Research Paper

Reverse-Phase High Performance Liquid Chromatography Method for Simultaneous Estimation of Enrofloxacin and Ketoprofen in Marketed Formulation

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A simple, fast, new, precise, sensitive, and accurate reverse-phase high performance liquid chromatography method was developed and validated according to International Council for Harmonisation guidelines for the estimation of enrofloxacin and ketoprofen in bulk and marketed formulation. Chromatographic separation was achieved using a Shimadzu high performance liquid chromatography system with an inertsil octadecylsilyl C_{18} column (4.6 mm×250 mm internal diameter, 5 µm particle size). The best results were obtained with the mobile phase composition consisting of 0.1 % trifluoroacetic acid, methanol, and acetonitrile in a ratio of 20:40:40 v/v/v. The system was regulated at a 0.7 ml/min flow rate at an optimized wavelength selected for detection at 262 nm. The retention time for enrofloxacin and ketoprofen were 2.941 and 5.756 min, respectively. The method has been validated for linearity, accuracy, precision, limit of detection, limit of quantification, and robustness as per International Council for Harmonisation guidelines. The calibration graphs were linear over the concentration range of 10-50 µg/ml for enrofloxacin and 6-30 µg/ml for ketoprofen. The values for enrofloxacin and ketoprofen were 1 µg/ml and 0.6 µg/ml for the limit of detection, and 0.3 µg/ml and 1.8 µg/ml for the limit of quantification, respectively. The analysis's conclusion indicates that for all of the validation parameters, the % relative standard deviation will be less than 2, and recovery studies revealed that the results were within the predetermined bounds. Therefore, it was determined that the suggested method was effective and that it could be utilized for the routine examination of enrofloxacin and ketoprofen in their marketed formulation.

Key words: Enrofloxacin, ketoprofen, reverse-phase high performance liquid chromatography, method development, method validation

Enrofloxacin (ENRO) is a synthetic compound from the fluoroquinolone family, with antibacterial activity commonly used in mammals. ENRO prevents bacterial Deoxyribonucleic Acid (DNA) from unwinding and duplicating by inhibiting the enzymatic actions of bacterial gyrase and topoisomerase IV. ENRO exhibits potent antibacterial activity against both gram-positive and gram-negative bacteria. The molecular formula and molecular weight of ENRO are $C_{19}H_{22}FN_3O_3$ and 359.4 g/mol. ENRO is a pale yellowish or light yellow crystalline powder that is partially insoluble in water, slightly soluble in methanol, soluble in acetonitrile, and freely soluble in methylene chloride. Fig. 1 depicts the structural composition of ENRO, which is 1-cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3-quinolonecarboxylic acid[1-3].

KETO is a Nonsteroidal Anti-Inflammatory Drug (NSAID) derived from propionic acid with anti-inflammatory, analgesic, and antipyretic properties for both animal and human use. KETO reduces the production of precursors to prostaglandins and thromboxanes by inhibiting the activity of the enzymes cyclooxygenase I and II. KETO also inhibits bradykinin, which is responsible for inflammation and pain. The molecular formula and molecular weight

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of KETO are $C_{16}H_{14}O_3$ and 254.3 g/mol. It is a white or almost white crystalline powder that is partially insoluble in water, soluble in acetonitrile, and freely soluble in ethanol (95 %), chloroform, ether, and methanol. Fig. 2 depicts the structural composition of KETO, which is (2-(3-benzolphenyl)-propionic acid[4-6].

According to a literature review, multiple analytical techniques have been described for the estimation of KETO and ENRO separately, including spectrophotometer[7-11], Ultraviolet (UV) spectrophotometer[12-14], UV-visible spectrophotometric method[15], kinetic spectrophotometer[16], electrokinetic chromatography[17], Infrared (IR) spectroscopy[18], derivative IR spectroscopy[19], Fourier Transform Infrared (FTIR) spectroscopy[20], capillary electrophoresis[21-23], High-Performance Liquid Chromatography (HPLC)[24-38], flow injection[38, 39], gas chromatography[40], and hyphenated techniques[41-44]. The present research is about the simultaneous estimation of KETO and ENRO by Reverse-Phase HPLC (RP-HPLC) in marketed formulation. The developed RP-HPLC method was validated for linearity, accuracy, precision, Limit of Detection (LOD), Limit of Quantitation (LOQ), and robustness as per International Conference on Harmonisation (ICH) Q2(R1) guidelines.

MATERIALS AND METHODS

Chemicals and reagents:

The standard drugs, ENRO and KETO, were obtained from Vijayanand Roadlines Pharma Tech Enterprises. The veterinary injection of the combination ENRO (100 mg) and KETO (60 mg) was obtained from a local pharmacy. Water (HPLC grade), methanol (HPLC grade), acetonitrile (HPLC grade), and Trifluoroacetic acid (TFA) used for mobile phase preparation were obtained from Thermo Fisher Scientific India Pvt. Ltd., Mumbai, India. The simultaneous estimation of KETO and ENRO was conducted using a Shimadzu HPLC series Liquid Chromatography (LC)-2030C chromatographic system, while wavelength selection was performed with a LABINDIA 3092 UV-visible spectrophotometer.

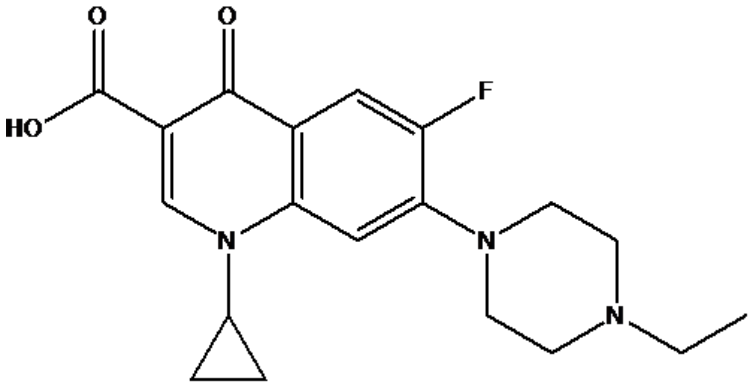

Fig. 1: Chemical structure of ENRO

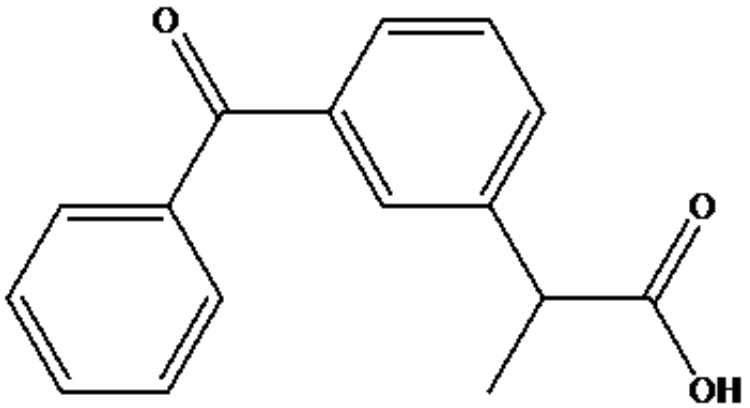

Fig. 2: Chemical structure of KETO

Method development by RP-HPLC:

Chromatographic conditions: Inertsil Octadecylsilyl (ODS) C_{18} column (4.6×250 mm, 5 µm) was used for separation, and the mobile phase of 0.1 % TFA, methanol, and acetonitrile in the ratio of 20:40:40 % v/v/v at the flow rate of 0.7 ml/min was chosen to provide appropriate peak resolution. The mobile phase was filtered through 0.45 µm membrane filter and degassed before use. The injection volume was 20 µl, and the elution was monitored at a wavelength of 262 nm.

Preparation of 0.1 % TFA: About 0.1 ml of TFA was pipetted out and transferred to a 100 ml volumetric flask, which was then filled to capacity with HPLC-grade water.

Preparation of mobile phase: A ratio of 20:40:40 % v/v/v mixture of 0.1 % TFA, methanol, and acetonitrile was used to make the mobile phase. The mobile phase prepared was filtered through a 0.45 µm membrane filter and sonicated to get rid of dissolved gases.

Diluent: As a diluent, a mixture of water and methanol in a ratio of 50:50 v/v was used.

Preparation of standard solutions:

Standard solutions of ENRO: A standard stock solution of ENRO was prepared by dissolving 10 mg of drug with diluent in a 10 ml volumetric flask and making up the volume to get a concentration of 1000 µg/ml. From the prepared stock solution, different volumes of standard solutions were taken and prepared at 10, 20, 30, 40, and 50 µg/ml concentrations of ENRO solutions.

Standard solutions of KETO: A standard stock solution of KETO was prepared by dissolving 20 mg of drug with diluent in a 100 ml volumetric flask and making up the volume to get a concentration of 200 µg/ml. From the prepared stock solution, different volumes of standard solutions were taken and prepared at 6, 12, 18, 24, and 30 µg/ml concentrations of KETO solutions.

Preparation of sample solution: A 1 ml solution containing 100 mg ENRO and 60 mg KETO from the formulation was taken, dissolved in diluent, and made up to 10 ml with diluent. Further dilution was made by taking 1 ml of the above solution and diluting it to 10 ml with diluent. Further dilutions were made according to the requirements.

Wavelength selection: A 10 µg/ml of ENRO and KETO working standard solutions were prepared separately with diluent. The detection was carried out in the UV range (200-400) nm. The prepared solutions of ENRO and KETO were scanned in a UV-visible spectrophotometer between the wavelength ranges of 200-400 nm. The isobestic point of the drugs was found to be 262 nm, and it was selected as the wavelength for simultaneous estimation of ENRO and KETO (fig. 3).

Method validation: Method validation was performed in terms of system suitability, specificity, linearity, accuracy, precision, LOD, LOQ, and robustness according to ICH Q2(R1) guidelines.

System suitability: To ensure the validity of the analytical procedure, the chromatographic system was subjected to a system suitability test. After injecting the standard preparation into the RP-HPLC 6 times in a replicate, the theoretical plate, resolution, tailing factor, and percentage Relative Standard Deviation (RSD) of peak area were all calculated.

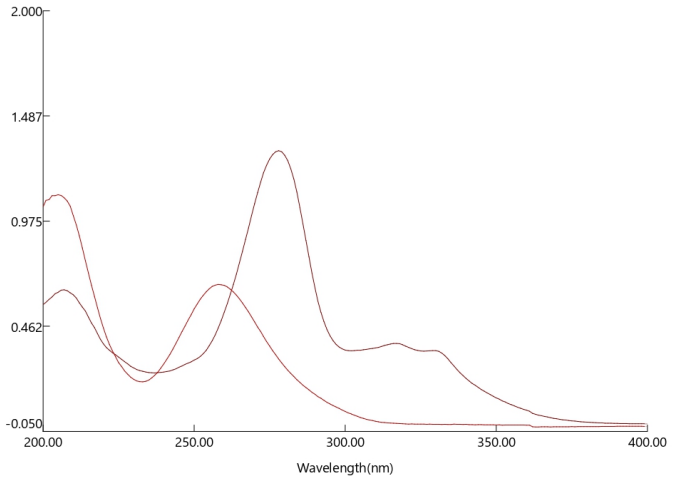

Fig. 3: Overlain UV spectra of ENRO (10 µg/ml) and KETO (10 µg/ml)

Specificity: The specificity of the analytical method is its ability to differentiate between the analytes and the other elements of the sample matrix. By individually injecting a 20 µl solution of the sample, standard, and blank into the chromatographic apparatus, specificity was evaluated.

Linearity: A method's capacity to deliver test findings that are directly proportional to sample concentration over a specified range of 10, 20, 30, 40, and 50 g/ml of ENRO and 6, 12, 18, and 24 g/ml of KETO was investigated. In order to make this determination, the relationship between sample concentration and detector response was used. Each method calibration curves were plotted, and the resulting data were then put through a regression analysis.

Accuracy: Accuracy is a metric for how closely the experimental value corresponds to the real concentration of the chemical in the matrix. In order to conduct the accuracy studies, multiple-level recovery studies were carried out by analysing standard additions at 3 levels. A known quantity of standard KETO and ENRO was added to a fixed equivalent quantity of the marketed formulation at levels of 50 %, 100 %, and 150 %, respectively. The percentage recovery was calculated.

Precision: System and method precision were used to assess the reproducibility of the proposed method. Precision experiments were carried out by preparing six determinations at concentrations of 30 µg/ml of ENRO and 18 µg/ml of KETO. System and method precision results were represented as a percentage of RSD.

LOD and LOQ: The standard calibration curve and the residual Standard Deviation (SD) of the regression lines of the y-intercept were used to determine the LOD and LOQ independently. LOD=3.3×D/S, LOQ=10×D/S, where, D is the standard deviation of the intercept of the regression line and S is the slope of the calibration curve.

Robustness: An analytical procedure's robustness is a measure of its ability to remain unaffected by small but intentional changes to the method parameters and offers a clue to its dependability under normal circumstances. Variations in the flow rate and mobile phase ratios were used to test the robustness. The outcome is given in percentage RSD.

Assay: An assay can be referred to as a quantitative measurement of the product's active pharmaceutical ingredient. The formulation contains 100 mg of ENRO and 60 mg of KETO. The sample solution was treated the same as the standard solution. The solutions were injected into RP-HPLC.

RESULTS AND DISCUSSION

Table 1 and Table 2 shows the results of the system suitability test. According to ICH guidelines, all the system suitability parameters were within the acceptable range. Fig. 4 illustrates the specificity of the current RP-HPLC method of analysis without any interference in retention time, the entire and clear separation of ENRO and KETO was seen. As the blank chromatogram (fig. 5) did not show any peak at the retention time of the analyte and the retention time was identical for both the sample (fig. 4) and standard (fig. 6) chromatograms, the developed analytical method was said to be specific.

TABLE 1: THE SYSTEM SUITABILITY TEST RESULTS

S. No.	ENRO	KETO
1	3 116 073	1 952 984
2	3 112 573	1 949 552
3	3 132 460	1 954 507
4	3 116 073	1 952 984
5	3 112 573	1 949 552
6	3 132 460	1 954 507
Mean	3 120 369	1 952 348
SD	9495.799	2072.309
% RSD	0.30	0.11

TABLE 2: THE SYSTEM SUITABILITY PARAMETERS OF ENRO AND KETO

Parameters	ENRO	KETO	Acceptance criteria
Number of theoretical plates (N)	3032	9831	>2000
Resolution (Rs)	-	12.73	>1.5
Tailing factor (T)	1.401	1.143	<2.0
% RSD	0.30	0.11	<2 %

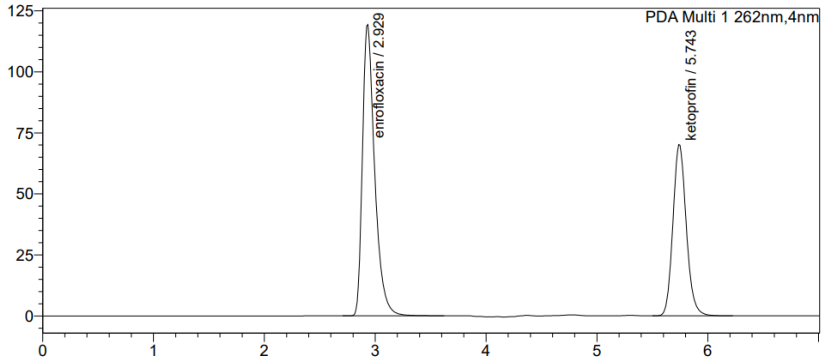

Fig. 4: Sample chromatogram

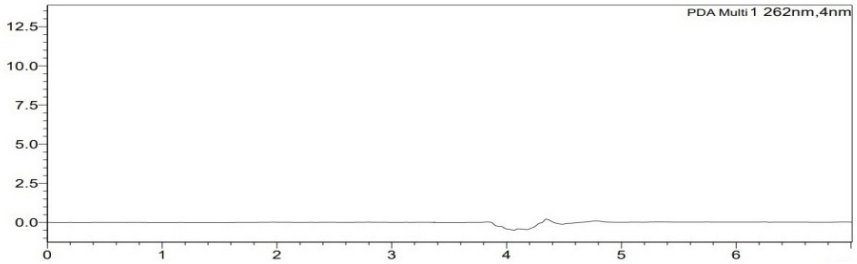

Fig. 5: Blank chromatogram

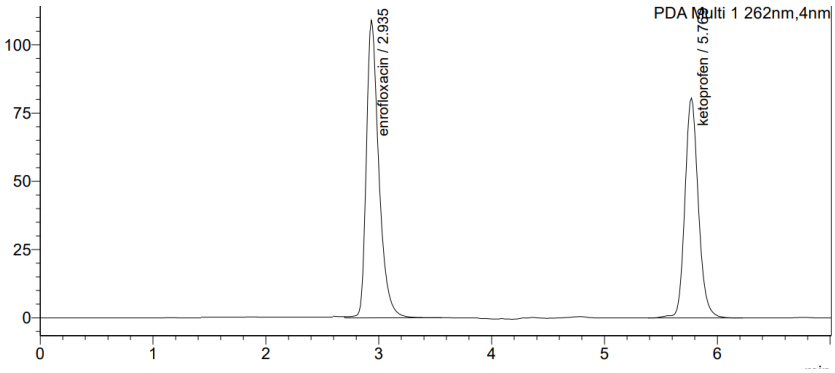

Fig. 6: Standard chromatogram

The linearity results are given in Table 3. The linearity ranges for ENRO and KETO were found to be 10-50 µg/ml and 6-30 µg/ml respectively. The calibration curve of ENRO is given in fig. 7, and that of KETO is given in fig. 8. The observed correlation coefficients for ENRO and KETO were found to be 0.9998 and 0.9996, respectively. Table 4 shows the HPLC area responses for accuracy determinations. For ENRO and KETO, the mean percentage recovery was found to be 99.79 % and 100.001 %, respectively. The results of the repeatability of system and method precision are given in Table 5. The developed analytical method was found to be precise, as the % RSD values of the system precision studies were 0.30 and 0.12 for ENRO and KETO, and the method precision study values were 0.29 and 0.13 for ENRO and KETO. Table 6 shows the LOD and LOQ study results. Serial dilutions of ENRO and KETO stock solutions were used for determining LOD.

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The percentage assay of ENRO and KETO was found to be 99.55 % and 99.65 %, respectively, as shown in Table 8. The present method established showed that it was easy, specific, particular, and capable of producing results that were exact and precise. A column made of inertsil ODS C18 (4.6 mm×250 mm internal diameter, 5 µm particle size) was used for the separation. At a flow rate of 0.7 ml/min and a detection wavelength of 262 nm, the mobile phase of 0.1 % TFA, methanol, and acetonitrile in the ratio of 20:40:40 % v/v/v was fed into the column. Additionally, the method's efficiency was demonstrated by its faster analytical time and lower mobile phase consumption. The analysis's conclusion indicated that for all of the validation parameters, the % RSD would be less than 2, and recovery studies revealed that the results were within the predetermined bounds. Therefore, it was determined that the suggested method was effective and that it could be utilised for the routine examination of ENRO and KETO in their marketed formulation.

TABLE 3: LINEARITY RESULTS OF KETO AND ENRO

ENRO		KETO	
Concentration (µg/ml)	Peak area	Concentration (µg/ml)	Peak area
10	1 200 132	6	664 091
20	2 122 510	12	1 345 125
30	3 096 827	18	1 945 510
40	4 080 939	24	2 636 046
50	5 158 677	30	3 338 760
Correlation coefficient (r^2) - 0.9998		Correlation coefficient (r^2) - 0.9996	

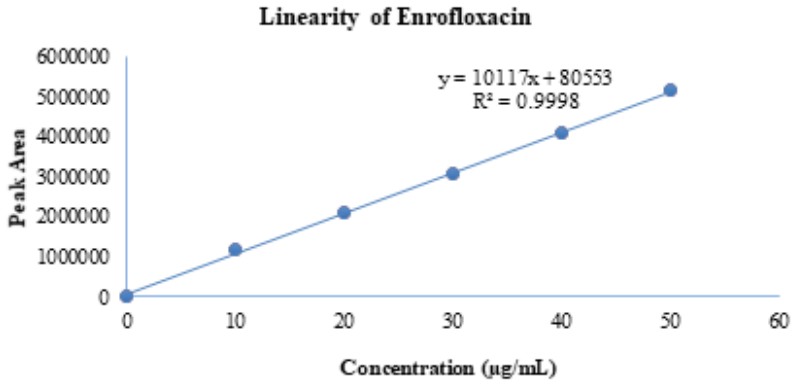

Fig. 7: Calibration curve of ENRO

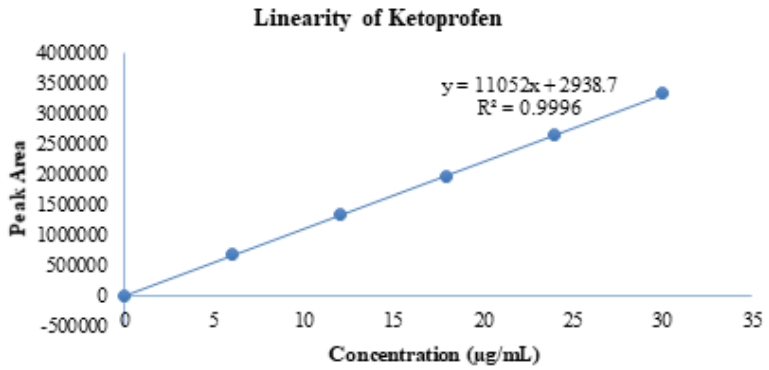

Fig. 8: Calibration curve of KETO

TABLE 4: ACCURACY RESULTS FOR ENRO AND KETO

% level	ENTO					KETO				
	Sample peak area	Standard peak area	% recovery (%)	Average % recovery (%)	Mean % recovery (%)	Sample peak area	Standard peak area	% recovery (%)	Average % recovery (%)	Mean % recovery (%)
	1 045 621	3 120 369	99.72			655 416	1 952 348	100.25		
50 %	1 051 256	3 120 369	100.26	100.08		654 899	1 952 348	100.13	100.21	
	1 051 489	3 120 369	100.28			655 658	1 952 348	100.25		
	3 135 647	3 120 369	99.26			1 956 245	1 952 348	99.7		
100 %	3 132 456	3 120 369	99.58	99.47	99.79	1 954 896	1 952 348	99.63	99.67	100
	3 132 789	3 120 369	99.59			1 955 891	1 952 348	99.68		
	5 233 694	3 120 369	99.83			3 272 514	1 952 348	100.07		
150 %	5 236 211	3 120 369	99.88	99.84		3 275 689	1 952 348	100.17	100.12	
	5 233 289	3 120 369	99.82			3 274 569	1 952 348	100.13		

TABLE 5: SYSTEM AND METHOD PRECISION RESULTS FOR ENRO AND KETO

S. No.	System precision		Method precision	
	ENRO	KETO	ENRO	KETO
1	3116132	1953232	3121132	1953691
2	3112637	1949613	3112741	1949824
3	3132506	1954601	3133631	1954839
4	3126147	1953016	3129293	1954822
5	3112653	1949621	3112925	1949371
6	3132489	1954521	3130721	1954628
Average	3122094	1952434	3123497	1952863
SD	9455.214	2275.873	9181.299	2567.982
% RSD	0.30	0.12	0.29	0.13

TABLE 6: LOD AND LOQ RESULTS FOR ENRO AND KETO

Detection wavelength (nm)	LOD (µg/ml)		LOQ (µg/ml)	
	ENRO	KETO	ENRO	KETO
262	1	0.6	3	1.8

TABLE 7: ROBUSTNESS RESULTS FOR ENRO AND KETO

S. No.	Condition	% RSD of ENRO	% RSD of KETO
1	Decrease in flow rate-0.6 ml/min	1.094	1.006
2	Increase in flow rate-0.8 ml/min	0.436	0.850
3	Mobile phase ratios of 50:10:40 v/v/v of methanol, 0.1 % TFA and acetonitrile	0.163	0.189
4	Mobile phase ratios of 40:10:50 v/v/v of methanol, 0.1 % TFA and acetonitrile	0.496	0.329

TABLE 8: ASSAY OF ENRO AND KETO

Drugs	Formulation contain	% assay
ENRO	100 mg	99.58
KETO	60 mg	99.65

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Conflict of interest:

The authors declared no conflict of interests.

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