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Analytical Method Development And Validation For The Simultaneous Estimation Of Flupirtine Maleate And Paracetamol In Bulk And Pharmaceutical Dosage Form Using RP-HPLC

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ABSTRACT: *A simple, specific, accurate, precise and economical reverse phase high performance liquid chromatographic (RP-HPLC) method has been developed for the simultaneous estimation of flupiritine maleate (FLU) and paracetamol (PARA) in bulk and pharmaceutical dosage form. The two components were separated using Inertsil ODS, C_{18} (250 mm × 4.6 mm id, 5 µm particle size) column by isocratic elution using mobile phase composition of potassium dihydrogen phosphate: methanol (70: 30) and pH 3.0 was adjusted with orthophosphoric acid. Flow rate used was 1ml/min and detection was carried out at 217nm. Injection volume is 5 µl. The retention time of flupiritine maleate and paracetamol is 2.553 min and 3.620 min respectively. As per ICH guidelines the method has been validated in terms of specificity, linearity, range, accuracy, precision, limit of detection, limit of quantitation, robustness. The method was found to be linear in the range of 50-150µg/ml (R^2=0.999) for flupiritine maleate and 10-30 µg/ml (R^2=0.999) for paracetamol. The limit of detection and limit of quantitation were found to be 2.14 and 7.13 for flupiritine maleate and 2.52 and 8.41 for paracetamol. Flupiritine maleate and paracetamol has the recoveries of 100.2% and 100.8% respectively and their relative standard deviations were less than 2%. All the validation parameters met the acceptance criteria. The proposed method can also be used for simultaneous estimation of these drugs in marketed dosage form.*

KEYWORDS :*Combined dosage form, Flupiritine maleate, Isocratic elution, Paracetamol, RP-HPLC.*

I. INTRODUCTION

Flupiritine maleate is an amino pyridine derivative with the chemical name of ethyl 2-amino-6-[(4 fluorobenzyl) amino] pyridin-3-yl carbamate maleate. It is soluble in methanol, ethanol and DMSO [1]. Flupirtine is a unique centrally –acting, non-opioid analgesic with muscle relaxant and neuro protective properties [2]. Flupirtine is used for treatment of acute and chronic pain, i.e., for painful increased muscle tone of the posture and motor muscles, primary headache, tumor pain, dysmenorrhea and pain after traumatologic/orthopedic operations and injuries [3]. It displays indirect N-methyl-D-aspartate (NDMA) receptor antagonism via activation of potassium channels [4]. Structure of flupiritine maleate (FLU) was shown in Fig.1.Paracetamol is amide derivative with the chemical name N-(4-hydroxyphenyl) acetamide. It is soluble in methanol and sparingly soluble in water. Paracetamol is a common analgesic and antipyretic drug that is used for the relief of fever, headaches and other minor aches and pains [5]. Paracetamol is a weak inhibitor of PG synthesis of COX-1 and COX-2 in broken cell systems, but, therapeutic concentrations of paracetamol inhibit PG synthesis in intact cells in vitro [6]. Structure of paracetamol (PARA) was shown in Fig.2 [7]. Flupirtine maleate and paracetamol in combined dosage form was used to relieve pain. Literature review reveals that various analytical methods like UVSpectrophotometry [8,9,10,11], HPLC [12,13,14,15,16,17], Human Plasma by HPLC [18,19], LC-MS [20, 21] and other analytical method have been developed for individually and combination with other drug. However new method has been reported for the estimation of PARA and FLU in their combine dosage form. Hence, it was proposed to develop simple, accurate and precise RP-HPLC method for estimation of PARA and FLU in their marketed formulation.Flupirtine maleate and paracetamol Tablets are available in the combination of 100 + 325 mg.

II. MATERIALS AND METHODS

2.1. Instrumentation

A Tech comp model double beam UV-visible spectrophotometer with 10mm matched quartz cells was used to measure absorbance. Separation and estimation was carried out using HPLC (Waters e2695 separations module with PDA detector), auto sampler, column used in experiment was Inertsil ODS, C_{18} (250mm × 4.6mm × 5µ), digital weighing balance used was Shimadzu, digital pH meter was Digisun digital pH meter -2001, sonicator used was Spectra lab. The mobile phase was prepared by mixing potassium dihydrogen phosphate

(pH-3.0): methanol in the ratio of (70:30) was filtered and degassed. Injection volume is 5µL, flow rate is 1.0 mL/min and the wavelength of detection is 217 nm.

2.2. Reagents and Chemicals

Flupirtine maleate (99% w/w) and paracetamol (99% w/w) was procured from Lara drugs pvt.ltd, Hyderabad, India. The commercial fixed dose formulation containing FLU 100 mg and PARA 325 mg, Ketoflam-P (Prakruthi products pvt.ltd) was procured from the local market. Orthophosphoric acid (AR grade, Qualigens), methanol (HPLC grade, Merck limited), Milli–Q water (HPLC grade), acetonitrile (HPLC grade, Merck limited), potassium dihydrogen phosphate (AR grade, Emplura) are used. All other chemicals are of the highest grade commercially available unless otherwise specified.

2.3. Preparation of diluent

Methanol and water were mixed in the ratio 70: 30 v/v. Sonicated to degas for 20min.

2.4. Preparation of standard drug solutions

Accurately weigh and transfer 50 mg of FLU working standard and 10 mg of PARA working standard in to 50 ml of volumetric flask, add about 30 ml of diluent, sonicate to dissolve, dilute to the mark with diluent and mix well to obtain stock solution. Filtered through 0.45 µ – nylon membrane filter. Further dilute 5.0 ml of above filtrate to 50 ml with diluent. Then the concentration of flupiritine maleate is about 100µg/ml and concentration of paracetamol is about 20µg/ml

2.5. Preparation of sample solution

Ten Tablets of marketed formulation Ketoflam-P (Prakruthi products pvt.ltd) were randomly selected which contains FLU 100 mg and PARA 325 mg formulation was weighed and finely powdered. Tablet powder equivalent to 100 mg FLU with relevant quantities of PARA was weighed and transferred to a 100 ml volumetric flask, extracted for 30mins with methanol and volume was made up to 100 ml with diluent. Then filtered through 0.45 µ – nylon membrane filter. Further dilute 5.0 ml of above filtrate to 50 ml with diluent and mix well. Marketed formulation was listed in Table 1.

2.6. Preparation of mobile phase

The mobile phase was prepared by mixing potassium dihydrogen phosphate (pH-3.0): methanol in the ratio of (70:30) and then filtered and degassed.

2.7. Assay

The procedure given in Indian Pharmacopoeia was followed for the assay of Tablets containing flupirtine maleate (100mg) and paracetamol (325mg). The active ingredients in each of 10 dosage units is taken by random sampling and analyzed by the developed method. Ten Tablets of marketed formulation Ketoflam-P (Prakruthi products pvt.ltd) were randomly selected and transferred separately into 100ml volumetric flasks and dissolved in 20 ml methanol. Then sonicated to dissolve for 10min and then make up the volume to 100ml with diluent. Then filtered through 0.45 µ – nylon membrane filter and diluted with diluent solution so that the resultant concentrations are within the calibration range of the developed method. The samples are then analyzed by using the validated method. The Tablets are said to be compliance if the each individual content is 90 – 110 % of the average content or labeled claim. The assay of marketed formulation Ketoflam-P was found to be within the limit. Results are shown in Table 2.

III. RESULTS

3.1. Method development and optimization

Several trials had been taken for the proper optimization of RP- HPLC method by changing different mobile phase composition with different ratio. Finally the mobile phase for the optimized condition was potassium dihydrogen phosphate (pH-3.0): methanol in the ratio of (70:30) was selected and chromatogram was shown in Fig.3. Several parameters like mobile phase pH, solvent, diluent, concentration of buffer solution, wavelength of detection were selected based on the sample. Wavelength of detection is 217nm. Less tailing and high theoretical plates are obtained with Inertsil ODS, C_{18} (250mm×4.6mm×5µ) column. The column temperature is maintained at 30ºC. The flow rate of the method is 1.0 ml/min. At the reported flow rate, peak shape was excellent; changes in flow rate resulted in unacceptable tailing factor and poor peak shape. Hence, 1.0 ml/min was optimized flow rate which decreased the consumption of the mobile phase, which in turn proves to be cost effective for long term routine quality control analysis. Retention times of the drugs obtained under these conditions were 2.553 and 3.620 min for FLU and PARA, respectively. Optimized chromatographic conditions were shown in Table 3 and peak results were shown in Table 4.

3.2. Method validation parameters [21, 22]

The optimized method was validated as per ICH (Q2) guidelines with respect to linearity, accuracy, precision, specificity, and robustness, limit of detection and limit of quantification.

3.2.1. System suitability System suitability test is a pharmacopoeial requirement and is used to verify, whether the resolution and reproducibility of the chromatographic system are adequate for analysis to be done. Standard solution was prepared as per the proposed test method and injected into the HPLC system in five replicates and chromatograms were recorded. It is defined as tests to measure the method that can generate result of acceptable accuracy and precision. Here plate count, tailing for each peak, resolution between the peaks and the % RSD of peak area of samples were measured. The system suitability was carried out after the method development and validation have been completed. System suitability results for FLU and PARA were shown in Table 5 and Table 6.

Linearity

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. A series of standard concentrations were prepared from 50% to 150% of the targeted concentration of flupirtine maleate and paracetamol. Linearity was performed by diluting standard stock solution. From stock solution aliquots of 2.5, 3.75, 5, 6.25, 7.5 ml was diluted to 50ml with diluent such that the final concentration of FLU in the range of 50 to 150µg/ml and PARA in the range of 10 to 30µg/ml. 10µl of each sample injected in duplicate for each concentration level and a linearity graph of concentration (µg/ml) versus average area response was plotted for flupirtine maleate and paracetamol peaks and the correlation coefficient was calculated. *The correlation coefficient should be NLT 0.999* and *statistical Y intercept should be ± 2.0%*. The observations of FLU and PARA were shown in Table 7 and Table 8. Linearity plot were shown in Fig. 4 and Fig. 5.

Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogeneous sample. The precision of analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of series of measurement.

System precision

Standard solution was prepared as per the proposed test method for system precision studies. Six replicate injections were injected into the HPLC system. The % RSD for the peak responses of six replicate injections should be NMT 2.0 %.

Method precision

In method precision, a homogenous sample of a single batch should be analyzed six times. This indicates whether a method is giving consistent results for a single batch. The % RSD for the six determinations should be *NMT 2.0* %. Results of method precision of FLU and PARA were listed in Table 9 and Table 10.

3.2.4. Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy study was conducted by spiking the known amount of active ingredients into the placebo at three different levels (50%, 100% and 150% of target concentration). The samples were analyzed as per the proposed test procedure and the % recovery for each spiked level was calculated. The % RSD at each spike level should be NMT 2.0. The overall % RSD for % recovery for all spike level should be NMT 2.0. The % recovery at each spike level should be NLT 97.0 %and NMT 103.0 % of the added amount. Results of accuracy and recovery were presented in the Table 11.

3.2.5. Specificity

Specificity is the procedure to detect quantitatively the analyte in presence of component that may be expected to be present in the sample matrix. For demonstration of specificity, 4 samples namely blank sample, sample containing flupirtine maleate alone, sample containing paracetamol alone and sample containing the mixture of flupirtine maleate and paracetamol were prepared separately. Specificity of the method was determined by comparing results of all the samples. The developed method is said to be specific if the % interference calculated as peak area (if any) at the retention time of each of the analytes in the blank sample is less than 20% of peak area at the corresponding retention times of each of the drugs in the lowest calibration standard. Specificity was determined by comparison of the blank chromatogram with that of the standard chromatogram. There is no interference of blank and placebo, it was also seen that there was no other interfering

peak at the retention time of flupirtine maleate and paracetamol. Hence the method is specific.3.2.6. Limit of detection and limit of quantitation Limits of detection (LOD) and Limit of quantification (LOQ) (Fig. 6) were estimated from both linearity calibration curve method and signal to noise ratio method. The limit of detection is the lowest concentration of analyte in a sample that can be detected but not necessarily determined in quantitatively using a specific method under the required experimental conditions. Such a limit is expressed in terms of concentration of analyte in the sample. The limit of quantitation is the lowest concentration of the analyte in a sample that can be determined with accepTable precision and accuracy under the stated experimental conditions quantitation limit is expressed as the concentration of analyte (e.g. Percentage, parts per million) in the sample.LOD and LOQ were calculated from the formula 3.3 x (σ/S) and 10 x (σ/S), respectively where, σ is standard deviation of intercept and S is the mean of slope. The results are shown in Table 12.

3.2.7. Robustness

Robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Effect of variation in flow rate of mobile phase (1.0 ± 0.1 mL/min), and effect of variation in temperature of column oven (30 ± 5°C) on chromatographic parameters such as retention time, theoretical plates, and tailing factor, were studied. At normal flow rate, the retention time of flupirtine maleate was 2.553 minutes while that of paracetamol was 3.620 minutes. At normal flow rate, the tailing factor for flupirtine maleate is 1.346 while that of paracetamol was 1.180. At higher flow rate 1.1 mL/min, the retention time of flupirtine maleate was 2.138 minutes while that of paracetamol was 3.024 minutes and tailing factor of flupirtine maleate and paracetamol were 1.168 and 1.032. At a lower flow rate of 0.9 mL/min, flupirtine maleate and paracetamol eluted at 3.193 and 4.514 minutes respectively. The tailing factor of flupirtine maleate and paracetamol were 1.209 and 1.121 respectively. At normal temperature of column oven, the retention times of flupirtine maleate and paracetamol were 2.553 and 3.620 minutes. At higher temperature (35°C), the retention times of flupirtine maleate and paracetamol were2.549 and 3.586 minutes and at lower temperature (25°C), the retention times of flupirtine maleate and paracetamol were 2.556 and 3.606 minutes.

IV. DISCUSSION

In this RP- HPLC method, HPLC conditions were optimized to obtain, an adequate separation of eluted compounds. Initially, various mobile phase compositions were tried to elute title ingredients. Mobile phase and flow rate selection was based on peak parameters (height, capacity, theoretical plates, tailing or symmetry factor), run time, resolution. According to USP, system suitability tests are an integral part of chromatographic method. They are used to verify the reproducibility of the chromatographic system. To ascertain its effectiveness, system suitability tests were carried out on freshly prepared stock solutions.Flupiritine maleate and paracetamol was determined by reverse phase HPLC using KH_2PO_4: Methanol as mobile phase and column Inertsil ODS C_{18}(250mmX4.6 mm, 5µ) or equivalent as a stationary phase. Detection was carried out using wavelength of 217nm for flupirtine maleate and paracetamol. After development of the method, it was validated as per ICH Q2 guidelines. The system suitability was found to be within the limits. The limits were not more than RSD <2%. The run time used here is 6min. The % recovery investigated at three levels and was in between 97% to103% indicates that the method was accurate. No interfering peaks were found in the chromatogram and do not interfere with the estimation of flupirtine maleate and paracetamol by the proposed RP-HPLC method. The precision was found to be within the limits. The %RSD was found to be Less than 2%, where as precision % RSD was 0.07% and 0.04% for flupirtine maleate and paracetamol. This indicates that the method is precise. The linearity was found in the concentration range of 50-150µg/ml for flupirtine maleate and 10-30µg/ml for paracetamol with regression 0.999, intercept of 16616 x and 19288x for flupirtine maleate and for paracetamol, slope is 37462.54 for flupirtine maleate and 91507.91 for paracetamol. The results are presented in Table 7-8. From the results shown in Table 12, it can be concluded that the LOD and LOQ of flupirtine maleate is 2.14 and 7.13 and for paracetamol is 2.52 and 8.41 respectively. Thus the method is simple, rapid, sensitive, specific, accurate, and precise and does not involve complicated sample preparation procedures. The method is robust for all parameter.

V. CONCLUSION

The proposed RP-HPLC method can be successfully used for the estimation of flupirtine maleate and paracetamol in their combined dosage formulations without any interferences form each other. The use of Inertsil ODS, C18 (250 mm × 4.6 mm id, 5 µm particle size) column in the present work has shown better elution of analytes with good resolution, improved plate count, capacity factor. The developed method gave good resolution between PARA and FLU with short analysis time (6 min). The accuracy of the methods was assessed by recovery studies at three different levels. The method was found to be precise as indicated by the repeatability analysis, showing % RSD less than 2.

The method shows good reproducibility and it is accurate, precise, specific and sensitive. No interference of additives, matrix etc. is encountered in this method. Further studies on other pharmaceutical formulations would throw more light on these studies. All the parameters for the drugs had met the criteria of ICH guidelines for method validation. However, RP-HPLC method is considered more specific and sensitive than the other chromatographic methods. The developed method may be recommended for routine and QC analysis of investigational drugs to provide simple, accurate and reproducible quantitative analysis.The method was found to be sensitive, reliable, reproducible, rapid and economic also.

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Tables

Table 1: Details of marketed formulation

Brand name	**Ketoflam-P**
Label claim	Flupiritine maleate : 100mg Paracetamol : 325mg
Manufactured by	Prakruthi products pvt.ltd, India.

Table 2: Assay report of formulation

S. No	Brand name	Content	Assay	%RSD
1.	KETOFLAM-P	Flupirtine maleate-100mg Paracetamol-325mg	99.2% 99.3%	0.04 0.01

Table 3: Optimized chromatographic conditions

Parameter/conditions	Description/Values
Column	Inertsil ODS C_{18}(250mm× 4.6mm × 5µ)
Detector	PDA- Detector
Flow rate	1.0 ml/min
Injection volume	5 µl
Wavelength	217 nm
Column temperature	30ºC
Sample temperature	25ºC
Run time	30 min
Buffer	Phosphate buffer pH 3.0
Mobile phase	Buffer : methanol (70:30)
Program	Isocratic

Table 4: Peak results for optimized method

S. no	Peak name	Retention time(min)	Area (µv*sec)	USP resolution	USP tailing	USP plate count
1 2	FLU PARA	2.553 3.620	3750935 9204015	 9.546	1.346 1.181	11249 13994

Retention time of FLU is 2.553 min and PARA is 3.620 min. USP tailing of both the drugs are less than 2.0, USP resolution is greater than 3.0 and USP plate count of both the drugs are greater than 2000. All parameters had been optimized.

Table 5: System suitability results for flupirtine maleate

Name	Injections	RT (min)	Area (µv*sec)	USP tailing	USP plate count
Flupirtine maleate	1	2.555	3752217	1.404	10971
Flupirtine maleate	2	2.554	3750686	1.404	11057
Flupirtine maleate	3	2.554	3750019	1.427	10799
Flupirtine maleate	4	2.555	3754687	1.374	10854
Flupirtine maleate	5	2.554	3753512	1.413	10595
Mean			3752224		
Std dev.			1933.445		
% RSD			0.1		

It was observed from the data tabulated above that all the system suitability parameters of FLU meet the predetermined acceptance criteria as per the test method and indicates the suitability of the selected system.

Table 6: System suitability results for paracetamol

Name	**Injections**	**RT (min)**	**Area (µv*sec)**	**USP resolution**	**USP tailing**	**USP plate count**
Paracetamol	1	3.620	9173592	9.337	1.271	13763
Paracetamol	2	3.621	9171404	9.432	1.205	13752
Paracetamol	3	3.621	9101911	9.377	1.226	13568
Paracetamol	4	3.618	9121184	9.327	1.208	13515
Paracetamol	5	3.610	9163394	9.248	1.251	13651
Mean			9146297			
Std dev.			32666.75			
% RSD			0.4			

It was observed from the data tabulated above that all the system suitability parameters of PARA meet the predetermined acceptance criteria as per the test method and indicates the suitability of the selected system.

Table 7: Calculations for linearity of flupirtine maleate

Linearity level	**No. of injections**	**Final conc. flupirtine maleate (µg/ml)**	**Retention Time (min)**	**Area (µv*sec)**
Linearity-50%	1	50	2.563	1878311
Linearity-75%	1	75	2.561	2814621
Linearity-100%	1	100	2.559	3757121
Linearity-125%	1	125	2.559	4696450
Linearity-150%	1	150	2.555	5624565
Mean				3754214
Std dev.				1482218
Correlation coefficient				0.999
Slope (m)				37462.54
Intercept (y)				16616

Table 8: Calculations for linearity of paracetamol

Linearity level	**No. of injections**	**Final conc. paracetamol (µg/ml)**	**Retention Time (min)**	**Area (µv*sec)**
Linearity-50%	1	10	3.612	4570007
Linearity-75%	1	15	3.616	6854697
Linearity-100%	1	20	3.624	9146275
Linearity-125%	1	25	3.620	11485733
Linearity-150%	1	30	3.616	13720798
Mean				9155502
Std dev.				3626032.862
Correlation coefficient				0.999
Slope (m)				91507.91
Intercept (y)				19288

The correlation coefficient for flupirtine maleate and paracetamol was found to be 0.999 and 0.999 respectively, which indicates that the peak responses are linear. This concluded that the method was linear throughout the range selected

Table 9: Results of method precision of FLU

Test no.	**Sample name**	**Retention time (min)**	**Area (µv*sec)**
1	Precision 1	2.556	3753596
2	Precision 2	2.557	3750008
3	Precision 3	2.557	3754071
4	Precision 4	2.557	3752256
5	Precision 5	2.556	3757416
6	Precision 6	2.557	3750690
Mean			3753006
Std dev.			2677.49
% RSD			0.07

From the above results, it is concluded that the method is precise.

Table 10: Results of method precision of PARA

Test no.	**Sample name**	**Retention time (min)**	**Area (µv*sec)**
1	Precision 1	3.618	9149541
2	Precision 2	3.622	9144541
3	Precision 3	3.617	9141186
4	Precision 4	3.617	9149773
5	Precision 5	3.617	9142074
6	Precision 6	3.616	9147658
Mean			9145796
Std dev.			3741.36
% RSD			0.04

Spiked level (%)	**Conc. of sample (µg/mL)**	**Sample area (µv*sec)**	**Retention time (min)**	**Amount spiked (µg)**	**Amount recovered (µg) Mean± S.D**	**% RSD**	**% Recovery**
FLUPIRTINE MALEATE							
50	50	1876213	2.562	49.47	49.54± 0.1020	0.2	100.13
100	100	3756750	2.558	98.95	99.21± 0.0461	0.04	100.3
150	150	5625096	2.554	148.37	148.56± 0.0672	0.04	100.12
PARACETAMOL							

50	10	4574305	3.613	9.89	9.968± 0.0075	0.06	100.8
100	20	9145774	3.616	19.79	19.93± 0.0057	0.01	100.7
150	30	13634178	3.619	29.67	29.93± 0.0376	0.12	100.9

From the above results, it is concluded that the method is precise.

Table 11: Peak results for accuracy of flupirtine maleate and paracetamol

Overall recovery of flupirtine maleate and paracetamol was found to be 100.2 and 100.8

Table 12: Result for LOD and LOQ of flupirtine maleate and paracetamol

Drug	**Sample name**	**Injection**	**Retention time (min)**	**Area (µv*sec)**	**Result**
FLU	LOD	1	2.568	988477	2.14
	LOQ	1	2.567	1983322	7.13
PARA	LOD	1	3.615	2475235	2.52
	LOQ	1	3.621	4895468	8.41

From the above results, it can be concluded that the LOD and LOQ of flupirtine maleate is 2.14 and 7.13 and for paracetamol is 2.52 and 8.41 respectively.

Figures

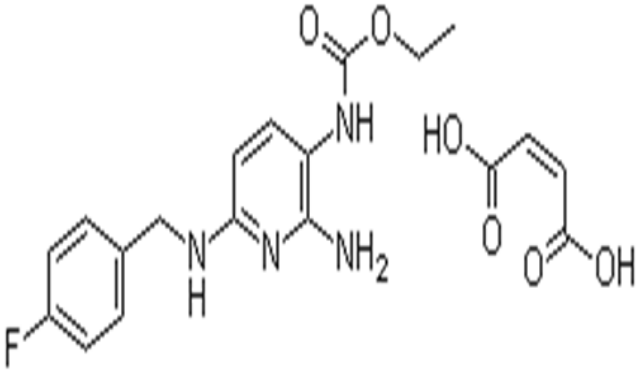

Fig. 1: Structure of flupirtine maleate

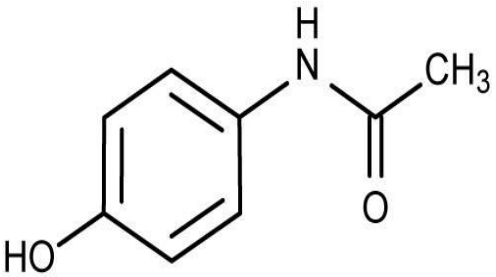

Fig. 2: Structure of paracetamol

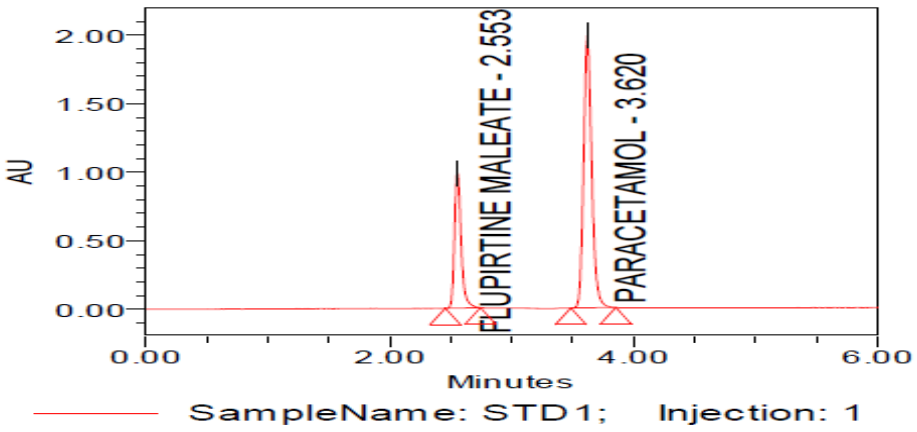

Fig. 3: Representative chromatogram of optimized method

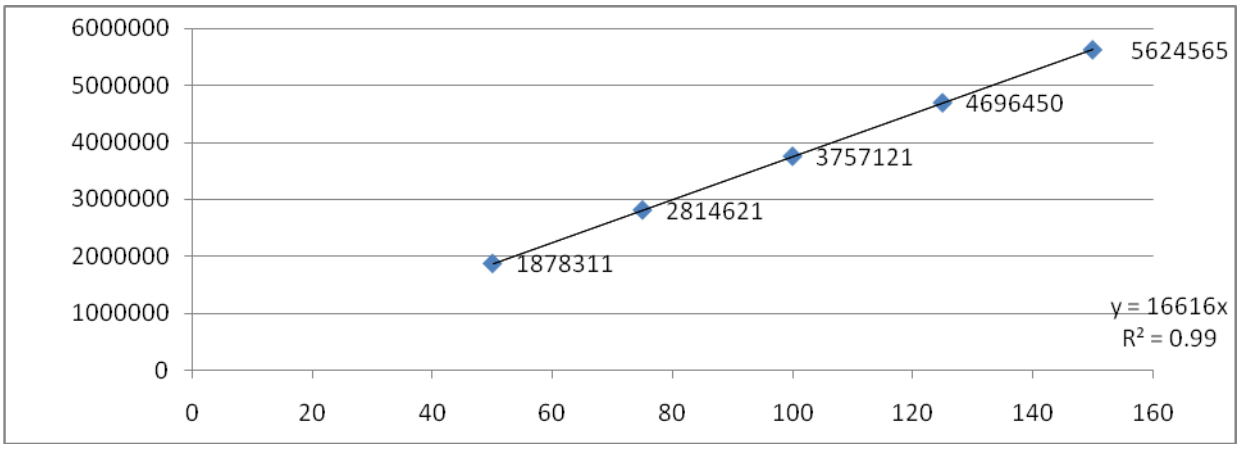

Fig. 4: Linearity plot of flupirtine maleate

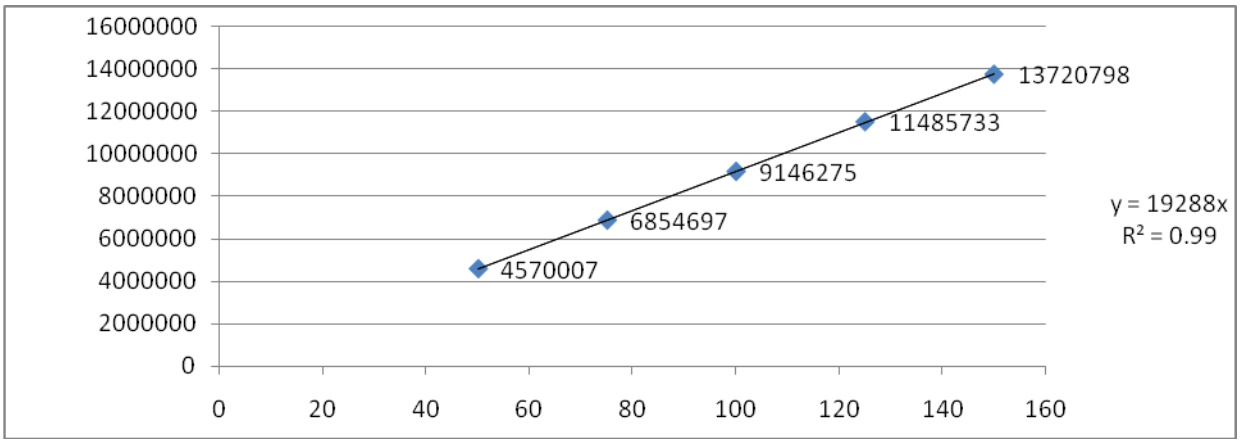

Fig. 5: Linearity plot of paracetamol

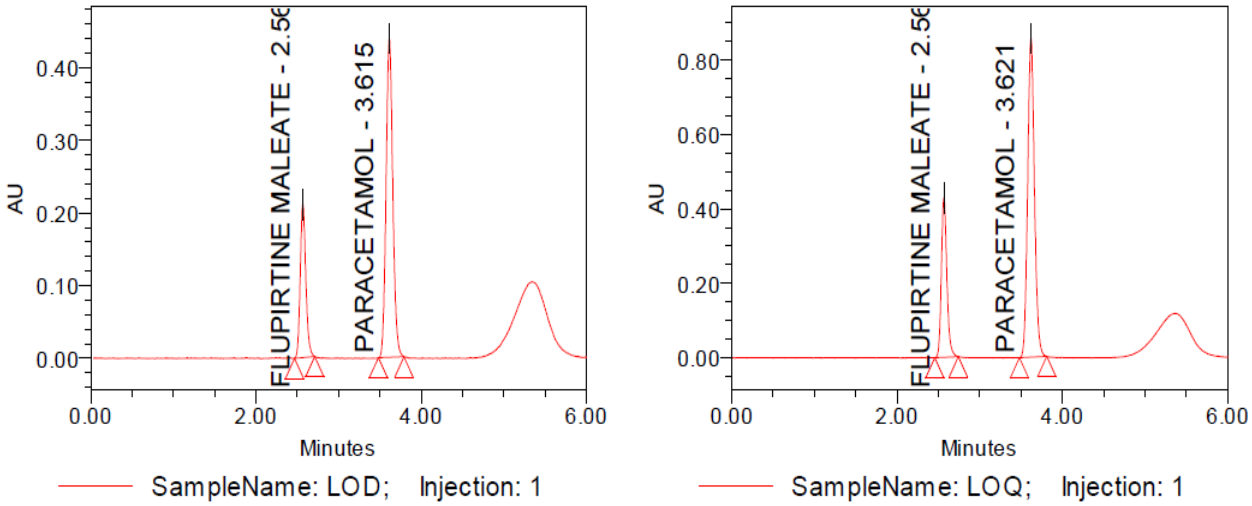

Fig. 6: Chromatograms for LOD and LOQ of FLU and PARA