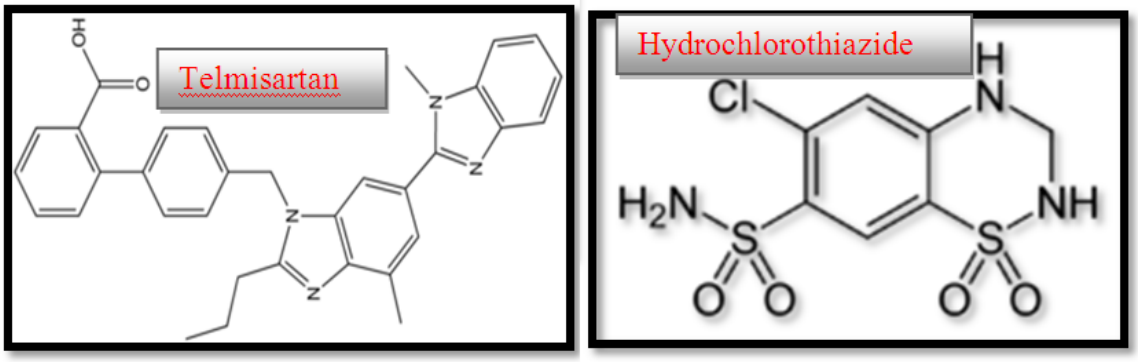
CHAPTER 6



RP - HPLC Method development and validation for simultaneous estimation of Telmisartan and Hydrochlorothiazide in formulation dosage forms

6.1: Introduction:

Detailed description of drugs Telmisartan and Hydrochlorothiazide were given in chapter 3.1

6.2 Literature review:

Detailed description of available literature for the estimation of Telmisartan and Hydrochlorothiazide using different analytical methods were given in chapter 3.2

6.3 Materials and Methods

6.3.1 Chemicals and solvents:

HPLC grade Methanol, Acetonitrile and water were purchased form Merck chemicals, Mumbai. Working standards of pharmaceutical grade Telmisartan and Hydrochlorothiazide were obtained as gift sample from Cipla pharmaceuticals, Mumbai, India and SUN pharmaceuticals, Hyderabad, India respectively. The market available brand TELISTA-H containing Telmisartan-40mg and Hydrochlorothiazide-6.5mg was purchased from local pharmacy.

6.3.2 Instrumentation:

Chromatographic separation was performed on a PEAK chromatographic system with kromasil C18 column (250mm×4.6mm; 5µm particle size) equipped with LC-P7000 isocratic pump; Rheodyne injector with 20µl fixed volume loop, variable wavelength programmable UV detector UV7000 and the output signal was monitored and integrated by PEAK Chromatographic Software version 1.06. Teccomp UV-2301 double beam UV-Visible spectrophotometer was used to carry out spectral analysis and the data was recorded by Hitachi software. Sonicator (1.5L) Ultrasonicator was used to sonicating the mobile phase and samples. Standard and sample drugs were weighed by using Denver electronic analytical balance (SI-234) and pH of the mobile phase was adjusted by using Systronics digital pH meter.

6.3.3 Chromatographic conditions:

Chromatographic operation was carried using a mobile phase of Methanol: Acetonitrile: water at a ratio of 40:40:20 (v/v), pH 5.2 at a flow rate of 1.0ml / min in isocratic condition. UV detection was carried out at 270 nm. Separation was achieved on C-18 Column (4.6mm x 250 mm, 5µm) at ambient temperature.

6.3.4: Preparation of Standard Stock Solutions

Standard stock solutions of concentration 1000 µg/ml of Telmisartan and 1000 µg/ml of Hydrochlorothiazide were prepared separately using methanol. From the standard stock solution, the working standard dilutions were prepared by diluting the stock solution with Methanol. 1ml from each of the standard dilutions was mixed with each other to get combined standard dilutions for constructing calibration curve

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6.3.5: Tablet formulation

Twenty tablets of each TELISTA-H containing Telmisartan-40mg and Hydrochlorothiazide - 6.5mg were weighed, and crushed into fine powder. A quantity of powder equivalent to 50mg of Telmisartan was dissolved in 25 ml of methanol and sonicate for 15 min. Then volume was made up to 50 ml with Acetonitrile and filtered through 0.45µ nylon membrane filter paper. From the obtained filtrate, a concentration of 120 µg/ml of Telmisartan was prepared by proper dilution. Based on the dosage of the two drugs in tablet, a concentration of 37.5µg/ml of Hydrochlorothiazide was obtained. The obtained mixed drug solution having a concentration of 120 µg/ml of Telmisartan and 37.5 µg/ml of Hydrochlorothiazide was used for the estimation of the drug in tablet dosage using the optimized method.

6.4 Optimization of HPLC Method

All drugs were subjected to chromatographic analysis using mobile phases of differing pH, flow rate using the under mentioned chromatographic conditions. The changes in the retention time of all drugs were noted as a function of changing mobile phase, pH, flow rate, strength and selectivity. Initially methanol: water in the ratio of (80: 20) was tried but both the peaks merged. After number of trials with different solvents with different ratios, methanol: Acetonitrile: water (40:40:20%) with ratio of (70: 30) was tried and it was found that both the peaks were well separated with acceptable resolution. Hence methanol: Acetonitrile: water (40:40:20) at flow rate of 1 ml/min was finalized which gave acceptable retention time, plates and good resolution for Telmisartan and Hydrochlorothiazide With the optimized chromatographic conditions a steady baseline was recorded. After the stabilization of the baseline for 20 min. Standard solutions containing 40-6.5µg /ml of Telmisartan and Hydrochlorothiazide were injected and chromatograms were recorded. Retention time of Telmisartan and Hydrochlorothiazide were found to be 4.63 and 6.14mins respectively. Optimized conditions were shown in table 6.1, chromatogram of Blank, standard and sample were shown in figure 6.A, 6.B and 6.C respectively.

Table: 6.1: Optimized method conditions:

S.NO	Parameter	Results
1	MP	Methanol: Acetonitrile: water 40:40:20 (v/v)
2	Wavelength	270nm
3	Stationary Phase	C-18
4	pH of MP	5.2
5	Flow Rate	1.0 ml/min
6	Pump Mode	Isocratic
7	Pump Pressure	6.6 ± 5MPa
8	Api Concentration	Telmisartan – 120 µg/ml Hydrochlorothiazide – 37.5 µg/ml
9	RT	Telmisartan – 4.64 min Hydrochlorothiazide - 6.14 min
10	Resolution	Telmisartan -------- Hydrochlorothiazide – 6.25
11	Area	Telmisartan – 579908 Hydrochlorothiazide -694331
12	Theoretical Plates	Telmisartan – 5784 Hydrochlorothiazide - 10733
13	Tailing Factor	Telmisartan – 1.63 Hydrochlorothiazide – 0.87

Figure: 6.A: Blank chromatogram

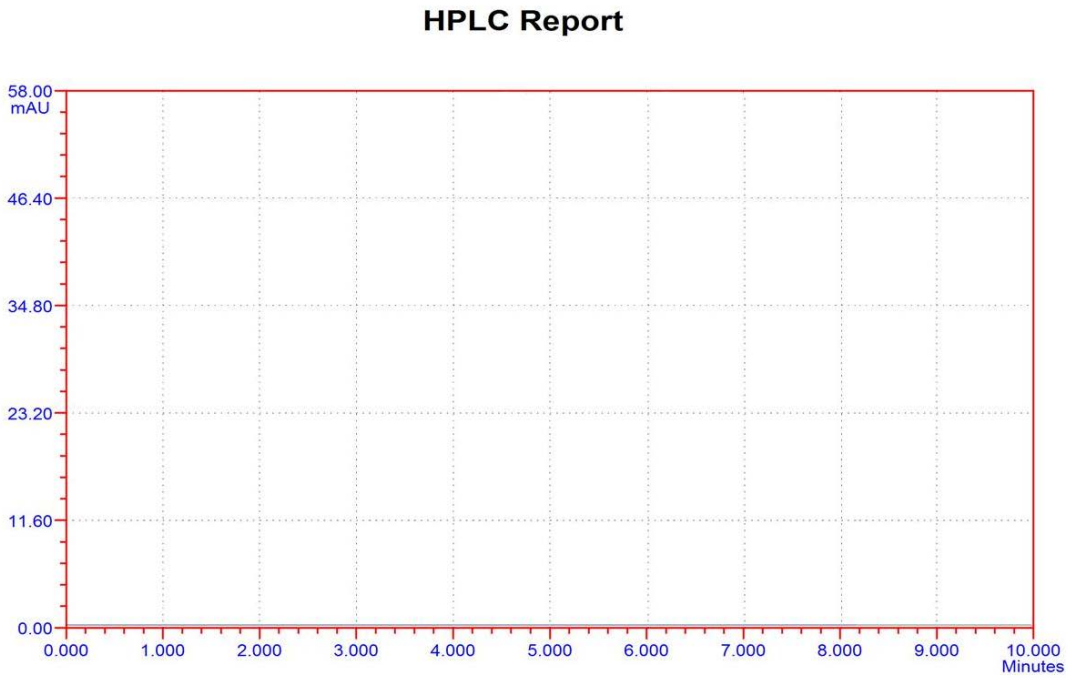


Figure: 6.B: Standard chromatogram

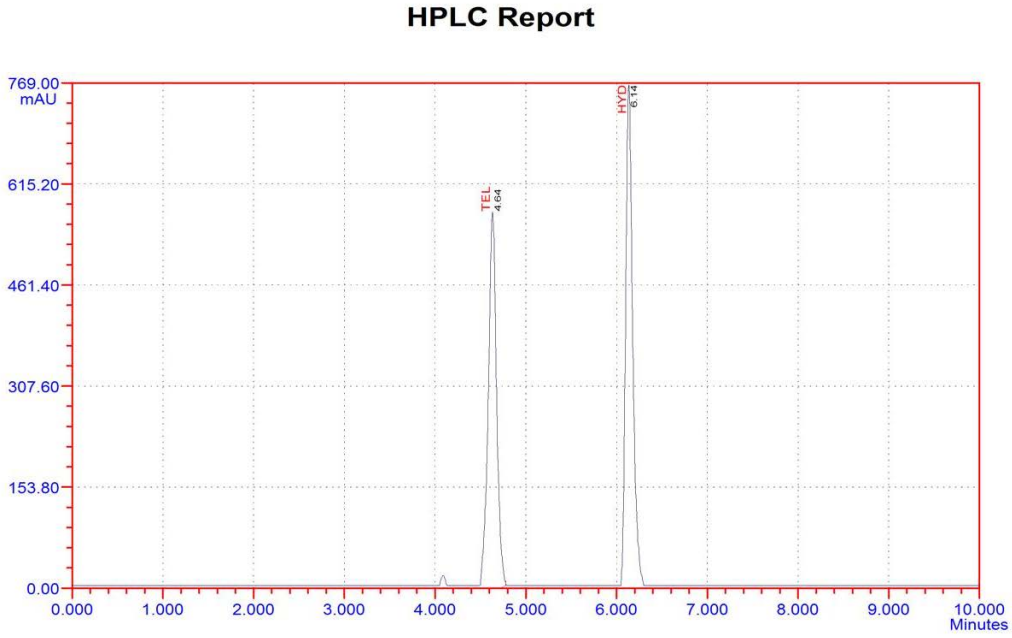
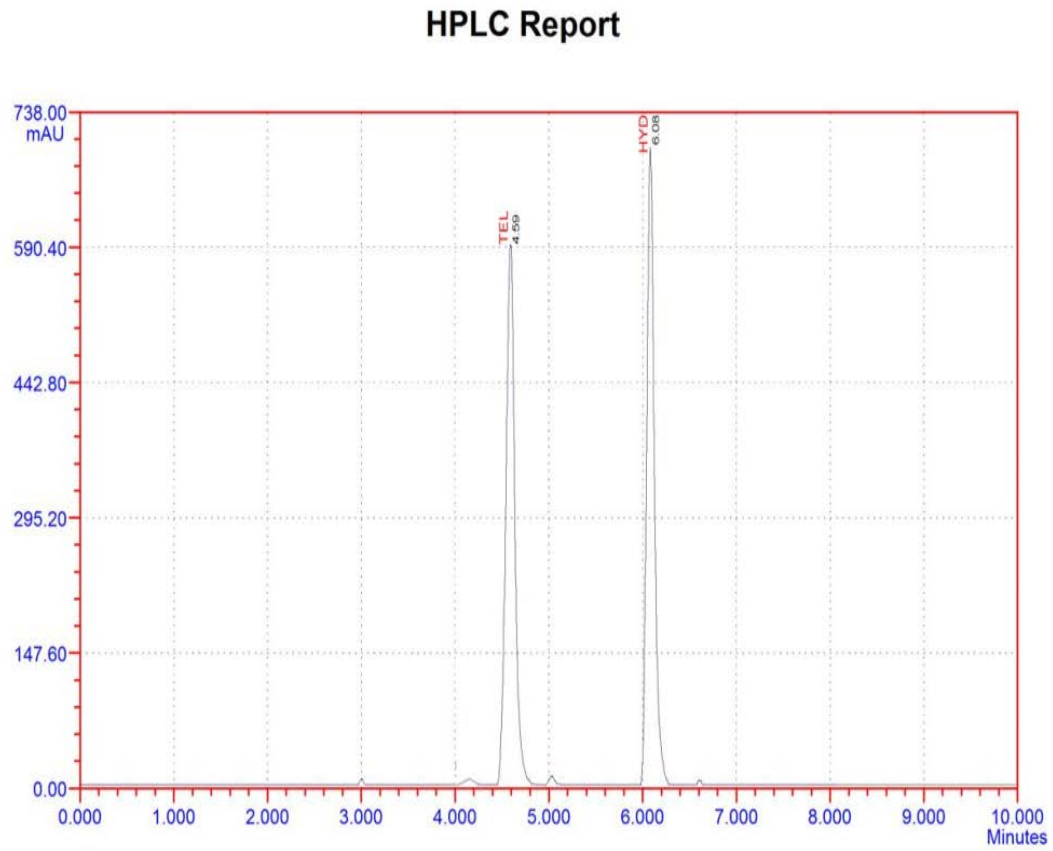


Figure 6.C Formulation chromatogram:



6.5 Method Validation

The developed method obeys validation parameters for the applicability of the method for estimation of drugs in pharmaceutical formulations. Different validation parameters like system suitability conditions, Linearity, Precision, Recovery, ruggedness, robustness, LOD and LOQ were studied. All these parameters were verified using ICH guidelines.

6.5.1: System Suitability parameters:

The column efficiency, resolution and peak asymmetry were for the standard solution was used to demonstrate the system suitability of the method. System suitability conditions were verified to ensure whether the developed chromatographic conditions give the best results or not. System suitability parameters were evaluated for six replicate

injections of the drug at a concentration of 120 µg/ml of Telmisartan and 37.5 µg/ml of Hydrochlorothiazide. In the optimized conditions, a well resolved peak with a resolution factor of 6.25 was observed at retention time of 4.64 min for Telmisartan and 6.14 min for Hydrochlorothiazide. Very high theoretical plates and very less tailing factor (1.57, 1.59) were observed for both the drugs. Hence the developed method obeys the system suitable criteria.

6.5.2 Specificity:

The specificity of an analytical method may defined as the ability to detect the analyte peak in the presence of the analyte by product, or other inactive components, such as dosage form excipient or impurities. The specificity of the method was determined by comparing the base line and retention times obtained when standard, blank and sample solutions were injected in to HPLC system using the optimized conditions. The results confirmed that the no spectral and chromatographic detection were observed at the retention of both the drugs in blank injection. A retention time of 4.64 min for Telmisartan and 6.14 min for Hydrochlorothiazide were observed for standard solution and similar retention times were observed for samples also. Hence the developed method was specific for Telmisartan and Hydrochlorothiazide.

6.5.3: Linearity:

The standard stock solution (1000µg/ml of Telmisartan and 100µg/ml of Hydrochlorothiazide) was further diluted to get Telmisartan and Hydrochlorothiazide concentration in the range of 40-280µg/ml and 6.5-87.5µg/ml respectively. Linearity of the method was studied by injecting seven concentrations of the drug prepared in the mobile phase into the LC system keeping the injection volume constant. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs. Linearity results were shown in table 6.2 and graph was shown in figure 6.E and 6.F

Table 6.2: Linearity results:

S.NO	Concentration in µg/ml	Telmisartan Peak Area	Concentration in µg/ml	Hydrochlorothiazide Peak Area
1	40	228045	6.5	228045
2	80	375044	25	457173
3	120	579908	37.5	694331
4	160	745442	50	878692
5	200	934721	62.5	1076590
6	240	1100389	75	1283970
7	280	1257359	87.5	1464879
		Slope:14293.14 Intercept:27288.58 Cc:0.999		Slope:16739.42 Intercept:28110.42 Cc:0.999

Figure: 6.D: Calibration curve of Telmisartan

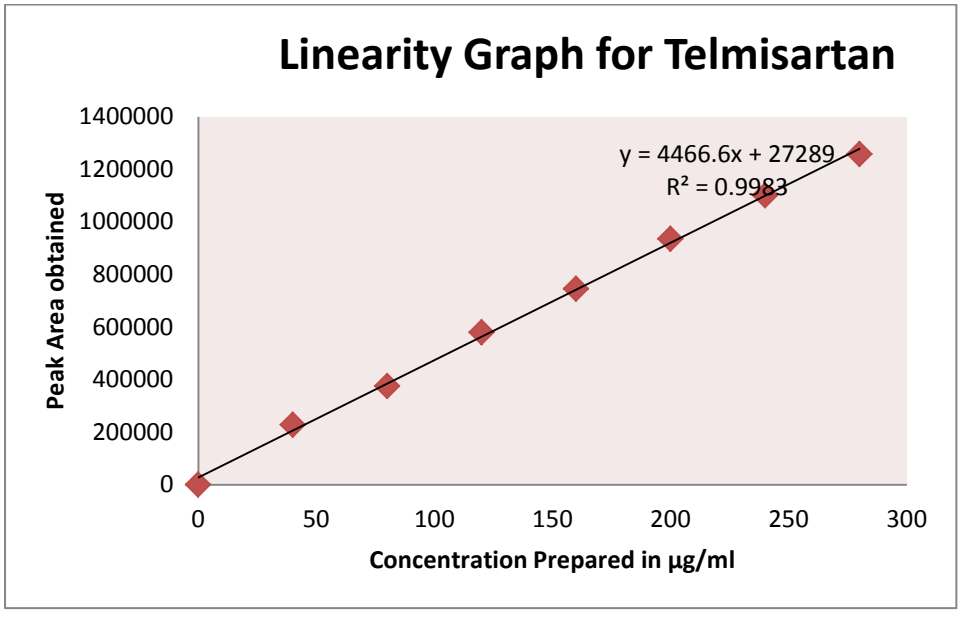
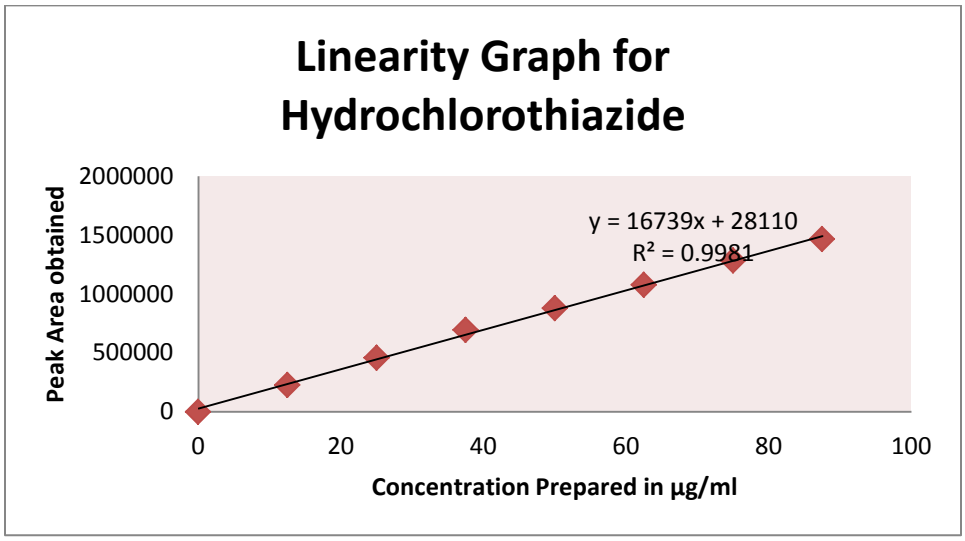


Figure: 6.E: Calibration curve of Hydrochlorothiazide



6.5.4: Precision

The precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analysis of three different concentrations 120 µg/ml for Telmisartan and 37.5 µg/ml for Hydrochlorothiazide six times on the same day. The intermediate precision of the method was checked by repeating studies on three different days. %RSD was found to be 0.55, 0.90 for Telmisartan and 0.72, 0.68 for Hydrochlorothiazide in Intraday and interday Precision respectively. The % RSD was found to be within the acceptance limit. Hence the method was found to be precise. Results of the precision studies were shown in table 6.3

Table 6.3: Results of Precision:

Intraday Precision		Interday Precision	
Telmisartan 120 µg/ml	**Hydrochlorothiazide 37.5 µg/ml**	**Telmisartan 120 µg/ml**	**Hydrochlorothiazide 37.5 µg/ml**
557710	682572	563824	694322
559163	694819	565703	694644
565938	689476	570006	698304
561906	691261	576776	685227
564381	686388	565464	688309
558185	697729	560750	686862
RSD:0.55	**RSD:0.72**	**RSD:0.90**	**RSD:0.68**

6.5.5: Robustness

To evaluate robustness of a HPLC method, few parameters were deliberately varied. The parameters included variation of wavelength; mobile phase ration and P^H were change to determine the Robustness of the method. Robustness of the method was done at levels 120µg/ml and 37.5 µg/ml for Telmisartan hydrochloride and Hydrochlorothiazide respectively. The % change in each of the changed condition was measured by comparing with calibration curve results. % change was found to be within the acceptance limit of less than 2. Hence small variations in the optimized conditions don't influence the results. Table 6.4 shows the robustness study results for Telmisartan and Hydrochlorothiazide.

Table 6.4: Robustness results:

Condition	Telmisartan		Hydrochlorothiazide	
	Mean area	% difference	Mean area	% difference
Standard	579908	…….	694331	……
Mp Changes-1 v/v M: A: W (35:45:20%) **Mp Changes-2 M: A: W (45:35:20%)**	572268 577278	1.31 0.45	700315 699648	0.86 0.76
WL Changes-272 **WL Changes- 268**	586362 576776	1.11 0.54	683941 693624	1.49 0.10
PH -5.0	580856	0.16	694312	0.002
PH -5.4	584587	0.80	686681	1.10

6.5.6 Accuracy:

Accuracy of the method was carried out by applying the method to drug sample (Telmisartan and Hydrochlorothiazide combination tablet) to which know amount of Telmisartan and Hydrochlorothiazide standard powder corresponding to 50, 100 and 150 % of label claim had been added (Standard addition method), mixed and the powder was extracted and analyzed by running chromatogram in

optimized mobile phase. High % recovery of more than 98% was observed for both the drugs in all the spiked level. Results of the recovery was given in table 6.5

Table 6.6: Accuracy results:

Recovery	Telmisartan				Hydrochlorothiazide			
	Concentration (µg/ml)		% Recovery	R.S.D	Concentration (µg/ml)		% Recovery	R.S.D
	Final	Obtain			Final	Obtain		
50%	120	118.78 ±0.39	98.98 ±0.46	0.46	37.5	37.48 ±0.33	99.96 ±0.89	0.89
100%	160	159.89 ±2.55	99.93 ±1.59	1.59	50	49.86 ±0.58	99.71 ±1.15	1.15
150%	200	198.74 ±1.69	99.37 ±0.84	0.85	62.5	62.61 ±0.57	100.81 ±0.91	0.90

6.5.7: Ruggedness:

Intraday precision at person to person variation was measured to express the ruggedness of the developed method. Standard drug solution at a standard concentration of 120 µg/ml and 37.5 µg/ml of Telmisartan and Hydrochlorothiazide was prepared by 6 different persons and the solution was injected in to HPLC system. Peak area response was noted and %RSD was calculated. Results of the Ruggedness were shown in the table 6.6.

Table 6.6: Ruggedness results

Concentration in µg/ml	Telmisartan Peak Area	Concentration in µg/ml	Hydrochlorothiazide Peak Area
120	598548	37.5	683799
120	581025	37.5	684273
120	585154	37.5	691386
120	595466	37.5	688011
120	587866	37.5	685037
120	599281	37.5	697530
	RSD:1.17		**RSD:0.77**

.5.8: Limit of detection and limit of quantization:

Limits of detection (LOD) and quantification (LOQ) represent the concentration of the analyte that would yield signal-to-noise ratios of 3 for LOD and 10 for LOQ, respectively. To determine the LOD and LOQ, serial dilutions of mixed standard solution of Telmisartan and Hydrochlorothiazide was made from the standard stock solution. The samples were injected in LC system and measured signal from the samples was compared with those of blank samples. Sensitivity results of Telmisartan and Hydrochlorothiazide were shown in table 6.7.

Table 6.7: Results of LOD and LOQ:

Parameter	Telmisartan	Hydrochlorothiazide
LOD	0.75µg/ml	2.5µg/ml
LOQ	2.5µg/ml	8.25µg/ml

6.5.9: Analysis of a marketed formulation:

To determine the content of Telmisartan and Hydrochlorothiazide in conventional tablet (Brand name: TELISTA-H, Label claim: 40mg Telmisartan and 100 mg Hydrochlorothiazide per tablet), twenty tablets were weighed, their mean weight determined and finely powdered. The weight of the tablet triturate equivalent to 40mg of Telmisartan and 6.5mg Hydrochlorothiazide was transferred into a 50 ml volumetric flask containing 30 ml methanol, sonicated for 30 min and diluted up to 50 ml with methanol. The resulting solution was determined (320 and 100µg/ml for Telmisartan and Hydrochlorothiazide, respectively). Supernatant was taken and after suitable dilution the sample solution was then filtered using 0.45-micron filter. The above stock solution was further diluted to get sample solution of 40 and 6.5µg/ml for Telmisartan and Hydrochlorothiazide respectively. A 20 µL volume of sample solution was injected into HPLC, six times, under the conditions described above. The peak areas were measured at 270 nm and concentrations in the samples were determined using multilevel calibration developed on the same HPLC system under the same conditions using linear regression equation. Results of the formulation analysis were shown in table 6.8 and formulation chromatogram was shown in figure 6.D

Table 6.8: Formulation assay results:

Brand name	Available form	Label claim	Concentration µg /ml	Amount found µg /ml	% Assay
TELISTA-H	Tablet	Tel -40mg Hyd-6.5mg	Tel -120 Hyd-37.5	Tel -118.59 Hyd-37.14	Tel -98.82 Hyd-99.04

6.6: Results and Discussion

The results of system suitable studies on simultaneous estimation method developed for Telmisartan and Hydrochlorothiazide in the current study involving methanol: acetonitrile: water in the ratio of 40:40:20 (v/v). Telmisartan and Hydrochlorothiazide showed good correlation coefficient (r^2 = 0.9991 for Telmisartan and 0.9990 for Hydrochlorothiazide) in given concentration range (40-280µg/ml for Telmisartan and 6.5-87.5µg/ml for Hydrochlorothiazide). The mean values of the slope and intercept were 4467 and 27289 for Telmisartan and 16739 and 28110 for Hydrochlorothiazide respectively. The results of the repeatability and intermediate precision experiments are shown in Table 8.8. The developed method was found to be precise as the RSD values for repeatability and intermediate precision studies were < 2 %, respectively as recommended by ICH guidelines13-15. Signal-to-noise ratios of 3:1 and 10:1 were obtained for the LOD and LOQ respectively. The LOD and LOQ were found to be 0.75µg/ml and 2.5µg/ml for Telmisartan and 2.5µg/ml and 8.25µg/ml for Hydrochlorothiazide, respectively.

To study the robustness of the proposed method one factor at the time was changed to estimate the effect. Thus, replicate injections of mixed standard solution were performed under small changes of three chromatographic parameters (factors). Insignificant differences in peak areas and less variability in retention time were observed. As shown from the data in Table 6.9 good recoveries of the Telmisartan and Hydrochlorothiazide in the range from 98.19 to 101.32% were obtained at various added concentrations. Tablets, expressed as a percentage of label claims were in good agreement with the label claims thereby suggesting that there is no interference from any of the excipients which are normally present. The drug content was found to be 98.82 % for Telmisartan and 99.04 % for Hydrochlorothiazide. Excipients from formulation were not interfering with the assay. Summary results for the analysis of Telmisartan and Hydrochlorothiazide were shown in table 6.9.

Table 6.10: Summary results for Telmisartan and Hydrochlorothiazide

	Parameter	Telmisartan	Hydrochlorothiazide
Method Developed	Elution	Isocratic	
	Mobile Phase	methanol: acetonitrile: water (40:40:20%) v/v	
	pH	5.2	
	Column	RP- C18 Column	
	Wave Length	270 nm	
	Flow	1.0 ml/min	
	Runtime	10 min	
	Temperature	Ambient	
Method validation	Retention Time	4.64 min	6.11min
	Tailing factor	1.28	1.03
	Theoretical plate	7412	96983
	Resolution	…….	6.20
	Linearity range	40-280 µg/ml	6.5-87.5 µg/ml
	Slope	14293.14	16739.42
	Intercept:	27288.58	28110.42
	r^2	0.999	0.999
	Intraday Precision	0.55	0.72
	Interday Precision	0.90	0.68
	Ruggedness	1.17	0.77
	RSD of Recovery	0.46-1.59	0.89-1.15
	Robustness difference	0.16-1.31	0.002-1.49
	LOQ	2.50 µg/ml	8.25 µg/ml
	Limit of Detection	0.75 µg/ml	2.5 µg/ml
	Formulation assay	98.82	99.04

.7 Conclusion

HPLC method was developed and validated as per ICH guidelines. UV detection allowed an accurate quantization of chromophoric compounds. The drug was analyzed by HPLC method using kromasil C-18 Column (4.6mm x 250 mm, 5µm), with isocratic conditions and simple mobile phase containing methanol: acetonitrile: water in the ratio of 40:40:20 v/v at flow rate of 1ml/min using UV detection at 270nm. The procedure has been evaluated for the linearity, accuracy, precision and robustness in order to ascertain the suitability of the analytical method. The method was also applied to marketed samples. It has been proved that the method is selective and linear between concentration range 40-280µg/ml for Telmisartan and 6.5-87.5µg/ml for Hydrochlorothiazide. LOD and LOQ were found to be 0.75 µg/ml and 2.5µg/ml for Telmisartan and 2.5µg/ml and 8.25µg/ml Hydrochlorothiazide, respectively. Statistical analysis proves that the method is suitable for the analysis of Telmisartan and Hydrochlorothiazide as in pharmaceutical formulation without any interference from the excipients. The method can be used for the routine analysis of Telmisartan and Hydrochlorothiazide in pharmaceutical formulations.

6.8 References

The references available for the drugs Telmisartan and Hydrochlorothiazide were given in chapter 3.8