

## DEVELOPMENT AND VALIDATION OF RP-CHIRAL HPLC METHOD FOR QUANTIFICATION OF (S)-ISOMER IN TENOFOVIR DISOPROXIL FUMARATE

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### ABSTRACT

**Objective:** The main objective of present study was to develop and validate a reverse phase enantioselective chiral high performance liquid chromatographic method was developed for enantiomeric resolution of Tenofovir disoproxil fumarate; it decreases the HIV infection in the human body. The method is specific, rapid, precise and accurate for the separation and determination of (S)-isomer in tenofovir disoproxil fumarate drug substance form.

**Methods:** The S-Isomer of Tenofovir disoproxil fumarate was resolved on a Chiral AGP (150 × 4.0 mm, 5 μm) column (L-41) using a mobile phase system containing 0.1 M ammonium acetate in water pH 6.8 with ammonia solution and methanol in the ratio of (85:15 v/v). The mobile phase was set at a flow rate of 0.8 ml/min and the volume injected was 10 μl for every injection. The detection wavelength was set at 260 nm and the column temperature was set at 15 °C.

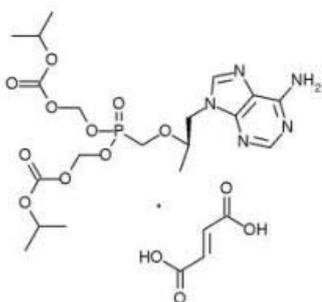
**Results:** The proposed method was productively applied for the quantitative determination of (S)-isomer in Tenofovir disoproxil fumarate drug substance form. The linear regression analysis data for calibration plots showed a good linear relationship over a concentration range of 0.125 to 3.75 μg/ml for (S)-isomer, 0.125-3.75 μg/ml for Tenofovir disoproxil fumarate. The mean values of the correlation coefficient were 0.999 and 0.999 for (S)-isomer and Tenofovir disoproxil fumarate. The method was validated as per the ICH guidelines. The detection limit (LOD) was about 0.05 μg/ml and quantitation limit (LOQ) was about 0.125 μg/ml for (S)-isomer and Tenofovir disoproxil fumarate. The relative standard deviation was found to be 0.78 % for (S)-isomer in Tenofovir disoproxil fumarate.

**Conclusion:** The developed and validated HPLC method and the statistical analysis showed that the method is repeatable and selective for the estimation of the (S)-isomer of the Tenofovir disoproxil fumarate drug substance.

**Keywords:** Tenofovir disoproxil fumarate, (S)-Isomer; RP-Chiral HPLC, Identification, Quantification and Validation

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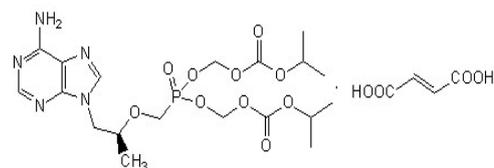
### INTRODUCTION



**Fig. 1: Chemical structure of tenofovir disoproxil fumarate**

Tenofovir disoproxil fumarate (TDF) is a fumaric acid, salt of bis-isopropoxycarbonyloxy methyl ester derivative of tenofovir. Chemically it is 9-[(R)-2-[[bis [[isopropoxy-carbonyl] oxy] methyl] phosphiny] methoxy] propyl] adenine fumarate [1-2]. TDF is the first nucleotide analog approved for HIV-1 treatment. Tenofovir is a nucleotide reverse transcriptase inhibitor [3] used in combination with other antiretrovirals for the treatment of HIV infections. TDF remains in cells for longer periods of time than many other antiretroviral drugs, thereby allowing for once-daily dosing. Literature survey reveals that there are several reports describing the determination of Tenofovir in plasma using HPLC coupled with fluorescence and UV detection [4-8]. Liquid chromatography coupled with tandem mass spectrometry was also reported [9-11]. Spectrophotometric [12]. The focus of the present study is to develop and validate a rapid,

stable, and economic high performance liquid chromatographic method for quality control of TDF in drug substance form. So it is essential to find an effective way to analyze the (S) enantiomer of Tenofovir disoproxil fumarate, the chemical structures of Tenofovir disoproxil fumarate and (S)-enantiomer are shown in fig. 1 and in fig. 2 The developed chiral RP-HPLC method was reproducible and accurate for the quantitative determination of (S)-enantiomer in Tenofovir disoproxil fumarate The newly developed method was validated as per ICH guidelines [13-16].



**Fig. 2: Chemical structure of tenofovir disoproxil fumarate (S) enantiomer**

### MATERIALS AND METHODS

#### Instruments

Chromatography was carried out by using Water's Alliance instrument equipped with column oven, UV detector, and the data was processed using a computer program (with Empower-2 software).

### Chemicals and reagents

Tenofovir disoproxil fumarate racemic mixture ( $\pm$ ) was obtained from the R&D department of Dr. Konda's life sciences (Hyderabad, India). Merck grade Ammonium acetate, Ammonia, and methanol were purchased from Merck (Mumbai, India). A stock solution of analyte was prepared in the diluent at a concentration of 0.1 mg/ml. Tenofovir disoproxil fumarate with 99.40 % purity and (S)-isomer (the undesired isomer (with 99.02 % purity) were prepared in the laboratory by using preparative HPLC.

### Chromatographic conditions

The chromatographic conditions were optimized using a chiral RP stationary phase, Chiral AGP column (150  $\times$  4.0 mm, 5  $\mu$ m, Supelco brand (a member of Sigma-Aldrich group). The isocratic mobile phase composition was a mixture of 0.1 M ammonium acetate in water pH 6.8 with ammonia solution and methanol (85:15 v/v), which was pumped at a flow rate of 0.8 ml/min. The temperature of the column was maintained at 15°C and the eluent was monitored at a wavelength of 260 nm. The injection volume was 10  $\mu$ l.

The chromatographic parameters, including the retention factor (k), the separation factor ( $\alpha$ ), and the resolution (Rs) were selected to evaluate the separation of compounds. All the chromatographic results were repeated three times.

### Sample preparation

Racemic mixture solution of Tenofovir disoproxil fumarate and (S)-enantiomer (100  $\mu$ g/ml each) prepared in mobile phase was used in the method development.

Stock solutions of Tenofovir disoproxil fumarate (0.1 mg/ml) and (S)-enantiomer (0.1 mg/ml) were prepared by dissolving an appropriate amount of the substance in the mobile phase. The analyte concentration of Tenofovir disoproxil fumarate was fixed as 250  $\mu$ g/ml. Tenofovir disoproxil fumarate solutions spiked with low levels of (S)-enantiomer were prepared by transferring calculated amount of undesired enantiomer stock solution with a pipette into the calculated amount of Tenofovir disoproxil fumarate stock solution, and then the solution was added to volume with mobile phase and mixed well.

### Validation of the method

The specificity of the method is performed by injecting both isomers and racemic mixture individually. The specificity determined by using peak purity, resolution.

The system suitability of the method performed by adding known concentration (2.5  $\mu$ g/ml) of undesired isomer to Tenofovir disoproxil fumarate. The system suitability is confirmed by using resolution, tailing factor, Tangent.

Method reproducibility was determined by measuring repeatability and intermediate precision of retention times and peak areas for each enantiomer. The repeatability of the method was determined by analyzing six replicate injections containing Tenofovir disoproxil fumarate (250  $\mu$ g/ml) spiked with (S)-enantiomer (1.0 %, 2.5  $\mu$ g/ml). The intermediate precision was determined over 2 d by performing six successive injections (n = 6) each day and also performed intermediate precision with different system, different analyst and with a different column by using six injections (n = 6).

The limit of detection (LOD) and limit of Quantitation (LOQ) for (S)-enantiomer was achieved by injecting a series of dilute solutions of by using standard deviation slope method (ICH Q2 (R1)). The LOQ level precision of the developed chiral method for (S)-enantiomer was checked by analyzing six solutions of (S)-enantiomer prepared at LOQ level and calculating the percentage relative standard deviation of the area.

Detector response linearity was assessed by preparing eight calibration sample solutions of (S)-enantiomer co-vering from 0.5

$\mu$ g/ml (LOQ) to 3.75  $\mu$ g/ml (0.125, 0.50, 1.00, 1.50, 2.00, 2.50, 3.00 and 3.75  $\mu$ g/ml) in mobile phase. The regression curve was obtained by plotting peak area versus concentrations, using the least square method. The percentage relative standard deviation of the slope and y-intercept of the calibration curve was calculated.

The accuracy of the method was carried out by injecting a known concentration of (S)-enantiomer to the Tenofovir disoproxil fumarate. The accuracy was calculated in terms of recovery (%). The study was carried out in triplicate at covering from LOQ, to 3.75  $\mu$ g/ml (LOQ, 1.25, 2.50 and 3.75  $\mu$ g/ml) in the mobile phase. The recovery of (S)-enantiomer was calculated.

To determine the robustness of the method, the flow rate was changed at the pace of 0.2 units from 0.6 to 1.0 ml/min. The effect of a change in the percent Buffer and Methanol ( $\pm$ 10%), and column temperature at 10°C and 20°C instead of 15°C were studied, and the other chromatographic conditions were held constant as stated previously.

The solution stability of Tenofovir disoproxil fumarate at analyte concentration was studied by keeping the solution in a tightly capped volumetric flask at room temperature on a laboratory bench for 48 h. The content of (S)-enantiomer was checked at 6 h interval up to the study period. Mobile phase stability was carried out by evaluating the content of (S)-enantiomer in Tenofovir disoproxil fumarate sample solutions prepared freshly at 6 h interval of 48 h. The same mobile phase was used during the study period.

## RESULTS AND DISCUSSION

### Optimization of chromatographic conditions

To develop a rugged and suitable reverse phase HPLC method for the separation of the two enantiomers, different stationary phases and mobile phases were employed. Chiralpak AGP column (150  $\times$  4.0 mm, 5  $\mu$ m) with a mobile phase consisting of 0.1 M ammonium acetate in water pH 6.8 with ammonia solution and methanol (85:15 v/v) was used. It was continued to select the best stationary and mobile phases that would give optimum resolution and selectivity for the two enantiomers. There was an indication of separation on Chiralpak AGP (150  $\times$  4.0 mm, 5  $\mu$ m) column using a mobile phase consisting of 0.1 M ammonium acetate in water pH 6.8 with ammonia solution and methanol (85:15 v/v). The composition of the mobile phase was optimized to enhance the chromatographic efficiency and resolution between the enantiomers. Based on the data obtained from the method development and optimization activities, Chiralpak AGP (150  $\times$  4.0 mm, 5  $\mu$ m) column with a mobile phase of 0.1 M ammonium acetate in water pH 6.8 with ammonia solution and methanol (85:15 v/v) was selected from the method development. The flow rate of the final method was 0.8 ml/min with injection volume 10  $\mu$ l. The column temperature was fixed and the detection wavelength was 260 nm. Under these conditions, the two enantiomers were separated well and the peak of (S)-enantiomer eluted after the main peak of Tenofovir disoproxil fumarate. In the optimized method, the typical retention time of Tenofovir disoproxil fumarate and (S)-enantiomer were 7.4 and 10.6 min respectively. Baseline separation of Tenofovir disoproxil fumarate and (S)-enantiomer was obtained with total run time of 20 min. The final optimized method was productively applied to separate (S)-isomer from Tenofovir disoproxil fumarate and was proven to be reproducible and accurate for the quantitative determination of (S)-isomer in Tenofovir disoproxil fumarate drug substance form. This is the first method to estimate (S) enantiomer in Tenofovir disoproxil fumarate drug substance form. The separation of an approximately 1:1 (wt/wt) mixture solution (in diluent) of the two enantiomers shown in fig. 3. HPLC chromatogram of Tenofovir disoproxil fumarate bulk drug sample (0.1 mg/ml) shown in fig. 4 and HPLC chromatogram of (S) enantiomer (0.1 mg/ml) was shown in fig. 5.

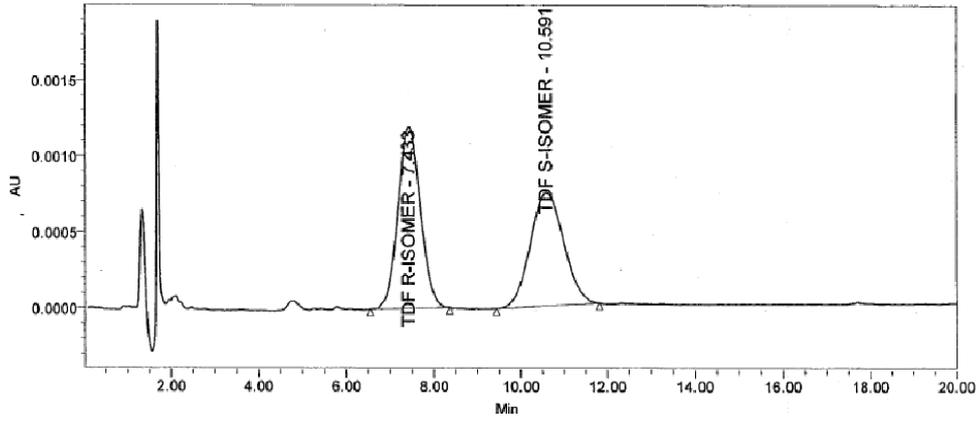


Fig. 3: Tenofovir disoproxil fumarate and (S) enantiomer

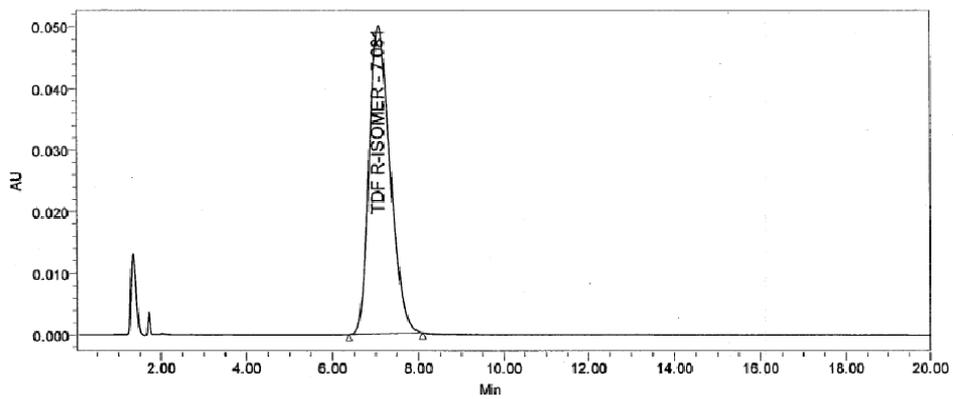


Fig. 4: Tenofovir disoproxil fumarate 0.1 mg/ml solution

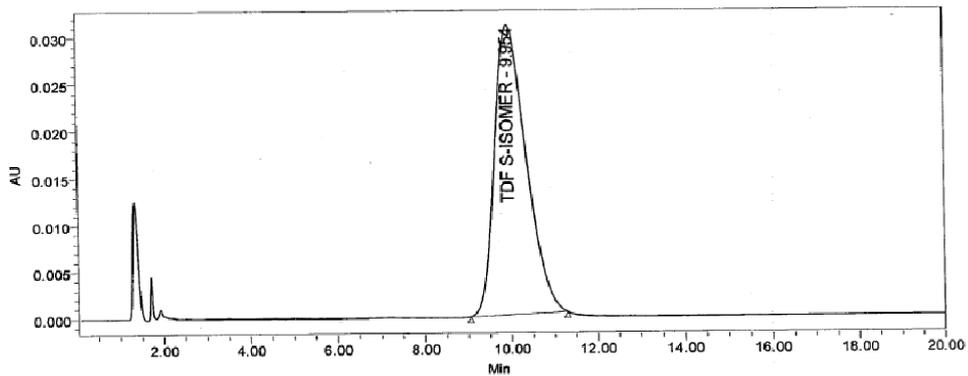


Fig. 5: (S) Isomer 0.1 mg/ml solution

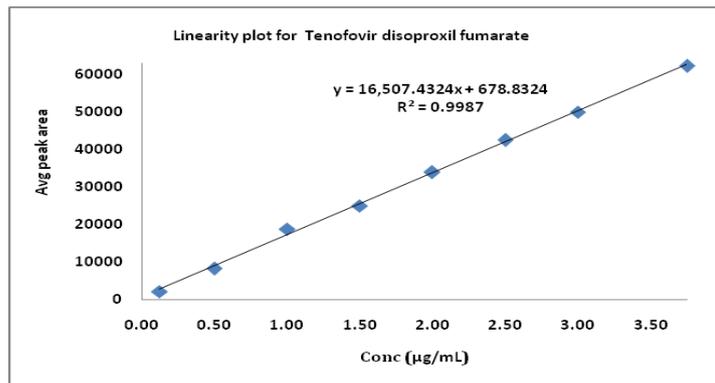


Fig. 6: Linearity plot for tenofovir disoproxil fumarate

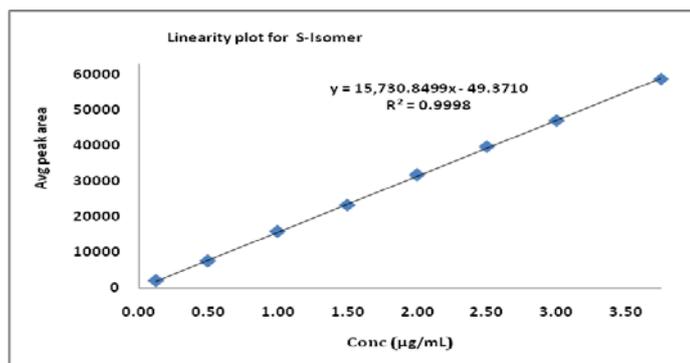


Fig. 7: Linearity plot for S-Isomer

**Validation results of the method**

The HPLC condition of the final method was evaluated for its

specificity, LOD, LOQ, linearity, accuracy, precision, robustness and stability. The specificity of the method was determined by using peak purity. The specificity results are given in table 1.

Table 1: Specificity

Compound	Purity angle	Purity threshold	Peak purity
S-Isomer	0.921	2.324	Pass
Tenofovir disoproxil fumarate	3.567	4.291	Pass

The limit of detection and limit of quantification of (S)-isomer was found to be 0.05 µg/ml and 0.125 µg/ml, respectively. Calculated the LOD and LOQ by using standard and slope method. The LOD and LOQ values for Tenofovir disoproxil fumarate were 0.05 µg and 0.125 µg. Method precision for (S)-enantiomer at 0.125 µg was less than 2.0% RSD. The resolutions between the two isomers were found more than 2.0. Therefore, this method had adequate sensitivity for the detection and estimation of (S)-enantiomer in Tenofovir disoproxil fumarate.

Good linearity of (S)-enantiomer was evaluated over eight levels of (S)-enantiomer solutions from 0.125 µg/ml to 3.75 µg/ml, with the linear regression equation  $y = mx + c$ , where x is the concentration in µg/ml, and y is the corresponding peak area of undesired enantiomer in mV/s. We observed linear results with respect to concentration for (S)-enantiomer. The correlation coefficient value is more than 0.999. The linearity results of Tenofovir disoproxil fumarate and (S) enantiomer are given in table 2 and in table 3. The linearity graph was shown in fig. 6 and in fig. 7 correspondingly.

Table 2: Linearity of tenofovir disoproxil fumarate

S. No.	Concentration (µg/ml)	R-Isomer peak *(n = 8)
1	0.125	2174
2	0.50	8270
3	1.00	18759
4	1.50	25005
5	2.00	33936
6	2.50	42456
7	3.00	49944
8	3.75	62181
Correlation coefficient		0.9993
Slope		16507.4324
Y-intercept		678.8324
r <sup>2</sup>		0.9987

\* Mean of eight determinations

Table 3: Linearity of (S)-isomer

S. No	Concentration (µg/ml)	S-Isomer peak *(n = 8)
1	0.125	1994
2	0.50	7502
3	1.00	15795
4	1.50	23311
5	2.00	31750
6	2.50	39656
7	3.00	47005
8	3.75	58723
Correlation coefficient		0.9999
Slope		15730.8499
Y-intercept		-49.3710
r <sup>2</sup>		0.9998

\* Mean of eight determinations

The standard addition and recovery experiments were conducted for (S)-enantiomer in bulk samples in triplicate at 0.125 µg/ml to 3.75 µg/ml (0.125, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.75 µg/ml). The accuracy was in terms of recovery (%). The

recovery was calculated by back-calculated concentration at each level in each preparation. The recovery is not less than 99.2% and not more than 102.8%. The recovery results are given in table 4.

Table 4: Accuracy

Added amount (µg)	Recovery (%)	*%RSD (n = 3)
1.5	99.33	1.01
3	101.67	0.76
4.5	102.00	0.33
7.5	101.87	0.26
9	100.33	0.68
10.5	99.24	1.13
12	100.75	0.25

\* Mean of three determinations

The repeatability and intermediate precision were expressed as relative standard deviation (RSD). For this study, a solution of Tenofovir disoproxil fumarate (250 µg/ml) spiked with (S)-enantiomer (1.0 %, 2.5 µg/ml) was analyzed in six injections to establish repeatability. RSD values were better than 0.5% for the

retention times of both the enantiomers. In the intermediate precision study results shown that RSD values were in same order of magnitude than those obtained for repeatability studies were captured in table 5. All these values indicated that the method was precise.

Table 5: Ruggedness

Name of the Interval	*%RSD (n = 6)
Day-1	0.3
Day-2	0.4
Day-3	0.5
Diff system	0.7
Diff column	0.6
Diff analyst	0.2

\* Mean of six determinations

As per ICH, the method robustness studies were demonstrated by adjusting flow rate, column temperature and mobile phase composition variations. The chromatographic resolution of

Tenofovir disoproxil fumarate and (S)-enantiomer was more than 2.0 under all separation conditions. The robustness results were captured in table 6.

Table 6: Robustness

Description	USP tailing	USP	USP
		Tangent	Resolution
Column flow: 0.80 ml/min	1.0	3829	2.5
Column flow: 1.20 ml/min	1.0	4362	2.2
Column Temp: 25°C	1.1	4125	2.3
Column Temp: 35°C	1.0	3829	2.3
Organic ratio: 110%	1.0	3242	2.1
Organic ratio: 90%	1.1	4457	2.3

The stability of the solution and mobile phase used in this method was tested over a long time. No significance change in (S)-enantiomer content was observed in Tenofovir disoproxil fumarate sample during solution stability and mobile phase stability experiments, and the RSD

values were less than 2.0% for (S)-enantiomer peak area. No unknown peak was observed in above stability conditions. Hence, the Tenofovir disoproxil fumarate sample solution and the mobile phase were stable for up to 48 h and the results were captured in table 7.

Table 7: Solution stability

S. No	Time interval	R-isomer peak *area (n=7)	S-isomer peak *area (n=7)
1	Initial	18752	15782
2	After 6 H	18648	15691
3	After 12 H	18729	15942
4	After 18 H	18658	15858
5	After 24 H	19022	15642
6	After 36 H	18926	16001
7	After 48 H	18451	15978
Average		18741	15842
STDEV		188.4537	141.6769
% RSD		1.01	0.89

\* Mean of seven determinations

**Batch analysis**

By using this method we can analyze and quantify (S)-isomer in

Tenofovir disoproxil fumarate in manufacturing batches and R and D samples. We get repeatable results in all samples at Quality control department and captured in table 8.

**Table 8: Batch analysis**

S. No	Batch No	S-isomer content *(% w/w)	Average *(% w/w)
1	PT001 Pre-01	0.12	0.13
2	PT001 Pre-02	0.14	
3	PT002 Pre-01	0.15	0.14
4	PT002 Pre-02	0.13	
5	RD001 Pre-01	0.08	0.07
6	RD001 Pre-02	0.06	
7	RD002 Pre-01	0.07	0.08
8	RD002 Pre-02	0.09	

\* Mean of two determinations

**CONCLUSION**

A simple, specific, linear, accurate and precise reverse phase chiral HPLC method was successfully developed, which was capable of separating the undesired enantiomer from Tenofovir disoproxil fumarate.  $\alpha$ 1-acid glycoprotein chiral Chiralpak AGP (L-41) column was found to be selective for the enantiomers of Tenofovir disoproxil fumarate. The developed and validated method can be used for the chiral purity testing of Tenofovir disoproxil fumarate. The developed method is also stable and can be used for the quantitative determination of chiral impurity in Tenofovir disoproxil fumarate drug substance form.

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**CONFLICT OF INTERESTS**

Declared none

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