurbiprofen in the dikamali gum-based matrix microspheres, and the method was validated for its accuracy, precision and linear and specificity. From the data obtained from this developed HPLC method, various pharmacokinetic parameters were calculated and were found that there is an increase in AUC and t_{max} values for test sample (FLS) compared to that of marketed formulation, which implies that the dikamali gum extends the release rate.

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CONFLICTS OF INTEREST: None of the authors has any conflicts of interest in the context of this work.

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