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DEVELOPMENT AND VALIDATION OF A REVERSED-PHASE HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF IRBESARTAN AND HYDROCHLOROTHIAZIDE IN PHARMACEUTICAL DOSAGE FORMS.

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Abstract:

A rapid and precise reverse phase high performance liquid chromatographic method has been developed for the validated of Irbesartan and Hydrochlorothiazide, in its pure form as well as in tablet dosage form. Chromatography was carried out on a Symmetry C18 (4.6 x 150mm, 5µm) column using a mixture of Methanol: Phosphate Buffer pH 3.5 (65:35) as the mobile phase at a flow rate of 1.0ml/min, the detection was carried out at 270 nm. The retention time of the Irbesartan and Hydrochlorothiazide was 2.456, 4.312±0.02min respectively. The method produce linear responses in the concentration range of 5-25mg/ml of Irbesartanand 2.5-12.5mg/ml of Hydrochlorothiazide. The method precision for the determination of assay was below 2.0%RSD. The method is useful in the quality control of bulk and pharmaceutical formulations.

Keywords: *Irbesartan, Hydrochlorothiazide, RP-HPLC, validation.*

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INTRODUCTION:

Analytical chemistry is the branch of chemistry involved in separating, identifying and determining the relative amounts of the components making up a sample of matter. It is mainly involved in the qualitative identification or detection of compounds and the quantitative measurement of the substances present in bulk and pharmaceutical preparation.

Measurements of physical properties of analytes such as conductivity, electrode potential, light absorption or emission, mass to charge ratio, and fluorescence, began to be used for quantitative analysis of variety of inorganic and biochemical analytes. Highly efficient chromatographic and electrophoretic techniques began to replace distillation, extraction and precipitation for the separation of components of complex mixtures prior to their qualitative or quantitative determination. These newer methods for separating and determining chemical species are known collectively as instrumental methods of analysis. Most of the instrumental methods fit into one of the three following categories viz spectroscopy, electrochemistry and chromatography.

Advantages of instrumental methods:

Small samples can be used
High sensitivity is obtained
Measurements obtained are reliable
Determination is very fast
Even complex samples can be handled easily

Limitations of instrumental methods:

An initial or continuous calibration is required
Sensitivity and accuracy depends on the instrument
Cost of equipment is large
Concentration range is limited
Specialized training is needed
Sizable space is required

High Performance Liquid Chromatography4,5:

HPLC is a type of liquid chromatography that employs a liquid mobile phase and a very finely divided stationary phase. In order to obtain satisfactory flow rate liquid must be pressurized to a few thousands of pounds per square inch.

The rate of distribution of drugs between Stationary and mobile phase is controlled by diffusion process. If diffusion is minimized faster and effective separation can be achieved .The techniques of high performance liquid chromatography are so called because of its improved performance when compared to classical column chromatography advances in column chromatography into high speed,efficient ,accurate and highly resolved method of separation.

For the recent study metformin and Sitagliptin was selected for estimation of amount of analyte present in formulation and bulk drug. The HPLC method is selected in the field of analytical chemistry, since this method is specific, robust, linear, precise and accurate and the limit of detection is low and also it offers the following advantages

Speed many analysis can be accomplished in 20min (or) less.
Greater sensitivity(various detectors can be employed).
Improved resolution(wide variety of stationary phases).
Re usable columns(expensive columns but can be used for many analysis).
Ideal for the substances of low viscosity.
Easy sample recovery, handling and maintenance.
Instrumentation leads itself to automation and quantification (less time and less labour).
Precise and reproducible.
Integrator itself does calculations.
Suitable for preparative liquid chromatography on a much larger scale.

HPLC components:

The essential components4 of a complete HPLC system are solvent delivery system (Pump), detector, fixed volume injector loop or autosampler, solvent reservoirs, packed column, data system and recorder. A schematic of a simplified HPLC system is shown in above Figure.

Column:

The column is probably the heart of HPLC system. The development of this column technology leads to the evolution of the HPLC instrumentation systems used today.The conventionally used HPLC columns are particle packed columns. The key of column selection when previous separation is not available resides in knowing the chemistry of the sample. Columns should never be dry. A dry column will eventually have voids because the packing will shrink away from the wall, which would result in band broadening. Before running a sample in HPLC the column should be equilibrated. Usually column equilibrium is achieved after passage of 10 – 20 column volumes of the new mobile phase through the column. Insufficient column equilibrium usually leads to retention difference.

Pump:

The solvent delivery system or as it is commonly called the pump includes two major types, constant volume or flow and constant pressure. Constant

volume pumps are mechanically driven systems, most commonly using screw driven syringes or reciprocating pistons. On the other hand, constant pressure pumps are driven or controlled by gas pressure.

Injector or Auto sampler:
Samples are usually introduced by syringe injection via a manual injector into the mobile phase stream or by the use of an auto sampler. The important aspects in sample introduction are precise and reproducible injections. This is especially important with quantitative analysis where the reproducibility of the peak response is dependant on the precision of the sample introduction. Direct syringe injection through a manual injector was the first popular method of sample introduction.
HPLC instrumentation evolved, many auto sampler techniques were applied so that sample introduction has become more precise and rapid.

Detector:
HPLC detectors include ultraviolet-visible, fluorescence, electrochemical, refractometer, mass spectrometer and others. The UV visible absorption detector is the most widely used detector in liquid chromatography, since most organic compounds show some useful absorption in the UV region. This detector is fairly universal in application, although sensitivity depends on how strongly the sample absorbs light at a particular wavelength.

Solvent reservoir:
Different containers are used as a solvent delivery system reservoir. The best material from which the containers are made is glass. Plastic containers are not recommended as it leads to plasticizer leaching. The container should be covered to prevent solvent evaporation. The tubing from the reservoir can be made of stainless steel or Teflon, and both are satisfactory.

Data handling and analysis:
Data handling in HPLC is as important to the success of any experiment or analysis as any other components in the system. It is part of good HPLC techniques to properly label and document the analytical results. The advanced computer softwares used now in data handling and analysis allow easy recording and storage of all chromatographic data.

Based on modes of chromatography8,9:
- Normal phase chromatography
- Reverse phase chromatography

Normal phase chromatography:
In normal phase mode the stationary base (eg; silica gel) is polar in nature and the mobile phase is non polar. In this technique, non polar compound travel faster and are eluted first. This is because less affinity between solute and stationary phase and take more time to elute.

Reverse phase chromatography:
The popularity of reversed phase liquid chromatography is easily explained by its unmatched simplicity, versatility and scope. Neutral and ionic analytes can be separated simultaneously. Retention in RPLC is believed to occur through nonspecific hydrophobic interaction of the solute with the stationary phase. The near universal application of RPLC stems from the fact that almost all organic compounds have hydrophobic regions in their structure and are capable of interacting with the stationary phase.

A decrease in the polarity of the mobile phase leads to a decrease in retention. It is also generally observed in RPLC that branched chain compounds are retained to a lesser extent than their straight chain analogues and that unsaturated compounds are eluted before their fully saturated analogs.A wide variety of RP-HPLC columns are available. Most columns are silica based.Silica offers good mechanical stability. A typical stationary phase is formed by chemically bonding a long-chain hydrocarbon group to porous silica. Typical ligands are n-octadecyl (C18), n-octayl (C8), n-butyl (C4), diphenyl (C2),and cyano propyl.

Parameters affecting separation:
Separation in reversed phase chromatography is affected by stationary phase type and column length. It is also affected by organic solvent type and percentage in the mobile phase and by mobile phase pH. Flow rate could also affect separation in reversed phase chromatography; however it is usually limited by the developed backpressure. Moreover temperature of the column also has an effect on separation.

MATERIALS AND METHODS:
Irbesartan-Sura labs, Hydrochlorothiazide-Sura labs, Water and Methanol for HPLC-LICHROSOLV (MERCK),Acetonitrile for HPLC-Merck, Phosphate buffer-Sura labs.

HPLC METHOD DEVELOPMENT:
TRAILS:
Preparation of standard solution:
Accurately weigh and transfer 10 mg of Irbesartan and Hydrochlorothiazide working standard into a 10ml of clean dry volumetric flasks add about 7ml of

Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.
Further pipette 0.15ml ofIrbesartan and 0.075ml of Hydrochlorothiazidefrom the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure:
Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Mobile Phase Optimization:
Initially the mobile phase tried was Methanol: Water with varying proportions. Finally, the mobile phase was optimized to Methanol: Phosphate Buffer in proportion 65:35 v/v respectively.

Optimization of Column:
The method was performed with various columns like C18 column, Symmetry and X-Bridge. Symmetry C18 (4.6×150mm, 5µ) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

Instrument used	:	Waters HPLC with auto sampler and PDA Detector 996 model.
Temperature	:	40ºC
Column	:	Symmetry C18 (4.6×150mm, 5µ)
Buffer	:	Accurately weighed 6.8 grams of KH2PO4 was taken in a 1000 ml volumetric flask, dissolved and diluted to 1000ml with HPLC water and the volume was adjusted to pH 3.5
pH	:	3.5
Mobile phase	:	Methanol: Phosphate buffer pH 3.5 (65:35v/v)
Flow rate	:	1ml/min
Wavelength	:	270nm
Injection volume	:	10 µl
Run time	:	7 min

VALIDATION:

PREPARATION OF BUFFER AND MOBILE PHASE:

Preparation of Phosphate buffer pH 3.5:
Accurately weighed 6.8 grams of KH2PO4 was taken in a 1000ml volumetric flask, dissolved and diluted to 1000ml with HPLC water and the volume was adjusted to pH 3.5.

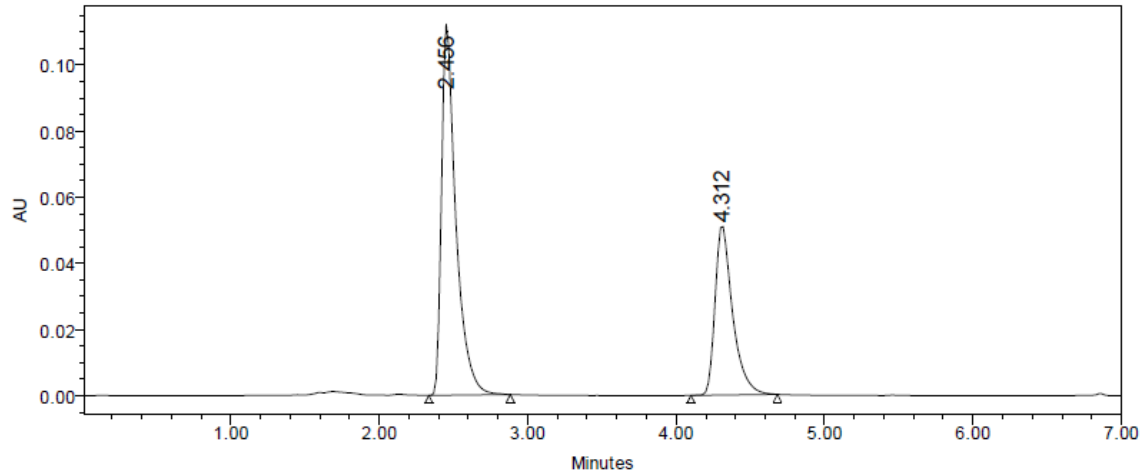
Preparation of mobile phase:
Accurately measured 650 ml (65%) of Methanol and 350 ml of Phosphate buffer (35%) a were mixed and degassed in digital ultrasonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:
The Mobile phase was used as the diluent.

RESULTS AND DISCUSSION:

Optimized Chromatogram (Standard)

Mobile phase	:	Methanol: Phosphate Buffer pH 3.5 (65:35)
Column	:	Symmetry C18 (4.6×150mm, 5.0 µm)
Flow rate	:	1 ml/min
Wavelength	:	270 nm
Column temp	:	40ºC
Injection Volume	:	10 µl
Run time	:	7 minutes



Optimized Chromatogram

Table: - peak results for optimized

S. No	Peak name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Irbesartan	2.456	600122	112157		1.6	5215
2	Hydrochlorothiazide	4.312	422042	51068	3.2	1.5	5648

Observation: From the above chromatogram it was observed that the Irbesartan and Hydrochlorothiazide peaks are well separated and they shows proper retention time, resolution, peak tail and plate count. So it's optimized trial.

Optimized Chromatogram (Sample):

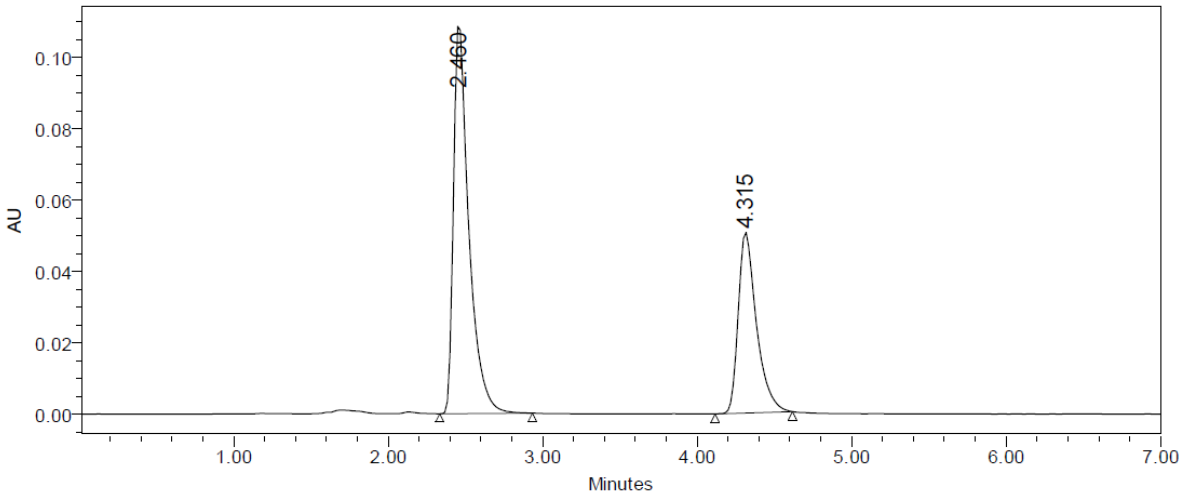


Figure: Optimized Chromatogram (Sample)

Table: Optimized Chromatogram (Sample)

S. No	Peak name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Irbesartan	2.460	600123	112157		1.6	5011
2	Hydrochlorothiazide	4.315	422041	51068	3.3	1.5	5947

Acceptance criteria:

- Resolution between two drugs must be not less than 2
- Theoretical plates must be not less than 2000
- Tailing factor must be not less than 0.9 and not more than 2.
- It was found from above data that all the system suitability parameters for developed method were within the limit.

Assay (Standard):

Table: Peak results for assay standard

Sno	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Irbesartan	2.456	600122	112157		1.5	5023	1
2	Hydrochlorothiazide	4.312	420842	51068	3.3	1.4	5946	1
3	Irbesartan	2.457	600205	112399		1.2	5149	2
4	Hydrochlorothiazide	4.308	422034	51511	3.3	1.4	5848	2
5	Irbesartan	2.456	600213	11201		1.5	5046	3
6	Hydrochlorothiazide	4.312	420191	52014	3.2	1.5	5941	3

Assay (Sample):

Table: Peak results for Assay sample

S.No	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Irbesartan	2.465	601812	110102		1.6	5028	1
2	Hydrochlorothiazide	4.337	414764	49842	3.2	1.5	5949	1
3	Irbesartan	2.474	600435	108333		1.6	5189	2
4	Hydrochlorothiazide	4.356	418130	48360	3.3	1.5	5818	2
5	Irbesartan	2.465	600212	112453		1.6	5061	3
6	Hydrochlorothiazide	4.337	413645	48641	3.2	1.5	5812	3

$$\%ASSAY = \frac{\text{Sample area}}{\text{Weight of standard}} \times \frac{\text{Weight of standard}}{\text{Dilution of sample}} \times \frac{\text{Dilution of sample}}{\text{Purity}} \times \frac{\text{Purity}}{\text{Weight of tablet}} \times 100$$

Standard area Dilution of standard Weight of sample 100 Label claim

= 600819.7/600180×10/15×15/0.1265×99.7/100×0.1265/10×100
= 99.8%
The % purity of Irbesartan and Hydrochlorothiazide in pharmaceutical dosage form was found to be 99.8 %.

FORCED DEGRADATION STUDIES:
Table: Results for degradation studies of

S.No	Type of degradation	Weight of sample (µg/ml)	Area of sample		Assay content (% w/w)	
			Irbesartan	Hydrochlorothiazide	Irbesartan	Hydrochlorothiazide
1	Acid (0.5N HCl)	15µg/ml of Irbesartanand 7.5µg/ml of Hydrochlorothiazide	600272	420188	99.7%	98.9%
2	Base (0.5N NaOH)	15µg/ml of Irbesartanand 7.5µg/ml of Hydrochlorothiazide	601837	420958	99.6%	100%
3	Peroxide (3% H_20_2)	15µg/ml of Irbesartanand 7.5µg/ml of Hydrochlorothiazide	600882	420911	100%	99%
4	Thermal (at 60^0 c)	15µg/ml of Irbesartanand 7.5µg/ml of Hydrochlorothiazide	600727	421058	100%	99.3%
5	Photolytic (sunlight)	15µg/ml of Irbesartanand 7.5µg/ml of Hydrochlorothiazide	600271	421844	99.5%	98.7%

CHROMATOGRAPHIC DATA FOR LINEARITY STUDY:
Irbesartan:

Concentration Level (%)	Concentration µg/ml	Average Peak Area
33.3	5	215760
66.6	10	417001
100	15	600435
133.3	20	791969
166.6	25	974736

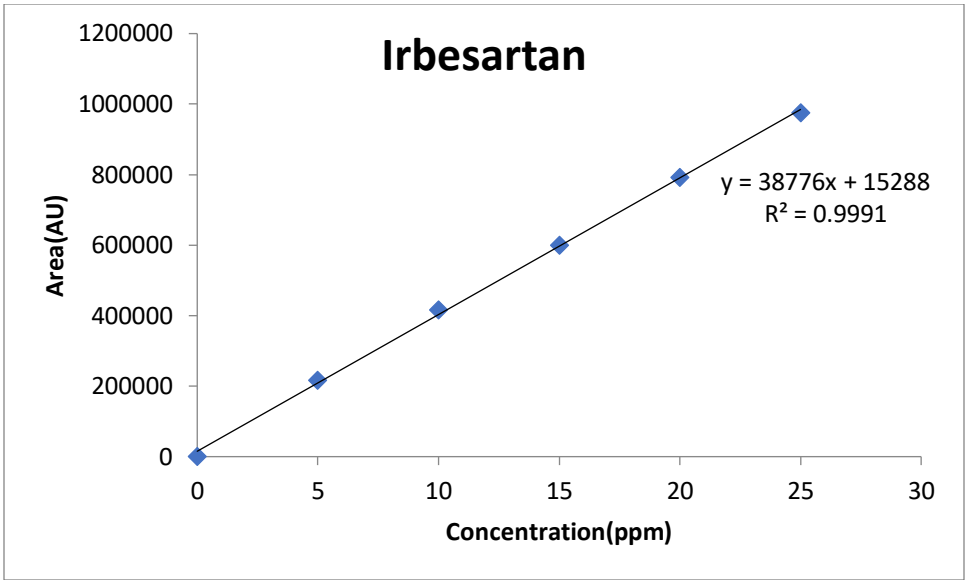


Figure:calibration graph for Irbesartan

Hydrochlorothiazide

Concentration Level (%)	Concentration µg/ml	Average Peak Area
33	2.5	145474
66	5	279372
100	7.5	421045
133	10	562151
166	12.5	721671

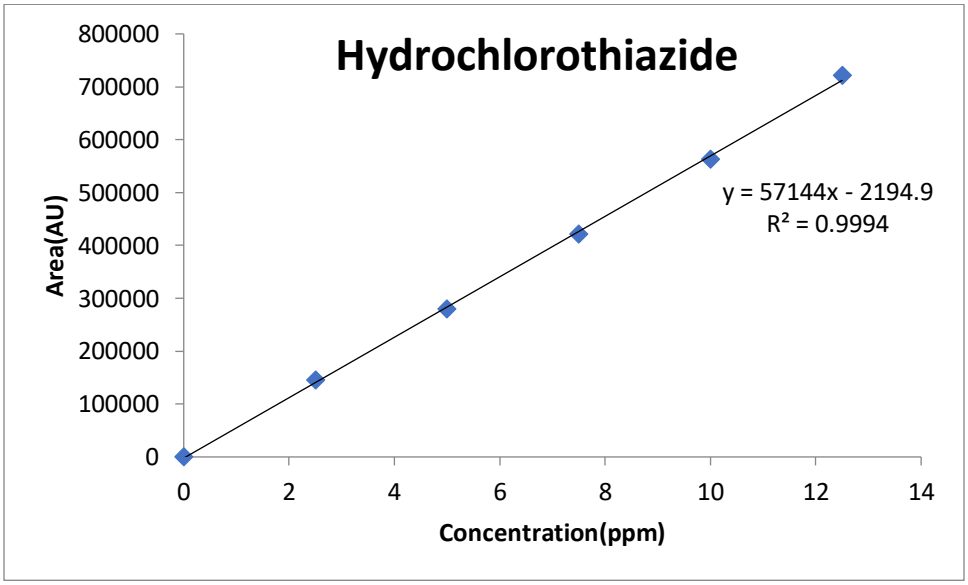


Figure 6.3.4 calibration graph for Hydrochlorothiazide

REPEATABILITY:

Table: Results of repeatability for Irbesartan:

S no	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Irbesartan	2.453	603403	112688	5881.3	1.4
2	Irbesartan	2.455	608107	113637	5844.1	1.3
3	Irbesartan	2.453	607266	112849	5918.1	1.3
4	Irbesartan	2.452	608776	112478	5847.3	1.4
5	Irbesartan	2.450	609758	111779	5801.8	1.5
Mean			607462			
Std. Dev			2445.82			
% RSD			0.40			

Acceptance criteria:

- %RSD for sample should be NMT 2
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Table: Results of method precession for Hydrochlorothiazide:

Sno	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Hydrochlorothiazide	4.289	429183	52411	5050.9	1.49	3.2
2	Hydrochlorothiazide	4.309	416643	52475	5084.8	1.5	3.2
3	Hydrochlorothiazide	4.306	424052	51841	5000.1	1.4	3.2
4	Hydrochlorothiazide	4.300	425235	51804	5026.4	1.51	3.2
5	Hydrochlorothiazide	4.295	416260	51274	5098.5	1.51	3.2
Mean			422274.6				
Std. Dev			5646.668				
% RSD			1.3				

Acceptance criteria:

- %RSD for sample should be NMT 2
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Intermediate precision:

Day 1:

Table: Results of Intermediate precision for Irbesartan

S no	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Irbesartan	2.465	602386	111226	5075.9	1.5
2	Irbesartan	2.472	608118	112497	5043.2	1.3
3	Irbesartan	2.467	605566	110347	5029.9	1.5
4	Irbesartan	2.466	608543	53992	5023.2	1.4
5	Irbesartan	2.472	609288	55420	5061.3	1.4
6	Irbesartan	3.424	607315	54154	5078.4	1.3
Mean			606869.3			
Std. Dev			2538.025			
% RSD			0.41			

Acceptance criteria:

- %RSD of six different sample solutions should not more than 2

Table: Results of Intermediate precision for Hydrochlorothiazide

S no	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Hydrochlorothiazide	4.323	422252	50991	5886.2	1.6	3.2
2	Hydrochlorothiazide	4.343	418090	50664	5947.5	1.5	3.2
3	Hydrochlorothiazide	4.324	424361	50295	5907.8	1.55	3.2
4	Hydrochlorothiazide	4.323	424692	49813	5890.0	1.50	3.2
5	Hydrochlorothiazide	4.342	411255	49826	5852.5	1.49	3.2
6	Hydrochlorothiazide	4.323	422252	50991	5756.8	1.50	3.2
Mean			420483.7				
Std. Dev			5096.974				
% RSD			1.2				

Acceptance criteria:

- %RSD of six different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is rugged.

Table: Results of Intermediate precision Day 2 for Irbesartan

S no	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Irbesartan	2.456	602581	112175	5013	1.7
2	Irbesartan	2.457	600985	112422	5007	1.7
3	Irbesartan	2.456	600145	114513	5198	1.8
4	Irbesartan	2.459	600332	111580	5246	1.7
5	Irbesartan	2.467	600566	110347	5096	1.8
6	Irbesartan	2.459	600332	111580	5178	1.8
Mean			600823.5			
Std. Dev			908.2622			
% RSD			0.15			

Acceptance criteria:

- %RSD of six different sample solutions should not more than 2

Table: Results of Intermediate precision for Hydrochlorothiazide

S no	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Hydrochlorothiazide	4.312	425263	50936	5981	1.5	3.2
2	Hydrochlorothiazide	4.308	427069	51400	5887	1.49	3.2
3	Hydrochlorothiazide	4.312	424231	51236	5928	1.5	3.2
4	Hydrochlorothiazide	4.322	423569	51084	5898	1.50	3.2
5	Hydrochlorothiazide	4.324	414361	50295	5887	1.5	3.2
6	Hydrochlorothiazide	4.322	413569	51084	5940	1.5	3.2
Mean			421343.7				
Std. Dev			5841.789				
% RSD			1.38				

Acceptance criteria:

- %RSD of six different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is rugged.

ACCURACY:

The accuracy results for Irbesartan

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	308408	7.5	7.55	100.6	100.3%
100%	600619	15	15	100	
150%	894293	22.5	22.6	100.4	

The accuracy results for Hydrochlorothiazide:

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	216092	3.75	3.8	101.3	99.7%
100%	423626	7.5	7.45	99.3	
150%	634469.7	11.25	11.1	98.6	

Acceptance Criteria:

- The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

Robustness

Irbesartan:

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	600122	2.456	5215	1.8
Less Flow rate of 0.9 mL/min	651206	2.741	5199	1.79
More Flow rate of 1.1 mL/min	546820	2.270	5234	1.8
Less organic phase	586420	3.266	5298	1.8
More organic phase	542813	2.147	5287	1.76

Acceptance criteria:
The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

Hydrochlorothiazide:

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	422042	4.312	5648	1.5
Less Flow rate of 0.9 mL/min	453012	4.830	5687	1.6
More Flow rate of 1.1 mL/min	398654	3.979	5602	1.5
Less organic phase	445983	3.266	5643	1.55
More organic phase	402315	2.147	5699	1.51

Acceptance criteria:
The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

CONCLUSION:

- In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative estimation of Irbesartan and Hydrochlorothiazide in bulk drug and pharmaceutical dosage forms.
- This method was simple, since diluted samples are directly used without any preliminary chemical derivatisation or purification steps.
- Irbesartan and Hydrochlorothiazide was freely soluble in ethanol, methanol and sparingly soluble in water.
- Methanol: Phosphate Buffer pH 3.5 (65:35) was chosen as the mobile phase. The solvent system used in this method was economical.
- The %RSD values were within 2 and the method was found to be precise.
- The results expressed inTablesfor RP-HPLC method was promising. The RP-HPLC method is more sensitive, accurate and precise compared to the Spectrophotometric methods.
- This method can be used for the routine determination of Irbesartan and Hydrochlorothiazide in bulk drug and in Pharmaceutical dosage forms.

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