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DEVELOPMENT AND VALIDATION OF NEW ANALYTICAL METHODS FOR THE SIMULTANIOUS ESTIMATION OF RAMIPRIL AND HYDROCHLOROTHIAZIDE IN BULK DRUG AND PHARMACEUTICAL FORMULATIONS BY RP-HPLC

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ABSTRACT

A HPLC method for simultaneous estimation of Ramipril and Hydrochlorothiazide in Drug and Pharmaceutical formulation was developed and validated. The developed method involves Merck Hitachi L-7100 double reciprocating pump, UV detector L-7400 (190-666nm), 150×4.6mm column with mobile phase composition orthophosphate buffer: Acetonitrile (60: 40 v/v) with neutral pH, at a flow rate of 0.8 ml/min and UV detection at 226 and 253 nm for first five minutes for Hydrochlorothiazide and 270nm for Ramipril. The method was validated as per ICH guidelines; Linearity was observed over concentration range of 1 to 5 µg/ ml for Ramipril and 2.5 to 12.5 µg/ ml Hydrochlorothiazide. The Accuracy of the proposed method was determined by recovery studies and found to be 97.95-102.3% and 97.98-102.66% for Ramipril and Hydrochlorothiazide respectively. The proposed method was extended for estimation of Ramipril and Hydrochlorothiazide in marketed pharmaceutical formulation (AltaceHCT) and it was found to be well within the acceptance limit. The developed and validated HPLC method for simultaneous estimation of Ramipril and Hydrochlorothiazide was found to be linear, accurate, precise, robust and rugged. Hence it can be used for routine analysis of Ramipril and Hydrochlorothiazide in tablet.

KEYWORDS: HPLC, Ramipril, Hydrochlorothiazide, Merck Hitachi.

INTRODUCTION

Quantitative chemical analysis is an important tool to assure that the raw material used and the intermediate products meet the required specifications. Drug analysis is the base for the determination of the product. Every year number of drugs is introduced into the market. Also, quality is important in every product or service but it is vital in medicines as it involves life.[1] Very often, there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, report of new toxicities, and development of patient resistance and introduction of better drugs by the competitors. Under these conditions, standard and analytical procedures for these drugs may not be available in Pharmacopoeias.[1] Quality control is a concept, which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stage of production. The decision to release or reject a product is based on one or more type of control action. With the growth of pharmaceutical industry during last several years, there has been rapid progress in the field of pharmaceutical analysis involving complex instrumentation. Providing simple analytical procedures for complex formulation is a matter of most importance.[2]

MATERIALS AND METHODS

REVERSE PHASE HPLC METHOD

INSTRUMENT USED

- Make : Merck Hitachi.
- Specification : Quaternary gradient system.
- Pump : L-7100 double reciprocating pump.
- UV-detector : L-7400 (190-666 nm).
- Column : C-18 column.
- Flow rate : 0.8 ml/min.
- Inj. volume : 10 µl
- Temperature : ambient

EXPERIMENTAL

A. Selection of sampling wavelength for analysis and preparation of standard calibration curves.

1. Reagents and Chemicals Used

- Acetonitrile (HPLC grade).
- Orthophosphate buffer.

2. Selection of mobile phase

The pure drug of Ramipril and Hydrochlorthiazide were injected into the HPLC system and run in different solvent systems. Different mobile phases like methanol and water, acetonitrile and water, methanol and acetonitrile, acetonitrile and 0.01M potassium dihydrogen orthophosphate buffer (KH_2PO_4) were tried in order to find the best conditions for the separation of Ramipril and Hydrochlorthiazide. It was found that Orthophosphate buffer and Acetonitrile gives satisfactory results as compared to other mobile phases. This mobile phase system was tried with different proportions and using different flow rates. Finally, the optimal composition of the mobile phase was determined to be Orthophosphate buffer: Acetonitrile (60: 40 v/v) with neutral pH.

3. Preparation of mobile phase

The HPLC grade Acetonitrile filtered through 0.45µm Nylon 47mm membrane filter paper. Orthophosphate buffer was also filtered through 0.45µm Nylon 47mm membrane filter paper. Both are ultrasonicated for 20 mins. Mobile phase was prepared by mixing 600 ml of Orthophosphate buffer and 400 ml of ACN.

4. Preparation of standard stock solution

10 mg each of standard Ramipril and Hydrochlorothiazide was weighed accurately and transferred to two separate 100 ml volumetric flasks. Both the drugs were dissolved in 50 ml of mobile phase with shaking and then volume was made up to the mark with mobile phase to get 100 µg/ml of standard stock solution of each drug. These stock solutions were filtered through 0.45 µm Nylon 47mm membrane filter paper.

5. Selection of analytical wavelength

By appropriate dilution of each standard stock solution with mobile phase, various concentrations of Ramipril and Hydrochlorthiazide were prepared separately. Each solution was scanned using double beam UV visible spectrophotometer 1700 in the “Spectrum mode” between the range of 400 nm to 200 nm and their spectra was overlaid. From the overlain spectra of Ramipril and Hydrochlorthiazide, 294.0 nm was selected as analytical wavelength for Multicomponent analysis using HPLC method.

6. Chromatographic condition

The mobile phase containing Orthophosphate buffer and ACN in the ratio of (60:40) was selected as the optimum composition of mobile phase, because it was found that this solvent system resolved both the components ideally. The flow rate was set to 0.8 ml/min and UV detection was carried out at 294 nm. The mobile phase and samples were degassed by ultrasonication for 20 min and filtered through 0.45 µm Nylon 47 mm membrane filter paper. All determinations were performed at constant room temperature.

7. Selection of analytical concentration range and preparation of calibration curve for Ramipril and Hydrochlorthiazide

❖ Ramipril

Appropriate aliquots were pipetted out from the standard stock solution (100 µg/ml) in to a series of 10 ml volumetric flasks. The volume was made up to the mark with the mobile phase to get a set of solutions having the concentration range, ranging from 50,75,100,125,150 µg/ml of Ramipril.

Triplicate dilutions of each of the above mentioned concentrations were prepared separately and from these triplicate solutions, 10 µl of each concentration of the drug were injected into the HPLC system two times separately and their chromatograms were recorded under the same chromatographic conditions as described above. Peak areas were recorded for all the peaks and a standard calibration curve of area against concentration was plotted.

❖ Hydrochlorzthiazide

Appropriate aliquots were pipetted out from the standard stock solution (100 µg/ml) in to a series of 10 ml volumetric flasks. The volume was made up to the mark with the mobile phase to get a set of solutions having the concentration range, ranging from 50,75,100,125,150 µg/ml of Hydrochlorthiazide.

Triplicate dilutions of each of the above mentioned concentrations were prepared separately and from these triplicate solutions, 10 µl of each concentration of the drug were injected into the HPLC system two times separately and their chromatograms were recorded under the same chromatographic conditions as described above. Peak areas were recorded for all the peaks and a standard calibration curve of area against concentration was plotted.

Both the drugs follow the Beer's & Lambert's law in the concentration range of 50-150

µg/ml for Ramipril and 50-150 µg/ml for Hydrochlorthiazide. The linearity of calibration curves and adherence of the system to Beer's & Lambert's law was validated by high value of correlation coefficient and less than 2% relative standard deviation (R.S.D.) for the intercept value.

B. Analysis of tablet formulation.

Twenty tablets of Ramipril and Hydrochlorthiazide in combination were weighed individually and their average weight was determined. The tablets were then crushed to fine powder and powder equivalent to 12.5 mg of Hydrochlorthiazide (5 mg of Ramipril) was weighed and transferred to 100 ml volumetric flask and dissolved in sufficient quantity of mobile phase. The contents were ultrasonicated for 20 minutes and the final volume was made up to the mark with mobile phase.

The above prepared solution was then filtered through 0.2 µm Nylon 47 mm membrane filter paper and was used as standard stock solution. Appropriate aliquot was pipetted out from the standard stock solution and was further diluted with the mobile phase to obtain a mixture containing Ramipril and Hydrochlorhiazide in the ratio of 1:2.5. Six different mixtures containing 50 µg/ml of Ramipril and 125 µg/ml were prepared as above from the standard stock solution. A 10 µl volume of each sample mixture was injected in to the sample injector of HPLC system and their chromatograms were recorded under the same chromatographic conditions as described above. The area of each peak was determined at 294 nm and the amount of drug present in the sample mixture was determined.

METHOD VALIDATION

A. LINEARITY AND RANGE

The linearity of analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in sample within a given range. The range of analytical method is the interval between the upper and lower levels of analyte that have been demonstrated to be determined within a suitable level of precision, accuracy and linearity.

The proposed method showed good linearity in the concentration range of 50 to 150 µg/ ml for Ramipril and 50 to 150 µg/ ml Hydrochlorthiazide.

B. ACCURACY

❖ **Procedure for determination of Accuracy**

Recovery studies were carried out by applying the method to drug sample present in tablet dosage form to which known amount of Ramipril and Hydrochlorthiazide corresponding to 80%, 100% and 120% level were prepared in the same manner as explained in method A. But in this method mobile phase i.e. orthophosphate buffer: ACN.

The solutions were filtered through 0.45 µm Nylon 47 mm membrane filter paper and then they were subjected to analysis by RP-HPLC method under the same chromatographic conditions as described above. At each level, three determinations were performed. The results obtained were compared with expected results and were statistically validated.

C. PRECISION

❖ **Procedure for determination of Repeatability (intra-day precision)**

Standard solutions of 50µg/ml Ramipril and 125 µg/ml of Hydrochlorthiazide was analysed six times at different time intervals in the same day. The concentration of sample mixture was determined as per the procedure given for the tablet formulation by determining AUC at selected analytical wavelength 294 nm. The variation of the results within the same day was analyzed and statistically validated.

❖ **Procedure for determination of Inter-day Precision**

In inter-day precision a set of five sample mixtures containing 50 µg/ml of Ramipril and 125 µg/ml of Hydrochlorthiazide were prepared and analyzed at same time on different days. The concentration of the sample mixture was determined as per the procedure given for the tablet formulation by determining area at selected analytical wavelength 294 nm. The variation of the results on different days was analyzed and statistically validated.

D. SPECIFICITY AND SELECTIVITY

The specificity of the RP-HPLC method was determined by complete separation of Ramipril and Hydrochlorthiazide with parameters like retention time (t_R), resolution (R_S) and tailing factor (T_f). Here tailing factor for peaks of Ramipril and Hydrochlorthiazide, was less than 2% and resolution was also more than 1%. The average retention time ± standard deviation for Ramipril and Hydrochlorthiazide were found to be respectively for five determinations. The peaks obtained for Ramipril and Hydrochlorthiazide were sharp and have clear baseline separation.

E. ROBUSTNESS

The evaluation of robustness should be considered during the development phase and depends upon the type of procedure under study. It should show the reliability of analysis with respect to deliberate variations in method parameters like different column temperate, different analytical wavelength, different flow rate. The solution containing 50µg/ml of Ramipril and 125 µg/ml of Hydrochlorthiazide was injected into sample injector of HPLC three times under different parameters like deliberate variations in flow rate, percentage of ethanol in the mobile phase and column temperature.

F. RUGGEDNESS

It should show the reliability of analysis with respect to deliberate variations in method parameters like different laboratories, different analysts. The solution containing 50 µg/ml of Ramipril and 125µg/ml of Hydrochlorthiazide was injected into sample injector of HPLC three times under different parameters like deliberate variations in flow rate and column temperature.

STEPS INVOLVED IN DEVELOPMENT OF HPLC METHOD:

- **Literature survey**

Here a detailed account of all analytical methods developed for the drug is collected to avoid duplication of the method developed. Details about the structure of the drugs and their physicochemical properties are also collected.

- **Selection of chromatographic method**

- First reversed phase should be tried.
- If not successful then normal phase should be taken into consideration.
- For ion exchange or ion pair chromatography, first ion suppression by pH control and reversed phase chromatography should be tried.

- **Selection of stationary phase**

Matching the polarity of sample and stationary phase and using a mobile phase of different polarity achieve a successful separation.

- **Selection of mobile phase**

Reversed phase bonded packing, when used in conjunction with highly polar solvents; approach is ideal and is a universal system for liquid chromatography. Mobile phase may be

either single liquid or combination of liquids, which are compatible with sample, column and instrument.

- **Selection of suitable detector**

Detector is the eye of HPLC system that measures the compounds after their separation on the column. There are basically two types of detectors- the bulk property detectors and solute property detectors. Detectors, in order of their popularity are UV, fluorescent, conductivity, polarimeter and refractive index detectors. UV detector is the first choice because of its convenience and applicability in case of most of the samples. The latest versions of equipments are available with photo diode- array detectors (PAD or DAD).

RESULTS AND DISSCUSSION

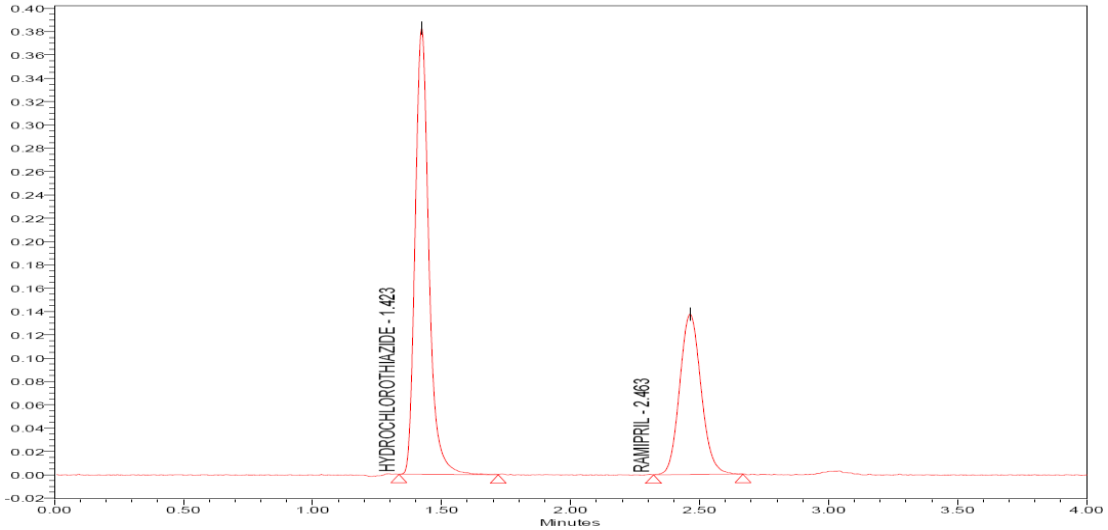

Fig. 1: Chromatogram showing retention times of Ramipril and Hydrochlorthiazide and respectively.

Table 1: Result of calibration curve for Ramipril at 294 nm by RP−HPLC Method.

Sr. No.	Concentration (µg/ml)	Area Under Curve
1	0	0
2	50	396658
3	75	594059
4	100	792897
5	125	990754
6	150	1180000

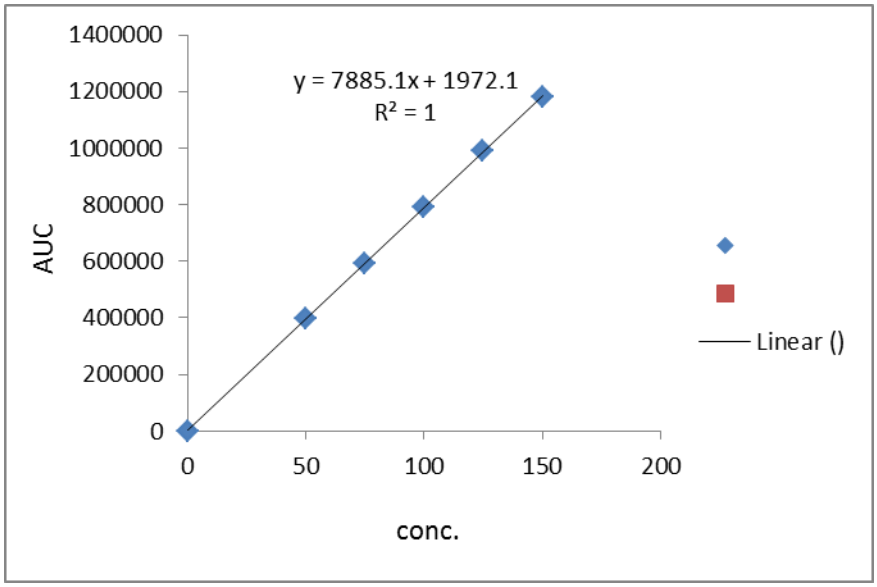

Fig. 2: Calibration curve of Ramipril 294.0 nm by RP-HPLC Method.

Table 2: Result of calibration curve for Hydrochlorthiazide at 294 nm by RP–HPLC Method.

Sr. No.	Concentration (µg/ml)	Area Under Curve
1	0	0
2	50	700716
3	75	1052560
4	100	1404987
5	125	1756887
6	150	2100419

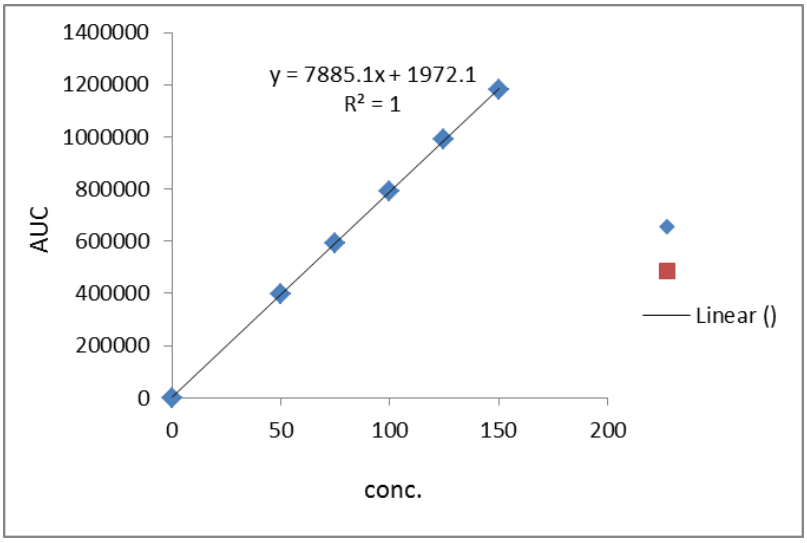

Fig. 3: Calibration curve of Hydrochlorthiazide at 294.0 nm by RP-HPLC Method.

Table 3: Statistical data of Ramipril and Hydrochlorthiazide at 294 nm by RP−HPLC method.

Parameter	RAM	HCZs
Linear Range (µg/ml)	50-150	50-150
Slope	7885	14024
Intercept	1972	578.7
Limit of Detection (µg/ml)	0.591	0.76
Limit of Quantification (µg/ml)	1.792	2.30

Table 4: Assay Results of Tablet Formulation.

Sr. No.	Amount present in (mg/tab)		Amount obtained in (mg/tab)		% Obtained	
	RAM	HCZ	RAM	HCZ	RAM	HCZ
1	5	12.5	4.9	12.375	98	99
2	5	12.5	4.9	12.25	98	98
3	5	12.5	4.95	12.375	99	99
4	5	12.5	5.05	12.625	101	101
5	5	12.5	4.95	12.37	99	99
6	5	12.5	5.05	12.5	101	100

Table 5: Statistical Validation Data for Tablet Formulation.

Components	Mean*	Standard Deviation*	Co-efficient of Variation*	Standard Error*
RAM	99	1.21	1.22	0.4745
HCZ	99	1.05	1.06	0.3354

*n = 6

Table 6: Determination of Accuracy of Ramipril and Hydrochlorthiazide.

Level of % recovery	Amount present (mg/tab)		Amount of standard drug added (mg)		Total amount recovered (mg)		% Recovery	
	RAM	HCZ	RAM	HCZ	RAM	HCZ	RAM	HCZ
80%	5	12.5	4	10	8.89	22.50	98.78	100.02
	5	12.5	4	10	9.05	22.58	100.61	100.37
	5	12.5	4	10	8.99	22.57	99.96	100.31
100%	5	12.5	5	12.5	9.89	25.01	98.91	100.05
	5	12.5	5	12.5	9.91	25.03	99.10	100.10
	5	12.5	5	12.5	10.05	25.09	100.53	100.35
120%	5	12.5	6	15	10.86	27.27	98.75	99.16
	5	12.5	6	15	10.88	27.46	98.90	99.86
	5	12.5	6	15	10.96	27.55	99.68	100.19

Table 7: Statistical Validation Data for Accuracy determination.

Level of (%) Recovery	Mean*		Standard deviation*		%Coefficient of Variation*		Standard Error*	
	RAM	HCZ	RAM	HCZ	RAM	HCZ	RAM	HCZ
80%	99.78	100.23	0.9277	0.1872	0.9297	0.1868	0.5356	0.1081
100%	99.51	100.17	0.8856	0.1607	0.8900	0.1604	0.5113	0.0928
120%	99.11	99.74	0.4993	0.5260	0.5038	0.5274	0.2883	0.3037

*n = 3

Table 8: Determination of Intra-day precision of Ramipril and Hydrochlorthiazide respectively.

Sr. no	Amount present (mg)		Amount found (mg)		Label Claim* %	
	RAM	HCZ	RAM	HCZ	RAM	HCZ
1	50	125	49.98	125.01	99.96	100.01
2	50	125	49.95	124.95	99.9	99.96
3	50	125	50.02	124.93	100.03	99.95
4	50	125	49.97	124.9	99.93	100.03
5	50	125	50.07	124.99	100.13	99.98
6	50	125	50.03	125.06	100.06	100.05

Table 9: Statistical validation data for determination of intra-day precision.

Components	Mean*	Standard deviation*	% Coefficient of Variation*	Standard Error*
RAM	100.00	0.0870	0.0870	0.0356
HTZ	99.99	0.0398	0.0398	0.0163

*n = 6

Table 10: Determination of inter-day precision of RAM and HTZ respectively.

Sr. no	Amount present (µg/ml)		Amount found (µg/ml)		Label Claim* %	
	RAM	HCZ	RAM	HCZ	RAM	HCZ
DAY-1						
1	50	125	49.86	124.8	99.72	99.84
2	50	125	49.95	125.5	99.9	100.4
3	50	125	49.87	124.9	99.74	99.92
4	50	125	49.95	125.5	99.9	100.4
5	50	125	49.95	124.9	99.9	99.92
6	50	125	49.95	124.9	99.9	99.92
DAY- 2						
1	50	125	49.89	124.8	99.78	99.84
2	50	125	49.77	124.9	99.54	99.92
3	50	125	49.98	125.3	99.96	100.24
4	50	125	49.99	124.9	99.98	99.92
5	50	125	49.78	124.8	99.56	99.84

6	50	125	49.88	124.8	99.76	99.84
DAY- 3						
1	50	125	49.80	125.5	99.6	100.4
2	50	125	49.98	124.9	99.96	99.92
3	50	125	49.78	124.8	99.56	99.84
4	50	125	49.69	125.5	99.38	100.4
5	50	125	49.88	124.9	99.76	99.2
6	50	125	49.96	124.9	99.92	99.2

Table 11: Statistical validation data for determination of inter-day precision.

Components	Mean*	Standard deviation*	% Coefficient of Variation*	Standard Error*
RAM	99.95	0.0967	0.0968	0.0394
HTZ	99.96	0.0505	0.0506	0.0206

Where n *= 3

Table 12: Linear regression data for Ramipril and Hydrochlorthiazide.

Component	Linear range (µg/ml)	Slope	Intercept	Regression coefficient (r^2)
RAM	50-150	7885	1972	1
HCZ	50-150	14024	578.7	1

Table 13: Summary of validation and System suitability parameters of Ramipril and Hydrochlorthiazide.

Parameters	RAM	HCZ
Linear range (µg/ml)	50-150	50-150
Slope	7885	14024
Intercept	1972	578.7
Regression coefficient (r^2)	1	1
Limit of Detection (µg/ml)	0.825	0.037
Limit of Quantification (µg/ml)	2.50	0.412
Retention time (min)	2.463	1.423
Tailing factor	1.1	1.2
Resolution factor	7.935	
Theoretical plate	4274	3668

Table 14: Robustness Results for variations in Flow Rate (ml/min).

Method Parameter	Level	Retention Time		Tailing factor	
Flow Rate (ml/min)		RAM	HCZ	RAM	HCZ
0.6	-1	2.6	1.5	1.244	1.1
0.8	0	2.463	1.423	1.1	1.2
1.0	+1	2.0	1.0	1.46	1.3

Table 15: Robustness Results for variations in Temperature.

Method Parameter	Level	Retention Time		Tailing factor	
Temperature		**RAM**	**HCZ**	**RAM**	**HCZ**
25^0 C	-1	2.468	1.424	1.147	1.193
27^0 C	0	2.436	1.420	1.125	1.179

Table 16: Statistical validation of Robustness Results for variations in Method Parameters.

Parameters	Mean		Standard Deviation		(%) Coefficient of variance	
	RAM	**HCZ**	**RAM**	**HCZ**	**RAM**	**HCZ**
Flow Rate						
Retention time	2.035	1.20	0.41114	0.211604	20,20014	17.52174
Temperature						
Tailing factor	1.268	1.202	0.1811	0.0970	14.28991	8.07117

The objective of the proposed work was to develop simultaneous methods for the determination of RAM and HTZ and to validate the methods according to ICH guidelines and applying the same for its estimation in marketed formulation. There is no official method for the simultaneous estimation of RAM and HTZ bulk and in combination.

UV Spectrophotometric and HPLC methods developed were found to be rapid, simple, precise, accurate and economic for routine estimation of RAM and HTZ simultaneously in commercial dosage forms.

In HPLC method

- The conditions were optimized to obtain an adequate separation of eluted compounds. Initially, various mobile phase compositions were tried, to separate titled ingredients. Mobile phase and flow rate selection was based on peak parameters (height, tailing factor, theoretical plates, resolution, run time, flow rate). The system with Orthophosphate buffer: ACN (neutral pH) (60:40 v/v) with 0.8 ml/min flow rate is quite robust. The optimum wavelength for detection was 294 nm at which better detector response for both the drugs was obtained. The retention times for RAM and HCZ was found to be 2.463 mins and 1.423mins respectively.
- According to USP XXIV (621), system suitability tests are an integral part of chromatographic method. They are used to verify the reproducibility of the chromatographic system. To ascertain its effectiveness, system suitability tests were carried out on freshly prepared stock solutions.

- The calibration was linear in concentration range of 50-150 µg/ml for both with regression 1 and 1, respectively. The low values of % RSD indicate the method is precise and accurate. The mean recoveries were found in the range of 98 – 101%.
- Sample to sample precision and accuracy were evaluated using five samples of five different concentrations, which were prepared and analyzed on same day. Day to day variability was assessed using five concentrations analyzed on three different days in a week. These results showed the accuracy and reproducibility of the assay.
- Ruggedness of the proposed method was determined by analysis of aliquots from homogeneous slot by different analysts, using similar operational and environmental conditions, the % RSD reported was found to be less than 2 %.
- The proposed method was validated in accordance with ICH parameters and the applied for analysis of the same in marketed formulations.

CONCLUSION

Spectrophotometric methods and one RP- HPLC method have been developed for the quantitative estimation of Ramipril and Hydrochlorthiazide individually in bulk as well as in combined pharmaceutical dosage form. New analytical methods were developed for the quantitative estimation of titled drugs.

The newly developed was performed by RP- HPLC and validated. It was validated for linearity, accuracy, precision, robustness and ruggedness. According to ICH criteria, all parameter values were found to be within the acceptable ranges. It can be concluded that this new HPLC method was found to be accurate, precise, robust, rugged, reliable andcost-effective. Hence, it can be employed for the routine quantitative analysis of ramipril and hydrochlorthiazide.

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