Original Article

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RP – HPLC Method Development and Validation for Simultaneous Estimation of Irbesartan, Amlodipine and Hydrochlorothiazide in Bulk and Tablet Dosage Forms

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Abstract

A reverse phase high performance liquid chromatographic method has been developed for the simultaneous estimation of Irbesartan, Amlodipine besylate and hydrochlorothiazide in bulk and Pharmaceutical formulation using RP-C_{18} Column. The mobile phase (Acetonitrile: methanol: 50mM phosphate buffer adjusted to pH 3.2 with orthophosphoric acid) was pumped at a flow rate of 1.2 mL/min in the ratio of 20:50:30% v/v and the eluent was monitored at 250 nm. Linearity was obtained in the concentration range of 5 - 30 µg/ml for Amlodipine Besylate, 20 - 120µg/ml for Irbesartan and 10 - 60 µg/ml for Hydrochlorothiazide. The method was statistically validated and RSD was found to be less than 2% indicating high degree of accuracy and precision of the proposed HPLC method. Due to its simplicity, rapidness, high precision and accuracy, the proposed HPLC method can be applied for determining Irbesartan, Amlodipine and hydrochlorothiazide in bulk and in pharmaceutical dosage form.

Keywords: RP-HPLC Method, Irbesartan, Amlodipine besylate, and Hydrochlorothiazide Development and Validation.

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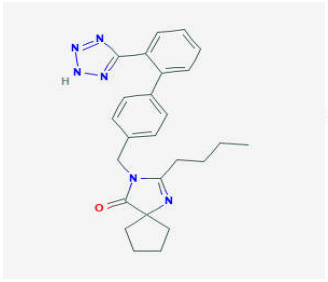
Introduction

Irbesartan (IRB)

Irbesartan is used mainly for the treatment of hypertension [1-7]. Irbesartan (INN) pronounced is an angiotensin II receptor antagonist. Irbesartan IAPUC name is 2-butyl-3-({4-[2-(2*H*-1,2,3,4- tetrazol-5-yl) phenyl]phenyl}methyl)-1,3-diazas- piro[4.4] non-1-en-4-one and molecular formula $C_{25}H_{28}N_6O$.

Amlodipine Besylate (AMB)

It is chemically 2- [(2 amino ethoxy) methyl] 4



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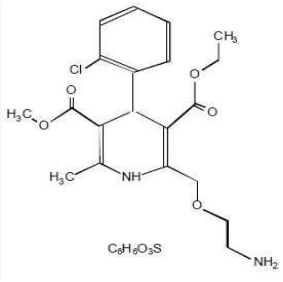

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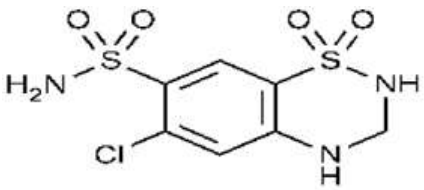

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(2 – chloro phenyl) 1, 4 dihydro 6 methyl 3, 5 pyridine dicarboxylic acid 3 ethyl 5 methyl ester, benzene sulfonate, is a potent dihydro calcium

channel blocker. Various analytical methods have been reported for the assay of AMB alone or in combination with other anti hypertensive agents in pharmaceutical formulations.

They include UV spectroscopy [2-4], high performance liquid chromatography [5-8], high performance thin layer chromatography [9, 10], LC MS [11] and LC MS/ MS [12].

Hydrochlorothiazide (HCT)

It is a chemically 6 chloro 3, 4 dihydro 7 sulfamoyl 2H 1, 2, 4 benzothia diazine 1, 1 – dioxide, is a thiazide diuretic. It increases sodium and chloride excretion in distilled convoluted tubule. Many analytical methods for HCT alone and in combination with other drugs by stability indicating method, RP- HPLC methods, spectro photometric methods and in plasma.

Literature survey revealed that numerous methods have been reported for estimation of Irbesartan in pharmaceutical formulations has been reported. Present study involves development of HPLC method [8-12] using simple mobile phase which is sensitive and rapid for all the three drugs are official in USP [32]. Amlodipine besylate and Hydrochlorothiazide are official in IP [33] and BP [34]. The chemical structures of IRB, AMB, and HCT are shown in figure 1.

Literature survey revealed that there are several methods were reported for the estimation of IRB, AMB and HCT individually as well as in combination with some other drugs. As no method is available for their simultaneous determination, however, it is essential to develop a suitable analytical method for simultaneous estimation of IRB, AMB and HCT in bulk and in pharmaceutical preparations, because HPLC methods have been widely used for routine quality control assessment of drugs, because of their accuracy, repeatability, selectivity, sensitivity and specificity.

We have developed a simple, precise, accurate and specific RP-HPLC method for the simultaneous determination of IRB, AMB and HCT in bulk and in pharmaceutical dosage forms.

Because analytical methods must be validated before use by the pharmaceutical industry, the proposed HPLC- UV detection method was validated in accordance with International conference in Hormonization (ICH) [5-6] guidelines, by assessing its selectivity, linearity, accuracy, and precision, limit of detection and limit of quantification.

Experimental

Instrumentation

Analysis was performed with a Schimadzu 3000 chromatograph equipped with LC solutions software and loop of injection capacity of 50µL- Visible detector set at 250 nm. The equipment was controlled by a LC Solution software. Compounds were separated on a (250mm × 4.6 mm i.d., 5µm particle size Primesil C_{18} column under reversed phase partition conditions. The mobile phase was a 20: 50: 30% (v/v) mixture of Acetonitrile: methanol: phosphate buffer (50Mm, pH 3.2± 0.1, adjusted with orthophosphoric acid). The flow rate was 1.2ml/min and the run time was 7min. before analysis both the mobile phase and sample solutions were degassed by the use of a sonicator (Ultra sonic bath sonicater, Bio-techies, India) and filtered through a 0.2 µm filter (Kshitij innovations, India). The identity of the compounds was established by comparing the retention times of compounds in the sample solution with those in standard solutions. Chromatography was performed in ambient temperature maintained at 20±1^{0}C. The UV spectrum of IRB, AMB and HCT for selecting the working wavelength of detection was taken using a Elico SL159, With UV Spectralthreats software UV-Visible spectrophotometer (shimadzu, Kyoto, Japan).

Reagents and Chemicals

Pharmaceutically pure samples of IRB IRB were obtained as a gift samples from Morpen laboratories, New Delhi. AMB and HCT were obtained as a gift samples from Dr. Reddy's, Hyderabad. A combination of IRB (300 mg), AMB (5 mg) and HCT (12.5 mg) in tablet formulation was procured from Local market. HPLC grade methanol, Acetonitrile, and water and potassium di hydrogen ortho phosphate (AR grade) were obtained from Merck Chemicals India Pvt. Limited, Mumbai, India.

Preparation of Stock Solution of IRB, AMB and HCT

About 31.25 mg of AMB, 125mg of IRB and 62.5mg of HCT were accurately weighed and transferred in to 25ml volumetric flasks separately. It was dissolved in methanol and the solution was made up to volume with same. From this standard stock solution, the mixed standard solution was prepared by pipetting each 1 ml of mother liquors into the same 25ml volumetric flask and made up to the volume with mobile phase to contain 50µg/ml, 150µg/ml and 100µg/ml of AMB, IRB and HCT, respectively.

Construction of Calibration Plots

Calibration standards for each analyte were prepared at the concentrations of 1, 2, 3, 4,5 and 6 µg/ml for AMB and HCT, IRB. All the solutions were chromatographed and the peak areas were measured. Peak areas were that plotted against their respective concentrations for IRB, AMB and HCT. From the plots it was found that all the drugs were linear in the concentration range of 5 - 30 µg/ml, 20 - 120µg/ml and 10 - 60 µg/ml for AMB, IRB, HCT, respectively. Unknown assay samples were quantified by reference to these calibration plots.

Assay of Tablet Formulation

The contents of twenty commercial tablets were weighed and their mean mass was determined. After grinding the tablets into a fine powder in a glass mortar, an accurately weighed quantity of the tablet powder equivalent to 50mg of IRB was quantitatively transferred in to a 50ml volumetric flask with about 45ml of methanol. The contents were sonicated for 15min, to ensure the complete solubility of drug. The mixture was then made up to 50ml with methanol. The solution was then centrifuged at 1000rpm for 10min and the clear supernatant was collected and filtered through 13mm membrane syringe filter (pore size 0.2µm). From the clear solution, further dilutions were made by diluting 1.0 ml into 25ml with mobile phase; from that 4.0ml in to 10ml with mobile phase to obtain 60 µg/ml of IRB which is also contains 30 µg/ml of AMB and 15µg/ml of HCT theoretically. Each sample solution was injected and the peak areas were measured for the determination of AMB, IRB and HCT in tablet formulation.

Statistical Calculations

Standard regression curve analysis was performed by use of Microsoft office excel 200 software (Microsoft,USA), without forcing through zero. Means, standard deviations and the other statistical parameters were calculated by use of SPSS software version 9.5 (SPSS, Cary, NC, USA).

Validation

The objective of method validation is to demonstrate that the method is suitable for its intended purpose as it is stated in ICH guidelines. The method was validated for linearity, precision (repeatability and intermediate precision), accuracy, selectivity and specificity. Accuracy was assessed by measuring recovery at three different levels. Precision assessed by measurement of intra and inter day precision. In the intraday study the concentrations of all the drugs were calculated six times on the same day at different time intervals. In the inter day study the concentrations of the drugs were calculated on six different days. Selectivity and specificity of the method were assessed by injecting solutions containing all the drugs; after chromatography three sharp peaks were obtained for all drugs. LOD and LOQ were measured to evaluate the detection and quantification limits of the method and to determine whether these were affected by use of the equations LOD= $3.3\sigma/S$ and LOQ = $10\sigma/S$, where σ is the standard deviation of the response and s is the slope of the calibration plot.

Results and Discussions

HPLC Method Development and Optimization

The multi component formulations have gained a lot of importance as there is greater patient acceptability, increased potency and decreased side effect. This work was focused on optimization of the conditions for the simple and rapid as well as low cost effective analysis including a selection of the proper column or mobile phase to obtain satisfactory results.

Solvent type, solvent strength (volume fraction of organic solvent(s) in the mobile phase and pH of the buffer solution), detection wavelength, and flow rate were varied to determine the chromatographic conditions giving the best separation. The mobile phase conditions were optimized so there was no interference from solvent and excipients. Other criteria, for example time required for analysis, appropriate k range ($1<k<10$) for eluted peaks, assay sensitivity, solvent noise, and use of the same solvent system for extraction of the drugs from the formulations during drug analysis were also considered. Method development was started with 50% v/v acetonitrile in water, but AMB not eluted up to 10mins. The mobile phase was changed to 50% v/v methanol in water, but resolution is less. The mobile phase was then adjusted by mixing acetonitrile with methanol and potassium dihydrogen orthophosphate buffer (50 μM) in the ratio 20:50:30 *v/v*. This resulted in distorted signals that were not well defined. Addition of 0.4 mL orthophosphoric acid and subsequent adjustment of the pH of buffer resulted in good separation and symmetrical peaks.

To determine the appropriate wavelength for simultaneous determination of AMB, IRB and HCT, solutions of these compounds in mobile phase were scanned in the range of 200 – 400nm. From the overlaid

UV spectra, suitable wavelength choices considered for monitoring the drugs were 225, 225 and 239 nm (*Figure 5*). Solutions of each substance in the mobile phase were also injected directly for HPLC analysis and the responses (peak area) were recorded at 225, 225 and 239 nm. It was observed that all analytes absorbed well at 250 nm, and at this wavelength there was no interference from the mobile phase or baseline disturbance, and it was, therefore, concluded that 250 nm was the most appropriate wavelength for analysis of all the drugs with suitable sensitivity.

The optimum mobile phase was, therefore, Acetonitrile: Menthanol: potassium dihydrogen orthophosphate buffer (50 mM; pH 3.2 µ0.1) in the ration of 20:50:30 (*v/v*). Under these experimental conditions sharp peaks were obtained for AMB, IRB and HCT at the retention times 3.60 min, 2.36 min and 5.24 min, respectively. The optimized

Table 1: Results from system suitability study

Parameters	IRB*	AMB*	HCT*
Peak area	58801	8460	30481
Retention Time	3.894	2.825	1.987
Tailing factor	1.231	1.323	1.382
Number of Theoretical plates	5669	4427	1835
Resolution	Between AMB and IRB 2.25		Between HCT and AMB 2.26

*Mean of six determinations

Table 2: Linearity data of irbesartan

S. No	Conc.	Peak Area
1.	20	213573
2.	40	332818
3.	60	434321
4.	80	542818
5.	100	652067
6.	120	760017

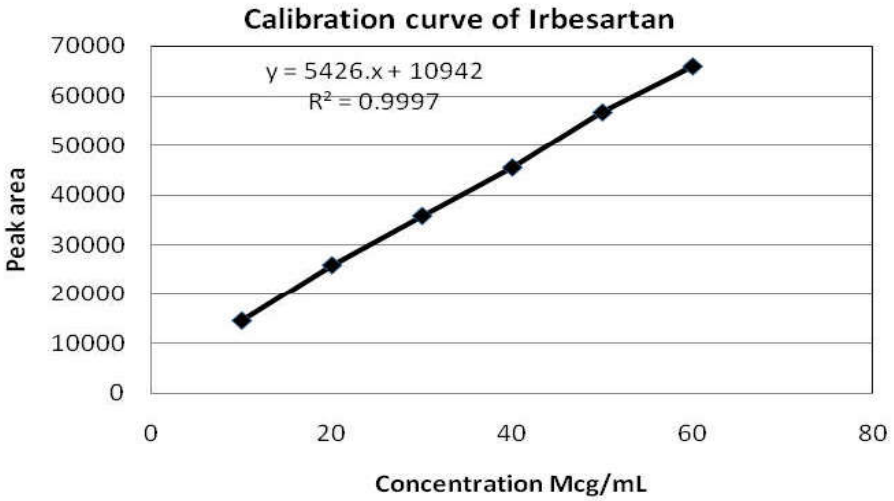

Fig. 2: Calibration curve of Irbesartan

Table 3: Linearity data of amlodipine

S. No	Concentration	Peak Area
1.	5	104885
2.	10	215117
3.	15	324399
4.	20	430976
5.	25	548730
6.	30	639020

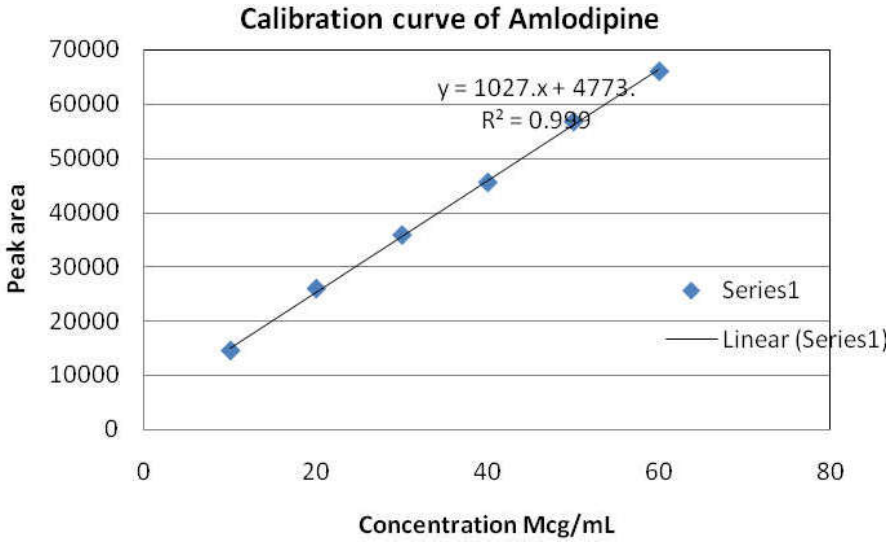

Fig. 3: Calibration curve of Amlodipine

Table 4: Linearity data of hydrochlorothiazide

S. No.	Concentration (Mcg/mL)	Peak area
1.	2. 10	3. 14484
4.	5. 20	6. 25905
7.	8. 30	9. 35790
10.	11. 40	12. 45484
13.	14. 50	15. 56699
16.	17. 60	18. 65965

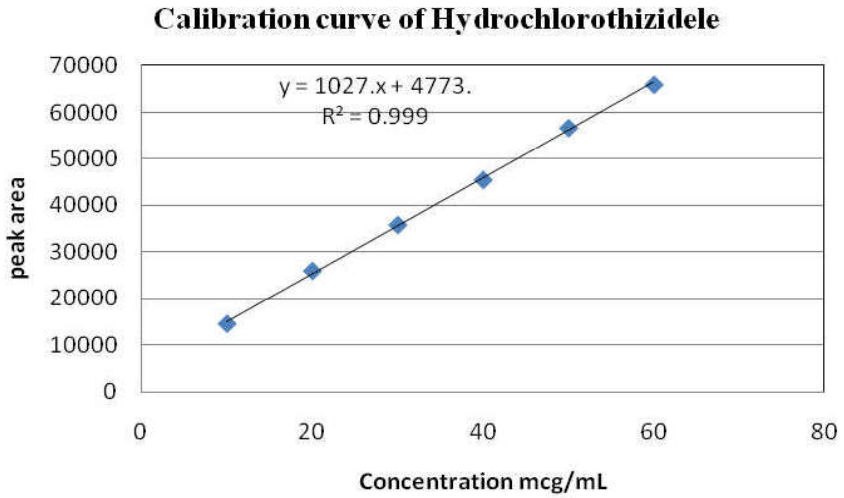

Fig. 4: Calibration curve of Hydrochlorothiazide

Table 5: Results from study of linearity

Parameters	IRB*	AMB*	HCT*
Detection wavelength	259nm	259nm	259nm
Correlation coefficient(r)	0.9997	0.9995	0.9993
Slope(b)	5426	1027	1027
Intercept(C)	10942	4773	4773
LOD(µg ml-1)	1.99	0.62	7.55
LOQ(µg ml-1)	6.07	1.89	22.89

*Mean of six determinations; **p > 0.05.

chromatogram for AMB, IRB and HCT (10 μg mL) were shown in *Figure. 6*. The resolution (RS) between HCT and AMB are 2.26; AMB and IRB are 2.25.

Method Validation

The system suitability parameters like capacity factor, number of theoretical plates, and USP tailing factor for all the analytes were found to be within the limit indicating the suitability of the system (Table 1). The values obtained for k' (1<k'<10) and RS (>2) showed these chromatographic conditions are appropriate for separation and quantification of all compounds. The number of theoretical plates and the USP tailing factor

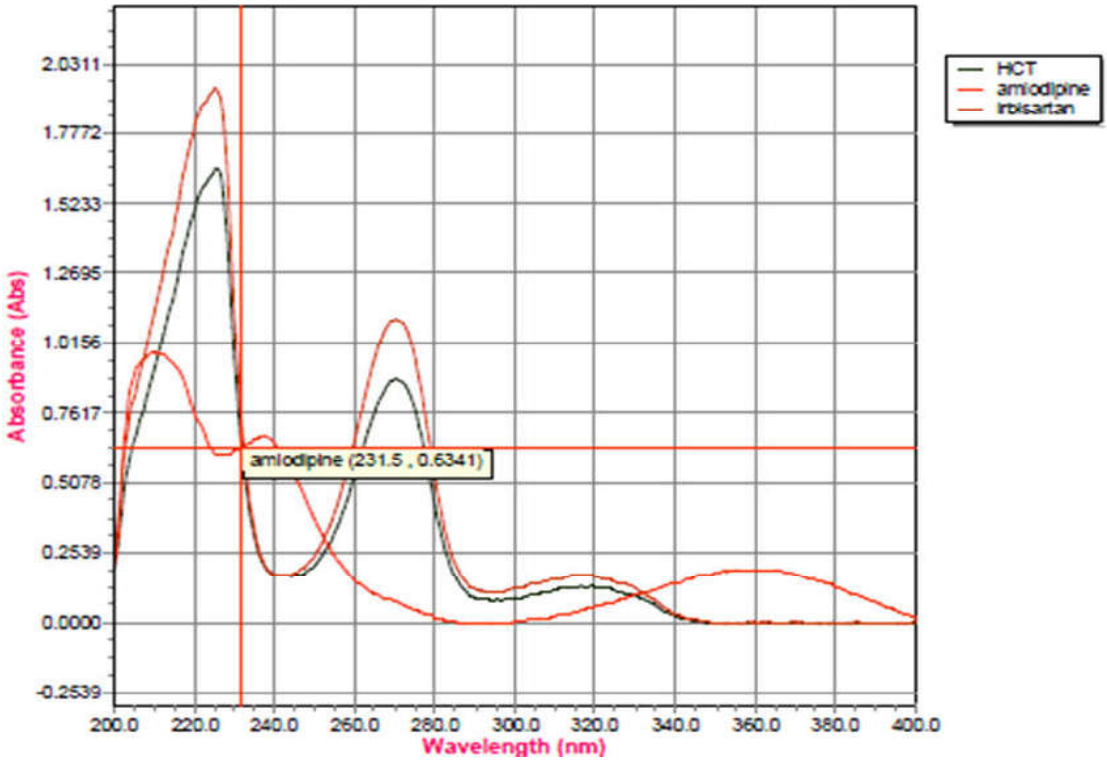

Fig. 5: Overlien uv spectra of IRB, AMP, and HCT

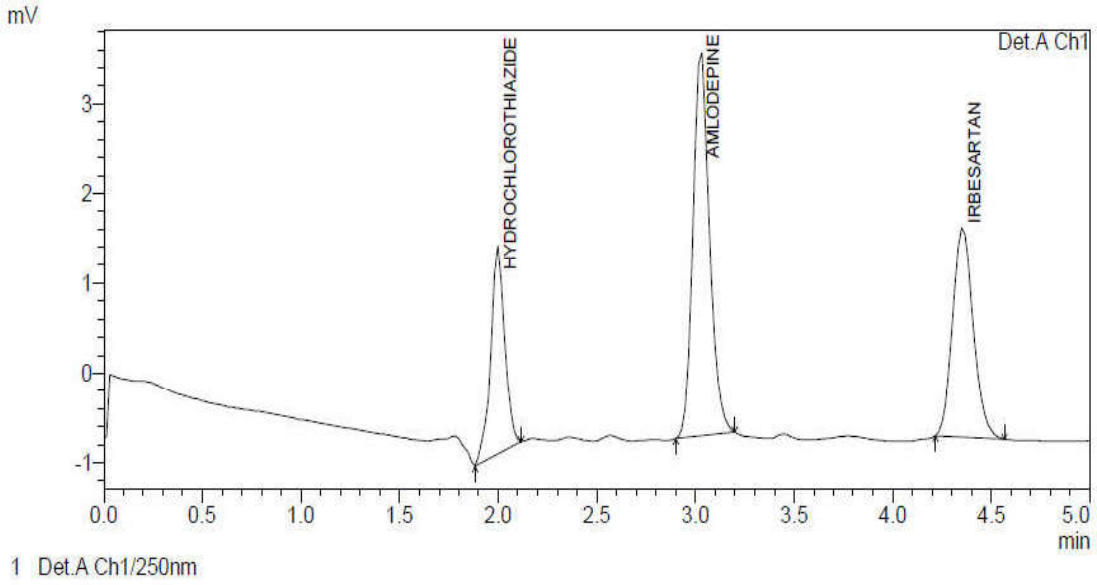

Fig. 6: Optimized chromatogram for IRB, AMB, and HCT (10 *mg* mL^{-1})

Table 6: Results from assay of tablet formulation

Drug	Label claim(µg/ tablet; n =6)	Amount found(µg)	Drug content (%)	S.D	COV (%)	S.E
AMB	10	9.995	99.95	0.3792	0.3794	0.1548
IRB	160	160.443	100.27	0.1708	0.1703	0.0697
HCT	25	25.007	100.03	0.0579	0.0578	0.0121

S.D., Standard deviation; COV, coefficient of variance; S.E., standard error

Table 7: Precission studies

Drug	Concentration(µg mL-1)	Intraday precision (n=6)		Interday precision (n=6)	
		Mean	RSD (%)	Mean	RSD (%)
AMB	3	27700	0.70	12415	1.65
IRB	1	284232	1.15	33192	0.6
HCT	2	115479	2.0	13605	1.0

Table 8: Results of recovery analysis

Drug	Sample No.	Amount present (µg/ml)	Amount added (µg/ml)	Amount estimated (µg/ml)	Amount recovered (µg/ml)	% Recovery	S.D	% R.S.D	S.E.
IRB	1	1.9866	1.2	3.1787	1.1921	99.34	0.2586	0.2597	0.1493
	2	1.9866	2.4	4.3830	2.3964	99.85			
	3	1.9866	3.6	5.5690	3.5824	99.52			
AMB	1	2.9822	2.4	5.3829	2.4007	100.29	0.6305	0.6330	0.3640
	2	2.9822	3.6	6.5651	3.5829	99.52			
	3	2.9822	4.8	7.7776	4.7954	99.04			
HCT	1	5.9503	2.4	8.3559	2.4056	100.24			
	2	5.9503	3.6	9.5520	3.6017	100.47	0.6189	0.6198	0.3573
	3	5.9503	4.8	10.7168	4.7665	99.30			

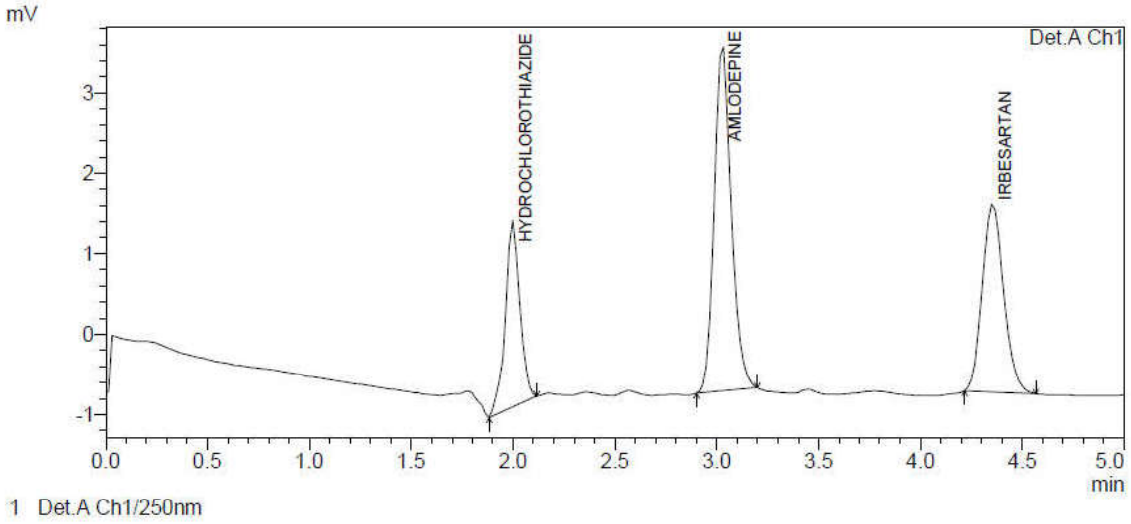

Fig. 7: Chromatogram obtained for IRB (1 µg mL-1), AMB (16 µg mL-1) and HCT (2.5 µg mL-1) in tablets

were within the acceptance criteria of >2000 and ≤ 1.5, respectively, indicating good column efficiency and optimum mobile phase composition.

Linearity

Linearity was tested in the concentration range 5 - 30 µg mL-1 for AMB, 10 – 60 µg ml-1 for HCT and 20 - 120 µg mL-1 for IRB. The solutions were chromatographed six times, in accordance with the International Conference on Harmonization. Separate calibration plots for AMB, IRB and HCT were constructed by plotting peak area against the respective concentrations and the method was

evaluated by determination of the correlation coefficient and intercept, calculated in the corresponding statistical study (ANOVA; $P < 0.05$), correlation coefficient r^2 values > 0.999 and intercepts very close to zero confirmed the good linearity of the method. The P values calculated for the calibration plots were greater than 0.05, indicating the variances were not significantly different (Table 5).

Assay of Tablet Formulation

The percentage label claim present in tablet formulation was found to be 99.93 ± 0.2092, 100.13 ± 0.1718, 100.01±0.0579 for AMB, IRB and HCT, respectively. The chromatogram for the analysis of formulation is shown in *Figure 7;* Precision of the method was confirmed by the repeated analysis of formulation for six times. The % COV values were found to be 0.3794, 0.1703 and 0.0578 for AMB, IRB and HCT, respectively. The low% COV values indicated that all the three drugs showed good agreement with the label claim ensures the precision of the method (Table 6).

Precision

Intraday and Interday precision was determined by repeating assay three times on same day for intraday and on three different days for inter day precision. (Table 7). The intraday precision ranges from 1.36 to 1.53, 0.48 to 0.75 and 1.03 to 1.70 for AMB, IRB and HCT, respectively. The interday precision ranges from 1.36 to 1.79, 0.52 to 1.15 and 1.38 to 1.76 for AMB, IRB and HCT, respectively

Accuracy

To check the accuracy of the developed methods and to study the interference of formulation excipients, analytical recovery experiments were carried out as per ICH guidelines. The results of recovery studies and its statistical validation data given in Table 8 indicate high accuracy of the proposed method. The percentage recovery was found to be in the range of 98.90 – 100.15% for AMB, 100.73 – 102.22% for IRB and 100.20 – 100.20% for HCT. The % COV values for AMB, IRB and HCT were found to be 0.6531, 0.7688 and 1.1305 respectively.

Robustness

As defined by ICH, The robustness of an analytical procedure describes to its capability to remain unaffected by small and deliberate variations in method parameters. Robustness was performed by small variation in the chromatographic conditions and found to be unaffected by small variations like ±2% variation in volume of mobile phase composition, ± 1% mL/min in flow rate of mobile phase, ± 1% variation in pH.

Specificity

The specificity of HPLC was ascertained by analyzing standard drug and sample solutions. The retention time of AMB, VAL and HCT was confirmed by comparing the retention time with that of the standard.

Conclusions

A simple isocratic RP – HPLC method with VU detection has been developed for simultaneous determination of IRB, AMB and HCT. The method was validated for accuracy, precision, specificity and linearity. The run time is relatively short (10 mins), which enables rapid quantification of many samples in routine and quality control analysis of tablets. The method also uses a solvent system with the same composition as the mobile phase for dissolving and extracting drugs from the matrices, thus minimizing noise. Thus the propose method is rapid, selective, requires a simple sample preparation procedure, Moreover, The lower solvent consumption leads to a cost effective and represents a good procedure of IRB, AMB and HCT determination in bulk and Pharmaceutical dosage forms.

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