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Development and Validation of RP -HPLC Method for the Determination of Gatifloxacin In Human Plasma

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ABSTRACT

A well developed and validated RP – HPLC method by UV- detection was used for the determination of Gatifloxacin in human plasma with metronidazole as internal standard. Solid phase extraction was involved in the process. The drug and the internal standard were eluted under isocratic mode using a 150 X 4.6 mm i.d, 5 µm Phenomenex ODS 2 C18 column. The mobile phase composed of a mixture of 5:95 % v/v methanol and 20mM mixed phosphate buffer (pH 3.5± 0.05) at a flow rate of 1.4 mL/Minute. The detection wave length of the detector was 268 nm. A volume of 50 µL was injected and the runtime of the method was 9 minutes. The method showed good linearity in the range of 50.1 to 7000.9 ng/mL. The recovery of gatifloxacin was 92.42 % with a standard deviation of 1.533 and recovery of internal standard was 87.6 %. The LOD of Gatifloxacin was 50.1 ng/mL. Matrix effects were not observed.

Keywords : Gatifloxacin, Antibacterial, Internal standard, RP – HPLC, Human plasma

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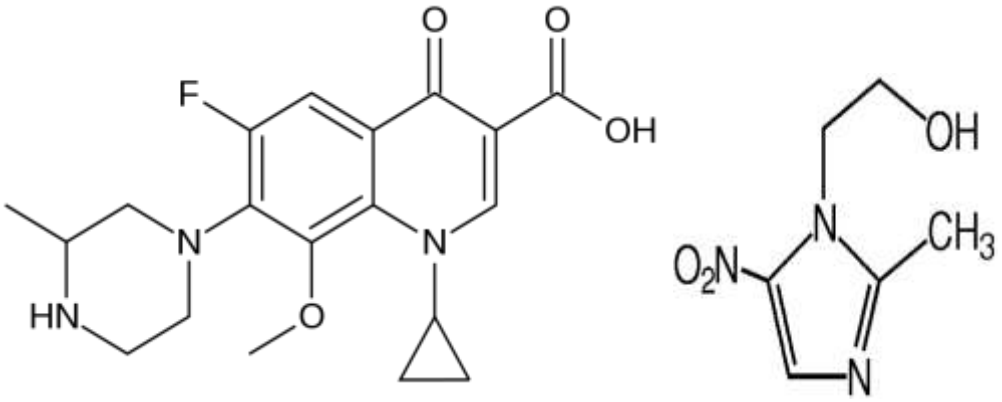
INTRODUCTION

Gatifloxacin sesquihydrate (GAT) is 1-Cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-(3-methyl-1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid sesquihydrate (Figure 1) is a well known Antimicrobial drug[1]. Gatifloxacin is a fluoroquinolone broad spectrum antibacterial agent that is active against both gram-positive and gram-negative organisms, anaerobes, as well as, Mycoplasma and Chlamydia.[2] Several methods were reported earlier for the etermination of GAT, alone and in combination with other drugs, by UV, HPLC, RP-HPLC and LC/MS [3-25]. Earlier reports on HPLC based bioanalytical estimation of GAT resulted in low sensitivity and high noise in the base line indicating a need to develop a more efficient ,sensitive, simple and rapid method in human plasma. Our present investigation aims to achieve the optimum chromatographic conditions for the determination of GAT using metronidazole (MTZ) as internal standard. The present method was developed and validated as per FDA guidelines [26].



Gatifloxacin **Metronidazole**

Figure 1 chemical structure of Gatifloxacin and Metronidazole

MATERIALS AND METHODS

Solvents and Chemicals

GATIFLOXACIN (GAT) and METRONIDAZOLE (MTZ) were gifted by M/s Hetero Drugs Pvt Ltd., Hyderabad and they were used as received. MCX OASIS 30mg/1cc cartridges, Methanol of HPLC grade and Potassium dihydrogen phosphate (GR grade) were used. Deionised water was processed through a Milli-Q water purification system (Millipore, USA). All other chemicals and reagents were of analytical grade.

Chromatographic System

The Chromatographic system consisted of a Shimadzu Class VP Binary pump LC-10ATvp, SIL-10ADvp Auto sampler, CTO-10Avp Column Temperature Oven, SPD-10Avp UV-Visible Detector. All the components of the system are controlled using SCL-10Avp System Controller.

LC Solutions software was used for data acquisition. The detector was set at a wavelength of 268 nm. Chromatographic separations were accomplished using a Phenomenex C_{18}, 5 µm, 150 mm×4.6 mm column. The mobile phase consisted of a mixture of 5 parts of Methanol and 95 parts of 20mM mixed phosphate buffer (pH3.5). The mixture was filtered through 0.45 µm membrane (Millipore, Bedford, MA, USA) under vacuum, and then degassed by flushing with nitrogen for 5 min. The mobile phase was pumped isocratically at a flow rate of 1.4ml/min during analysis, at ambient temperature. The rinsing solution consists of a mixture of 50: 50 % v/v of methanol : HPLC Grade Water.

Preparation of Standard Solutions

Gatifloxacin stock solution was prepared in methanol such that the final concentration is approximately 1.0 mg/mL. Stock solution of Metronidazole (approx 1 mg/mL) is prepared in HPLC Grade methanol. The solutions were stored below 10°C and they were stable for at least two weeks. Aqueous stock dilution of Gatifloxacin was prepared in diluent solution (mixture of 20: 80 % v/v of methanol: HPLC Grade water). About 2.72 grams of potassium dihydrogen phosphate was weighed accurately and transferred into a 1000 mL reagent bottle and dissolved in 200mL of Milli-Q water. The above solution was sonicated for 5 min and its pH was adjusted to (3.5 ± 0.05) with ortho phosphoric acid solution and made up to volume with Milli-Q water. The solution was stored at room temperature and used within 3 days from the date of preparation.

Sample Preparation

Aqueous stock dilutions were prepared initially. 0.5 ml of each aqueous stock dilution is transferred into a 10 mL volumetric flask. The final volume is made up with screened drug-free K_2EDTA human plasma and mixed gently for 15 minutes to achieve the desired concentration of calibration curve standards. The final calibration standard concentrations are 0.0 (Blank; no Gatifloxacin added), 50.1, 150.0, 750.2, 1500.4, 3000.8, 4001.0, 5250.7 and 7000.9 ng/ml. Each of these standard solutions was distributed in disposable polypropylene micro centrifuge tubes (2.0 ml, eppendorf) in volume of 0.7 ml and stored at -70°C until analysis. Similarly quality control samples were prepared in plasma such that the final concentrations were 50.3, 150.6, 3301.6 and 5240.7 ng/ml respectively and labeled as Lower limit of quantification (LLOQ), Low quality control (LQC), median quality control (MQC) and high quality control (HQC) respectively.

Extraction Procedure

Five hundred micro liters of the spiked plasma calibration curve standards and the quality control samples were transferred to a set of pre-labeled polypropylene tubes containing 50 µL of

Metronidazole dilution (internal standard; 20 µg/mL). The tubes were vortexed for ten seconds. Five hundred micro liters of 5% ortho phosphoric acid solution was added to each tube and vortexed for another ten seconds. Each of the MCX 30mg/1CC cartridges was conditioned with 1mL of methanol followed by equilibrating with 1mL of 0.5% ortho phosphoric acid in water on the solid phase extraction chamber. The above samples were loaded on to the cartridges and the cartridges were again washed with 1mL of 0.5 % ortho phosphoric acid in water followed by 1mL of 10% methanol in water. The cartridges were dried for about one minute and eluted with 1mL of 1% ammonia in methanol. The eluents were evaporated in a stream of nitrogen for 20 min at 500C and the residues in the dried tubes were reconstituted with 0.3mL of the mobile phase. The contents of the tubes were vortexed and transferred into auto-sampler vials and then analyzed by HPLC. An aliquot of 50 µL of the sample was drawn each time from the vials in the auto sampler.

Validation of quantitative HPLC method

The quantitative HPLC-UV method was validated to determine selectivity, calibration range, accuracy and precision, limit of detection (LOD), limit of quantitation, % recovery, freeze–thaw, and auto sampler stability. The initial assay was fully validated for Gatifloxacin analysis in human plasma according to FDA guidelines.

Selectivity

The selectivity of the method was evaluated by analyzing six independent drug-free K_2EDTA human plasma samples with reference to potential interferences from endogenous and environmental constituents.

Calibration curve

Calibration curves were generated to confirm the relationship between the peak area ratios and the concentration of GAT in the standard samples. Fresh calibration standards were extracted and assayed as described above on three different days and in duplicate. Calibration curves for GAT were represented by the plots of the peak-area ratio (GAT/MTZ) versus the nominal concentration of the GAT in calibration standards. The regression line was generated using 1/concentration ^{2} factor as the mathematical model of best fit. GAT concentrations in QC samples, recovery, and stability samples were calculated from the resulting area ratio and the regression equation of the calibration curve.

Accuracy and precision

The accuracy and precision of intraday and inter-day samples were determined by analysis of QCs at four levels (LLOQ, LQC, MQC and HQC; n = 6 at each level) along with three separate

standard curves done in duplicates. The Inter-day analysis was performed on 3 separate days.

The accuracy of an analytical method describes how close the mean test results obtained by the method are to the nominal concentration of the analyte. Accuracy was calculated by the following equation, expressed as a percentage:

Accuracy (%) = mean observed concentration/nominal concentration × 100

The precision was expressed by co-efficient of variation (CV). The CV % indicates the variability around the mean in relation to the size of the mean, and is defined as:

CV (%) =standard deviation/mean observed concentration× 100

Stability Studies

Autosampler, and freeze–thaw stability of GAT was determined at low, medium and high QC concentrations. To determine the impact of freeze–thaw cycles on GAT concentration, samples were allowed to undergo 3 freeze (−70 ^{o}C) thaw (room temperature) cycles. Following sample treatment/storage conditions, the GAT concentrations were analyzed in triplicates and compared to the control sample that had been stored at −70 ^{o}C. Autosampler stability of extracted samples was determined by comparing GAT concentration in freshly prepared samples and samples kept in autosampler at 4 ^{o}C for 24 h.

Recovery

Recovery was determined by comparing the area under the curve (AUC) of extracted QC samples (LQC, MQC and HQC) with direct injection of extracted blank plasma spiked with the same nominal concentration of GAT as in the QC samples. This should highlight any loss in signal due to the extraction process. IS recovery was determined for a single concentration of 200 µg/mL.

Data analysis

HPLC data acquisition and processing was performed by Shimadzu LC Solutions Ver 1.23 SP 1 software. Standard curves for quantitation of GAT were constructed using a 1/concentration ^{2} weighted linear regression of the peak area ratio versus GAT concentration. Unknown and QC sample concentrations were back-calculated from the standard curves.

RESULTS AND DISCUSSION

Method Development

The HPLC procedure was optimized with a view to develop a sensitive and reproducible method for the determination of GAT in Human Plasma. Since both GAT and internal standard are highly non-polar [27,28] we employed the usage of solid phase extraction process. To get a better

response the pH of the mobile phase is set to the acidic side. During our observation, a pH value around 3 resulted in better peak shape for the internal standard while that of the drug is not acceptable. Also, alkaline mobile phase characteristics causes deterioration of the bonded phase in the column due to alkaline hydrolysis of end-capped silica [29,30]. Compared to acid catalyzed hydrolysis, the hydrolysis of end-capped silica in alkaline conditions is usually very rapid. Therefore experiments were performed using Potassium Dihydrogen phosphate in a limited pH range of 3.0 to pH 5.5. The response was checked at the detector using a connector (without the column). A pH value of 3.5 ± 0.05 gave maximum response for the analyte at 268 nm. A similar response was observed with the usage of 20mM mixed phosphate buffer. Therefore the final mobile phase consisted of 5: 95 % v/v methanol and 20mM mixed phosphate buffer. The run time of analysis is higher when a longer normal phase column (250 X 4.6 mm id) is used. The resolution between the peaks was decreased and peaks were not acceptable peak shape when the experiment is performed using a shorter column (50 X 4.6 mm id). However better resolution, less tailing and high theoretical plates are obtained with a Phenomenex column C_{18} 150 X 4.6 cm 5 µm column.

The flow rate of the method is 1.4 ml/min. The column temperature is maintained at ambient. At the reported flow rate, peak shape was acceptable, however increasing or decreasing the flow rate increased the tailing factor and resulting in poor peak shape and decreased resolution between the drug and internal standard. There was no interference in the drug and internal standard, from the extracted blank. The peak symmetry were found to be good when the mobile phase composition of 5: 95 v/v methanol and 20mM mixed phosphate buffer leading to better resolution of the drug and internal standard. Increasing the organic portion of the mobile phase caused MTZ to elute early. A mobile phase containing aqueous portion greater than 95% led to very late elution and very poor peak shape for GAT. The peaks were also broad with unacceptable asymmetry factor.

Table 1 Method Parameters.

Parameter	Gatifloxacin	Metronidazole
Theoretical Plates	5150 ± 20	11305 ± 37
HETP	29.2 ± 1.4	13.37 ± 0.8
Tailing Factor	1.02 ± 0.02	0.86 ± 0.02
Resolution	------	7.78 ± 0.04
K^1	0	0.92

Since the noise effects in solid phase extraction (SPE) method are similar to that of liquid-liquid extraction, we have done the final analysis using solid phase extraction (SPE). The method parameters like resolution, HETP, peak asymmetry and selectivity are included in Table 1.

Detection and chromatography

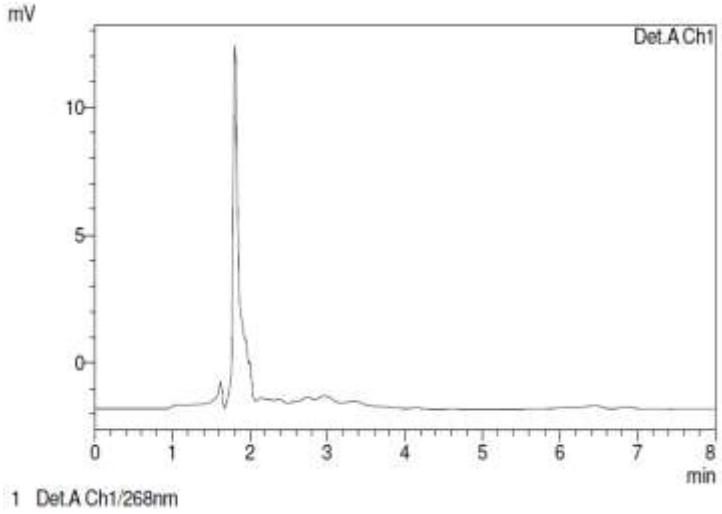


Figure 2 chromatogram of the extracted blank plasma sample

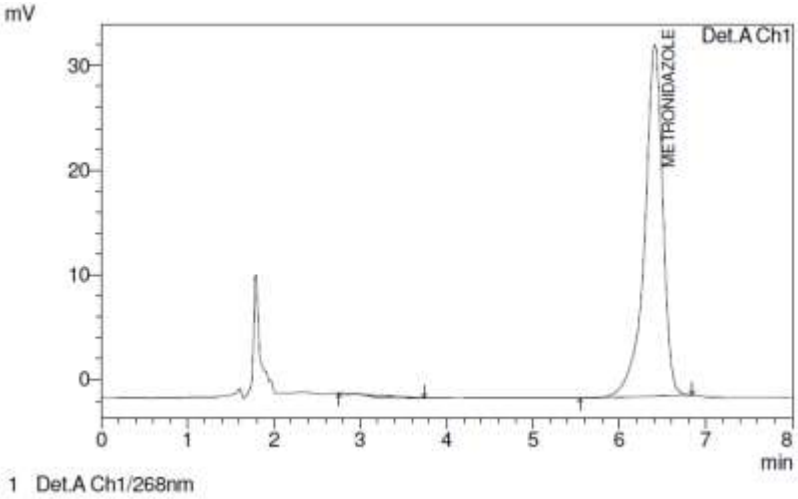


Figure. 3 Chromatogram of Metronidazole (IS) spiked blank plasma sample

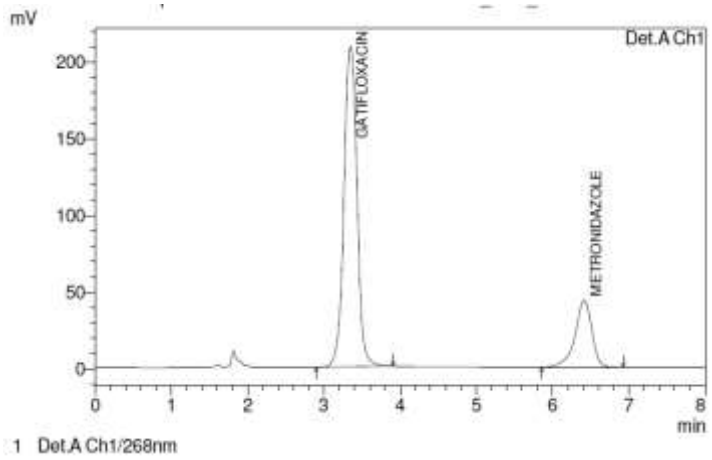


Figure .4 A chromatogram of GATIFLOXACIN (drug) and Metronidazole (IS) spiked plasma sample

The chromatogram of blank Human Plasma was represented in figure 2. This chromatogram was obtained when Human Plasma was not spiked with IS and sample. Figure 3 represents the typical chromatogram obtained for Human Plasma spiked alone with IS and not with sample. The chromatogram in figure 4 was obtained when Human Plasma wasspiked with IS and GAT in their ULOQ. The retention times for GAT and IS were 3.33 and 6.43 min, respectively.

Method validation

Selectivity

The method was found to have high selectivity for the analytes; since no interfering peaks from endogenous compounds were observed at the retention time for GAT in any of the six independent blank plasma extracts evaluated.

Calibration curves

A system suitability exercise was performed before the initiation of the validation. A system was assumed to be suitable for analysis if and only if the % CV for the retention times of GAT and internal standards is less than 2 %. The results are tabulated in Table 2.

Table 2: System Suitability Study

	Metronidazole Internal Standard (200 µg/mL)		Gatifloxacin (7000.9 ng/mL)	
	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area
Mean (n = 6)	6.43	433085.7	3.33	708872.8
S. D.	0.03	3488.17	0.02	5460.22
% CV	0.61	2.64	0.19	3.26

Table 3: Results of regression analysis of the linearity data

Linearity parameters	Mean ± SD (n = 6)
Slope	0.0001
Intercept	0.0026
Correlation coefficient (r^2)	0.9992

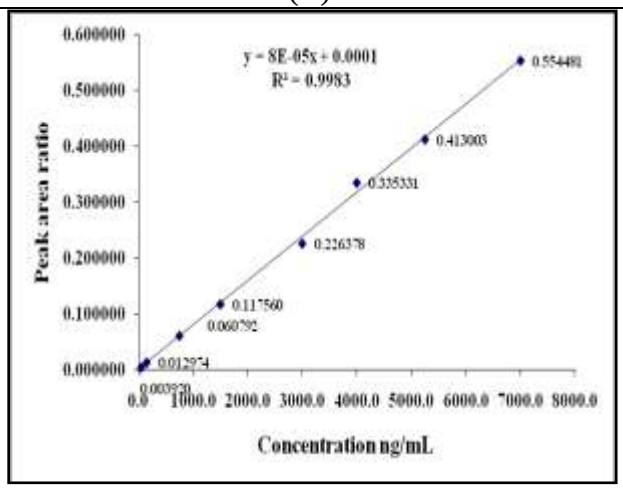


Figure. 5 Calibration curve for gatifloxacin (GAT)

Calibration curves for GAT in human plasma were fitted by weighted 1/concentration ^{2} quadratic regression, with the r ^{2} values of >0.99 for all curves generated during the validation. The calibration curve accuracy for plasma was presented in Table 3 demonstrating that measured concentration is within ± 15% of the actual concentration point (20% for the lowest point on the standard curve, the LLOQ). Results were calculated using peak area ratios. A representative calibration curve showing the regression equation and r^2 value is depicted in Figure – 5.

Accuracy and precision

A detailed summary of the intra-day and inter-day precision and accuracy data generated for the assay validation was presented in Table 4. Inter-assay variability was expressed as the accuracy and precision of the mean QC concentrations (LLOQ, LQC, MQC, and HQC) of three separate assays. Intra-assay variability was determined as the accuracy and precision of the six individual QC concentrations within one assay. The inter- and intra-assay accuracy and precision was <5% for all QC concentrations, which was within the general assay acceptability criteria for QC samples according to FDA guidelines .

Table 4: Intra and Inter day accuracy and precision of HPLC assay

	Nominal Concentration (ng/mL)			
	50.3	150.6	3301.6	5240.7
DAY 1				
Mean	51.6	150.9	3217.7	5268.5
S.D.	6.11	10.09	282.08	377.76
% CV	11.8	6.7	8.8	7.2
DAY 2				
Mean	47.9	149.0	3357.9	5176.0
S.D.	1.81	8.18	282.33	442.13
% CV	3.8	5.5	8.4	8.5
DAY 3				
Mean	47.2	149.4	3295.2	5077.3
S.D.	3.86	2.54	312.52	478.41
% CV	8.2	1.7	9.5	9.4
DAY 4				
Mean	47.4	146.9	3254.3	5211.6
S.D.	1.93	4.66	215.71	204.32
% CV	4.1	3.2	6.6	3.9

Each mean value is the result of triplicate analysis

Limit of detection and limit of quantification

LOD was defined as the lowest concentration that produced a peak distinguishable from background noise (minimum ratio of 3:1). The approximate LOD was 25 ng/mL. The LLOQ has been accepted as the lowest points on the standard curve with a relative standard deviation of less than 20% and signal to noise ratio of 5:1. Results at lowest concentration studies (50 ng/mL) met

the criteria for the LLOQ (Table 3). The method was found to be sensitive for the determination of GAT in human plasma samples. The ULOQ has been accepted as the highest points on the standard curve with a relative standard deviation of less than 15%.

Carryover test

A critical issue with the analysis of many drugs was their tendency to get absorbed by reversed phase octa-decyl-based chromatographic packing materials, resulting in the carryover effect. However in this analysis no quantifiable carryover effect was obtained when a series of blank (plasma) solutions were injected immediately following the highest calibration standard.

Stability studies

The results of short-term, long term and freeze–thaw stability are presented in Table 5. Determination of GAT stability following three freeze–thaw cycles showed that for all QC samples there was a minor change in the GAT concentration.

Table 5: Short Term, long term and Freeze Thaw stability of GAT

	Nominal Concentration (ng/mL)	
	150.6 (LQC)	**5240.7 (HQC)**
Bench Top Stability(7 Hours)		
Mean Accuracy (%)	97.9	94.2
S.D.	3.09	246.98
% CV	2.1	5.0
Long-term stability (9 Days)		
Mean Accuracy (%)	96.4	93.2
S.D.	7.81	123.64
% CV	5.4	2.5
Freeze – Thaw stability(3 Cycles)		
Mean Accuracy (%)	99.4	97.7
S.D.	4.79	200.14
% CV	3.2	3.9

Each mean value is the result of triplicate analysis

Recovery

Percentage recovery of GAT was measured by dividing the peak area values of extracted QC samples with direct injection of solution containing the same nominal concentration of compounds as the QC samples in extracted blank plasma. The mean recovery of GAT from plasma spiked samples of GAT at LQC, MQC and HQC levels was 93.6 %, 92.9 % and 90.7 % respectively. The overall recovery is 92.42 % with a % Coefficient of variation of 1.7 %. IS recovery at 200 µg/mL of MTZ was 87.6 % with a % Coefficient of variation of 8.6 %.

CONCLUSION

A HPLC method was developed and validated for the determination of GAT in human plasma.

The extraction process involved solid phase extraction procedure final analysis. This assay requires only a small volume of plasma (500 µL). There is no carryover effect. In this SPE method of extraction, baseline noise is minimal. Matrix effects were not observed. In conclusion, method validation following FDA guideline indicated that the developed method had high sensitivity with an LLOQ of 50.3 ng/mL, acceptable recovery, reliability, specificity and excellent efficiency with a total running time of 9.0 min per sample, which is important for large batches of samples. Thus this method can be suitable for pharmacokinetic, bioavailability or bioequivalence studies of GAT in human subjects. This method has been successfully applied to analyze GAT concentrations in human plasma.

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