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DEVELOPMENT OF NEW LIQUID CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS ESTIMATION OF HYDROCHLOROTHIAZIDE AND CANDESARTAN CILEXETIL AND ITS APPLICATION IN SOLUBILITY ENHANCEMENT STUDIES

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ABSTRACT
A liquid chromatographic method was developed for the estimation of hydrochlorothiazide (HCT) and candesartan cilexetil (CSC) in solid dispersions as a part of solubility enhancement studies. In this method drugs were resolved by using C18 column and mobile phase consisting of phosphate buffer pH 5 and acetonitrile (30:70 %v/v). The mobile phase was pumped at a flow rate of 1 mL/min and UV-detection was carried out at 255 nm. The quantification of drugs was achieved by using external standard method. The method was validated as per ICH Q2 (R1) guidelines. Linearity was observed over concentration range of 2-10 µg/mL and the coefficient of determination was found to be 0.99. The limit of detection and limit of quantitation were found to be 1.31 µg/ml and 3.97µg/mL for hydrochlorothiazide and 1.72µg/ml and 4.39µg/mL for candesartan cilexetil. The liquid chromatographic method was proved to be acuurate and precise. The validated method was successively applied to study the enhanced solubility of both drugs in the prepared solid dispersions.

Keywords: Hydrochlorothiazide, Candesartan cilexetil, Solid dispersion, Limit of detection, ICH guidelines.

INTRODUCTION:

Hydrochlorothiazide (HCT) is a diuretic and a BCS class IV drug with low solubility and low permeability, exhibiting poor oral absorption. The chemical name of hydrochlororthiazide is 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide. It reduces the reabsorption of electrolytes from the renal tubules. This results in increased excretion of water and electrolytes, including sodium, potassium, chloride and magnesium. Candesartan is an a ngiotensin-Receptor Blocker, chemically known as 2-ethoxy-1-({4-[2-(2H-1, 2, 3, 4-tetrazol-5-yl) phenyl] phenyl} methyl)-1H-1, 3-benzodiazole-7-carboxylic acid that was used alone or with other agents to treat hypertension. It selectively blocks the binding of Angiotensin-II to AT1 in many tissues including vascular smooth muscle and the adrenal glands. [1-2]

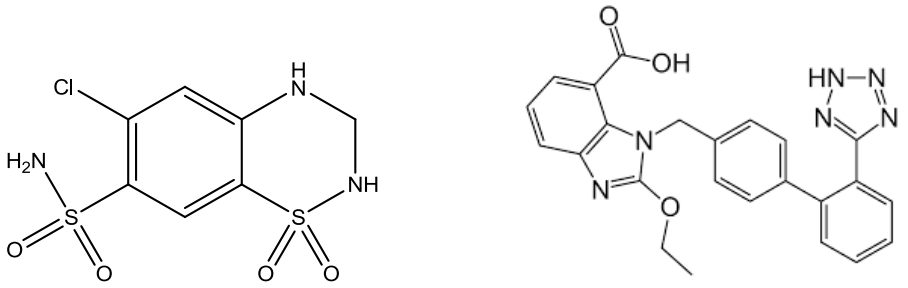

Fig. 1: Structure of hydrochlorothiazide and candesartan cilexetil

The literature review reveals that there are HPLC and UV Spectrophotometric methods for estimation of these two drugs in formulations. [3-11]

UV spectrophotometric methods are commonly used methods for the estimation of drug concentrations during solubility enhancement studies. HPLC methods are more sensitive than spectrophotmetric methods. The present study focuses on development of a simple liquid chromatographic method for the simultaneous estimation of hydrochlorothiazide and candesartan cilexetil for application in solubility enhancement studies.

MATERIALS AND METHODS

Chemicals and reagents

Hydrochlorthiazide and candesartan cilexitel pure drug samples are from Hetero laboratories, Hyderabad. All other chemicals and reagents used are obtained from SDFCL and are of analytical grade.

Instrument

An isocratic high performance liquid chromatograph (SHIMADZU, HPLC), HPLC pump LC-20AT with LC solution software, UV-visible detector SPD-10A and Enable-18H C18 column was used (250 x 4.6 mm, 5 µm).

Method Development

A liquid chromatographic method was developed in reverse phase mode by optimising wavelength, mobile phase and its pH and composition, column type and flow rate.[12]

Preparation of standard solutions

100 µg/mL hydrochlorothiazide and candesartan cilexetil solutions were prepared in HPLC grade water.

Selection of wavelength

The sensitivity of the HPLC method that uses UV detection depends upon the proper selection of the detection wavelength. An ideal wavelength is one that gives good response for the drugs to be detected. In order to ascertain the optimum wavelength (λ_{max}) suitable dilutions of the drugs were made with methanol and scanned on UV-Visible spectrophotometer in the range of 200 to 400 nm against methanol as blank. The λ_{max} obtained from the spectrum was used as a detection wavelength in HPLC analysis.

Selection of mode of separation

In reverse phase mode, the mobile phase is comparatively more polar than the stationary phase. For the separation of polar or moderately polar compounds, the most preferred mode is reverse phase. The nature of the analyte is the primary factor in the selection of the mode of separation. A second factor is the nature of the matrix. Reverse phase mode is selected for separation of these two drugs.

Selection of mobile phase and its composition

In RP-HPLC method, water-organic solvent is generally used as the mobile phase. Acetonitrile and methanol are preferred organic modifiers. The proportions of buffer (0.1 M phosphate buffer pH =5.0 adjusted with orthophosphoric acid) and acetonitrile (50:50, 40:60, 30:70) were varied and studied.

Selection of buffer and pH

Buffer selection was done based on pKa value of the drug.

$$\mathbf{pH = pKa \pm 1.5}$$

For the present work mobile phase pH was selected between 2.5 to 6.0 based on pKa value of the drug. Buffer like 0.1M phosphate buffer (pH =5.0 adjusted with orthophosphoric acid) was used.

Column selection

C18 column was selected as the method is reverse phase mode and the nature of the drug is polar.

Selection of column temperature

Temperature variation has a significant effect on separation of analytes which affect the other chromatographic factors such as resolution, capacity factors. All the method development work was done at ambient temperature (25˚C).

Selection of flow rate

Flow rate can sometimes be useful and readily utilized to increase the resolution, although its effect is very moderate. Flow rates of 0.9, 1.0 and 1.1 mL/min were tried and 1 mL/min was suitable for separation and elution of drug peak.

All the above chromatographic conditions were optimized with the trails by varying mobile phase composition and flow rate. The optimized chromatographic conditions are given in table 1 and the respective chromatogram is shown in fig. 2.

Method validation

The optimized RP-HPLC method was validated according to ICH Guidelines Q2 (R1): Validation of Analytical Procedures: Text and Methodology.[13]

The developed method was validated for the following parameters - specificity, linearity, range, LOD, LOQ, accuracy, precision, robustness and system suitability parameters. Stability of analyte solutions was checked.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of the components which may be expected to be present; typically these might include impurities, degradation products and matrix components. Blank and standard sample solutions were prepared and injected into HPLC system and were analyzed as per the test method.

From stock solutions of 100 µg/mL, 10 µg/mL solutions of both drugs were prepared. Blank run and sample run were performed.

Linearity and Range

The linearity of the analytical method is its ability to elicit test results which are directly proportional to the concentration. The range of an analytical procedure is the interval between the upper and lower concentrations (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

A series of calibration standard solutions in the concentration range 2 µg/mL to 10 µg/mL were prepared and injected into HPLC system and were analyzed as per method. From the chromatograms peak areas were recorded, calibration curves were constructed and analyzed statistically for proving linearity.

Limit of detection and Limit of quantitation

The limit of detection (LOD) and the limit of quantitation (LOQ) were determined using the standard deviation method. From the linearity data, the LOD and LOQ were calculated using the following formulae:

$$\mathbf{LOD= 3.3\sigma/S}$$

Where, σ = standard deviation of the response

S= slope of the calibration curve of the analyte

$$\mathbf{LOQ= 10\sigma/S}$$

Where, σ = standard deviation of the response

S= slope of the calibration curve of the analyte.

Accuracy

Accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as conventional true value or an accepted reference value and the value found. A series of sample solutions were prepared in triplicate by spiking the hydrochlorothiazide and candesartan cilexetil API in the range of 80% -120% of test concentration and injected into HPLC system and were analyzed as per method.

Recovery studies (drug product)

Recovery studies were carried out by using standard addition method, known amount of standard drug (2 µg/mL, 4 µg/mL, 6 µg/mL) to the sample solution (2 µg/mL) and the samples were analyzed and total amount of drug present was calculated from the calibration curve using regression equation. The percent recovery of the drugs was calculated and tabulated.

Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the homogeneous sample under prescribed conditions. Precision of the method was determined as repeatability and intermediate precision.

Repeatability

From the mixed stock solution of 100 µg/mL (containing candesartan cilexetil and hydrochlorothiazide), 6 µg/mL concentration solution was prepared. The above solution was filtered using Whatman filter

paper. Six successive injections of working standard solution were injected and chromatograms were recorded. From chromatograms, peak areas were recorded and %RSD was calculated.

Intermediate precision

To evaluate intermediate precision, replicate samples were prepared and analyzed as per test method by using HPLC system and on same and different days. From stock solution, 4.8 µg/mL, 6 µg/mL and 7.2 µg/mL solutions were prepared.

Intermediate precision of the proposed method was determined on samples solutions at varying concentration levels by analyzing three replicates of each sample as a batch. Successive injections of working standard solutions were injected and chromatograms were recorded. From chromatograms, peak areas were recorded and %RSD was calculated.

Robustness

The 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. Robustness was carried out by varying flow rate, mobile phase composition and pH.

Flow rate variation

Sample solution was prepared and injected into the HPLC system with flow rates 0.9 mL/min and 1.1 mL/min.

Mobile phase composition variation

Samples were analyzed with mobile phases consisting of buffer (phosphate buffer pH -5) and acetonitrile in the ratio of 64:36 % v/v and 56:44 %v/v.

pH variation in mobile phase

Two different mobile phases with phosphate buffer pH 4.9 and pH 5 were prepared and samples were analysed.

In all the cases from chromatograms, peak areas were recorded and sample concentrations were calculated.

Stability of analyte solutions

It is necessary to study stability of sample in the solvent used to prepare the sample solutions for injection in order to establish that the sample solution composition, especially the analyte concentration does not change with the time elapsed between the preparation of the solution and its analysis by HPLC. It is done to determine the period of time; a solution can be used before analysis without compromising accuracy. A minimum of 24 hrs, 48 hrs or 72 hrs is routinely recommended for chromatographic conditions.

Standard samples were prepared as per methodology and analyzed at initial and at different time intervals (0-72 hrs) by keeping at room temperature. %RSD was calculated at each interval against freshly prepared standard from the peak areas of the samples.

System suitability tests

The system suitability tests were carried out during the method development for optimizing the method conditions for chromatographic evaluation. For this parameters like plate number (N), resolution (R), tailing factor, RSD of peak area for repetitive injections were calculated.

FORMULATION DETAILS

Preparation of solid dispersions

Solid dispersions were prepared by using fusion technique. They are prepared with 1:2, 1:3 and 1:6 ratios of drug: polymer. Required quantities of the drug is weighed and kept aside. Then accurately weighed amounts of the polymer were taken in a lean crucible. The crucible along with the polymer was placed on a thermostatic hot plate which is at a temperature of 60°C. Keep stirring continuously until the polymer changes its state. Then the drug was added with continuous stirring. After thorough mixing, the medium was transferred onto a clean tile which is then placed on an ice bar. The homogenous mixture now solidifies and the formed mixture was then scrapped out. Blank or placebo was prepared in the same manner omitting the drug.

Evaluation of prepared solid dispersions of hydrochlorothiazide and candesartan

The prepared solid dispersions were evaluated to see the polymer and drug interactions. This is done by FTIR studies. Initially the pure drugs and the polymers used were subjected to FTIR studies. Then the prepared solid dispersions were subjected to FTIR studies and comparisons were drawn to see any undesirable interactions between the drugs and the polymers used.[14-15]

Quantitative analysis of solid dispersions of hydrochlorothiazide and candesartan

Sample solution were prepared in triplicate by transferring 0.6 mL of prepared solid dispersion solution (1000 µg/mL) and the dilutions were made with HPLC water to get 6 µg/mL sample solution. Prepared solutions were injected into HPLC system and were analyzed as per the developed method. From the chromatograms, peak areas were recorded and % purity was calculated.

RESULTS AND DISCUSSION

A liquid chromatographic method was developed and validated for the determination of hydrochlorothiazide and candesartan in solid dispersions using a gradient high performance liquid chromatograph.

Both the drugs have good absorption characteristics in UV region and the absorption maximum of hydrochlorothiazide and candesartan cilexetil was found to be 255 nm This wavelength was selected as a detection wavelength in HPLC- UV detector for the determination of both drugs.

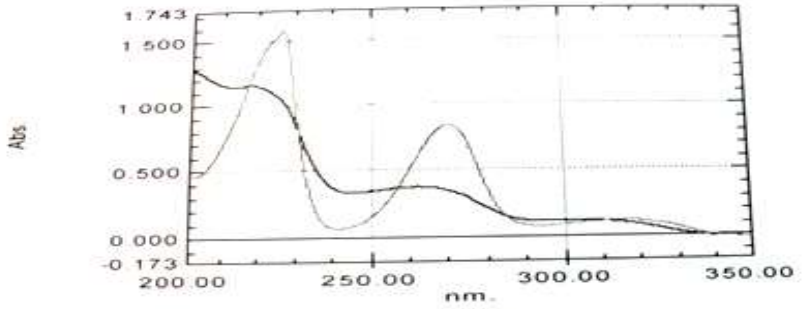

Fig. 2 :Overlay absorption spectrum of hydrochlorothiazide and candesartan cilexetil

The method was developed using C18 column with a mobile phase consisting of acetonitrile and phosphate buffer, at a flow rate of 1 mL/min. The method was optimized in seven trials with variation in mobile phase composition, type of solvents and flow rate.

Best results were obtained with phosphate buffer pH-5: ACN in the ratio (30:70) as mobile phase and 1.0mL flow rate using C_{18} column and the peak shape was good with minimum baseline noise. The optimized chromatographic conditions are shown in Table 1 and the chromatogram is shown in Fig 3.

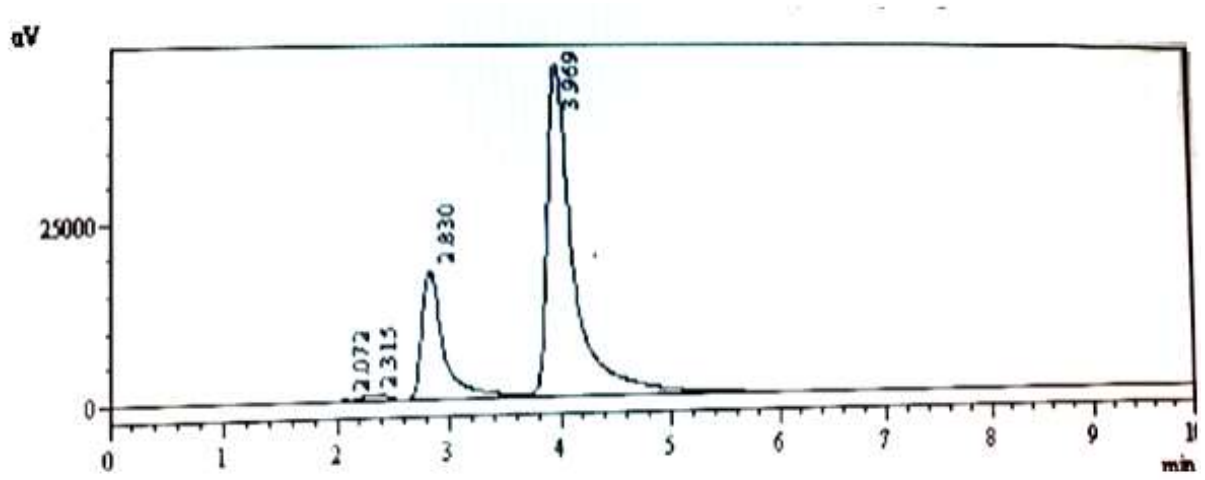

Fig. 3: Chromatogram for optimized chromatographic conditions

Table 1: Chromatographic conditions of optimized method

Parameters	Conditions
Column	C18 (250 mm x 4.6 mm, 5 µ)
Mobile phase	Phosphate Buffer pH-5:ACN (30:70)
Flow rate	1.0 mL/min
Run time	10 min
Column temperature	Ambient
Loop volume	20 µL
Detection wavelength	255 nm
Drug retention time	2.8 and 3.9 min

The drug peaks were eluted under the optimized conditions at the retention time of 2.8 and 3.9 minutes for hydrochlorothiazide and candesartan cilexetil respectively.

All the parameters determined as part of validation were within the acceptance crtiteria. The data for different validation parameters was discussed below.

Specificity was demonstrated using standard solutions of two drugs and blank. The chromatograms of hydrochlorothiazide and candesartan cilexetil and blank are shown in Fig. 4,5 and 6.

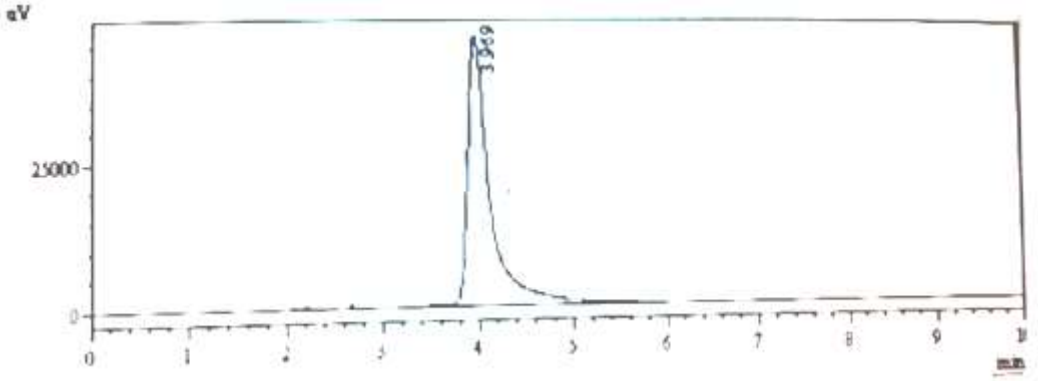

Fig. 4: Chromatogram of pure hydrochlorothiazide

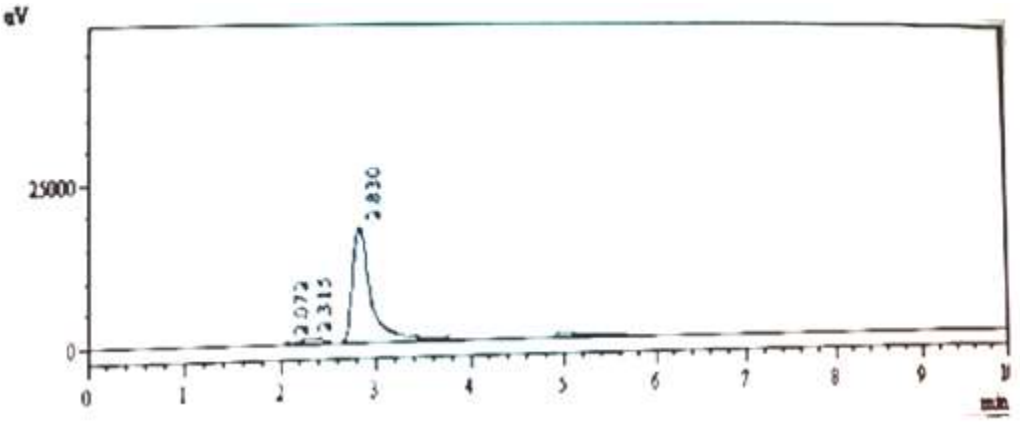

Fig. 5: Chromatogram of pure candesartan cilexetil

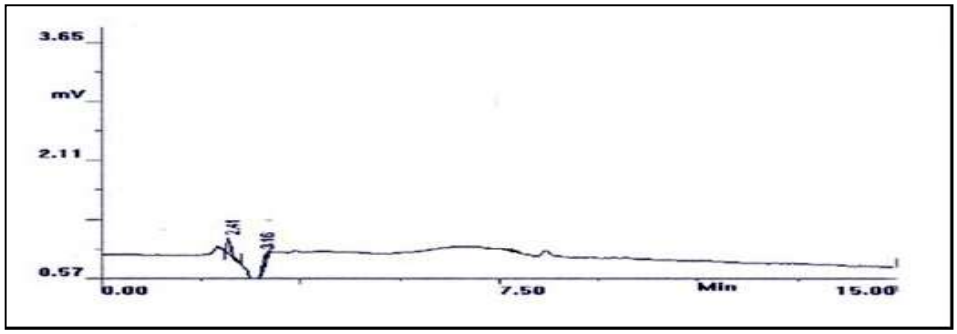

Fig.6: Chromatogram of blank

It was observed that no peaks were eluted at the retention times of both drugs, in the blank chromatogram thus showing that no interference existed. This indicates the specificity of the method for elution of analytes within the defined experimental conditions.

The five calibration standards solutions of hydrochlorothiazide and candesartan cilexetil were analysed and the calibration curves were plotted separately for hydrochlorothiazide and candesartan cilexetil

between the peak area and concentration by replicate analysis (n=3) at all concentration levels and the linear relationship was evaluated using the least square method using Microsoft Excels program. Both cilexetil and hydrochlorothiazide were found to be linear in the concentration range of 2 – 10 µg/mL.

Table 2: Calibration curve data of hydrochlorothiazide and candesartan cilexitel

S. No	Concentration(µg/mL)	Peak area[a]	
		HCT	CSC
1	2	72142	252895
2	4	132423	404260
3	6	190031	605347
4	8	260231	814570
5	10	323011	1004750

[a]Average of 3 determinations

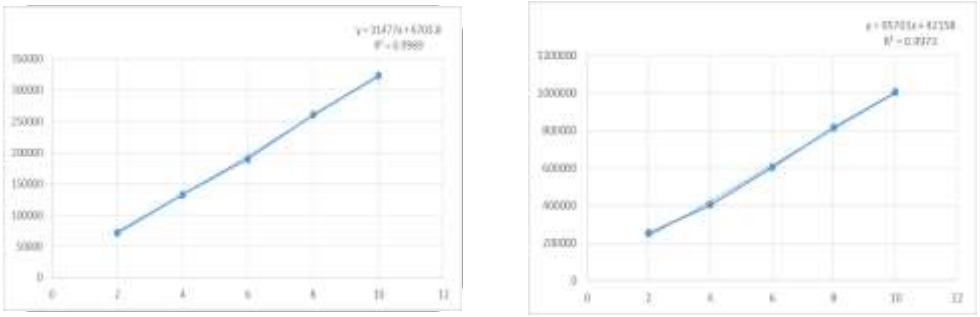

Figure.7: Calibration curve of hydrochlorothiazide and candesartan cilexitel

The LOD and LOQ values calculated by standard deviation method are found to be 1.31µg/mL and 3.97µg/mL respectively for hydrochlorothiazide. For candesartan cilexetil, the calculated values of LOD and LOQ were found to be 1.72µg/ml and 4.39µg/mL respectively.

Recovery studies (drug product)

The peak area and %RSD values for accuracy determination of drug product (solid dispersions) The results indicate best recovery (98.7-101.6%) of the added drug at different concentration levels. Recovery values indicate that the method is accurate for the determination of drug in the solid dispersions.

Table 3: Recovery study data for Hydrochlorothiazide and candesartan cilexetil

S.no	Spike level	Con. added (µg/mL)	Hydrochlorothiazide				Candesartan cilexetil			
			Peak area	Con. Recovered	%Recovery	Mean±SD[b]	Peak area	Con. Recovered	% Recovery	Mean±SD[b]
1	80	4	409852	4.0	100.0	100.1±0.88	138477	4.1	101.2	99.9±0.76
2	100	6	608135	6.1	101.6		190935	6.0	100.0	
3	120	8	815680	7.9	98.7		256978	7.9	98.6	

[a,b] Average of three determinations

Precision of the method was established by reporting repeatability and intermediate precision. The samples were analyzed at different sessions of the day and on different days and the samples didn't show any variations in the measured peak areas and the calculated %RSD values are very low indicating that the method was precise.

Table 4: System suitability data for hydrochlorothiazide and candesartan cilexetil

System suitability parameter	Results		Acceptance criteria
	HCT	CSC	
Tailing factor	1.2	1.8	Not more than 2.0
Theoretical plates	7195	8250	Not less than 5000
%RSD for area of six injections of standard	0.49	1.04	Not more than 2.0

Quantitative analysis of solid dispersions

Analysis of solid dispersions was carried out by developed RP-HPLC method. The assay data are given in tables 5 and chromatograms of assay are shown in Fig 8.

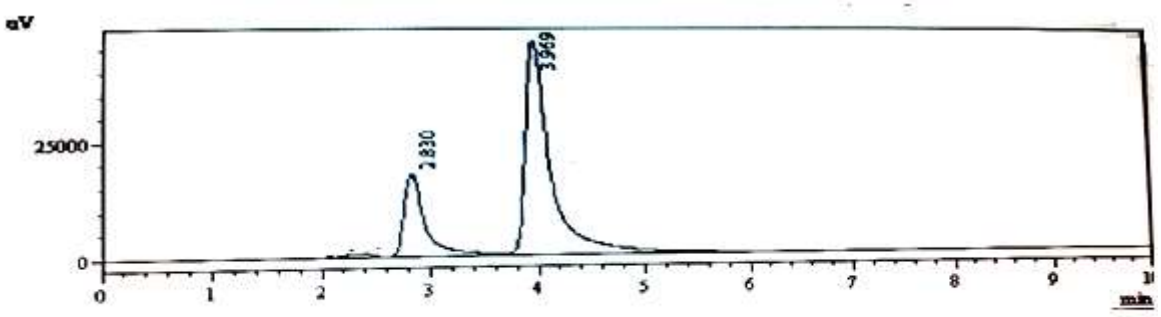

Fig.8: Chromatogram of candesartan cilexetil and hydrochlorothiazide solid dispersion (2 µg/mL)

Table 5: Assay data of hydrochlorothiazide and candesartan cilexetil

S. No	Concentration (µg/mL)	Peak area		Assay (%)		Mean[a]	
		HCT	CSC	HCT	CSC	HCT	CSC
1	2	252389	71853	99.8	99.6		
2	2	250871	71276	99.2	98.8	99.16	99.43
3	2	249101	72069		99.9		

[a] Average of three determinations

Percent purity or assay value of candesartan cilexetil and hydrochlorothiazide in solid dispersions were found to be 99.16% and 99.43% respectively.

CONCLUSION

In most of the solubility enhancement studies of poorly soluble drugs by different techniques like solid dispersions, using hydrotropic reagents, quantification of drugs was done by UV methods for monitoring the enhancement in solubility of drugs in different solvents.

The liquid chromatographic method developed in the present study was applied successfully for the determination of concentrations of hydrochlorothiazide and candesartan cilexetil in marketed formulations and prepared solid dispersion as a part of solubility enhancement studies.

The developed chromatographic method was found to be specific because there was no interference in the blank and placebo chromatograms near the retention time of the analytes.

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