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VIEWS

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Pharmacophore Modeling and Virtual Screening for Searching New P38 Kinase Inhibitors: *In Silico* Studies

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ABSTRACT

In search new anti-inflammatory agents, p38 kinase a proline directed serine threonine mitogen activated protein kinase has been known as attractive target for anti-inflammatory drug discovery. An attempt has been made here to search new scaffold with added attributes as p38 kinase inhibitor employing ligand based Pharmacophore development and virtual screening. A best Pharmacophore was developed using thirty two compounds with know IC_{50} values as training set by HypoGen with r^2 0.89 and root mean square deviation (RMSD) 1.282 consisting features like hydrogen bond acceptor and ring aromatics and cost difference of 48.4. The aforementioned model was validated by predicting activity of training set consisting 150 diverse set of molecules which showed predictive r^2 0.6. With the high confidence the Pharmacophore was explored for screening a database of 0.3 million molecules which resulted in 560 new hits and among them fifty two compounds have been identified as best active structures.

Key words: p38 kinase/ anti-inflammatory/Pharmacophore/HypoGen

INTRODUCTION

Proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are known to involve in numerous inflammatory diseases and are expressed in higher levels in human body during inflammation.[1] Protein based anticytokines offered limited clinical application in containing the aforementioned elevated cytokines and need for nonprotein small molecules for the treatment of inflammatory diseases still persists till today. [2, 3]

P38 MAP kinase is member of proline directed serine threionine protein kinase plays an important role in the stability and production of TNF-α and IL-1β. Four isoforms (α, β, γ, and δ) of p38 kinase have been reported and α isoform being highly implicated in inflammatory diseases by forming cardinal member of signalling pathway which leads to excessive production of pro-inflammatory cytokines.[4, 5] Thus p38 kinase has been considered as one of the most important and vital target for the development of new anti-inflammatory agents.[6]

A pragmatic shift in the approach of medicinal chemist has been witnessed towards the search of new potent and clinically effective p38 kinase inhibitors. Numerous compounds have been reported to possess potent p38 kinase inhibitory activity and were tried in clinical trials (**Figure 1**).[7] QSAR based and Structure based approaches have already been reported to understand and design new effective p38 kinase inhibitors.[8-11] and only few pharmacophore based virtual screening are reported.[12] In continuation to our previous efforts to design new p38 kinase inhibitors and antiinflammatory agents[13-15], here we wish to report *in silico* pharmacophore development and virtual screening of data base of compounds for the search of new p38 kinase inhibitors and the picturesque scheme of the planned work is represented in **figure 2**.

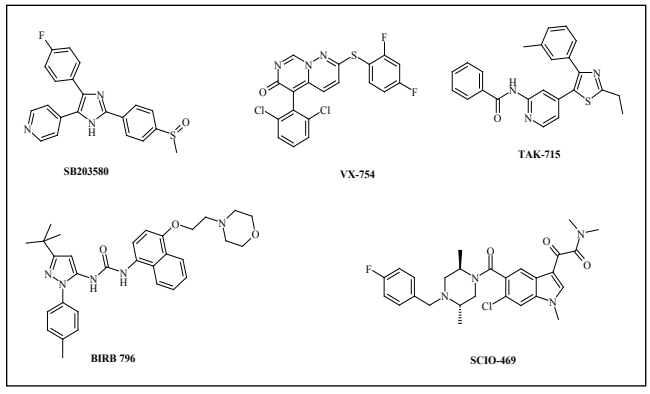

Figure 1: Structure of some of p38 kinase inhibitors

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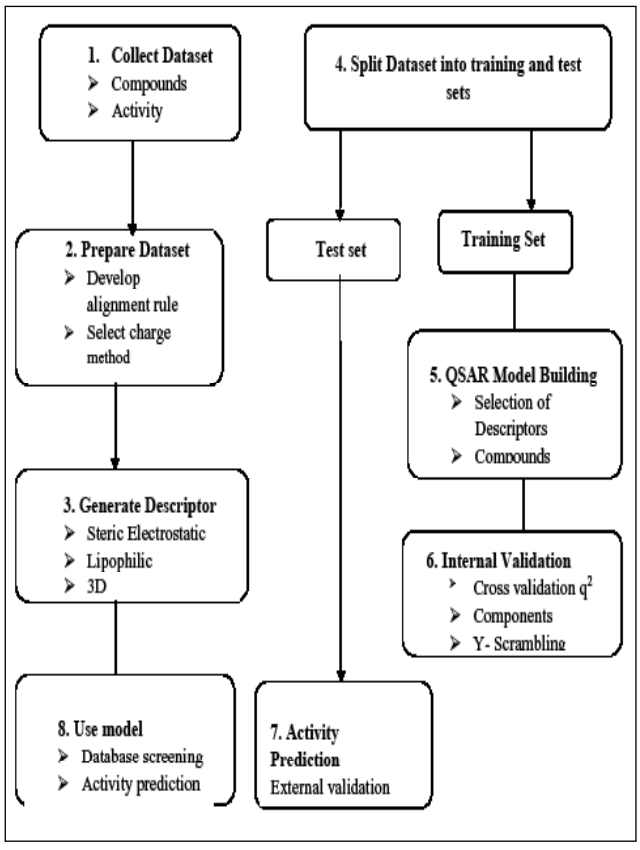

Figure 2: Flow chart of QSAR

MATERIALS AND METHODS

All the *insilico* molecular modeling work has been carried on linux based PC with 3 Gb ram and sufficient space in hard disc at GVK biosciences, Hyderabad using discovery studio (DS).

Selection of Molecules and modeling

Upon thorough literature survey of p38 kinase inhibitors we have selected 182 reported compounds which possess p38 kinase inhibitory activity in the range of 0.08 nM to 4570 nM.[16-22] These selected compounds have been divided into training set (32 molecules) and test set (150 molecules) based on structural diversity and activity profile by keeping most active and least active compounds in training set. Further, all the compounds were grouped into most active, moderately active and low active which was done based on their reported activity in a hope to get initial information.

Structures were drawn using sketch module of DS and were refined before subjected to minimization. Upon refinement of initial structures, all the structures were subjected to minimization using standard protocols of DS and saved as .sd file format. Activity values of each compound were inserted and were converted to their logarithmic scale along with uncertainty value was set to 3. Multiple conformations of molecules were generated at 20.0 kcal/mol above the global energy minima and set maximum 250 such conformers for each molecule.

Upon generation of conformation of the molecules, the training set molecules were submitted to Catalyst hypothesis generation using HypGen module in which four common features included viz. hydrogen bond donor (HBD), hydrogen bond acceptor (HBA), ring aromatic (RA) and hydrophobic (HY) to generate statistically significant pharmacophore model. Top ten best pharmacophores were intended to be generated and best one of them was selected based on the high correlation, lowest total cost and RMSD and this model was further subjected for external validation using 150 test set molecules. After garnering the predictability of the pharmacophore model, 0.3 million molecules from Asinex (Asinex Gold and Asinex Synergy) data base were screening for getting best new hits as probable p38 kinase inhibitors.

RESULT AND DISCUSSIONS

Best ten pharmacophore models were generated by HypGen module in Discovery Studio[23] and were exported. Most of the generated hypothesis possessed statistically high correlation coefficient r^2 value of 0.9. Maximum number of hypothesis described HY, HBA, ring aromatics and HBD features. Numerous training set compounds mapped with hydrogen bond acceptor and ring aromatics which indicated that HBA and RA are major pharmacophores for the potent p38 kinase inhibitory activity thus are considered cogently for screening new p38 kinase inhibitors. On the basis of statistical parameters one best pharmacophore was selected as highlighted by lowest RMSD and highest correlation coefficient. The best selected model exhibited correlation coefficient r^2 0.89 and RMSD 1.282. The most active compound of all the series considered during the pharmacophore development has been mapped effectively with the best pharmacophore which offers required pharmacophores for p38 kinase inhibitors as depicted in the **figure 3**.

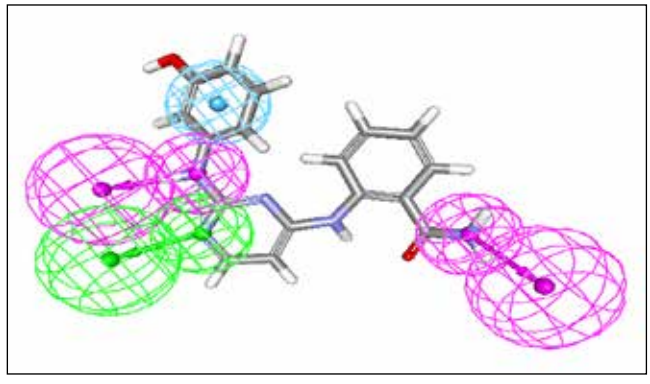

Figure 3: Features of best pharmacophoric model

HypoGen also propose two types of costs which offer a great help in deciding the best pharmacophores. One of the costs is total cost or fixed cost which explains the pharmacophore with maximum data fit in and second cost is null cost which infers highest cost of pharmacophore without

any features. On the basis of cost, a best pharmacophore is the one which the difference cost between null cost and total cost is lesser than null cost. Usually a difference of cost in the range of 40-60 assumes to correlate the data 80-90% well. In our studies total cost was 120 and null cost was found to be 168.4 and the difference was 48.4 with r^2 0.89 and RMSD 1.282.

In order to gain confidence about the model thus generated, activity of all the training set molecules were predicted for as internal validation. Usually in the pharmacophoric studies, activities of training set are grouped in three bin system like most active (<25 nM), moderately active (25-500 nM) and low or least active (>500 nM). Few compounds exhibited the difference of activity in single magnitude which shows the robustness of the pharmacophore. This shows that several molecules out of 32 training set molecules, errors found to be less than 10 which hints that predicted activity of training set was in the range of 10 fold greater and 1/10 of actual activity.

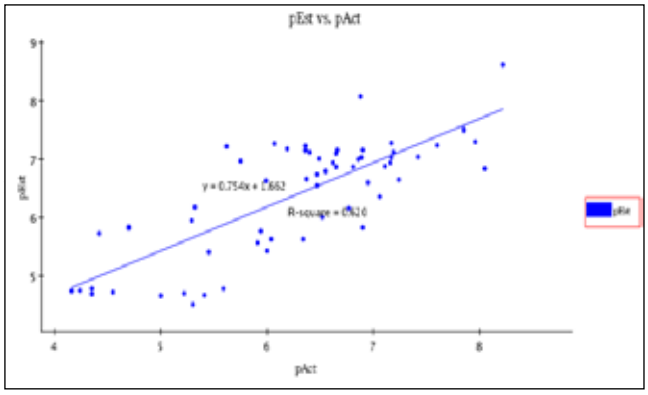

Figure 4: A plot of estimated activity (Y axis) verses actual activity (X axis)

One of the best application of pharmacophore modeling and other QSAR studies is that pharmacophore model offers prediction of large data base of compounds apart from prediction of activities of training and test set molecules accurately and classifying them active or inactive. All the prediction of data base of compounds was performed by employing Flexible search option. Numerous attributes were considered for selection of hits, such as hit list, percentage ratio and fit score upon virtual screening of data base of compounds. Two data bases Asinex Gold and Asinex Synergy containing three hundred thousand molecules have been employed for virtual screening. Over all 560 new hits were identified and among the new hits fifty two compound were grouped highly active, two hundred forty compounds in moderately active and rest were in low active bins.

CONCLUSION

In the concluding remarks, afore discussed research work was tasked to explore the features required for p38 kinase inhibitors. HypGen model of Discovery Studio was employed to correlate features of compounds with activity of training set and developed a statistically fit pharmacophore hypothesis. A best highly predictive pharmacophore was resulted from training set with hydrogen bond acceptor, ring aromatic and hydrogen bond donor was obtained. This pharmacophore model was cross validated by predicting the activities of training set molecules and with this encouragement activity of test set compounds comprising 150 molecules were predicted. To our confidence in the developed model, the predicted activity of tested compounds was well within the acceptable range with predicted r^2 value of 0.6.

Thus the statistically fit model was further employed to screen data base of three hundred thousand molecules in order to search new compounds with diverse chemical structures considering the pharmacophoric features as mentioned earlier. Over all fifty two molecules found to be highly active and two hundred forty molecules were in moderately active range. Further synthesis of compounds resulted from virtual screening and subsequent screening against p38 inhibitory activity will further boost confidence of this pharmacophore model.

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Formulation Development and *In Vitro* Evaluation of Nizatidine Floating Microspheres

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ABSTRACT

The aim of the present research study was to develop and evaluate Nizatidine loaded floating microsphere for controlled release of drug at the gastric mucosa. Gastro retentive floating microspheres containing Nizatidine were prepared by ionic gelation technique using sodium alginate as polymer, HPMC K4M, HPMC K15M, HPMC K100M as drug release modifier, and sodium bicarbonate as gas generating agent. Calcium chloride was used as cross linking agent. The prepared microspheres were characterized by FTIR, DSC and SEM analysis and evaluated by means of micromeritics, *in vitro* buoyancy, percentage yield, swelling studies, drug entrapment efficiency, and drug release studies. Among the total 18 formulations F17 was optimized. The % yield of F17 formulation was found to be 95.47%. On the basis of optical microscopy the particle size was 50.67µm. The % buoyancy, % entrapment efficiency and swelling index of F17 formulation was 94.23%, 93.62% and 92.13%, respectively. The Cumulative % drug release of F17 formulation was 96.54% in 12h when compared with marketed product 94.15% in 1h. SEM studies showed the particles were in spherical shape. Stability studies were conducted as per ICH guidelines. On the basis of obtained results, Floating microspheres were of good candidate for targeting to GIT. Nizatidine floating microspheres can be successfully used for controlled drug release to the gastric mucosa in treatment of upper GIT infection.

Keywords: Nizatidine, Peptic ulcer, Floating microspheres, Release order kinetics.

INTRODUCTION

Conventional oral drug delivery system is the most preferable route because of ease in administration, patient compatibility and flexibility [1]. To design an oral drug delivery system, it is essential to optimize both drug release from the formulation and residence time within the GIT [2]. Drugs that have short half life and easily absorbed from the gastrointestinal tract require frequent dosing to circumvent these problems, the oral controlled release formulations have been developed in attempt to slow the drug release into the GIT and maintain the constant plasma drug concentration [3]. Dosage forms that can be retained in the stomach are called gastroretentive DDS. This system have a bulk density less than that of gastric contents and thus remain buoyant in the stomach for a extended period without altering the gastric emptying rate [4]. The gastroretentive drug delivery system (GRDDS) is used in enhancing the bioavailability of drugs that are poorly soluble, not stable at intestinal and colonic p^H and having absorption window in stomach [5]. Floating microspheres are gastroretentive DDS formulated by non effervescent approach. Microspheres size range is 1-1000µm. Floating microspheres are spherical and free flowing powder particles [6]. When microsphere particle come in contact with gastric contents the polymers hydrate to form a colloidal gel barrier that restricts both the fluid penetration rate into the device and drug release [7]. As the external dosage form surface dissolves, the gel layer is maintained by the hydration of the next hydrocolloid layer [8]. The air trapped by the swollen polymer lowers the density and leads to buoyancy to the microspheres. Minimum gastric fluid is needed to permit attainment of buoyancy [9].

Peptic ulcer damages the inner lining of the stomach, duodenum etc. Nizatidine is a competitive, reversible inhibitor of H_2-receptors, mainly in the gastric parietal cells. It is used for the treatment of gastric and duodenal ulcers. Nizatidine had a half-life of 1-2 h. The Nizatidine bioavailability is around 85% with slight inter subject variations [10]. Nizatidine undergoes hepatic metabolism by N_2-monodes-methylnizatidine, N_2-oxide, S-oxide.

In present research study an attempt has been made to design and develop gastroretentive Nizatidine floating microspheres for controlled drug delivery in stomach. Effect

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of polymer and their concentration in formulation will be studied.

MATERIALS AND METHODS

Materials

Nizatidine was obtained from Dr. Reddy's laboratories, Hyderabad., India as a gift sample. Sodium alginate and sodium bicarbonate was purchased from Pruthvi Chemicals, Mumbai, India. Calcium chloride was obtained from SD Fine ltd, Mumbai., India. HPMC K4M and HPMC K15M, HPMC K100M were purchased from Rubicon Labs, Mumbai, India. Remaining all chemicals used in this research study was of analytical grade.

Formulation of Nizatidine Floating microspheres

Nizatidine Microspheres was prepared by using various excipients includes sodium alginate as microsphere core forming agent, HPMC K4M, HPMC K15M and HPMC K100M as rate controlling agent, sodium bicarbonate as gas generating agent, and calcium chloride as cross linking agent.

Floating microspheres Preparation

Nizatidine Microspheres were formulated by ionotropic gelation technique mentioned in Table 1. Initially, 2% sodium alginate solution was prepared by dissolving in distilled water and stirred thoroughly by magnetically. On complete solution, accurately weighed quantity of drug followed by HPMC K4M, K15M and HPMC K100M and sodium bicarbonate carbonate of different weights were added to the above dispersion. Then the above dispersion was stirred at 500rpm, maintained room temperature. The mixture was sonicated for 30min to eliminate air bubbles that may have been formed during the stirring process. The homogenous dispersion was extruded using a 20G needle fitted with a 10ml syringe into 100ml of 1% of calcium chloride solution, being stirred at 100rpm for 10min into the gelation medium. Then microspheres were collected, washed with distilled water and oven dried at 60°C [11].

Evaluation parameters

Micromeritic properties

The characterization of prepared microspheres were

Table - 1

Formulation trials of Nizatidine Floating microspheres

Formulation code	Nizatidine (mg)	Sodium alginate (%)	HPMCK4M (mg)	Sodium bicarbonate (mg)	Calcium Chloride (%)
F1	150	2	150	25	4
F2	150	2	200	50	4
F3	150	2	250	75	4
F4	150	2	300	100	4
F5	150	2	350	120	4
F6	150	2	400	150	4
Formulation code	**Nizatidine (mg)**	**Sodium alginate (%)**	**HPMC K15M (mg)**	**Sodium bicarbonate (mg)**	**Calcium chloride (%)**
F7	150	2	150	25	4
F8	150	2	200	50	4
F9	150	2	250	75	4
F10	150	2	300	100	4
F11	150	2	350	120	4
F12	150	2	400	150	4
Formulation code	**Nizatidine (mg)**	**Sodium alginate (%)**	**HPMC K100M (mg)**	**Sodium bicarbonate (mg)**	**Calcium chloride (%)**
F13	150	2	150	25	4
F14	150	2	200	50	4
F15	150	2	250	75	4
F16	150	2	300	100	4
F17	150	2	350	120	4
F18	150	2	400	150	4

carried out for particle size, angle of repose, bulk density, tapped density, carr's index and % buoyancy [12]. The prepared floating microspheres were also evaluated for swelling index [13], % yield [14], entrapment efficiency [15], and test for buoyancy [16].

In vitro drug release

Nizatidine floating microspheres release studies of were conducted in 900ml of simulated gastric fluid (0.1N HCl pH 1.2) at 37±0.5°C by using USP dissolution apparatus II. Accurately weighed quantity of 100mg floating microspheres was transferred into 900ml of 0.1NHCl medium and stirring at 100rpm. Aliquots of samples were withdrawn at prespecified time intervals, filtered and diluted with similar medium finally assayed at 224nm using double beam spectrophotometer. The samples withdrawn were replaced with same dissolution medium and all the samples were analyzed in triplicate [17].

Release order kinetics

Drug release data of optimized floating microspheres formulation were fitted to various kinetic models to reveal the drug release mechanism from the microspheres. Those consist of Zero order, first order, Higuchi model and Korsmeyer-Peppas exponential equation and r^2 values were determined.

Drug-excipient compatibility studies

Fourier Transform Infrared Spectroscopy [18] and scanning electron microscopy (SEM) [19] studies were performed.

Stability studies

Optimized formulation such as F17 floating microspheres were subjected to stability testing at 40°C ± 2°C/75% RH ± 5% RH for 6months using stability chamber (Thermo Lab, Mumbai). Samples were withdrawn at predetermined intervals 0, 30, 60, 120, and 180days period according to ICH guidelines. Various *in vitro* parameters like % yield, entrapment efficiency and *in vitro* release studies were determined [20].

RESULTS AND DISCUSSION

Floating microspheres

Figure 1: Nizatidine Floating Microspheres

Table 2
Micromeretic properties of Nizatidine floating microspheres

Formulation Code	Particle Size (µm)	Bulk density	Tapped density	Angle of repose	Carr's Index (%)	% Buoyancy
F1	55.45±0.04	0.59 g/ml	0.58 g/ml	27˚.93	14.56	50.13
F2	60.12±0.08	0.66 g/ml	0.59 g/ml	23˚.91	9.34	64.42
F3	65.29±0.13	0.74 g/ml	0.62 g/ml	29˚.67	8.34	78.86
F4	73.43±0.04	0.76 g/ml	0.73 g/ml	30˚.54	13.36	69.53
F5	62.35±0.04	0.59 g/ml	0.57 g/ml	27.94	8.12	91.24
F6	79.67±0.09	0.89 g/ml	0.83 g/ml	30˚.15	9.23	67.12
F8	75.45±0.09	0.67 g/ml	0.72 g/ml	25˚.54	13.95	90.17
F9	55.23±0.14	0.51 g/ml	0.63 g/ml	22˚.91	10.32	65.08
F10	63.22±0.11	0.79 g/ml	0.75 g/ml	23.70	11.04	52.05
F11	83.34±0.10	0.68 g/ml	0.65 g/ml	30˚.24	12.34	66.74
F12	78.45±0.21	0.67 g/ml	0.55 g/ml	22˚.91	10.98	87.29
F13	65.32±0.09	0.82 g/ml	0.82 g/ml	25˚.54	13.95	70.18
F14	55.23±0.14	0.56 g/ml	0.63 g/ml	22˚.91	10.32	75.30
F15	73.22±0.11	0.72 g/ml	0.77 g/ml	21.70	8.08	75.64
F16	81.34±0.10	0.68 g/ml	0.65 g/ml	30˚.24	12.34	80.47
F17	50.67±0.13	0.47 g/ml	0.51 g/ml	20˚.74	7.67	94.23
F18	74.35±0.32	0.80 g/ml	0.72 g/ml	29˚.67	11.43	85.16

The particle size, bulk density, tapped density, angle of repose, Carr's index and % buoyancy were determined and found to be within the limits. The results are summarized in Table 2.

Entrapment efficiency, % yield and swelling index

The % yields ranged from 62.75% to 95.47% with the % entrapment efficiency being between 65.27% to 93.62%. The swelling index results from 69.27% to 92.13%. The better results were observed in F17 formulation with HPMC K100M as rate retarding polymer (Table 3).

Table - 3

% yield, % swelling index, and entrapment efficiency of Nizatidine Floating microspheres formulations

Formulation Code	Percentage Yield (%)	Swelling index (%)	Entrapment Efficiency (%)
F1	90.35±0.12	82.24±0.24	70.23±0.31
F2	84.35±0.35	78.24± 0.16	89.14±0.22
F3	77.95±0.27	80.15±0.31	87.63±0.17
F4	92.45±0.21	70.51± 0.28	83.45±0.34
F5	68.75±0.32	87.31±0.25	78.29±0.12
F6	83.92± 0.28	80.19±0.17	67.83±0.35
F7	65.45±0.19	76.17± 0.23	73.16±0.30
F8	74.35±0.17	82.93±0.36	65.27±0.21
F9	88.65±0.36	85.31±0.24	78.13±0.15
F10	78.35± 0.33	69.27± 0.19	75.52±0.28
F11	86.98±0.29	89.11±0.33	82.94±0.11
F12	91.23±0.12	83.34±0.27	71.11±0.32
F13	62.75± 0.25	73.92±0.12	78.25±0.33
F14	82.34±0.31	88.92±0.26	75.16±0.14
F15	76.95±0.11	81.62±0.31	70.19±0.26
F16	85.45±0.24	77.24±0.32	68.10±0.15
F17	95.47±0.36	92.13±0.17	93.62±0.29
F18	80.42±0.29	79. 19±0.30	84.73±0.13

Table - 4

***In vitro* Cumulative % drug release of Nizatidine floting microspheres from F1 to F6 and Marketed product**

Time (h)	F1	F2	F3	F4	F5	F6	Marketed product
0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
1	11.06±0.15	12.22±0.14	12.98±0.23	13.54±0.18	13.78±0.14	13.05±0.12	94.53±0.26
2	18.07±0.11	21.32±0.22	25.52±0.16	24.76±0.21	26.19±0.17	25.24±0.15	--
4	33.06±0.10	38.34±0.16	39.75±0.15	37.32±0.12	39.60±0.23	38.56±0.12	--
6	50.35±0.13	52.42±0.09	48.72±0.17	52.75±0.39	53.50±0.19	54.60±0.20	--
8	64.31±0.22	65.62±0.11	63.61±0.10	68.54±0.17	65.56±0.13	69.78±0.23	--
10	78.03±0.31	68.86±0.17	72.43±0.18	83.81±0.31	75.20±0.19	74.76±0.16	--
12	80.42±0.19	82.42±0.12	84.18±0.14	87.16±0.17	88.41±0.21	90.64±0.17	--

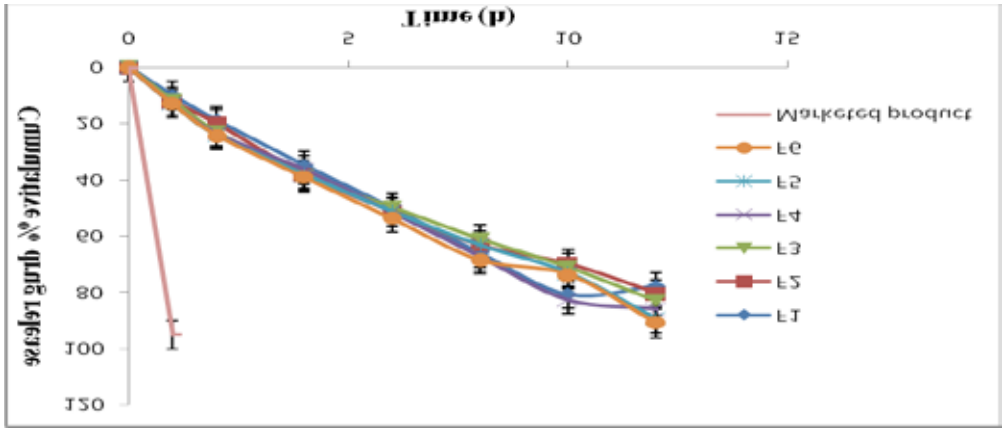

Figure 2: *In vitro* Cumulative % drug release of Nizatidine floting microspheres F1 to F6 and marketed product

Table - 5
***In vitro* Cumulative % drug release of Nizaidine floating microspheres formulation from F7 to F12**

Time (h)	F7	F8	F9	F10	F11	F12
0	0±0	0±0	0±0	0±0	0±0	0±0
1	11.15±0.16	12.34±0.15	13.42±0.12	14.19±0.12	15.09±0.13	11.92±0.13
2	24.63±0.09	25.76±0.13	25.85±0.14	26.42±0.23	25.52±0.23	24.92±0.11
4	38.48±0.18	38.96±0.12	45.76±0.22	37.20±0.16	38.62±0.16	40.61±0.15
6	53.61±0.12	48.21±0.14	52.64±0.23	52.37±0.19	56.55±0.11	55.89±0.16
8	64.54±0.17	63.22±0.16	63.32±0.12	64.30±0.25	69.82±0.17	68.32±0.12
10	68.89±0.13	71.42±0.18	71.62±0.18	68.91±0.26	82.90±0.19	74.29±0.13
12	83.92±0.16	85.72±0.15	88.73±0.13	84.43±0.21	86.11±0.32	89.72±0.14

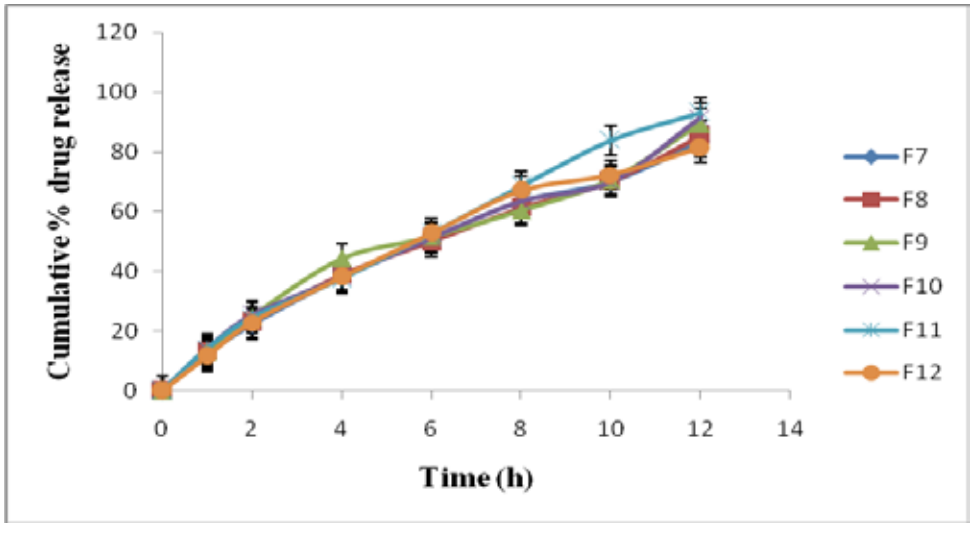

Figure 3: *In vitro* Cumulative % drug release of Nizatidine floating microspheres from F7 to F12

Table - 6
***In vitro* Cumulative % drug release of Nizaidine floating microspheres formulation from F13 to F18**

Time (h)	F13	F14	F15	F16	F17	F18
0	0±0	0±0	0±0	0±0	0±0	0±0
1	10.83±0.13	11.042±0.12	12.65±0.21	14.87±0.32	15.16±0.13	13.02±0.14
2	18.32±0.16	21.63±0.20	24.15±0.16	25.52±0.34	25.96±0.11	23.06±0.13
4	34.06±0.12	39.24±0.15	37.83±0.23	38.55±0.21	39.03±0.27	38.02±0.16
6	51.74±0.11	52.36±0.17	48.93±0.15	53.52±0.32	54.76±0.22	51.02±0.26
8	67.26±0.23	64.72±0.11	63.09±0.10	68.43±0.18	67.82±0.12	65.02±0.23
10	78.91±0.26	68.02±0.13	73.76±0.13	78.80±0.32	79.71±0.31	70.62±0.11
12	80.21±0.14	81.97±0.23	82.17±0.27	84.17±0.22	96.54±0.72	86.32±0.23

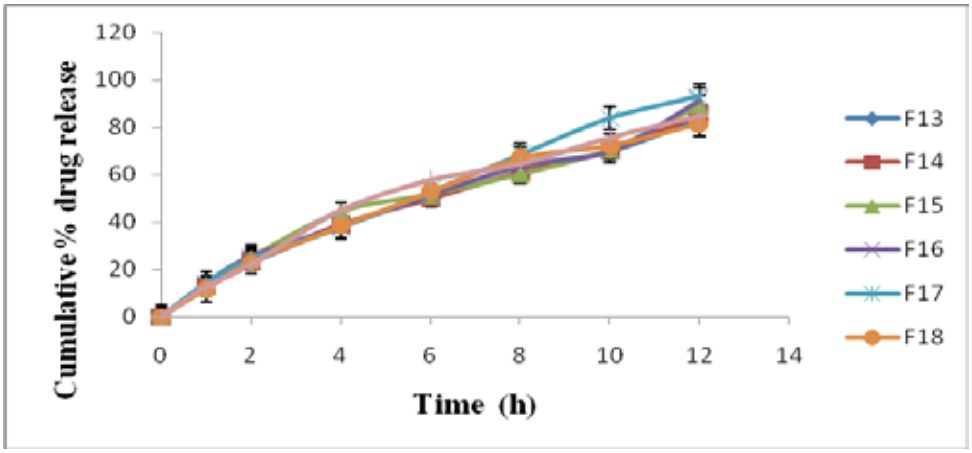

Figure 4: *In vitro* Cumulative % drug release of Nizatidine floating microspheres from F13 to F18

***In vitro* drug release studies**

The drug release from the microspheres was controlled over a period of 12h at pH 1.2 as mentioned in the Tables 4, 5 & 6 and in Figures 2, 3 & 4. The Cumulative % drug release of optimized formulation F17 was found to be 96.54±0.72% at the end of 12h where as marketed product noted 94.53±0.26% within 1h.

Mathematical modeling of optimized formulation (F17)

The *in vitro* drug release profiles were fitted to several kinetic models and release data followed by their R^2 and n values shown in the Table 7. The optimized formulation was best fitted in Zero Order and Korsmeyer-Peppas. The optimized formulation n value was 0.827 indicating non Fickian (anomalous) transport thus it projected that delivered its active ingredient by coupled diffusion and erosion. The marketed conventional formulation followed the first order kinetics indicating drug release is directly proportional to the concentration of drug.

Table - 7
Release kinetics of optimized formulation of floating microspheres

Formulation code	Zero order	First order	Higuchi	Korsmeyer-Peppas	
	R^2	R^2	R^2	R^2	n
F17	0.989 ±0.0121	0.926 ±0.0125	0.971 ±0.0231	0.993 ±0.0352	0.827
Marketed product		0.894±0.0216			

Drug excipient compatibility studies

FTIR spectroscopy of Nizatidine microspheres

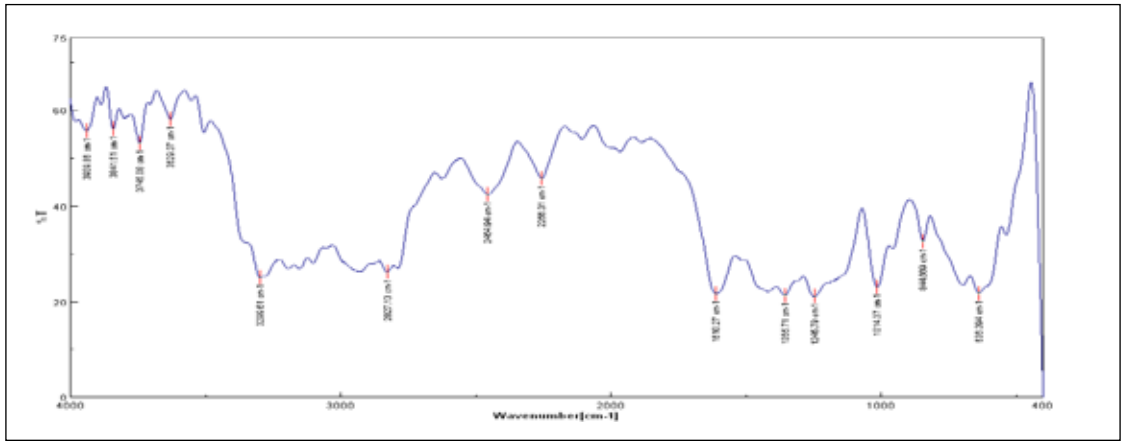

Figure 5: FTIR spectrum of pure drug Nizatidine

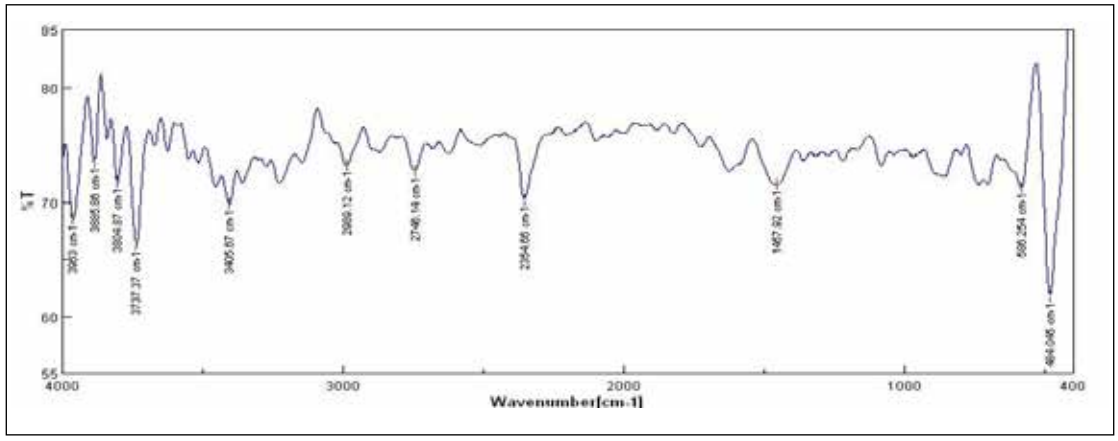

Figure 6: FTIR Spectrum of Floating Nizatidine microspheres

The FTIR spectrum of pure drug (Figure 6) showed characteristic sharp peaks at 3421cm^{-1}(C-N stretch), 2951cm^{-1}(C-H stretch),1436cm^{-1}(C=H deformation in NCH,CH), 1500cm^{-1}(CH & OCH groups),1587cm-^{1}(Conjugated with NO),1419cm^{-1}for CH_2 bond. There were no new significant bonds observed in the pure drug (Figure 5) and optimized formulation (Figure 6), which indicates that no interaction observed between the drug and excipients.

SEM studies of Nizatidine microspheres

The microspheres surface was rough and spherical in shape as seen in Figure 7. The surface of the Nizatidine microspheres was rough due to higher concentration of drug consistently discreted at the molecular level in the matrices.

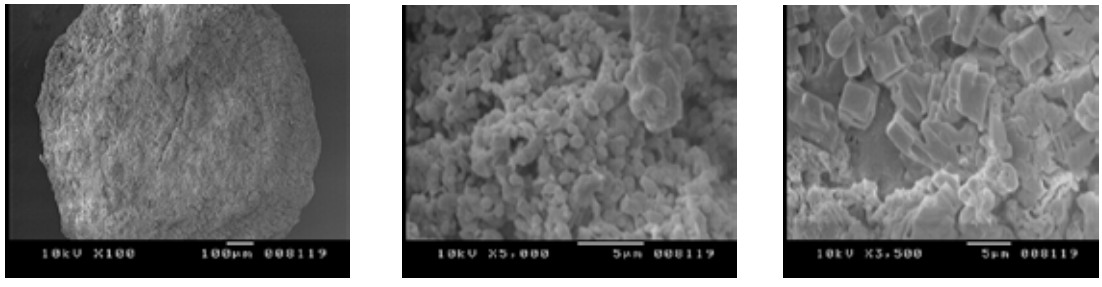

Figure 7: Scanning electron micrographs of optimized floating microspheres

Stability studies

Stability studies of optimized Nizatidine microspheres as per ICH guidelines was carried out for 6months at 40°C ± 2°C/75% RH ± 5% RH. At predetermined time intervals samples were withdrawn and subjected to % yield, entrapment efficiency and *in vitro* drug release analysis. Significant change was not observed in results before and after stability studies. Indicating the optimized formulation (F17) was stable.

CONCLUSION

From the above results, it could be concluded that Nizatidine microspheres prepared by ionotropic gelatin method showed controlled release effect compared to marketed product. The prepared microspheres were evaluated for micromeritic properties, particle size, % buoyancy, swelling index, % yield, drug entrapment, and drug release and stability studies. Among all the formulations F17 was selected as optimized formulation based on the physicochemical and release studies. The *in vitro* release study of formulation F17 showed 96.54±0.72%, after 12h. Optimized formulation best fit with zero-order and Korsmeyer-peppas model and the '*n*' value of it was found to be 0.827 indicating that the drug release was followed by non-fickian (anomalous) diffusion. FTIR and scanning electron microscopy revealed no drug excipient interaction takes place. On the basis of results, floating microspheres were of good candidate for targeting to GIT. Nizatidine floating microspheres can be successfully used for controlled drug release to the gastric mucosa in treatment of upper GIT infection.

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Evaluation of Analgesic and Anti-Inflammatory Activity of Selected Plants with Individual and Polyherbal Extraction

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ABSTRACT

Current study was undertaken to investigate the analgesic and anti-inflammatory effects of root and leaf of the *Bauhinia rufescens Lam*, leaves of *Cassia auriculata L, leaves* and flowers of *Mimosa pudica L* by Maceration method. For evaluating of analgesic and anti-inflammatory activity, we used Acetic Acid Induced Writhing Method, Eddy's Hot Plate Method, Carrageenan induced hind paw oedema, Formalin induced paw oedema model. Results of the phytochemical screening show the presence of alkaloids, flavonoids and triterpenoids in the extract. The PHME 400 mg/kg have exhibited significant increase in reaction time in Eddy's hot plate method in mice at different time intervals, exhibited significant increase percentage of inhibition in writhing method in mice at different time intervals. Exhibited a significant reduction in paw oedema volume in Carrageenan induced paw oedema and exhibited a significant reduction in paw oedema volume in Formalin induced paw oedema in rats at different time intervals. Conclusion the data obtained also suggest that the anti-inflammatory and analgesic effects of the extract may be mediated via both peripheral and central mechanisms. The role of alkaloids, flavonoids and triterpenoids will evaluate in future studies.

Keywords: Analgesic, Anti-inflammatory activity, *Bauhinia rufescens Lam, Mimosa pudica L*, *Cassia auriculata L,*

INTRODUCTION

Inflammation is considered as a primary physiologic defence mechanism that helps body to protect itself against infection, burn, toxic chemicals, allergens or other noxious stimuli. An uncontrolled and persistent inflammation may act as an etiologic factor for many of these chronic illnesses [1]. Although it is a defence mechanism, the complex events and mediators involved the inflammatory reaction can induce, maintain or aggravate many diseases [2]. Currently used anti-inflammatory drugs are associated with some severe side effects. Therefore, the development of potent anti-inflammatory drugs with fewer side effects is necessary. In recent years, schiff bases are widely used in formulating various types of drugs for their diversive biological activities [3, 4]. *Bauhinia rufescens Lam. (Fabaceae) i*s a shrub usually 1-3 m high and sometimes reaching 8 m. The bark is ash-grey, smooth and very fibrous. The leaves are very small with greenish-yellow to white and pale pink flowers. Fruits aggregated with 4-10 seeds each [5] *Cassia auriculata L. (Fabaceae)* is a much branched shrub with smooth cinnamon brown bark and closely pubescent branchlets. The leaves are alternate, stipulate, paripinnate compound, very numerous, closely placed. Its flowers are irregular, bisexual, bright yellow and large. The fruit is a short legume, oblong, obtuse, tipped with long style base, flat, thin, papery, undulately crimpled, pilose, pale brown [6]. *Mimosa pudica L. (Fabaceae)* it is a prickly perennial herb that grows up between 0.5- 0.9m high; leaves are bi-pinnate, opposite, compound in nature and sensitive to touch; flowers are axillary positioned, clustered in fluffy balls, radially symmetrical and campanulate; fruits occur in aggregate of 2-8 pods [7].

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MATERIALS AND METHODS

Collection of plant and authentication

Root and leaf of the *Bauhinia rufescens Lam*, leaves of *Cassia auriculata L, leaves and flowers of Mimosa pudica L* was procured from Madhavachetti botanical garden, Thirupathi and was authenticated by Dr. K. Madhavachetti, Assistant Professor in Department of Botany at Sri Venkateswara University, Tirupathi.

Extraction by Simple Maceration

Root and leaf of the *Bauhinia rufescens Lam*, leaves of

Cassia auriculata L, leaves and flowers of Mimosa pudica L individually and poly herbal of both plant materials are made into powder and then gone for the Maceration with sufficient quantity of methanol for 7 days. During maceration, it was shaked twice daily. On 7th day it was filtered and the filtrate was concentrated. The remaining solvent was evaporated by heating on a water bath (50°C) to get methanolic extract and the extract was stored in desiccator.

Preliminary Phytochemical Screening

The crude methanolic extract of Root and leaf of the *Bauhinia rufescens Lam*, leaves of *Cassia auriculata L, leaves and flowers of Mimosa pudica L* individually and poly herbal extraction were tested for its different chemical groups such as alkaloids, flavanoids, tannins, steroids, saponins ,fixed oils, gums and mucilages, tri-terpenoids, carbohydrates and glycosides, phytosterols.

Experimental Animals:

Albino mice of either sex weighing between 16-25 g, Wister rats of either sex weighing between 180-250g. Then the animals were acclimatized for 7 days under standard husbandry conditions. Room temperature 26 ± 2^0 C, Relative humidity 45-55%, Light/ dark cycle - 12: 12hr, all animal studies were performed as per the guidelines of CPCSEA and Institutional Animal Ethical Committee (IAEC). CPCSEA Approval Number: **VIP/GVS/Ph.D./IAEC/2016-17/15.**

Analgesic Activity:

Acetic Acid Induced Writhing Method [8]

This study was carried out using acetic acid induced abdominal writhing reflex pain model. Thirty five mature mice were randomly divided into 4 groups (1-4) of 6 mice per group, fasted for 12 hours and treated as follows, Group 1 (control group) received 10ml/kg normal saline, group 2 (standard) received 30mg/kg of Diclofenac sodium; groups 3, 4, 5 and 6 received MEBR (400mg/kg), MECA (400mg/kg).MEMP (400mg/kg) and PHME (400mg/kg). respectively using gastric gavage. One hour after drug and extract administration, 0.6% glacial acetic acid (10ml/kg) was administered intraperitoneally (I.P) to all the mice to induce abdominal contractions or writhings. The analgesic effect was assessed in each mouse for 30, 60,120 minutes and recorded. The degree of analgesia was calculated using the following formula.

$$\frac{\text{Mean of control group- Mean of treated group}}{\text{Mean of control group}} \times \frac{100}{1}$$

This represents the percentage of inhibition of writhing

Eddy's Hot Plate Method: (Thermal stimulus) [9]

In the hot plate method albino mice (18-28) were divided into four groups each consisting of six animals. All the animal selected for the studied were under gone the normal basal reaction time and then separated as different groups like Group I served as a control (Formailin 1% W/V vehicle), Group II served as a standard (received Diclofenac sodium 30mg/kg) while the Group III, IV, V and VI received the MEBR (400mg/kg), MECA (400mg/kg). MEMP (400mg/kg) and PHME (400mg/kg).. All animals were lowered onto the surface of a hot plate (50±1.00ºC) enclosed with cylindrical glass and the time for the animal to jump or lick the fore limb was noted as the reaction time (RT). Cut off time in the absence of a response was 15 sec to prevent the animals from being burnt. The observations were made before and after administration of respective drugs at 60 min, 120 min, 180 and at the end of 240 min.

Anti-inflammatory activity

Carrageenan induced hind paw oedema [10]

Albino rats of either sex weighing 150-200 grams were divided into four groups of six animals each. The dosage of the drugs administered to the different groups was as follows. Group I - Control (normal saline 0.5 ml/kg), Group II – Diclofenac sodium (30 mg/kg, p. o.), Group – III, IV, V, VI, MEBR (400mg/kg), MECA (400mg/kg). MEMP (400mg/kg) and PHME (400mg/kg), all the drugs were administered orally. Diclofenac sodium served as the reference standard anti inflammatory drug. After one hour of the administration of the drugs, 0.1 ml of 1% W/V carrageenan solution in normal saline was injected into the sub plantar tissue of the left hind paw of the rat and the right hind paw was served as the control. The paw volume of the rats were measured in the digital plethysmograph (Ugo basile, Italy), at the end of 0 min., 60min., 120min., 180min. and 240min. The percentage increase in paw oedema of the treated groups was compared with that of the control and the inhibitory effect of the drugs was studied. The relative potency of the drugs under investigation was calculated based upon the percentage inhibition of the inflammation.

Formalin induced paw oedema model in rats: [11, 12]

Experimental Procedure:

Male wister rats (150-200 g) of 6 groups were housed as groups of 6, fasted overnight prior to and during the experiment but have free access to water. Group A was served as toxicant control treated with toxicant Formalin; group B with Diclofenac (30 mg/kg i.p.) that served as standard. Groups C, D, E and F MEBR (400mg/kg), MECA (400mg/kg).MEMP (400mg/kg) and PHME (400mg/kg), Respectively, The rats of groups B, C, D, E and F were administered with 1% of Formalin into sub plantar region of right hind paw of rats 1hr after administration of Diclofenac/ extracts. Immediately thereafter the oedema volumes of the injected paws were measured plethysmographically at prefixed time intervals.

For comparison purpose, the volume of oedema was measured at prefixed time intervals. The difference between paw volumes of the treated animals was measured and the mean oedema volume was calculated. Percentage reduction in oedema volume was calculated by using the formula

$$\text{Percentage reduction} = \frac{Vo - Vt}{Vo} \times 100$$

Where, Vo = Volume of the paw of control at time't'.

Vt = Volume of the paw of drug treated at time't'.

RESULTS AND DISCUSSION

Analgesic activity by Eddy's hot plate test in mice:

The PHME 400 mg/kg have exhibited significant increase in reaction time in Eddy's hot plate method in mice at different time intervals. Results were tabulated in **Table 1.** Diclofenac sodium (30mg/kg) was used as standard and it has significantly increased the reaction time at the end of 1st and 2nd h which was found to be a time dependent effect. During 1st h of the study MEBR, MECA, MEMP and PHME the reaction time was3.21±0.17, 3.3 0±0.17, 3.41±0.18, 3.50±0.17 seconds respectively. During 2ndh of the study MEBR, MECA, MEMP and PHME the reaction time was 4.34±0.21, 4.74±0.19, 5.44±0.21 5.74±0.19 respectively which was time dependent effect. During 3rd h of the study MEBR, MECA, MEMP and PHME the reaction time was 6.17±0.23, 7.11±0.10, 8.10±0.13 and 8.17±0.10respectively. During 4th h of the study MEBR, MECA, MEMP and PHME the reaction time was 7.06±0.19, 7.46±0.16, 7.51±0.29, and 7.81±0.16 respectively. Diclofenac sodium showed a significant reaction time during 1st and 2nd hr whereas test groups have shown during 2nd and 4th hr and the results were tabulated and represented graphically. Showed in **figure 1 .**

Table - 1

Analgesic effect of MEBR, MECA, MEMP and PHME in Eddy's hot plate method in mice

Groups	Treatment	Hot plate reaction time			
		60 min	120 min	180 min	240 min
Control	Formalin (1%w/v)	2.512±0.3182	2.386±0.381	2.176±0.36	2.07±0.351
Standard	Diclofenac sodium (30mg/kg)	3.545±0.470	6.076±1.14**	9.93±0.45**	7.93±0.32**
MEBR	MEBR 400mg/kg	3.21±0.17	4.34±0.21	6.17±0.23**	7.06±0.19**
MECA	MECA 400 mg/kg	3.30±0.17	4.74±0.19*	7.11±0.10**	7.46±0.16**
MEMP	MEMP 400mg/kg	3.41±0.18	5.44±0.21	8.11±0.13**	7.51±0.29**
PHME	PHME 400mg/kg	3.50±0.17	5.74±0.19*	8.17±0.10**	7.81±0.16**

Values are expressed in mean±SEM, where n = 6, Significant at P < 0.05, 0.01** and 0.001***, compared to control group.*

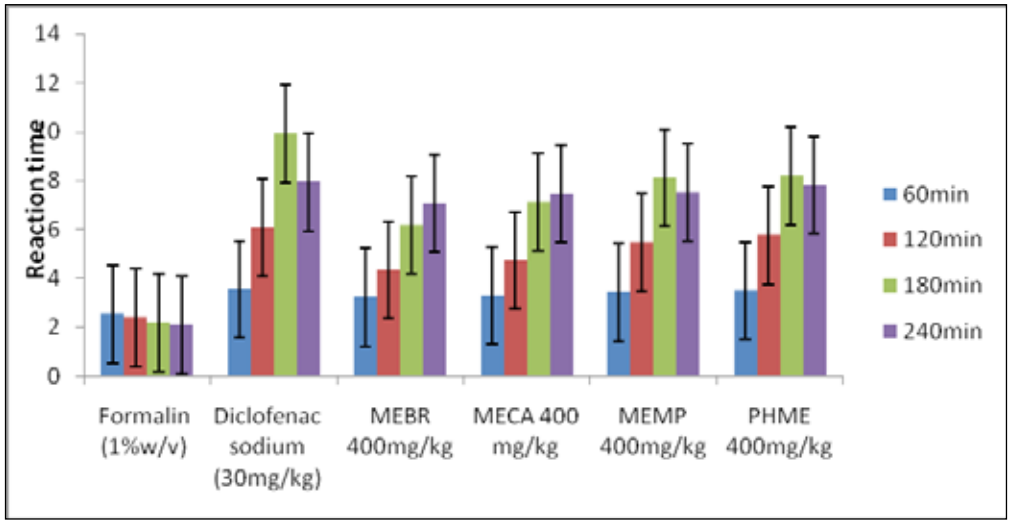

Figure 1: Analgesic effect of MEBR, MECA, MEMP and PHME in Eddy's hot plate method in mice

Acetic acid induced writhing test in mice:

The PHME 400 mg/kg exhibited significant increase percentage of inhibition in writhing method in mice at different time intervals. Results were shown in **Table 2.** Diclofenac sodium (30mg/kg) was used as standard and it has significantly increased the percentage inhibition of writhing by 89.7% at the end of 2 hr which was found to be a time dependent effect. During first 30 min of the study MEBR, MECA, MEMP and PHME % of inhibition of writhing were 54.12, 49.5, 57.1, and 64.1 respectively. During 1hr of the study MEBR, MECA, MEMP and PHME the % of inhibition of writhing was 66.11, 62.5, 69.1, and 72.5 % respectively which was time dependent effect. During 2hr of the study MEBR, MECA, MEMP and PHME % of inhibition of writhing was 76.08, 73.7, 79, and 83.72% and the results were tabulated and graphically represented in **figure 2**.

Table - 3
MEBR, MECA, MEMP and PHME on Acetic acid induced writhing response in mice

Groups	Treatment	Number of writhing (Mean± SEM)			% Inhibition of Writhing		
		30 min	60 min	120 min	30 min	60 min	120 min
Group- I	Control 0.1ml	26.66±3.602	27.66±4.356	35.83±4.199	-	-	-
Group-II	Standard 30 mg/kg	13.33±1.33**	8.66±1.02**	3.66±1.25**	62.79	75.83	89.78
Group- III	MEBR 400mg/kg	12.22±1.81**	7.14±1.67**	2.57±1.34**	54.17	66.11	76.08
Group-IV	MECA 400mg/kg	14.5±0.99**	9.83±0.30**	5.83±1.40**	49.54	62.53	73.72
Group- IV	MEMP 400mg/kg	16.42±1.81**	12.14±1.87**	8.57±1.64**	57.13	69.12	79.04
Group-V	PHME 400mg/kg	14.5±0.99**	9.83±0.30**	5.83±1.40**	64.53	72.56	83.72

Values indicate mean± SEM (ANNOVA test followed by Dunnet's t- test)

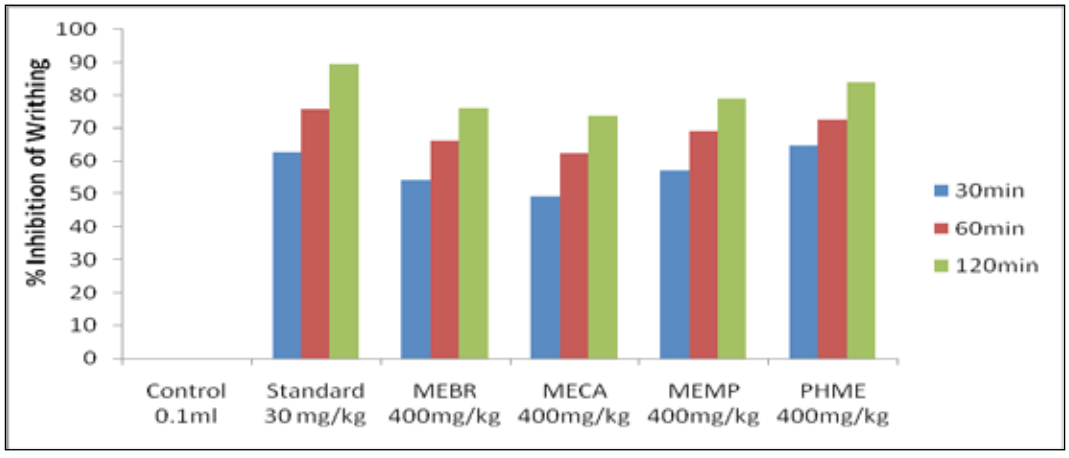

Figure 3: MEBR, MECA, MEMP and PHME on Acetic acid induced writhing response in mice

Anti-inflammatory:

Anti-inflammatory effect of PHME on paw volume in Carrageenan induced paw oedema in rats

The PHME 400 mg/kg exhibited a significant reduction in paw oedema volume in Carrageenan induced paw oedema in rats at different time intervals. Results are tabulated in **Table 3.** Diclofenac sodium (30 mg/kg) was used as standard reference and it has significantly reduced paw oedema volume by 17.81% at 1st h, 27.58% at 2nd h, 50.09% at 3rd h and74.13 % at 4th h, which was found to be a time dependent effect.

During 1st h of study MEBR, MECA, MEMP and PHME have significantly reduced oedema volume by 3.43, 10.72, 3.43, 7.72 % respectively noted as time dependent effect.

During 2nd h of study MEBR, MECA, MEMP and PHME have significantly reduced oedema volume by 13.9, 24.28, 15.11, 17.29 % respectively noted as time dependent effect.

During 3rd h of MEBR, MECA, MEMP and PHME have significantly reduced oedema volume by 32.07, 34.33, 38.08, 47.46 % respectively noted as time dependent effect.

During 4th h of study MEBR, MECA, MEMP and PHME have significantly reduced oedema volume 51.72, 56.89, 55.17, 60.34 % respectively which was recorded as time dependent effect and result are graphically represented in **Figure 3.**

Table - 4
Anti-inflammatory effect of MEBR, MECA, MEMP and PHME on paw volume in Carrageenan induced paw oedema in rats

Groups	Treatment	Paw oedema volume			
		60 min	120 min	180 min	240 min
Control	Carrageenan	0.466±0.042	0.4833±0.047	0.533±0.04	0.58±0.030
Standard	Diclofenac sodium (30mg/kg)	0.383±0.030	0.35±0.02236	0.266±0.033	0.15±0.02236
MEBR	400mg/kg	0.25±0.0228	0.2166±0.033	0.267±0.032	0.1833±0.030
MECA	400 mg/kg	0.216±0.017	0.266±0.034	0.25±0.012	0.15±0.012
MEMP	400mg/kg	0.45±0.0428	0.4166±0.047	0.366±0.033	0.2833±0.030
PHME	400mg/kg	0.416±0.047	0.366±0.033	0.35±0.022	0.25±0.022

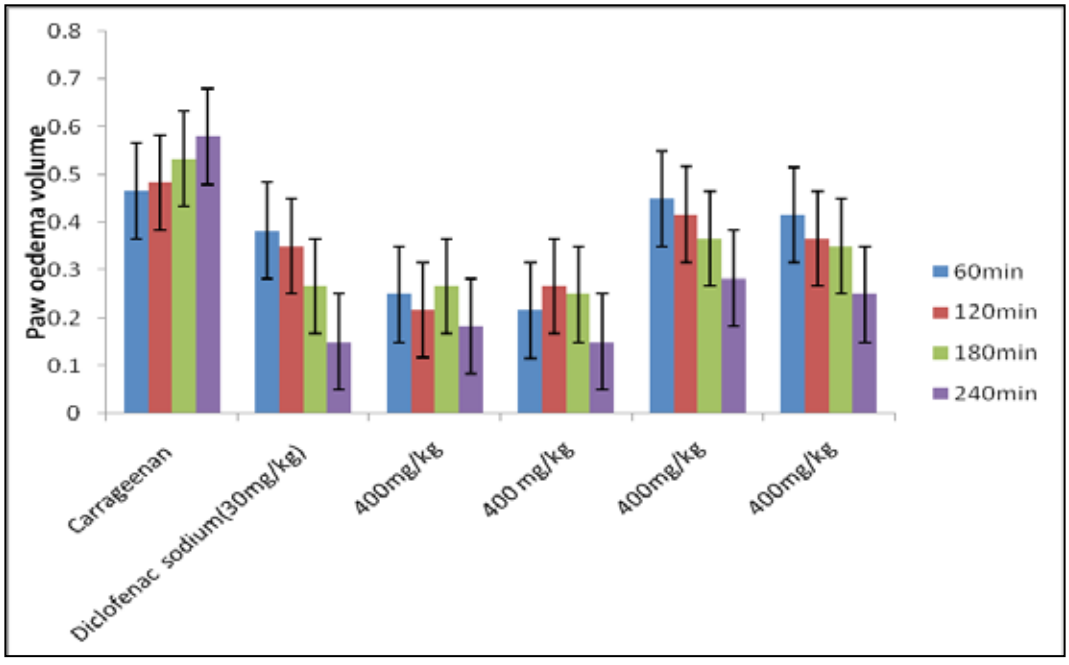

Figure 4: Anti-inflammatory effect of MEBR, MECA, MEMP and PHME on paw volume in Carrageenan induced paw oedema in rats

Anti-inflammatory activity of MEBR, MECA, MEMP and PHME in Formalin induced paw oedema model in rats:

The PHME with 400 mg/kg have exhibited a significant reduction in paw oedema volume in Formalin induced paw oedema in rats at different time intervals. Results are tabulated in **Table 4.** Diclofenac sodium (30 mg/kg) was used as standard reference and it has significantly reduced paw oedema volume by 30.55% at 1st h, 57.08% at 2nd h, 75.83% at 3rd h and 86.40% at 4th h, which was found to be a time dependent effect. During 1st h of study MEBR, MECA, MEMP and PHME have significantly reduced oedema volume by 13.1, 14.6, 16.77, and 21.29%, respectively noted as time dependent effect. During 2nd h of study MEBR, MECA, MEMP and PHME have significantly reduced oedema volume by 22.4, 25.7, 29.75, 45.49% respectively noted as time dependent effect. During 3rd h of study MEBR, MECA, MEMP and PHME have significantly reduced oedema volume by 52, 53.2, 60.22, 61.40% respectively noted as time dependent effect. During 4th h of study MEBR, MECA, MEMP and PHME have significantly reduced oedema volume by 55.5, 58.6, 65.25, 72.27% respectively which was recorded as time dependent effect and result are graphically represented in **Figure 4.**

Table - 5
Anti-inflammatory activity of MEBR, MECA, MEMP and PHME in Formalin induced paw oedema model in rats

Group	Treatment	Paw oedema volume			
		60 min	120 min.	180 min	240min
Control	Formalin(1%w/v)	0.46±0.02	0.52±0.03	0.58±0.02	0.60±0.02
Standard	Diclofenac sodium (30mg/kg)	0.39±0.02	0.36±0.01	0.29±0.03	0.23±0.02
MEBR	400mg/kg	0.42±0.02	0.40±0.02	0.39±0.01	0.36±0.01
MECA	400 mg/kg	0.45±0.02	0.42±0.02	0.38±0.01	0.33±0.02
MEMP	400mg/kg	0.37±0.02	0.32±0.02	0.26±0.01	0.26±0.01
PHME	400mg/kg	0.35±0.02	0.31±0.02	0.21±0.01	0.22±0.02

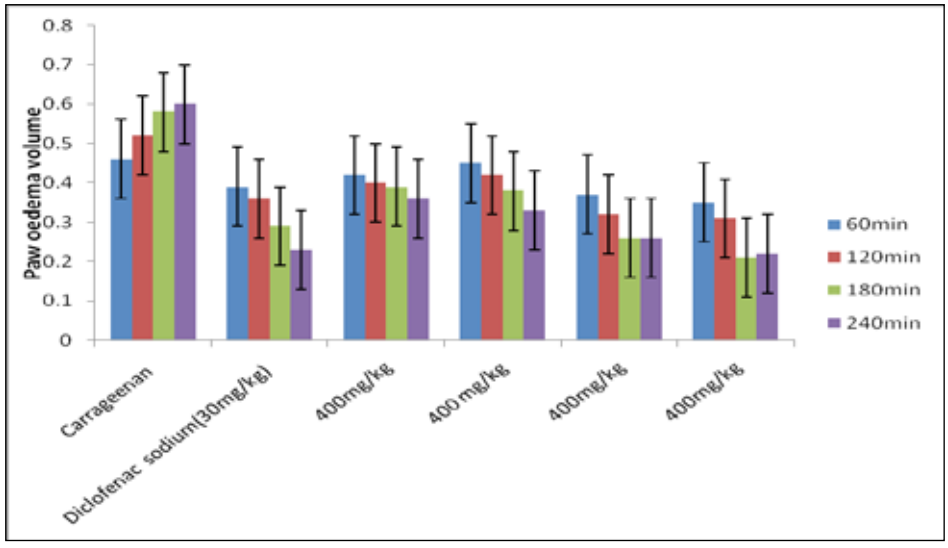

Figure 5: Anti-inflammatory activity of MEBR, MECA, MEMP and PHME in Formalin induced paw oedema model in rats

CONCLUSION

It is concluded that the aqueous extract of root and leaf of the *Bauhinia rufescens Lam*, leaves of *Cassia auriculata L, leaves and flowers of Mimosa pudica L* possesses analgesic and anti inflammatory properties, which are probably mediated via inhibition of prostaglandin synthesis as well as central inhibitory mechanisms. The extract will, therefore, be of potential benefit in the management of pain and inflammatory disorders.

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A New RP-HPLC Method Development and Validation for The Simultaneous Estimation of Febuxostat and Ketorolac in Bulk and Tablet Dosage Form

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ABSTRACT

A simple, fast, precise, selective and accurate RP-HPLC method was developed and validated for the simultaneous determination of Febuxostat and Ketorolac from bulk and formulations. Chromatographic separation was achieved isocratically on a Waters Novapak RP C_{18} column (150×3.9 mm, 4 µ particle size) using a mobile phase, Phosphate buffer and Methanol (adjusted to pH 5.8 with 1% Ortho-phosphoric acid) in the ratio of 40:60 v/v. The flow rate was 1.2 ml/min and effluent was detected at 321 nm. The retention time of Febuxostat and Ketorolac were 1.923 min and 3.104 min. respectively. Linearity was observed in the concentration range of 20-60 µg/mL and 5-15 µg/mL for Febuxostat and Ketorolac respectively with correlation coefficient 0.999 for both the drugs. Percent recoveries obtained for both the drugs were 100.16-101.49% and 99.35-101.75%, respectively. The method was validated according to the ICH guidelines with respect to specificity, linearity, accuracy, precision and robustness. The results were within the acceptance criteria. The proposed methods were found to be satisfactory and could be used for their routine analysis of Febuxostat and Ketorolac in their formulations.

Key words: RP-HPLC Method; UV-VIS detection; Febuxostat and Ketorolac; Tablet dosage forms.

INTRODUCTION

Febuxostat chemically is 2-[3-cyano-4-isobutoxy phenyl)-4-methyl-1, 3-thiazole -5-carboxylic acid) (molecular formula C16H16N2O3S)[FEB] [Figure-1]. It is a novel xanthine oxidase inhibitor indicated for the chronic management of hyperuricemia in patients with gout [1, 2, and 3]. FEB is a non-purine analogue inhibitor of both oxidized and reduced forms of xanthine oxidase.

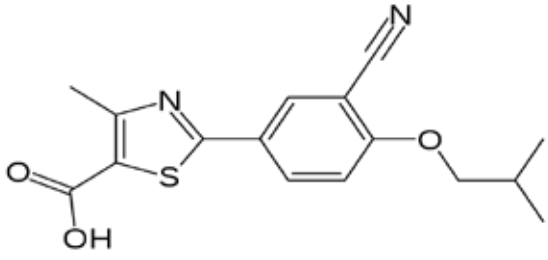

Figure 1: structure of Febuxostat

Ketorolac chemically is (±)-5-benzoyl-2,3-dihydro-1H-pyrrolizine-1-carboxylic acid,2- amino-2 (hydroxymethyl)-1,3- propanediol [4]. (Molecular formula C15H13NO3) [KET] [Figure-2]. It is a non steroidal anti inflammatory drug, when administered systemically has demonstrated analgesic, anti inflammatory and anti pyretic activity [5]. Ketorolac acts by inhibiting the bodily synthesis of prostaglandins [6, 7]

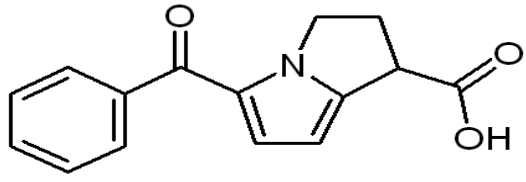

Figure 2: structure of Ketorolac

Literature survey reveals that few spectrophotometric [8] and HPLC [9] methods have been reported for simultaneous estimation of Febuxostat and Ketorolac. A successful attempt has been made for simultaneous determination of Febuxostat and Ketorolac in combined dosage form by using HPLC. Therefore, it was thought worthwhile to develop simple, accurate, precise and reliable RP-HPLC method for simultaneous estimation of both the

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drugs Febuxostat and Ketorolac in combined dosage form and validated in accordance with ICH guidelines [10]

EXPERIMENTAL

Materials and reagents

Pure API (Active Pharmaceutical Ingredient) sample of Ketorolac, 99%, Molecular Weight =255.26gms/mole was acquired from Mylan Laboratories Pvt. Ltd, Hyderabad, India and Febuxostat, 99%, Molecular Weight=316.37gms/mole was acquired from Sun pharmaceuticals private limited ,Chennai, India., Water HPLC grade (Millipore Corporation – Milli-Q), Ortho-phosphoric acid HPLC Grade (S.D Fine-Chem Ltd), Acetonitrile HPLC Grade (Qualigens Fine Chemicals Pvt Ltd), Methanol HPLC Grade (Millipore Corporation – Milli-Q), Sodium Di Hydrogen Orthophosphate AR grade (S.D Fine-Chem Ltd).

Method optimization

Buffer Preparation: Accurately weighed 1.19grams of Di Sodium hydrogen phosphate Dihydrate and 8.25gms of Potassium Dihydrogen Phosphate in HPLC water and dilute to 1000 ml with the same solvent , pH (5.8) was adjusted to with Ortho phosphoric acid, filtered through 0.45 µm nylon membrane filter and degassed.

Mobile Phase: Buffer and Methanol were mixed in the ratio of 40:60 v/v and sonicated to degas.

Preparation of working standard solution:

Aliquots ranging from 0.5mL to 1.5mL of KET and 0.2mL to 0.6mL were taken from working stock solution (100µg/ml-KET, 1000µg/ml-FEB) and diluted to 10mL with mobile phase to give final concentration of 5-15µg/ml of ketorolac and 20-60µg /ml of Febuxostat.

Preparation of sample drug solution for pharmaceutical formulations:

The marketed formulations containing 10mg of Ketorolac and 40 mg of Febuxostat was taken. From the above solution subsequent concentrations of 5-15µg/ml and 20 to 60µg/ml were prepared with mobile phase, sonicated and filtered through 0.45µ membrane filtered

Procedure for calibration curve:

The contents of the mobile phase were filtered before use through 0.45micron membrane and pumped from the respective solvent reservoirs to the column at a specified flow rate. Prior to injection of the drug solutions, the column was equilibrated for at least 30min with the mobile phase flowing through the system. The chromatographic separation was achieved using a mobile phase consisting of Buffer and Methanol at 40:60v/v the eluent was monitored using UV detector at a wavelength of 321nm. The column was maintained an ambient temperature (25^0c) and an injection volume of 20 µl of each of standard and sample solutions were injected into the HPLC system to get the chromatograms. The retention time, peak area of drug was recorded graph was plotted by taking concentration of the drug on x-axis and peak area on y-axis. (Table-1).

Recommended Procedure

After systematic and detailed study of the various parameters involved as described under the results and discussion in this chapter, the following procedure was adopted for the determination of Febuxostat & Ketorolac in bulk sample and pharmaceutical formulations.

Procedure

Initially the mobile phase was pumped for about 30 minutes to saturate the column thereby to set the baseline corrected. Then 20 µl of the standard and sample solutions were injected separately. A quantitative determination of the active ingredients was made by comparison of the peak area of the sample injection with the corresponding peak area of the standard injection. The amount of Febuxostat & Ketorolac present in the sample was calculated through the standard calibration curve.

Table - 1
Optimized chromatographic conditions

Parameter	Content
Column	An Novapak RP-C_{18} column (150x3.9 µm i.d;particle size 4 µm)
Mobile Phase	Phosphate buffer in water pH-5.8 adjusted with O-Phosphoric Acid: Methanol in the isocratic mode (40:60 v/v)
Flow Rate	1.2 ml/min
Run time	8 minutes
Temperature	Ambient
Injection Volume	20 µl
Detection & Wavelength	PDA Detector, 321 nm
Retention times	1.9302 minutes for Febuxostat and 3.104 minutes for Ketorolac

Analytical method validation

The appropriate wavelength in the UV-region (321 nm), was selected for the measurement of the active ingredients in the proposed method. The method was validated by linear fit curve and all the other parameters were calculated similar to the spectrophotometric method and were discussed in the following pages. The typical chromatogram indicating the separation of Febuxostat and Ketorolac with a Novapak-C_{18} RP column and mobile phase consisting of Buffer and Methanol were mixed in the ratio of 40:60 v/v and pH was adjusted to 5.8 with Ortho-Phosphoric acid.

Parameters Fixation

In developing this method, systematic study of the effects of various parameters were undertaken by varying one parameter at a time and controlling all other parameters. The following studies were conducted for this purpose.

Mobile Phase Characteristics

In order to get sharp peaks and baseline separation of the components, the author has carried out a number of experiments by varying different components like composition of organic phase in mobile phase, pH of the aqueous phase, total pH of the selected mobile phase, modifiers and flow rate by changing one at a time and keeping all other parameters constant respectively. The optimum conditions evolved from the above studies were incorporated in the recommended procedure.

Detection Characteristics

The schematic experimentation has been carried out to test whether Febuxostat and Ketorolac has been linearly eluted, from the column successively and systematically. In this method, different amounts of the active ingredients were taken and all the solutions were analyzed by respective procedures separately. Quantitative determinations were made by comparing the peak area from a sample injection to the corresponding peak area from the standard injection in the method.

RESULTS AND DISCUSSION

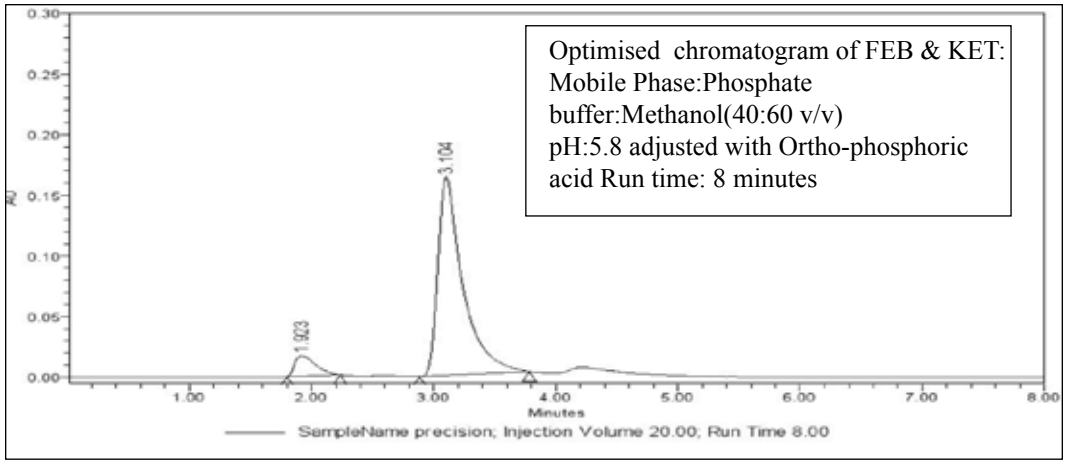

Figure 3: Optimized Chromatogram of Febuxostat and Ketorolac

The following parameters were used to validate the method for the proposed assay procedure of Febuxostat and ketorolac in pharmaceutical dosage forms.

Precision

The precision of the method was ascertained separately from the peak area obtained by actual determination of six replicas of a fixed amount of the drug and formulation. The HPLC systems were set up, describing chromatographic conditions, mentioned as above and following the system equilibration of then working standard solution containing 40µg/ml of Febuxostat and 10µg/ml of Ketorolac by injecting six times and recording the response peak areas.

The precision was repeated with the formulated sample for the same concentrations by injecting the working sample solutions containing 40µg/ml of Febuxostat and 10µg/ml of Ketorolac. The sample Febuxostat and Ketorolac was processed six times for the response of peak area. The % Relative Standard Deviation (RSD), were calculated and presented in **Tables: 2-3** respectively.

Acceptance criteria:

The individual assays of Febuxostat and Ketorolac should be not less than 98% and not more than 102% and %RSD of assay should be NMT 2.0% by both analysts.

Table -2
Precision of Recommended Procedure Using Standard Drugs (Febuxostat & Ketorolac)

S. No	Injection Number	Name of the Drug & Concentration (40 µg/mL)	Retention time in minutes	Peak Area	Name of the Drug & Concentration (10 µg/mL)	Retention time in minutes	Peak Area
1	1	Febuxostat	1.932	202686	Ketorolac	3.107	2341653
2	2	Febuxostat	1.923	201719	Ketorolac	3.104	2330075
3	3	Febuxostat	1.936	202770	Ketorolac	3.114	2327554
4	4	Febuxostat	1.934	205665	Ketorolac	3.114	2334942
5	5	Febuxostat	1.926	208243	Ketorolac	3.114	2335520
7	Mean		1.9302	204216.6		3.1106	2333949
8	Standard Deviation			2691.8			5447.697
9	% RSD			1.32			0.23

Table - 3
Precision of Recommended Procedure Using Sample (Febuxostat & Ketorolac)

S. No	Injection Number	Name of the Drug & Concentration (40 µg/mL)	Retention time in minutes	Peak Area	Name of the Drug & Concentration (10 µg/m)	Retention time in minutes	Peak Area
1	1	Febuxostat	1.997	217118	Ketorolac	3.210	2350782
2	2	Febuxostat	2.009	216128	Ketorolac	3.228	2304212
3	3	Febuxostat	2.002	214423	Ketorolac	3.221	2312475
4	4	Febuxostat	2.001	217780	Ketorolac	3.215	2305900
5	5	Febuxostat	2.008	217780	Ketorolac	3.214	2311894
7	Mean		2.0034	215547		3.2176	2317053
8	Standard Deviation			2218.636			19199.61
9	% RSD			1.03			0.83

Accuracy

To determine the accuracy of the proposed method, different amounts of bulk sample of Febuxostat and Ketorolac within linearity limits was taken and analyzed by the proposed method.

Calculations:

$$\frac{A_t \text{ X } W_s \text{ X Avg. } W_t \text{ X P}}{A_s \text{ X } W_t \text{ X Claim. Wt}}$$

Where,

A_t = Average area due to Formulation peak in sample preparation

A_s = Average area due to peak in the Standard preparation

W_s = Weight of the working standard

W_t = Weight of the sample Formulation

P = Potency of the working standard

Avg.Wt = Average Weight.

Assay Results: (Febuxostat)

$$\frac{20776210}{206453} \times \frac{3.0}{100} \times \frac{10}{10} \times \frac{10}{27.8} \times \frac{99.9}{1.2} \times \frac{111.23}{100} \times 100 = 100.56\%$$

Assay Results : (Ketorolac)

$$\frac{2286541}{2302475} \times \frac{10}{10} \times \frac{1.2}{10} \times \frac{10}{27.8} \times \frac{10}{1.2} \times 100 \times \frac{111.23}{40} \times \frac{99.9}{100} = 99.24\%$$

Linearity:

Aliquots of primary standard Febuxostat& Ketorolac stock solution was taken in different 10 ml volumetric flask and diluted up to the mark with the mobile phase such that the final concentrations of Febuxostat are in the range of 20 - 60µg/mL and 5 -15µg/mL respectively Each of these drug solutions (20µL) was injected three times into the column, and the peak areas and retention times were recorded. Evaluation was performed with PDA detector at 321nm.

Acceptance criteria:

Correlation Coefficient should be not less than 0.9990.

% of y- Intercept should be ±2.0.

% of RSD for level 1 and Level 6 should be not more than 2.0%.

Analysis of Formulations:

To find out the suitability of the method for the assay of pharmaceutical formulation containing Febuxostat and Ketorolac were analyzed by the proposed method. It was found that the proposed method do not differ significantly in the precision and accuracy from the reference method.

Recovery Studies:

Recovery studies were conducted by analyzing the formulations in the first instance for the active ingredients in the concentration of 50% of the working standard solution, 100% of the working standard solution (40µg/ml of Febuxostat and 10µg/ml of Ketorolac) and 150% of the working standard solution by the proposed method. Each concentration was injected three times and the peak areas were recorded. The known amount of the pure drug of the working standard solution contains was added to each three previously analyzed formulations and the total amount of the drug was again determined by the proposed method (each concentration was injected three times) by keeping the active ingredient concentration within the linearity limits. Results are showed in **Table 4 and 5.**

Table - 4
Recovery data for Febuxostat

% Concentration (at specification Level)	Area	Amount Added (mg)	Amount recovered (mg)	% Recovery	Mean Recovery
50%	1164128	5.0	5.07	101.34%	101.00%
100%	2331828	10.0	10.15	101.49%	
150%	3451918	15.0	15.02	100.16%	

Table - 5
Recovery data for Ketorolac

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount recovered (mg)	% Recovery	Mean Recovery
50%	93780	5.0	5.07	101.42 %	100.84%
100%	188160	10.0	9.99	101.75 %	
150%	275594	15.0	14.90	99.35%	

ROBUSTNESS:

Effect of variation of flow rate:

A study was conducted to determine the effect of variation in flow rate. Standard solution prepared as per the test method was injected into the HPLC system using flow rates, 1.0mL/min and1.2mL/min. The system suitability parameters were evaluated and found to be within the limits for 1.0mL/min and 1.2mL/min flow.

Febuxostat (FEB) and Ketorolac (KET) was resolved from all other peaks and the retention times were comparable with those obtained for mobile phase having flow rates 1.0mL/min.

Acceptance criteria:

The Tailing Factor of FEB and KET standards should be NMT 2.0 for Variation in Flow.

Limit of detection (LOD) and limit of quantification:

The detection limit of the method was investigated by injecting standard solutions into the HPLC column. By using the signal-to-noise (S/N) method, the peak–to-peak noise around the analyte retention time is measured. Subsequently, the concentration of the analyte which would yield a signal equal to certain value of noise to signal ratio was also estimated. A signal-to-noise ratio (S/N) of 3 was generally accepted for estimating LOD and signal-to-noise (S/N) ratio of 10 was used for estimating LOQ.

The LOQ can be determined by signal-to-noise ratio of 10:1, or even approximated by multiplying the LOD by 3. This method is commonly applied to analytical methods that exhibit the baseline noise. The LOD was found to be 0.04µg/ml for Febuxostat and 0.12µg/ml for Ketorolac. The LOQ was found to be 0.16µg/ml for Febuxostat and 0.42µg/ml for Ketorolac.

Table - 6

Summary of Validation data of Febuxostat & Ketorolac

Parameters	Results	
	Febuxostat	**Ketorolac**
Linearity range(µg /ml)	20-60	5-15
Wave Length (λ max)	321 nm	321 nm
Coefficient of determination	0.9991 ± 0.01	0.9991± 0.01
Limit of detection (µg/ml)	0.04	0.12
Limit of quantification (µg/ml)	0.16	0.42
% Recovery (n = 3)	101.00	100.84
Precision (RSD [%])	1.32	0.23
LOD	3.02	2.98
LOQ	9.97	9.98

SUMMARY AND CONCLUSION

There are only few reported methods on the RP-HPLC determination of Febuxostat and Ketorolac in Tablet dosage form in the literature prior to the commencement of this work. The author has developed a sensitive, accurate and precise RP-HPLC procedure for the estimation of Febuxostat and Ketorolac in bulk drug and also in pharmaceutical formulations.

From the typical chromatogram of Febuxostat and Ketorolac it was found that the retention times were 1.923 minutes for Febuxostat and 3.104 minutes for Ketorolac. A mixture of Disodium hydrogen phosphate dehydrate and Potassium Dihydrogen Phosphate in Water HPLC grade (pH adjusted to 5.8 with 1% Ortho-Phosphoric Acid: Methanol (40:60 v/v) was found to be the most suitable solvent for elution to obtain well defined peaks, free from tailing as per the ICH guidelines. In the HPLC method, the standard and sample preparations required less time and no tedious extraction were involved thereof. A good linear relationship (r =0.9998) was observed between the concentration range of linearity in the range of 20-60µg/ml for Febuxostat and 5-15µg/ml for Ketorolac respectively.

The low values of standard deviations are indicative of the high precision of the method developed. The assay of Febuxostat and Ketorolac was found out to be 99.24% and 100.56% respectively. Based upon the recovery studies it was found that about 101.00% of Febuxostat and 100.84% of Ketorolac were recovered which indicate high accuracy of the method.

The absence of additional peaks in the chromatogram indicated non-interference of the common excipients used in the tablets. It is thus, demonstrated that the developed RP-HPLC method is simple, linear, accurate, sensitive and reproducible. Thus, the developed method can easily be used for the routine quality control of bulk and pharmaceutical formulations of Febuxostat and Ketorolac with a short analysis time.

It can be seen from the results presented that the proposed procedure has good precision and accuracy. The results of the analysis of pharmaceutical formulations revealed that the proposed methods are suitable for their analysis with virtually no interference of the usual additives present in the pharmaceutical formulations.

The above proposed method obviates the need for any preliminary treatment and is simple, sensitive and reliable. It can be used for the routine determination of Febuxostat and Ketorolac in bulk samples and also in pharmaceutical formulations.

Thus, the present procedures constitute the RP-HPLC method with good precision, accuracy and sensitivity for the simultaneous estimation of Febuxostat and Ketorolac in pure stage and also in pharmaceutical formulations.

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Formulation of Sublingual Tablets by Using Solid Dispersion Technique: An Overview

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ABSTRACT

Improving oral bioavailability of drugs those given as solid dosage forms remains a challenge for the formulation scientists due to solubility problems. The dissolution rate could be the rate-limiting process in the absorption of a drug from a solid dosage form of relatively insoluble drugs. Therefore increase in dissolution of poorly soluble drugs by solid dispersion technique presents a challenge to the formulation scientists. Solid dispersion techniques have attracted considerable interest of improving the dissolution rate of highly lipophilic drugs thereby improving their bioavailability by reducing drug particle size, improving wettability and forming amorphous particles. Formulation of solid dispersion as sublingual tablets not only improves dissolution characteristics of drug but also provides ease of administration and quicker onset of action. This paper presents a review of the latest developments about sublingual tablets prepared by solid dispersion technique.

Keywords: Sublingual, solubility, solid dispersion, bioavailability

Introduction

Sublingual drug delivery (SL) of the medication implies arrangement of the medication under the tongue and drug comes to straightforwardly into the circulation system through the ventral surface of the tongue and floor of the mouth. The fundamental system for the retention of the medication into oral mucosa is by means of latent dissemination into the lipoidal film. The retention of the medication through the sublingual course is 3 to 10 times more prominent than oral course and is just surpassed by hypodermic infusion. For these details, the little volume of spit is generally adequate to bring about tablet crumbling in the oral cavity. [1-4]

Drug delivery through the sublingual route had emerged from the desire to provide immediate onset of pharmacological effect. Dysphasia (difficulty in swallowing) is a common problem of all age groups, especially geriatrics, pediatric, and patients who are mentally retarded, uncooperative, nauseated or on reduced liquid intake/diets have difficulties in swallowing these dosage forms. [24-30]

Drugs that are given sublingually reach directly in to the systemic circulation through the ventral surface of the tongue and floor of the mouth. The drug is rapidly absorbed into the reticulated vein that lies underneath the oral mucosa, and transported through the facial veins, internal jugular vein, and braciocephalic vein and then drained in to systemic circulation. [5-11]

The solubility of certain drugs presents a challenge to the formulator for developing a suitable oral formulation. The bioavailability of poorly water-soluble drug is often limited by its dissolution rate, which in turn is controlled by the surface area available for dissolution. For such drugs solid dispersion is a vital approach to achieve reduction in size and increase in solubility and hence dissolution characteristics Solid dispersion when exposed to aqueous media, the carrier is dissolved; the drug is released as very fine colloidal particles, increasing dissolution, absorption, and therapeutic efficacy of drugs. Formulating sublingual tablet using solid dispersion of drug shall not only improve solubility and consequent bioavailability but also reduce drug related side effects along with quick onset of action and improved patient compliance and convenience. Sublingual tablets of oxazepam, glyburide, tenoxicam, valdecoxib, rofecoxib, itraconazole, furosemide, diazepam, artemether, clonazepam, celecoxib, meloxicam, oxcarbazepine, ibuprofen and aceclofenac were developed using SD technique. [11, 14, 15, 17, 23]

The sublingual tablet satisfies the patient's need that can be used by those having problem in swallowing; the need of industry to provide improved solubility, stability,

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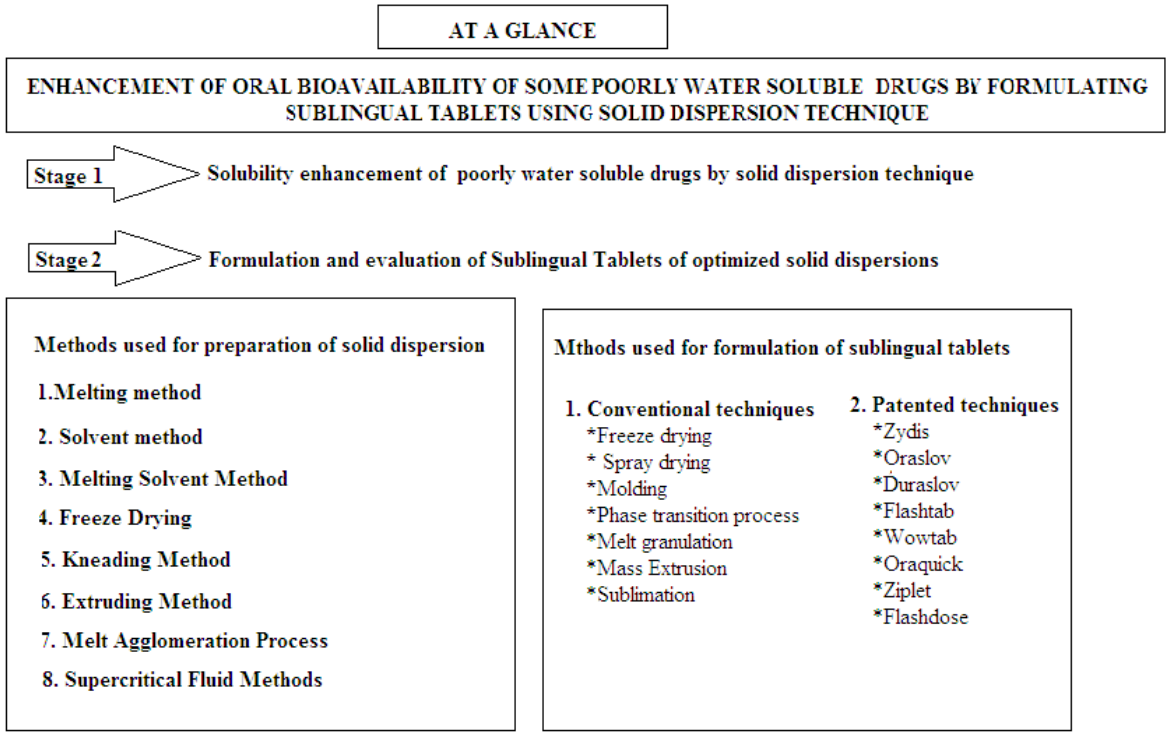

bioavailability along with safety and compliance; as well as need of market to develop new formulation of drugs whose patent has expired into new and improved form so as to extend market exclusion. [19-24]

Suitability of drug for preparation of sublingual tablet

No bitter taste. Dose lowers than 20 mg, Good solubility, stability in water and saliva. Many drug properties could potentially affect the performance of sublingual tablets like solubility crystal morphology, particle size, hygroscopicity, compressibility and bulk density of drug. Some drugs undergoes extensive first pass metabolism which results in poor bioavailability of its oral dosage forms, that kind of drugs are suitable for sublingual dosage form. Drugs that are unstable in parenteral preparation are suitable for sublingual dosage form. Many pharmaceuticals are designed for sublingual administration, including cardiovascular drugs, steroids, barbiturates, enzymes, antiemetics, vitamins, minerals and vaccines. [39-41]

Methods used for formulation of sublingual tablets by solid Dispersion (SD) Technique

The combination of solid dispersion and technology are required to address the problem of low bioavailability. The SD approach has been widely and successfully applied to improve the solubility, dissolution rates and consequently bioavailability of poorly water soluble drugs. An obstacle of SD technology in pharmaceutical product development is that a large amount of carrier, ie, more than 50% to 80% wt/wt, was required to achieve the desired dissolution. This high percentage of carrier causes consistency of product performance at the time of manufacturing. This is a major consideration in that the number of market products arising from this approach has been less than expected. [40] carriers used for SD formulations, many carriers such as polyethylene glycol (PEG), polyvinylpyrrolidone(PVP), hydroxypropylm ethylcellulose(HPMC),hydroxypropylcellulose, Gelucires, Eudragits, chitosans, hydroxypropylmethylcellulose and poloxamer have been reported to improve the solubility and bioavailability of poorly water soluble drugs. [31-37]

Sublingual tablets of Glyburide have developed by solid dispersion technique. Glyburide is a poorly water soluble oral hypoglycaemic agent, with problems of variable bioavailability and bioequivalence related to its poor water solubility. Solid dispersion of Glyburide was prepared by co-fusion method using PEG 6000 as carrier. The Glyburide dissolution profile from the newly developed tablets was clearly better than those from various commercial tablets at the same drug dosage. [9, 38]

Solid dispersions of oxazepam using different ratios of Gelita collagel as the carrier and lactose were prepared by the spray drying method. Dissolution studies have shown that by preparing solid dispersions, the dissolution rate and the solubility of oxazepam increase markedly, independent of the ratio of drug, carrier and lactose. Tablets of solid dispersions of oxazepam: Gelita collagel, physical mixtures and the drug alone were prepared. The best results from the dissolution test were obtained for tablets containing solid dispersions. They remained in good physical properties when stored for one year in normal conditions. [8]

Celecoxib is a poorly water-soluble drug, and bioavailability from its crystalline form is very low. Increase

List of carriers used for solid dispersion technology [12-18]

Type of carrier	Examples
Enteric polymer	Poly(meth)acrylates (EUDRAGIT® L 30 D-55 EUDRAGIT® L 100, EUDRAGIT® S 100), hydroxypropyl methyl cellulose phthalate (HPMCP), cellulose acetyate phthalate (CAP)
Hydrophilic polymers	starch, sodium carboxymethyl cellulose, sodium alginate, polyethylene glycol (PEG), polyvinyl pyrollidone (PVP), hydroxy propyl methyl cellulose (HPMC), polyvinyl alcohol (PVA), β- cyclodextrin, mannitol, chitosan, carrageenan
Surfactant	polyethylene - polypropylene glycol, lecithin, bile salt, Lauroyl polyoxyl-32 glycerides
Amphiphilic polymers	polyethylene oxides (PEO,PEO/polypropylene glycol (PPG) copolymers, PEG-modified starches, vinyl acetate/vinylpyrrolidone random copolymers, polyacrylic acid and polyacrylates

solubility and dissolution rate of celecoxib by preparing a solid dispersion with PVP K30 using a solvent-evaporation method. Concerning the optimization study, multiple regression analysis reveals that an optimum concentration of croscarmellose sodium and a higher percentage of pearlitol are required for obtaining rapidly disintegrating tablets. [19]

Clonazepam is a poorly water soluble drug, therefore to enhance the solubility and release of drug, solid dispersion of drug and PVP K30 was prepared by solvent evaporation method. The tablets were prepared by direct compression technique on rotary tablet machine. [18]

Conclusion

Formulation of Sublingual by using solid dispersion of drug is unique technique by which solubility of the drug can be enhanced which is most challenging aspect of drug delivery. Solid dispersion technique offered excellent means for increasing the solubility of drugs which have potential to better absorb from oral mucosa. But still the marketed preparations associated with solid dispersions are few. Many researches are already done in relation to formulation of sublingual with solid dispersion that let us know the potential of using solid dispersion along with sublinguals. But yet researches are to be carried to form a basis of commercialization of poorly water soluble and water insoluble drugs in sublingual using solid dispersion technique in the near future. It can be concluded that combination of solid dispersion and super-disintegrants is a promising approach to prepare efficient sublingual tablets. The tablets containing SD exhibited better dissolution profile than commercial tablets.

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