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Original Article

New Antihypertensive Tablets Formulation and HPLC Analyses Using New Generation Core Shell Column

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ABSTRACT

Objectives: Formulation of triplet combined solid dosage film coated tablet containing amlodipine besylate (equivalent to 5 mg amlodipine), hydrochlorothiazide (12.5 mg) and losartan potassium (50 mg) for the treatment of severe hypertension. Development and validated of a simple, fast, precise, selective and accurate HPLC method for the simultaneous determination of amlodipine besylate, hydrochlorothiazide and losartan potassium in the tablets.

Methods: The formulation of the tablets was carried out as per standard protocols. The various steps involve in formulation were dispensing of raw materials, sieving, preparation of granulating solvent, mixing, granulation, drying (In FBD), lubrication, compression and coating. The separation of these three drugs was achieved on a Sun Shell C_8 column (150 mm x 4.6 mm, 2.6 µm) with phosphate buffer-acetonitrile (70:30% v/v) as mobile phase at 1.0 mL/min flow rate and 230 nm detection.

Results and Conclusion: The physical parameters of tablets were satisfactory with average weight deviation from 3.23 to 3.29%, friability 0.04%, disintegration time 8.3 minutes, average hardness 85.43N and thickness from 3.92 to 4.01 mm. The assay was found to be 99.89%, 99.99% and 99.97% of amlodipine, hydrochlorothiazide and losartan potassium, respectively. The dissolution was found to be 98.8 to 99.70%, 97.85 to 98.95% and 97.98 to 99.99% of amlodipine, hydrochlorothiazide and losartan potassium, respectively. The uniformity of content was 99.85 to 99.99% and 99.60 to 99.99% of amlodipine and hydrochlorothiazide, respectively. The retention times observed were to be 7.338, 2.097 and 10.675 minutes for amlodipine besylate, hydrochlorothiazide and losartan potassium, respectively. The method was statistically validated for linearity, recovery, limit of detection, limit of quantification, accuracy,

precision, robustness, stability of drugs in pure form and in presence of matrices and forced degradation study. The method was successfully applied for analysis of combined dose tablet.

Keywords: Anti-hypertensive tablet formulation, Amlodipine besylate, Hydrochlorothiazide, Losartan potassium, HPLC analyses, Core shell column.

INTRODUCTION

World Health Organisation (WHO) confirmed that about 33.3% populations are having cardiac problems (data of 194 countries)[1]. The most common cardiac diseases are hypertension, cardiac arrhythmias, glaucoma, angina pectoris, thyrotoxicosis and migraine headaches[2,3]. Among various medications for hypertension amlodipine besylate, hydrochlorothiazide and losartan potassium are considered as quite effective (Figure 1)[4-8]. There are various routes of drugs administrations including topical, oral, sublingual, transdermal, rectal, parenteral etc. The oral route has received the most attention because of more flexibility in dosage form design, patient acceptance and relatively safe mode. Moreover, the constraints of sterility and potential damage at the site of administration are avoided. About 70% of the total medicines are dispensed in the form of tablets due to various advantages[9]. Generally, amlodipine besylate, hydrochlorothiazide and losartan potassium are prescribed as single dosage or in combination of two only[10-14]. It is very useful, inexpensive and psychological accepted for treating hypertension with three active ingredients in single dosage form.

HPLC is considered as the best technique for developing precise, accurate, linear, robust, stable and rugged analytical methods in pharmaceutical dosage forms[15-18]. The speed and economy are the most crucial aspects in quality control laboratories and other pharmaceutical analyses to increase throughput and reduce expenses. This is because of hiking prices of all chemicals and man power globally. Recently, special type core shell columns are available; called as new generation columns. These columns have superficially porous particles (shell particles; 2.7 µm) giving ultra fast speed and 70% reduction in run time. Recently, Ali *et al*.[19,20] reviewed the applications of core shell columns. The authors observed these columns are suitable for ultra fast analyses using simple HPLC instrument; without costly UPLC. Literature survey indicates some papers describing HPLC analyses of amlodipine besylate, hydrochlorothiazide and losartan potassium as single constituents or in combination of two in tablets[21-36]. It was observed that all these methods have used classical C_{18} columns. These methods are costly chemical and time consuming. Moreover, the limits of detection and quantification are high.

Keeping all these facts into consideration, it was considered worthwhile to develop a new formulation of coated tablets containing amlodipine besylate, hydrochlorothiazide and losartan potassium ingredients for fast, ready and inexpensive cardiovascular medication. The tablets were formulated and prepared to increase the drugs release, enhance the drugs absorption and bioavailability, reduce dose and side effects, improve the patients compliance, more efficacious hypertension therapy, perform preformulation studies for drug excipient compatibility. Besides, the efforts were made to study the effects of varying concentrations of polymer on drug release

and evaluate the physicochemical characterization of developed formulation. The simultaneous estimation of amlodipine besylate, hydrochlorothiazide and losartan potassium in the tablets and other assays of tablets were carried out by developing and validated new HPLC method using core shell column. The results of these findings are discussed herein.

EXPERIMENTAL

Chemicals and reagents

HPLC grade solvent such as acetonitrile was purchased from Qaligens India. Triethylamine and phosphoric acid were purchased from Merck India. Sodium dihydrogen phosphate dihydrate of SQ grade was purchased from Qualigen India. Water used was prepared by Adrona Crystal, Latvia. The other chemicals and reagents for tablets formulation are given in the Table 1.

Instruments used

HPLC system used was of Shimadzu, Japan (UFLC XR, LC-20ADXR) consisting of solvent delivery pump, auto sampler, absorbance detector (UV-Vis.) and Lab. solution software. The columns used were Sushell(s) C_8 (150 x 4.6mm, 2.6 µm) of Chromanik Japan. The other instruments used in this study are given in Table 2.

Formulation of pharmaceuticals dosage

The formulation of the tablets was carried out as per standard protocol[37-40]. The various steps involve in formulation were dispensing of raw materials, sieving, preparation of granulating solvent, mixing, granulation, drying (In FBD), lubrication, compression and coating as shown in Figure 2. The raw materials were weighed in required quantities and passed through different sizes of sieves. The different ingredients such as hydrochlorothiazide, amlodipine besylate, lactose monohydrate, MCC P^H 101, SSG, losartan potassium, were sieved by mesh no. 40 except Aerosil, PVP K 30 and Magnesium stearate, which were sieved by mesh no. 60. The given quantity of PVP K 30 was dissolved in 80 g of IPA. The mixing was performed manually as follows:

- Mixed amlodipine besylate and SSG together.
- Mixed hydrochlorothiazide.
- Mixed lactose monohydrate.
- Mixed losartan potassium.
- Mixed MCC P^H 101.
- Tumbled powder in polybag for 3 minutes.

The granulation was performed manually until required situation was obtained. The damped mass was sieved through sieve number 14. The wet granules were loaded in the trolley of FBD. The moisture content was maintained between 2 to 3% at 80^0C. After drying, the dry granules were sieved through sieve number 20. The temperature ranged from 28 to 45ºC for 5-25 minutes. The mass of dried granules was mixed with magnesium stearate and aerosil then tumbled in poly bag for 45 seconds. The compression was performed using punching machine. The punching tool was round, biconvex, 8 mm in plain diameter. The RPM of machine was 13. Only three formulations were developed using above cited procedures as given in Tables 3-6.

Coatings of the tablets f the best formulation

The coating suspension was prepared as given below.

- PEG 6000 was dissolved into hot purified water at 50-55^0C.
- HPMC was dispersed into above solution and left it for soaking overnight.
- Benzyl alcohol was added.
- Purified talc was added.
- Titanium dioxide was passed through mesh 100 with the help of purified water and then added.

- Brilliant blue lake and tartrazine lake colours were passed through mesh 300 with the help of purified water and then added.

The formulation of coating composition is given in Table 6.

Parameters evaluation of coated tablets

The formulated tablets were evaluated by using various parameters as discussed below briefly.

Shape of tablets

The compressed and film coated tablets were examined under the magnifying lens for the shape of the tablets as per standard method[50].

Tablet dimensions

Thickness and diameter were measured using a calibrated Vernier Calliper. Ten tablets of each formulation were picked randomly and thickness was measured individually[41].

Hardness

Hardness indicates the ability of a tablet to withstand mechanical shocks while handling. The hardness of tablets was determined using Tablet Hardness Tester. It is expressed in Kg/cm^2 or N. Ten tablets were randomly picked and hardness of the tablets was determined[42].

Friability test

This test is applicable to compressed tablets and is intended to determine the physical strength of tablets (measured in %). For tablets with an average weight of 6.5 g or less should take a sample of whole tablets corresponding to about 6.5 g For tablets with an average weight of more than 6.5 g should take a sample of 10 whole tablets. The friability of tablets was determined using Friability Test Apparatus of Aastha International, New Delhi, India. Thirty three tablets were initially weighed (W1) and transferred into friability test apparatus. The friabilator was operated at 25 rpm for 4 minutes or run up to 100 revolutions[43]. The tablets were weighed again (W2). The percentage friability was calculated by the following formula.

Friability (%) = [(W1-W2)/W1] x 100

The percentage (%) friability less than 1% was considered acceptable.

Weight variation test

Twenty tablets were selected randomly from each batch and weighed individually to check for weight variation. A little variation was allowed in weight of a tablet according to US Pharmacopoeia. The variations include ±10% if average weight is less than 80 mg. If it is more than 80 mg and less than 250 mg the variations should be ±7.5. If average weight is more than 250 mg variation should be less than ±5%[44]. The following percentage deviation in weight variation was allowed. It was calculated by the following formula.

Upper Deviation (%) =
[Maximum individual weight of a tablet - Average weight of a tablet/Average weight of a tablet] x 100

Lower Deviation (%) =
[Maximum individual weight of a tablet - Average weight of a tablet/Average weight of a tablet] x 100

In all formulations, the tablets weights were between 80 to 250 mg (about 210 mg) and, hence, ±7.5% maximum differences are allowed.

Disintegration test

This test determines whether dosage forms such as tablets, capsules etc.

disintegrate within a prescribed time when placed in a liquid medium under the prescribed experimental conditions (15 and 30 minutes for uncotaed and coated tablets, respectively). Water at 37± 2^0C was used as the liquid for the disintegration of uncoated and film coated tablets. A disc was added to each tube and apparatus was operated. Six tablets were used, which disintegrated within prescribed time (30 minutes for film coated tablets)[45].

Uniformity of content test

The test for uniformity of content was determined on the basis of assay of individual contents of active substances of a number of single dose units. It was determine to ascertain whether the individual contents were within limits set with reference to the average content of the sample or not. This was performed only for the tablets, which contain 10 mg or less than 10 mg or less than 10 percent active ingredient with respect to the average weight of a tablet. Hence, uniformity of content was performed for amlodipine besylate and hydrochlorothiazide because it was less than 10 mg or 10%. The contents of active ingredients (amlodipine and hydrochlorothiazide only in each of 10 tablets) were taken at random were determined using the method given in the assay[46].

In vitro dissolution

Dissolution of amlodipine besylate and losartan potassium

The standard solutions of amlodipine besylate were prepared by weighing accurately amlodipine besylate working standard equivalent to amlodipine 28 mg (39 mg of amlodipine besylate) and diluted to 100 mL with methanol. Similarly, the standard solutions of losartan potassium were prepared by weighing accurately 55.5 mg of losartan potassium working standard and diluted to 50 mL with methanol. The final concentration of combined standard solutions were prepared by diluting 2.0 mL of stock solution of amlodipine besylate and 5.0 mL of stock solution of losartan potassium to 100 mL with dissolution medium. In vitro dissolution was carried out by dissolving amlodipine besylate and losartan potassium in 900 mL 0.01 M sodium acetate solution (pH 4.5). The apparatus used was Paddle and operated at 75 rpm for 30 minutes at 37^0C $\pm$ 0.5^0 C. The injection volume was 10 µL. One tablet was kept in 900 mL dissolution medium. After completion of dissolution, a suitable volume of medium was sampled and filtered through 0.2 µm membrane filter paper. The dissolution was determined by HPLC conditions developed herein[47].

Dissolution of hydrochlorothiazide

The standard solution of hydrochlorothiazide was prepared by weighing accurately 28 mg of hydrochlorothiazide working standard and diluted to 100 mL with acetonitrile. Further, 5.0 mL of resulting solution was diluted to 100 mL with medium. In vitro dissolution was carried out by dissolving hydrochlorothiazide in 900 mL distilled water. The apparatus used was Paddle and operated at 100 rpm for 30 minutes at 37^0C $\pm$ 0.5^0C. The injection volume was 10.0 µL. One tablet was kept in 900 mL dissolution medium. After completion of dissolution, a suitable volume of medium was sampled and filtered through 0.2 µm membrane filter paper. The dissolution was determined by HPLC conditions developed herein.

Inter and intraday assays

The inter- and intra-day assays were carried out to determine the degradation of APIs in tablet. These experiments were carried out for 24 h and 7 days for inter and

intraday assays, respectively. These experiments were carried out at pH 7.0 being blood pH. Weight of 20 tablets was taken and average weight of tablets was determined. All weighted tablets were crushed with the help of mortar and pestle. Weight of powder equivalent to average wt. of tablets (about 211 mg) was taken in 100 mL volumetric flask and about 70 mL of 50 mM phosphate buffer of 7.0 pH was added. All volumetric flasks containing samples were sonicated for 15 minutes. These samples were allowed to stand for few minutes to equilibrate with room temperature. Then phosphate buffer was added up to mark and shacked well. The samples were kept undisturbed for 24 hrs and 7 days, respectively. These samples were centrifuged for 10 minutes at 2000 rpm. Further, 5.0 mL of supernatant liquid was diluted to 50 mL with same diluent and filtered through 0.2 µm membrane filter paper. Finally the concentrations of API were determined by newly developed HPLC method[48].

High performance liquid chromatography

Preparation of standard solutions

The standard solutions of amlodipine were prepared by weighing accurately amlodipine besylate working standard equivalent to amlodipine 10.0 mg (14.0 mg) and diluted to 100 mL with diluent. Similarly, the standard solutions of hydrochlorothiazide were prepared by weighing accurately 25.0 mg of hydrochlorothiazide working standard and diluted to 100 mL with diluent. The standard solutions of losartan potassium were prepared by weighing accurately 50.0 mg of losartan potassium working standard and diluted to 50.0 mL with diluent. The final concentration of combined standard solution was obtained by diluting 5.0 mL of each stock solution to 100 mL with diluent.

Preparation of test solutions for assay

Twenty tablets were weighed and average weight of tablet was determined. All weighed tablets were crushed with the help of mortar and pestle. The powder equivalent to the average weight was weighed accurately in 100 mL volumetric flask. About 70 mL of diluent was added and sonicated for 10 minute. The samples were allowed to stand for some times to equilibrate with environmental temperature. The volume up to 100 mL was made with same diluent. The samples were centrifuged for 10 minutes at 2000 rpm. Further, 5.0 mL of supernatant liquid was diluted to 50 mL with same diluent. All samples were filtered through 0.2 µm nylon membrane filter paper.

Test solution preparation for UOC

One tablet was taken in 100 mL volumetric flask and about 70 mL of diluent was added. Then it was sonicated for 10 minutes and allowed to stand for few minutes to equilibrate with room temperature. All samples were centrifuged for 10 minutes at 2000 rpm. Further, 5.0 mL of supernatant liquid was diluted to 50 mL with same diluent. These were filtered through 0.2 µm nylon membrane and performed as the method of assay.

HPLC conditions

All the experiments were carried out by HPLC system as described above. The aliquots of 5.0 µL for assay and uniformity of content and 10.0 µL for dissolution of standard solutions of each drugs and their mixture in tablets were loaded onto HPLC instrument, separately and respectively. The mobile phase used was phosphate buffer (pH 2.5)-acetonitrile (70:30, v/v) in isocratic mode (1.0 mL/min.). Buffer solution was prepared containing 0.15% sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$) and 0.4% Triethylamine

(TEA) with pH adjusted to 2.5 with 85% phosphoric acid. The mobile phase was prepared, filtered and degassed daily before use. All the experiments were carried out at 45±1 °C temperature with detection at 230 nm. The chromatographic parameters such as retention (k), separation (α) and resolution (R_s) factors were calculated. The order of elution was ascertained by running individual drug. The qualitative and quantitative analyses were carried out using retention times and peak areas, respectively. The chromatographic method was optimized and validated by carrying out an extensive experimentation followed by applied analyses of drugs molecules in tablet formulation[49].

VALIDATION

HPLC method was validated by calculating different HPLC parameters. The different parameters studied were linearity and range, limit of detection (LOD), limit of quantitation (LOQ), specificity, precision, accuracy, robustness, ruggedness, system suitability test, forced degradation study solution of drugs and reagent stability study. The limits of detection (LOD) and quantitation (LOQ) were determined on the basis of the slope and standard deviation of y-intercepts of the calibration curve of amlodipine besylate, hydrochlorothiazide and losartan potassium. The results of the statistical analyses of the experimental data such as relative standard deviation, correlation coefficients and confidence limits were calculated by Microsoft Excel software program. Good linearity of the calibration graphs and the negligible scatter of experimental points were considered for calculations of correlation coefficients and relative standard deviations[50]. Robustness of method was determined by versatility of the experimental factors that affected the peak areas.

Linearity and range

The linearity was confirmed by least squares linear regression analysis of calibration curve[50]. The linearities of calibration curves (peak area *vs.* concentration) for amlodipine besylate, hydrochlorothiazide and losartan potassium standards were checked over the concentration ranges of 5.960-8.315 μgmL^{-1}, 10.275-15.510 μgmL^{-1} and 39.85-60.79 μgmL^{-1}, respectively. Equal volume (5.0 µL) of the standards as described above was loaded onto HPLC instrument. The chromatograms were developed separately and respectively. The calibration curves of amlodipine besylate, hydrochlorothiazide and losartan potassium were constructed using the observed peak areas versus nominal concentrations of amlodipine besylate, hydrochlorothiazide and losartan potassium. The range of an analytical procedure was determined by taking the lowest and highest concentration in the linearity range.

Detection and quantitation limits

The limits of detection (LOD) and quantitation (LOQ) were determined as three and five times to the baseline noise, respectively, following the United States Pharmacopoeia[50].

Specificity

Specificity of method was determined by observing any interference in chromatographic parameters due to the presence of some impurities in the standard samples. The standard samples were mixed with little amount of crude amlodipine besylate, hydrochlorothiazide and losartan potassium tablet contents to make them impure.

Precision

Precision data was calculated at three different concentrations i.e. 5.5, 7.29 and

8.37 µgmL^{-1} for amlodipine besylate, 10.21, 12.31 and 14.88 µgmL^{-1} for hydrochlorothiazide and 40.66, 50.33 and 59.50 µgmL^{-1} for losartan. Five sets of the chromatographic runs were carried out for all three concentrations.

Accuracy

Accuracy of HPLC method was ascertained using different concentrations of amlodipine besylate, hydrochlorothiazide and losartan potassium. Three concentrations used were 5.5, 7.29 and 8.37 µgmL^{-1} for amlodipine besylate, 10.21, 12.31 and 14.88 µgmL^{-1} for hydrochlorothiazide and 40.66, 50.33 and 59.50 µgmL^{-1} for losartan. The chromatographic runs were carried out five times (n = 5). Accuracy was determined by interpolation of five replicates peak areas of these molecules.

Robustness

Robustness of HPLC method was determined by carrying out a slight variation in the chromatographic conditions. The varied experimental conditions were flow rate, temperature, mobile phase composition, different column and pH. The retention time, peak area and shape were analyzed under the established and slightly varied experimental conditions.

Ruggedness

Ruggedness of the method was ascertained by changing the experimental environment such as different instruments and different days (i.e. intermediate precision).

System suitability test

System suitability was evaluated by replicate (n=5) injection of the same standard solution containing AML, HCT and LOS at 7.175, 12.575 and 50.37 µg/mL, respectively.

Forced degradation study

Forced degradation study is required to demonstrate specificity of stability indicating methods. It also provides information of degradation pathways and degradation products of the drugs. Besides, forced degradation study is useful to elucidate the structures of the degradation products. Forced degradation study was carried out by injecting standard and test solutions in duplicate. Assay was calculated with respect to the area of the peak. The test solutions were prepared as the standard solution except the addition of placebo according to the average weight of tablets. The solutions were subjected to the five stress conditions viz. acidic, basic, oxidative, thermal and photolytic conditions.

Solution of drugs stability

For this study, the samples were used from the linearity study up to 48 hours. The assays were determined at first day, 24 hours and 48 hours. For study of drugs solution with placebo mixture, the samples were used from the recovery study up to 48 hours. It was used to know the effect of excipients on the stability of drugs. The standard solution was prepared freshly. The assays were carried out at first day, 24 hours and 48 hs.

Reagent Stability Study

All the mobile phase and diluents were used for 48 hours during the stability study of drugs.

RESULTS AND DISCUSSION

The results and discussion of this manuscript is divided into two parts. First part describes the formulation of the tablets while second part deals with the HPLC analyses of amlodipine besylate, hydrochlorothiazide and losartan potassium in standard solutions and tablet formulation.

Formulation of pharmaceuticals dosage

It is clear from Tables 3-5 that formulation 3 is the best one due to the test results of friability, disintegration, and hardness, uniformity of content, assay and dissolution rate. These were found to be more accurate than 1 and 2 formulations. These values were acceptable as per US Pharmacopia.

Evaluation Parameters of coated tablets

The evaluation parameters are given in Tables 7 and 8. It is clear from these tables that the shapes of tablets were almost similar with standard deviation of 0.05-0.2%. The dimensions of tablets were almost similar with standard deviation of 0.1-0.2%. The harnesses were 111.2-131.40N, 102-110.23N and 80.01-91.43 N for formulation 1, 2 and 3, respectively. It is clear that formulation 3 is the best one. The friability (%) values for formulation 1, 2 and 3 were 0.1, 0.08 and 0.04, respectively (for uncoated tablet). Therefore, formulation 3 was considered as the best one by friability point of view (lowest among all the three). The weight variation values for formulation 1 were -2.26 and +3.26% as minimum and maximum, respectively. Similarly, these values for formulation 2 and 3 were -2.15 and +2.66% and -3.23 and +3.29%, respectively. All three formulations were acceptable by weight variation values but 3rd was considered the best due to other reasons. The disintegration values (min) were 13.5, 12.5 and 8.3 for formulations 1, 2 and 3. Of course, all three values are acceptable. The uniformity of contents of amlodipine in three formulations were found to be 97.50-99.50%, 98.56- 101.24% and 99.85-99.99% for formulations 1, 2 and 3, respectively. Similarly, the uniformities of contents of hydrochlorothiazide in three formulations were 98.76-99.99%, 97.45-99.50% and 99.60-99.99% for formulations 1, 2 and 3, respectively. Over all, these results indicated that formulation 3 was the best one due narrow range of UOC.

In vitro Dissolution

In vitro dissolution values (%) of amlodipine besylate for formulations 1, 2 and 3 were 89.00 to 98.00, 91.00 to 98.00 and 98.80 to 99.70, respectively. Similarly, these values for hydrochlorothiazide were 87.50 to 98.60, 93.50 to 98.50 and 97.85 to 98.95, respectively. The values for losartan potassium were 83.00 to 95.00, 91.50 to 97.00 and 97.98 to 99.99, respectively. These values indicated that all three formulations are acceptable as per dissolution values.

HPLC Analyses

The separation and identification of amlodipine besylate, hydrochlorothiazide and losartan potassium were carried out on new generation core shell columns and mobile phase as described into experimental section. The separated amlodipine besylate, hydrochlorothiazide and losartan potassium in tablets were confirmed by running standards of these molecules. The retention times were compared for qualitative purpose. For quantitative estimation the peak areas were considered. The calibration curves were plotted for these three molecules and used to determine their concentrations in newly formed tablets. The capacity (k), separation (α) and resolution (R_s) factors for these molecules in standard solutions and tablets were calculated. The values of these parameters are given in Tables 9. The chromatograms of amlodipine besylate, hydrochlorothiazide and losartan potassium in standard solutions and tablets are given Figures 3 and 4. It is clear from Table 9 and Figures 3 and 4 that all three molecules are base lined separated with sharp peak within 11 min. The order of elution was hydrochlorothiazide > amlodipine besylate > losartan potassium. A

perusal of Table 9 indicates that the values of separation (α) and resolution (R_s) factors are greater than 1.0, indicating complete separation.

HPLC Method Optimization

HPLC conditions were optimized by changing composition of acetonitrile in mobile phase. Besides, pHs and flow rates of mobile phase were also varied. The optimization was also ascertained by fixing detector wave lengths. In addition, other mobile phases containing phosphate buffer, acetate buffer and different organic modifiers were also tested. As a result of exhaustive experimentation, the best HPLC conditions were optimized and reported herein. The optimizations of important chromatographic parameters are discussed in the following paragraphs.

VALIDATION OF HPLC METHOD

HPLC method was validated with respect to various parameters including linearity and range, limit of detection (LOD), limit of quantitation (LOQ), specificity, precision, accuracy, robustness, ruggedness, system suitability test, forced degradation study, solution of drugs and reagent stability study[50].

Linearity and range

The linearity of calibration curves (peak area *vs.* concentration) for hydrochlorothiazide, amlodipine besylate and losartan potassium standards as well as in newly formed tablet were checked over the concentration ranges of 10.28-15.51 μgmL^{-1}, 5.96-8.315 μgmL^{-1} and 39.85-60.79 μgmL^{-1} respectively. The plotted curves were linear over these concentration ranges (n = 5) for three amlodipine besylate, hydrochlorothiazide and losartan potassium. The peak areas of amlodipine besylate, hydrochlorothiazide and losartan potassium were plotted versus their respective concentrations. The linear regression analysis was performed on the resultant curves. The correlation coefficient (r) for amlodipine besylate, hydrochlorothiazide and losartan potassium were found to be 0.9995, 1.0000 and 0.9993 respectively for all three molecules. The values of RSD and confidence levels were in the range of 0.39-0.58% and 98.88-101.59% across the concentration ranges studied.

Detection and Quantitation Limits

The values for LOD and LOQ of hydrochlorothiazide were 0.0608 and 0.1843 µg, respectively. These values for amlodipine besylate were 0.4366 and 1.3232 µg, respectively. On the other hand, these values for losartan potassium were 3.5102 and 10.6369 µg, respectively. The resultant RSDs for these studies were in the range of 0.39-0.58%.

Specificity

The method was quite good specific as can be seen from Figure 3. The retention times of all molecules were almost similar in both standard solutions and tablet formulation. There was no effect of the added impurities in standards on the retention times and peak shape of these molecules. These findings indicated good specificity of the reported method.

Precision

The precision data was calculated by taking three different concentrations (80%, 100% and 120%) of hydrochlorothiazide, amlodipine besylate and losartan potassium (010.21, 12.31 and 14.88 μgmL^{-1} for hydrochlorothiazide, 5.5, 7.29 and 8.37 μgmL^{-1} for amlodipine besylate and 40.66, 50.33 and 59.50 μgmL^{-1} for losartan potassium). Six chromatographic runs were carried out for all the molecules at all three concentrations. The RSDs values were

calculated and ranged from 0.4326-0.6614%.; indicating HPLC method precise.

Accuracy

The accuracy of the method was tested by analyzing different extracted samples of various tablets. The accuracy was determined by interpolation of replicates (n = 5) peak areas of three accuracy standards. In each case, the percent errors were calculated and ranged from -0.68 to 1.74%, -0.74 to 0.78% and -0.68 to -0.35% for amlodipine besylate, hydrochlorothiazide and losatran potassium, respectively. These ranges indicated good accuracy of the developed method.

Robustness

The small changes made were in mobile phase compositions, flow rates, oven temperature, different column and pH of mobile phase. It was observed that there were no remarkable variations in HPLC results. No change in HPLC results were observed by varying above experimental conditions, which indicated the reported method as robust.

Ruggedness

The ruggedness assessment was performed during the development of HPLC method. The RSD (%) values for intra- and inter-days of hydrochlorothiazide, amlodipine besylate and losartan potassium in the range of 0.38, 0.43, 0.57 and 0.572, 0.429, 0.477 indicating the robustness of the method. Besides, the results obtained with different operators were unaffected, which also indicated ruggedness of the method.

System suitability test

System suitability was ascertained by running five replicates of all three drugs. The RSD (%) of retention time, peak area, number of theoretical plates, resolution, capacity factor and tailing factor for all the analytes were within 2%, indicating the suitability of the system. The number of theoretical plates and the tailing factor were within the acceptance criteria of > 2000 and ≤ 2, respectively, representing good column efficiency and optimum mobile phase composition.

Forced degradation study

The results of forced degradation study were quite interesting. It was observed that assays values were in the range of 97.19 to 99.58% for all three APIs. These values clearly indicate that that tablet ingredients are quite stable under varied experimental conditions.

Solution of drugs stability

The assays were determined at first day, 24 hours and 48 hours. RSD (%) was found to be less than 2%.

Reagent Stability Study

All the mobile phase and diluents were used for 48 hours during the stability study of drugs and the results were found to be linear, accurate and precise.

Inter- and Intraday Assays

The release of the drugs in blood is crucial factor for their actions. Besides, the stabilities and degradations of the residual drugs are also important to determine. For this purpose intra- and inter-days assays were ascertained for API of the developed tablets. It was observed that the release of hydrochlorothiazide, amlodipine besylate and losartan potassium were 98.45, 92.44 and 97.40% after 24 hrs. Contrarily, these values were 56.38, 88.28 and 96.78%. These values indicate that the drugs are quickly released in phosphate buffer at pH 7.0, necessary requirement for fast drug action. It can be observed that these API degradate moderately after 7 days. This is a good

feature indicating APIs absence in blood and body tissues after their curing action.

CONCLUSION

A successful coated tablet formulation (210 mg) was achieved for hypertensive patients for immediate release of drugs and improves bioavailabilities. The newly developed tablet contains amlodipine besylate (equivalent to 5 mg amlodipine), hydrochlorothiazide (12.5 mg) and losartan potassium (50 mg) along with excipients. The formulated tablets showed compliance for various physico-chemical parameters *viz.* thickness, friability, hardness, disintegration, assay of active ingredients, uniformity of content and in-vitro dissolution test. The formulation F3 was found to be the best one. This formulated dosage is very convenient and economic for treating pypertensive patients in the place of individual three ingredients. The developed and validated HPLC method using core shell column is very useful, precise, accurate, robust and economic to estimate the content of amlodipine besylate, hydrochlorothiazide and losartan potassium simultaneously. The drugs release and degradation studies at pH 7.0 indicate this combination ideal due to fast release and degradation of residual drugs after 24 hrs.

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Table 1. List of raw materials

S.N.	Raw materials	Manufacturer	Supplier
1	Amlodipine besylate	Cadila Health Care Pvt. Ltd.	Quest Pharmaceuticals Pvt. Ltd.
2	Hydrochlorothiazide	CTX Life Sciences Pvt. Ltd.	Quest Pharmaceuticals Pvt. Ltd.
3	Losartan K	Vadusa Pharma Chem Ltd.	Quest Pharmaceuticals Pvt. Ltd.
4	Microcrystalline cellulose pH 101	Mingtai Chem Co. Ltd.	Quest Pharmaceuticals Pvt. Ltd.
5	Sodium starch glycollate	Amishi Drug and Chemicals Pvt. Ltd.	Quest Pharmaceuticals Pvt. Ltd.
6	Lactose monohydrate	Moder Dairies Ltd.	Quest Pharmaceuticals Pvt. Ltd.
7	PVP K30	BASF Corporation	Quest Pharmaceuticals Pvt. Ltd.
8	Magnesium stearate	Nitika Pharma Specialities Pvt. Ltd.	Quest Pharmaceuticals Pvt. Ltd.
9	Aerosil	Evonik Industries	Quest Pharmaceuticals Pvt. Ltd.
10	Isopropyl alcohol	Avantor Performance Materials India Ltd.	Quest Pharmaceuticals Pvt. Ltd.
11	Hydroxypropyl methyl cellulose	Taian Ruitai Cellulose Co. Ltd.	Quest Pharmaceuticals Pvt. Ltd.
12	Brilliant blue lake	Roha Dyechem Pvt. Ltd.	Quest Pharmaceuticals Pvt. Ltd.
13	Tartrazine lake	Roha dyechem Pvt. Ltd.	Quest Pharmaceuticals Pvt. Ltd.
14	Purified talc	Nitika Pharma Specialities Pvt. Ltd.	Quest Pharmaceuticals Pvt. Ltd.
15	Titanium dioxide	G. B. Nitrochem Pvt. Ltd.	Quest Pharmaceuticals Pvt. Ltd.
16	Benzyl alcohol	Sabari Chemicals	Quest Pharmaceuticals Pvt. Ltd.
17	Polyethylene glycol (PEG) 6000	India Glycol Ltd.	Quest Pharmaceuticals Pvt. Ltd.

Table 2. List of instruments used

S.N.	Instruments	Company/Brand	Model
1	Chromatography (UFLC XR)	Shimadzu	LC-20ADXR
2	Pump	Shimadzu	Not applicable
3	Detector	Shimadzu	Not applicable
4	Column	Chromanik technologies	Not available
5	Injection	Shimadzu	Not applicable
6	Column oven	PCI analytics	HCO-02
7	P^H meter	Eutech instruments	PC510
8	Centrifuge machine	Remi	R8C
9	Ultrasonic bath	PCI analytics	20L400H/DTC
10	Water bath	Equiron	6806 DI
11	Refrigerator	Whirlpool	WRDR-161J20
12	UV -Visible spectrophotometer	Shimadzu	1700
13	Analytical balance	Denver	TB2150

Table 3. First Formulation (F1)

S.N.	Materials	Quantity/ tab (mg)	Quantity (%)	Quantity required (g)	Wt. taken (g)
1	Amlodipine Besylate	6.935	3.468	13.870	13.870
2	Hydrochlorothiazide	12.500	6.250	25.000	25.000
3	Losartan Potassium	50.000	25.000	100.000	100.000
4	Microcrystalline cellulose pH 101	65.000	32.500	130.000	140.000
5	Sodium starch glycollate	3.500	1.750	7.000	10.130
6	Lactose monohydrate	55.065	27.533	110.130	99.000
7	PVP K30	3.000	1.500	6.000	8.000
8	Magnesium stearate	2.500	1.250	5.000	2.000
9	Aerosil	1.500	0.750	3.000	2.000
10	Isopropyl alcohol	40.000	20.000	80.000	80.000
	Total	200.000	100.000	400.000	400.000

Table 4. Second Formulation (F2)

S.N.	Materials	Quantity/tab (mg)	Quantity (%)	Quantity required (g)	Wt. taken (g)
1	Amlodipine Besylate	6.935	3.468	13.870	13.870
2	Hydrochlorothiazide	12.500	6.250	25.000	25.000
3	Losartan Potassium	50.000	25.000	100.000	100.000
4	Microcrystalline cellulose pH 101	68.000	34.000	136.000	140.000
5	Sodium starch glycollate	4.200	2.100	8.400	10.130
6	Lactose monohydrate	53.365	26.683	106.730	99.000
7	PVP K30	3.000	1.500	6.000	8.000
8	Magnesium stearate	1.000	0.500	2.000	2.000
9	Aerosil	1.000	0.500	2.000	2.000
10	Isopropyl alcohol	40.000	20.000	80.000	80.000
	Total	200.000	100.000	400.000	400.000

Table 5. Third Formulation (F3) (Final and Excellent formulation)

S.N.	Materials	Quantity/ tab (mg)	Quantity (%)	Quantity required (g)	Wt. taken (g)
1	Amlodipine Besylate	6.935	3.468	13.870	13.870
2	Hydrochlorothiazide	12.500	6.250	25.000	25.000
3	Losartan Potassium	50.000	25.000	100.000	100.000
4	Microcrystalline cellulose pH 101	70.000	35.000	140.000	140.000
5	Sodium starch glycollate	5.065	2.533	10.130	10.130
6	Lactose monohydrate	49.500	24.750	99.000	99.000
7	PVP K30	4.000	2.000	8.000	8.000
8	Magnesium stearate	1.000	0.500	2.000	2.000
9	Aerosil	1.000	0.500	2.000	2.000
10	Isopropyl alcohol	40.000	20.000	80.000	80.000
	Total	200.000	100.000	400.000	400.000

Table 6. Formulation of coating

S.N.	Materials	Quantity/tab (mg)	Quantity (%)	Quantity required (g)	Wt. taken (g)
1	HPMC	4.500	46.154	31.500	31.500
2	Brilliant blue lake	0.020	0.205	0.140	0.140
3	Tartrazine lake	0.050	0.513	0.350	0.350
4	Purified talc	2.700	27.692	18.900	18.900
5	Titanium dioxide	1.800	18.462	12.600	12.600
6	Benzyl alcohol	0.450	4.615	3.150	3.150
7	PEG 6000	0.230	2.359	1.610	1.610
8	Water	45.000	461.538	315.000	315.000
	Total	**9.750**	**100.000**	**68.250**	**68.250**

Table 7. Evaluation Parameters of Uncoated Tablets

S.N.	Evaluation Parameters	Formulation (F1)	Formulation (F2)	Formulation (F3)
1	Average Wt./tab. (mg)	199.5	200.1	199.8
2	Weight variation (%)	Max. 3.66, Min. 2.33	Max. 2.56, Min. 2.25	Max. 2.91, Min. 2.25
3	Friability (%)	0.1	0.08	0.04
4	Disintegration (minutes)	11.12	9.54	5.23
5	Hardness (N)	118.49 (110.5-128.32)	103.24 (100-109.54)	83.23 (78.03-89.76)
6	Thickness (mm)	3.85 to 3.85	3.65 to 3.85	386 to3.95

Acceptance criteria:

- Average wt. variation: ±7.5%
- Friability: Not more than 1.0%
- Disintegration time: 15 minutes
- Hardness: Not less than 40N

Table 8. Evaluation Parameters of Coated Tablets

S.N.	Evaluation Parameters	Formulation (F1)	Formulation (F2)	Formulation (F3)
1	Description:	Round and Biconvex in shape and light green in colour	Round and Biconvex in shape and light green in colour	Round and Biconvex in shape and light green in colour
2	Average Wt./tab. (mg)	210.25	210.85	209.98
3	Weight variation (%)	Max. 3.26, Min. 2.26	Max. 2.66, Min. 2.15	Max. 3.29, Min. 3.23
4	Disintegration (minutes)	13.25	12.5	8.3
5	Hardness (N)	119.49 (111.2-131.40)	105.34 (102-110.23)	85.43 (80.01-91.43)
6	Thickness (mm)	3.94 to 4.08	3.85 to 3.99	3.92 to 4.01
7	**Assay:**			
	Amlodipine besylate	99.52	99.12	99.89
	Hydrochlorothiazide	98.22	99.45	100.20
	Losartan potassium	99.38	99.93	99.97
8	Uniformity of content:			
	Amlodipine besylate	97.5-99.5	98.56-101.24	99.85-100.65
	Hydrochlorothiazide	98.76-100.05	97.45-99.50	99.60-100.96
9	Dissolution:			
	Amlodipine besylate	89.00-98.00	91.00-98.00	98.80-99.70
	Hydrochlorothiazide	87.50-98.60	93.50-98.50	97.85-98.95
	Losartan potassium	83.00-95.00	91.50-97.00	97.98-100.05

Acceptance criteria:

- Average wt. variation: +7.5%
- Disintegration time: 30 minutes
- Hardness: Not less than 40N
- Assay of Amlodipine: 90-110%
- Assay of Hydrochlorothiazide: 92.5-107.5%
- Assay of Losartan potassium: 90-110%
- UOC of Amlodipine: 85-115%
- UOC of Hydrochlorothiazide: 85-115%

Table 9. The capacity, separation and resolution factors of amlodipine besylate, hydrochlorothiazide and losartan potassium in tablets

Sl. No.	Compounds	k	α	R_s	RSD	CC	CL
1.	Amlodipine besylate						
	Standard	0.30	11.80 (peaks 1 & 2)	11.19 (peaks 1 & 2)	0.433	0.9995	99.41±0.53
	Tablet	0.30	11.79 (peaks 1 & 2)	11.18 (peaks 1 & 2)	0.661	0.9995	100.24±0.51
2.	Hydrochloro-thiazide						
	Standard	3.54	1.60 (peaks 2 & 3)	3.67(peaks 2 & 3)	0.387	1.0000	100.03±0.48
	Tablet	3.53	1.60 (peaks 2 & 3)	3.66(peaks 2 & 3)	0.43	0.9997	99.90±0.33
3.	Losartan potassium						
	Standard	5.63	1.60 (peaks 2 & 3)	3.67(peaks 2 & 3)	0.575	0.9993	100.87±0.72
	Tablet	5.62	1.60 (peaks 2 & 3)	3.66 (peaks 2 & 3)	0.48	0.9999	99.04±0.37

Experimental Conditions:

Columns: Sushell C_8 (150 x 4.6mm, 2.6 µm) column of Chromanik Japan.
Mobile Phase: Phosphate buffer (pH 2.5)-acetonitrile (70:30, v/v).
Buffer solution was prepared containing 0.15% sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H2O$) and 0.4% Triethylamine (TEA) with pH adjusted to 2.5 with 85% phosphoric acid.
Flow Rate: 1.0 mL/min.
Detection: UV at 230 nm.
Temperature: 45±1ºC
n = 5
SD: Standard deviation of Rs.
CC: Correlation coefficient, CL: Confidence level (%)

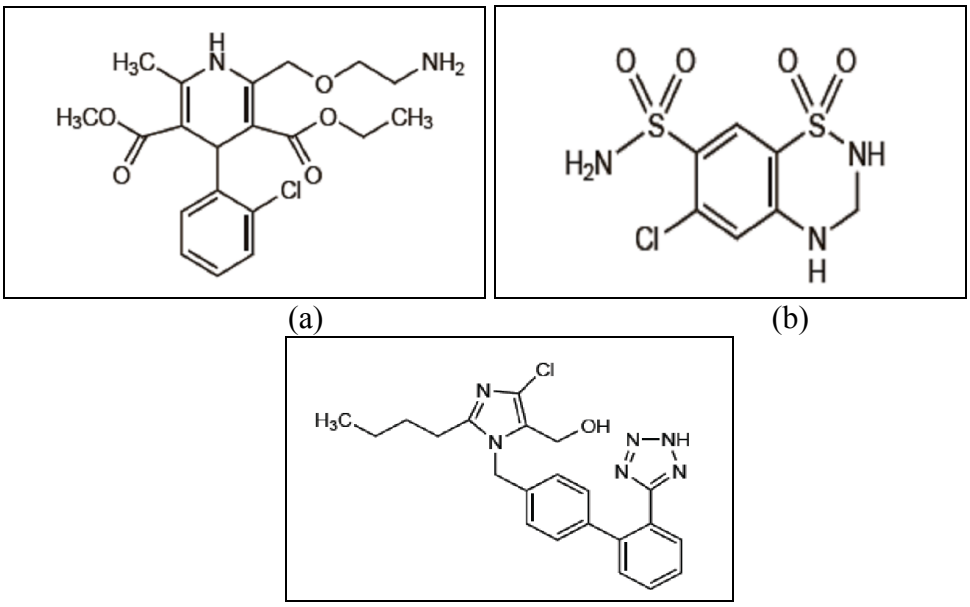

Figure 1. Chemical structures of (a): Amlodipine besylate, (b): Hydrochlorothiazide and (c): Losartan potassium

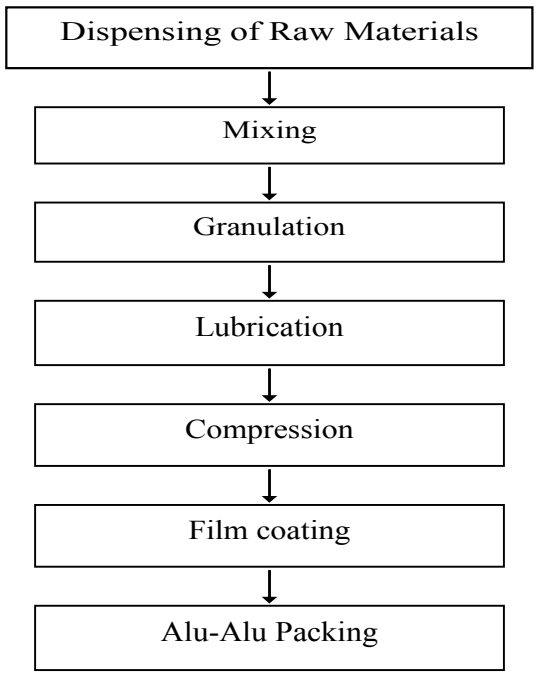

Figure 2. Process flow chart

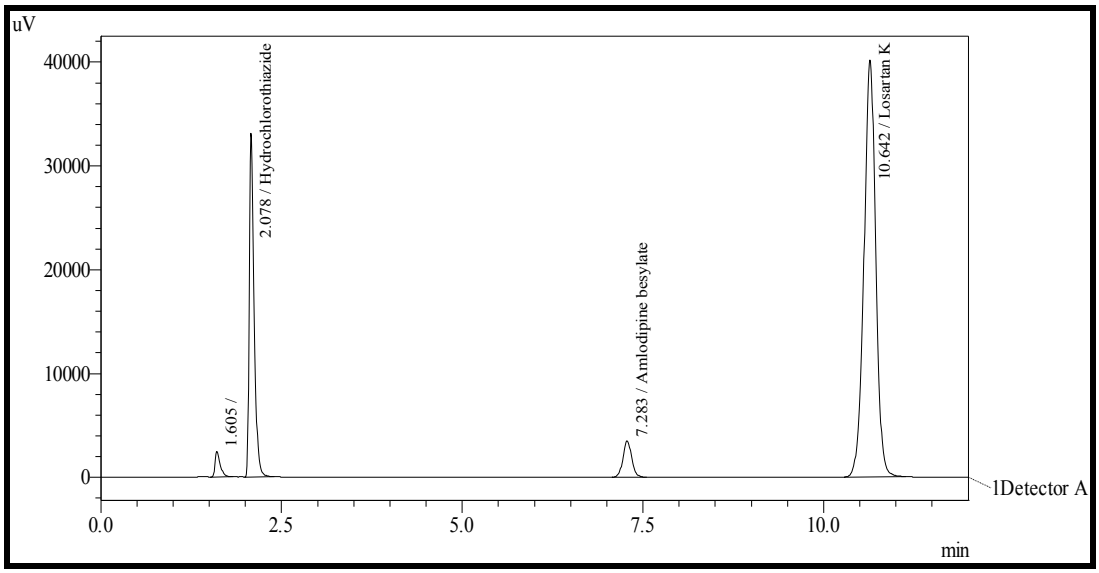

Figure 3. The chromatograms of hydrochlorothiazide, amlodipine besylate and losartan potassium in standard solutions

Experimental Conditions:

Columns: Sushell C_8 (150 x 4.6mm, 2.6 µm) column of Chromanik Japan.
Mobile Phase: Phosphate buffer (pH 2.5)-acetonitrile (70:30, v/v).
Buffer solution was prepared containing 0.15% sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H2O$) and 0.4% Triethylamine (TEA) with pH adjusted to 2.5 with 85% phosphoric acid.
Flow Rate: 1.0 mL/min.
Detection: UV at 230 nm.
Temperature: 45±1ºC
n = 5

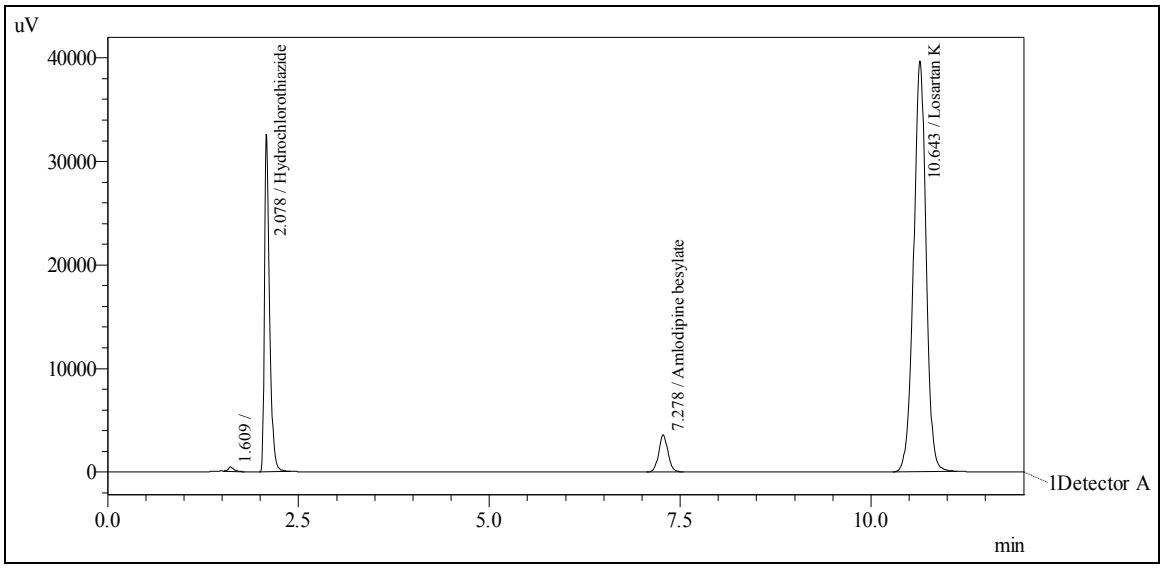

Figure 4. The chromatograms of hydrochlorothiazide, amlodipine besylate and losartan potassium of test solution in newly formulated tablets

Experimental Conditions:

Columns: Sushell C_8 (150 x 4.6mm, 2.6 µm) column of Chromanik Japan.

Mobile Phase: Phosphate buffer (pH 2.5)-acetonitrile (70:30, v/v).

Buffer solution was prepared containing 0.15% sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H2O$) and 0.4% Triethylamine (TEA) with pH adjusted to 2.5 with 85% phosphoric acid.

Flow Rate: 1.0 mL/min.

Detection: UV at 230 nm.

Temperature: 45±1ºC

n = 5