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BIO ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF LERCANIDIPINE AND ATENOLOL IN HUMAN PLASMA BY USING RP-HPLC

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ABSTRACT

A simple, sensitive, precise and accurate high performance liquid chromatographic (HPLC) method was developed and validated for the estimation of Lercanidipine and Atenolol in human plasma. The chromatographic separation was achieved with the reverse phase column Phenomenox Gemini C18 (150mmX4.6, 5µ) and the mobile phase consisted of acetonitrile and 10mM ammonium acetate buffer (80:20 v/v) at ph-3.2(adjusted with acetic acid) at a flow rate of 0.2mL/min. Haloperidol was used as an internal standard. The wavelength used for the detection of Lercanidipine and Atenolol was 275nm with total run time of 10 minutes. The retention times of Lercanidipine, Atenolol and Haloperidol was found to be 6.6, 3.5 and 4.3 minutes respectively. The calibration curve was linear (r^2> or =0.99) ranging from 5 to 2000ng/mL for Lercanidipine and 10 to 3000ng/mL for Atenolol. The lower limit of quantification was 15ng/mL for Lercanidipine and 30ng/mL for Atenolol. Interday precision were lower than 5% (CV) and accuracy ranged from 98 to 102 % for Lercanidipine and 96 to 102% for Atenolol in terms of percent accuracy. Mean extraction recovery was found to be 75 to 80% for both Lercanidipine and Atenolol respectively. The method was successfully developed and validated in human plasma for excellent selectivity, accuracy, precision, recovery and stability in compliance to international regulatory guidelines.

KEYWORDS: HPLC, Lercanidipine, Atenolol, internal standard, human plasma, validation.

INTRODUCTION

Lercanidipine (LER), (+-)-2-[(3,3-diphenylpropyl) methyl amino]-1, 1-dimethylethyl methyl 1,4- dihydro-2, 6-dimethyl-4-(m-nitrophenyl)-3, 5-pyridinedicarboxylate, is a third generation lipophilic and vaso-selective dihydropyridine calcium channel blocker by selectively inhibiting the transmembrane influx of calcium ions into vascular smooth muscle through L-type calcium channels. Because of its voltage –dependent calcium antagonist activity, LER can lower the incidence of peripheral edema and reduce the cardiovascular morbidity and mortality in hypertensive patients. In addition, once-daily LER represents a good tolerability and a useful therapeutic option in the management of patients with mild-to-moderate hypertension. Overall LER has a comparable antihypertensive efficacy but with significantly less vasodilatory induced side-effects compared to the first- and second-genaration DHPs, such as nifedipine, nicardipine, amlodipine and fleodipine.

Atenolol is a b-1 selective (cardioselective) b adrenergic receptor blocking agent. it does not have membrane stabilizing and intrinsic sympathomimetic (partial agonist) activities. Atenolol may be chemically described as a benzene acetamide, 4-[21-hydroxy-31-[(1-methyl ethyl)amino]propoxy]. Atenolol is a beta-adrenoceptor blocking agent which acts preferentially on beta-receptors in the heart. It has little intrinsic sympathomimetic activity and no membrane stabilizing activity. It reduces raised blood pressure by an unknown mechanism and also inhibits exercise induced tachycardia and decreases plasma rennin concentration. It causes slight airways obstruction but less than that seen with non-selective beta-blockers. The inhibition of exercise induced tachycardia is correlated with blood levels but there is no correlation between plasma concentrations and antihypertensive effect. The possible mechanism of the anti-anginal activity of atenolol appears to be due to a reduction in left ventricular work and oxygen utilization resulting(mainly) from the decrease in heart rate and contractility. The antiarrhythmic effect of atenolol is apparently due to its anti-sympathetic effect. There is no evidence that membrane stabilizing activity or intrinsic sympathomimetic activity are necessary for antiarrhythmic efficacy. By its anti-symapathetic effect, atenolol depresses sinus node function, atrioventricular node function and prolongs atrial refractory periods. It has no direct effect on eletrophysiological properties of the His-Purkinje system. selectivity decreases with increasing doses.

Literature survey has revealed that there is no method were reported for simultaneous estimation of Lercanidipine and Atenolol in plasma by liquid chromatography. Methods

reported for simultaneous estimation of Lercanidipine and Atenolol in combined dosage forms and bulk pharmaceutical formulations include a method development and validation of stability indicating RP-HPLC method for simultaneous estimation of Atenolol and Lercanidipine in bulk and its pharmaceutical formulations by using mobile phase consists of acetonitrile:methanol:0.02M potassium dihyrogen phosphate buffer(50:10:40),V/V,pH5.0) at a flow rate of 1mL/min with uv detection of 226nm.C18 column used as stationary phase. retention time of atenolol and lercanidipine were 2.0min and 3.5min respectively. In another literature method have reported development and validation of a RP-HPLC method for simultaneous estimation of Atenolol and Lercanidipine hydrochloride in pharmaceutical dosage forms. chromatographic separation achieved isocratically on Luna C18 column (5µm, 150mmX4.60mm) and acetonitrile :phosphate buffer(60:40 V/V,pH-3.6)as mobile phase, at a flow rate of 0.5mL/min.detection was carried out at 235nmn.retention times of Lercanidipine and Atenolol was found to be 5.97 min and 2.27 min respectively. Another method reveals that simultaneous spectrophotometric estimation of atenolol and lercanidipine hydrochloride in combined dosage form by ratio derivative and dual wavelength method.

From the literature survey, various analytical method developments have been reported for estimation of Lercanidipine and Atenolol in aqueous by RP-HPLC and UV-methods. Here an attempt was made to develop the bio-analytical method for estimation of Lercanidipine and Atenolol from human plasma using Haloperidol as internal standard and to validate as per international regulatory guidelines.

MATERIALS AND METHODS

Chemicals and reagents used: Acetonitrile of HPLC grade were purchased from sigma-aldrich chemicals pvt.ltd, India. Methanol of hplc grade were purchased from himedia laboratories pvt.ltd. The reference standard of lercanidipine, atenolol and haloperidol (Internal standard) were purchased from Sigma Aldrich India. Acetic acid, hplc were purchased from himedia laboratories pvt.ltd.Tert-butyl methyl ether. HPLCgrade were obtained from lobachemiepvt.ltd, India. Ammonium acetate were purchased from thermo fisher scientific india, pvt.ltd, India. Blank human plasma were obtained from blood bank services PSG Hospitals, Coimbatore, Ultra pure water (0.45µm) was obtained from a mille Q water purification system from Millipore (Millford, USA),(table-1).

Instrumentation and Chromatographic Conditions

The Instrument selected for this method was HPLC waters system which consists of Waters pump control module II, Waters 515 solvent delivery system (pump), Rheodyne injector (20µ loop), Waters 2489 UV-Visible detector, Empower-2 software from Waters Corporation as data processor. Analytical column was Phenomenexgemini C18(150 x 4.60 mm),5µ by using the mobile phase consists of 80% Acetonitrile and 20% of 10mM ammonium acetate having pH 3.2 (adjusted by adding acetic acid). The mobile phase only used after the mobile phase was filtered through a 0.45 µm membrane filter and degassed by ultra sonicator. Apart from this Sartorius single pan digital balance(BSA 224S-CW) was used for weighing of the samples. Eppendorf Centrifuge, Thermo scientific micropipettes of 200µL and 1000µL were used, tarson 7596 vortex mixer used for the mixing purpose of samples, Nitrogen evaporator used for evaporating the samples before injections. Ultra sonic cleaner used for the process of proper dissolving as well as mixing. Shimadzu 1600 pc UV spectrum used for the analysis of λmax of the selected drugs.

Preparation of Standard Stock Solution of lercanidipine

Weighed about 10.40 mg of lercanidipine reference standard and transfer in to 10 mL volumetric flask and kept for sonication for 5 minutes. Make up the volume with methanol. The concentration was approximately 1000 µg/mL.

Preparation of standard stock solution of atenolol

Weighed about 10.40 mg of atenolol reference standard and transferred in to 10 mL volumetric flask and sonicated for 5 minutes. Make up the volume with methanol. The concentration of solution approximately was 1000 µg/mL.

Preparation of Mixture of working standard of lercanidipine and atenolol

Transfer the 0.400 µL of LER and 0.400 µL of ATN solutions in to 2mL eppendorf tube then add 0.200 mL of 50% methanol in water**.** From the stock mixture, stock calibration curve were prepared range from 5-2000 ng/mL for LER and 10-3000 ng/mL for ATN.

Preparation of internal standard solution

Internal standard stock solution of haloperidol (1mg/mL) was prepared using methanol. From this solution, working internal standard solution was prepared which contain 10 µg/mL of internal standard using 90% acetonitrile.

Extraction Procedure

Transfer 225 µL of blank plasma, add 25µL of drug mixture (LER+ATN) from each level separately, and vortex for 1 min, then add 20 µL of internal standard solution (haloperidol10 µg/mL) to a polypropylene vial vortex for 1 min, and then add 1.5ml of tert-butyl methyl ether as extracting solvent vortex for 3 minutes and kept for centrifuged at 10,000 rpm for 3 minutes (at 4^0 C). Separate the organic layer. Separated organic layer transferred in to clean test tubes and kept for evaporation at 40 ^{0}C in nitrogen evaporator for 15 min and reconstitute with 250µL mobile phase.

Bioanalytical Method Validation

Preparation of calibration curve: The linearity of the method was evaluated by a calibration curve in the range of 5-2000ng/mL for Lercanidipine and 10-3000ng/mL for Atenolol, including lower limit of quantitation(LLOQ). The calibration curve was achieved by plotting the peak area ratios of Lercanidipine, Atenolol and internal standard versus the concentration of Lercanidipine and Atenolol by least-squares linear regression analysis. The calibration curve requires a correlation coefficient (R^2) of >0.99. the acceptance criteria for each back-calculated standard concentration should be within 15% of nominal concentration,except it should not exceed 20% for the LLOQ.

System suitability

System suitability solution (equivalent to middle concentration) was prepared and inject 6 replicates in to the chromatographic system before each batch. Then the coefficient of variation for peak area response and retention time for the peaks of the test substance and internal standard was determined separately. The coefficient of variation of peak area responses and retention times for the peaks of test substances and the internal standard from 6 replicate injections of system suitability should not be more than 5.00% and 2.00% respectively.

Carry over

Blank Plasma screening during validation, blank plasma samples from 4 different lots were processed according to the extraction procedure and evaluate the interference at the retention times of analytes and internal standard. The 3 free interference lots were selected from the 4 lots.

Selectivity and specificity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Evidence should be provided that the substance quantified is the intended analyte. Analyses of blank samples of the appropriate biological matrix (plasma, urine, or other matrix) should be obtained from at least six sources. Each blank sample should be tested for interference, and selectivity should be ensured at the lower limit of quantification (LLOQ). Potential interfering substances in a biological matrix include endogenous matrix components; metabolites; decomposition products; and, in the actual study, concomitant medication and other xenobiotics. If the method is intended to quantify more than one analyte, each analyte should be tested to ensure that there is no interference. The selectivity was evaluated by using chromatograms of 6 blank plasma samples obtained from 6 different sources were compared with the chromatograms obtained from standard solutions.

Sensitivity

Blank plasma and lowest level of calibration curve was processed in triplicate. The mean noise (h) for the blank plasma samples in terms of height to be determined the mean signal (H) for the peaks of samples for the lowest calibration level in terms of height need to be determined. Then the signal to noise ratio for the peaks for the drugs should not be more than the acceptance criteria.

Calibration Curve

A calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. The relationship between response and concentration should be continuous and reproducible. A calibration curve should be generated for each analyte in the sample. The calibration standards can contain more than one analyte. A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte.

A Seven–point calibration curve for Lercanidipine and Atenolol was constructed by plotting the peak area ratio of the Lercanidipine: IS and Atenolol :IS against the nominal concentration of calibration standards in blank human plasma. Following the evaluation of different weighing factors, the result were fitted to linear regression analysis with the use of a $1/x^2$ (x= concentration), Weighting factor. The calibration curve should have the correlation coefficient (r^2=0.99) or better. The acceptance criteria for each back calculated standard

concentrations were ± 15 % deviation from the nominal value except at LLOQ which was set at ± 20%.

The standard solution and sample solution were injected with the above chromatographic conditions, and the chromatograms were recorded. The retention time of Lercanidipine and Atenolol were 6.67 and 3.52 min, respectively. The response factor (peak area ratio of drug peak area and the internal standard peak area) of the standard solution and the sample were calculated and the concentration of the Lercanidipine and Atenolol present in the plasma samples was calculated.

Accuracy and precision

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the actual value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected study sample concentrations is recommended. The mean value should be within 15% of the nominal value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the nominal value serves as the measure of accuracy.

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected study sample concentrations is recommended. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV.

Precision is further subdivided into within-run and between run precision. Within-run precision (intra-batch precision or within-run repeatability) is an assessment of precision during a single analytical run. Between-run precision (inter-batch precision or between-run repeatability) is an assessment of precision over time and may involve different analysts, equipment, reagents, and laboratories. Sample concentrations above the upper limit of the standard curve should be diluted. The accuracy and precision of these diluted samples should be demonstrated in the method validation. The recovery of an analyte in an assay is the

detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the analyte in solvent.

RESULTS

The **accuracy** of the optimized methods was determined by relative and absolute recovery experiments. Accuracy was determined by replicate analysis of samples containing known amounts of the Analyte. A minimum of three concentrations in the range of expected study sample concentrations was recommended. The mean value should be within 15% of the nominal value. The coefficient of variation (%) of these values was less than 5 %. The accuracy values ranged for Lercanidipine was 98.37 % to 101.71% and for Atenolol was 95.77% to 101.66 %. It is therefore, derived that the developed methods are accurate and reliable.

The optimized methods for the estimation of the drugs were found to be **precise**. This was evident from the coefficient of variation values, which were less than 10 % at all concentrations (Table 2&3).

The six blank plasma samples obtained from six different volunteers were analyzed and the chromatograms were recorded and compared with the chromatograms obtained from standard solutions. Endogenous interferences were not detected at the retention time of selected drugs and internal standard.These observations show that the developed assay method is **specific and selective**.

It was observed that the optimized methods were **linear** within a specific concentration range for individual drugs. The calibration curves were plotted between response factor and concentration of the standard solutions.The linearity range for Lercanidipine was found to be 5,15, 150, 300, 600, 900, 1500 and 2000 ng/mlrespectively and their slope (k) and the intercept values (B) were 9.18 and -1.38 with regression coefficient 0.998 respectively. The linearity range for Atenolol was found to be 10, 30, 90, 250, 500, 1000, 1500, 2000, 2500 and 3000 ng/mlrespectively and their slope (k) and the intercept values (B) were 3.18 and -1.45 respectively with the regression coefficient 0.999(The goodness of fit (Correlation coefficient 'r^2') was consistently greater than 0.99 during the course of validation for Lercanidipine and Atenolol.

The accuracy (% nominal) of the mean back calculated concentrations of calibration curve levels ranged from 98.14 % to 101.63 % for Lercanidipine and 99.11 to 102.85 % for atenolol.

The **stability** of the drug spiked human plasma samples at three levels were studied for three freeze thaw cycles. The mean concentrations of the stability samples were compared to the theoretical concentrations. Similarly, short term (3 h), post preparative stability, freeze thaw stability and standard solution stability were evaluated. The stability of the internal standards was also performed. The results showed that the selected drugs were stable in plasma for about one month when stored at frozen state (Table 4&5).

The **limit of detection** (LOD) value was found to be 5.0 ng/ml for Lercanidipine and 10ng/ml for Atenolol. The lower limit of quantity (LLOQ) was defined as the Lowest concentration the calibration standard with lowest concentration (signal to noise ratio should be less than (10 : 1) that can be calculated with an acceptable precision and accuracy. **limit of quantification** (LOQ) value was 15.0 ng/ml for lercanidipine and 30ng/ml for Atenolol. This observation showed that the developed methods have adequate sensitivity. These values, however, may be affected by the separation conditions (e.g., column, reagents, and instrumentation and data systems), instrumental changes (e.g., pumping systems and detectors) and use of non HPLC grade solvents and may result in changes in signal to noise ratios.

The **ruggedness and robustness**of the methods were studied by changing the experimental conditions. No significant changes in the chromatographic parameters were observed when changing the experimental conditions (operators, instruments, source of reagents and column of similar type) and optimized conditions (pH, mobile phase ratio and flow rate).

There was No **Carry over** effect caused by injecting the highest concentration of the standard solution of the drugs just before the plasma.

By using the liquid -liquid extraction method it gave adequate **recovery** and clean samples. The recovery was evaluated at three different levels of analyte low, medium and high quality control standards area were compared against the mean area of respective un-extracted quality control standards area. The mean recovery for Lercanidipine was founded as (Low-

77.55% Medium –78.48 % High- 76.91 %) and for Atenolol(Low-74.68% Medium- 78.43% High-76.36%) (Table 5 & 6).

System suitability Solution (equivalent to middle concentration) was prepared and injected six replicates in to the chromatographic system before each batch. Then the coefficient of variation for peak area response and retention time for the peaks of the test substance and internal standard determined separately. The coefficient of variation of peak area responses and retention times for the peaks of test substances and the internal standard from the six replicate injections of system suitability should not more than 5.00 % and 2.00 % respectively. The results obtained are within the acceptance criterion as mentioned above. System suitability parameters such as column efficiency (theoretical plates), resolution factor and peak asymmetry factor of the optimized methods were found satisfactory (Table 5).

In conclusion, the developed method for the estimation of Lercanidipine and atenolol in plasma is accurate, precise, selective and linear and is therefore, can be employed for a comparative bioavailability study to evaluate its applicability.

Table. 2: intra – and inter-day precision and accuracy of LER in human plasma in vitro.

intra – and inter-day precision and accuracy of LER in human plasma in vitro						
Nominal concentration (ng/mL)	Intra-day(n=6)			Inter-day(n=6)		
	Measured concentration (mean±sd)	Cv (%)	Accuracy (%)	Measured concentration (mean±sd)	Cv (%)	Accuracy (%)
LER						
15.97	15.92±0.13	0.92	99.69	15.94±0.10	0.95	98.96
684.97	684.49±6.03	0.88	99.83	683.59±6.01	0.89	99.59
1585.58	1588.05±16.68	1.05	100.16	1579±16.56	1.12	99.89

Table. 3: intra – and inter-day precision and accuracy of ATN in human plasma in vitro.

intra – and inter-day precision and accuracy of ATN in human plasma in vitro						
Nominal concentration (ng/mL)	Intra-day(n=6)			Inter-day(n=6)		
	Measured concentration (mean±sd)	Cv (%)	Accuracy (%)	Measured concentration (mean±sd)	Cv (%)	Accuracy (%)
ATN						
31.93	31.8±1.47	4.63	99.59	30.93±1.23	4.84	99.23
1095.96	1091.68±10.60	0.97	99.61	1085.67±10.15	1.12	99.12
2536.83	2532.28±17.63	0.7	99.82	2526.38±17.34	0.89	99.24

Table 4: stability of LER in human plasma in vitro under different conditions.

Nominal concentration (ng/mL)	Short term stability		Post preparative stability		Freeze thaw stability	
	Measured concentration (mean±SD,ng/mL)	% CV	Measured concentration (mean±SD,ng/mL)	% CV	Measured concentration (mean±SD,ng/mL)	% CV
LER						
15.97	16.08±0.56	3.54	15.72±0.50	3.2	15.72±0.59	3.8
1585.58	1559.07±32.70	2.1	1578.46±29.87	1.89	1550.17±51.66	3.33

Table. 5: stability of ATN in human plasma in vitro under different conditions.

Nominal concentration (ng/mL)	Short term stability		Post preparative term stability		Freeze thaw stability	
	Measured concentration (mean±SD,ng/mL)	%CV	Measured concentration (mean±SD,ng/mL)	%CV	Measured concentration (mean±SD, ng/ mL)	%CV
ATN						
31.93	31.27±0.76	2.45	31.74±1.09	3.47	31.36±1.04	3.33
2536.93	2513.84±32.44	1.47	2516.35±60.97	2.42	2523±32.44	1.29

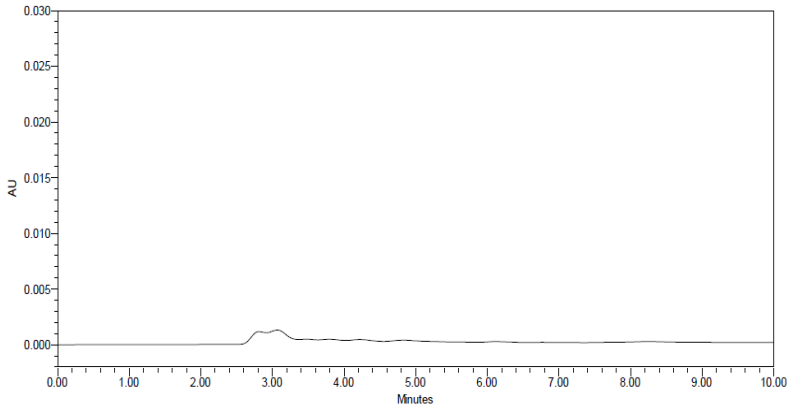

Fig. no. 1: Representative chromatogram of Blank Plasma.

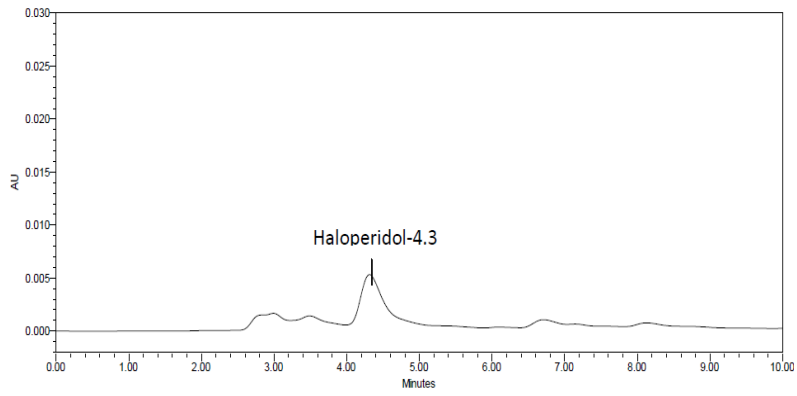

Fig. no. 2: Representative chromatogram of Blank Plasma with IS.

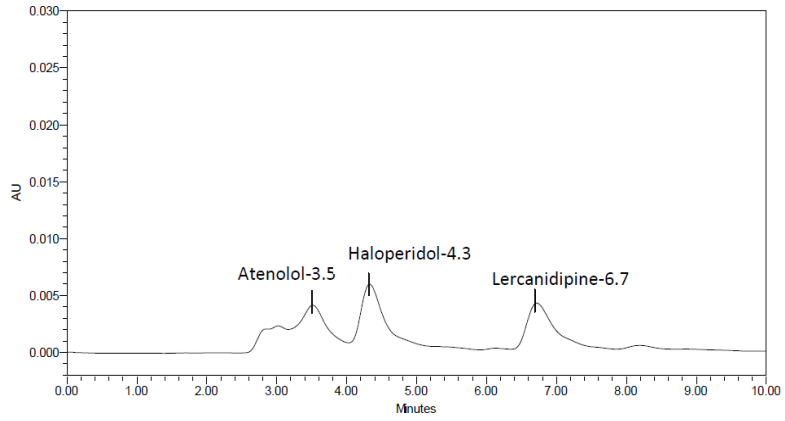

Fig. no. 3: Representative chromatogram of LQC(Lower Quality Control).

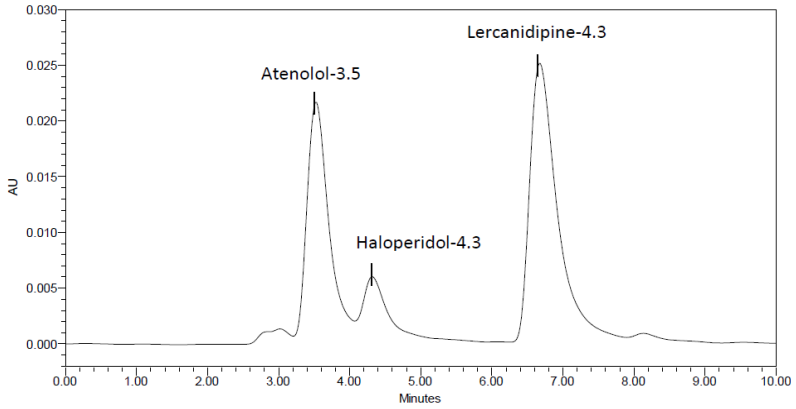

Fig. no. 4: Representative chromatogram of HQC(High Quality Control).

DISCUSSION

A simple, novel, Precise, economical RP-HPLC method was developed for the simultaneous estimation of lercanidipine and atenolol in human plasma. Different mobile phases were tried for selecting the ideal mobile phase. Among the various mobile phases Acetonitrile: 10mM ammonium acetate (ph-3.2 adjusted with acetic acid) with the ratio of 80:20 was found to be ideal as mobile phase, Since it gave good resolution and peak shapes with perfect symmetry. The flow rate was found to be optimized at 0.2 mL/min Detection was carried out by uv detector at 275 nm. Quantification was done by internal standard calibration method within 10 minutes. The linearity and range for lercanidipine was 5-2000 ng/mL and for atenolol was from 10-3000 ng/mL. The correlation coefficient for lercanidipine and atenolol were 0.999 and 0.998 respectively which indicates a perfect correlation. The developed method was validated for accuracy, precision, system suitability. The Percentage recovery of the drugs were 75-79 %. The good percentage recovery of the sample clearly indicates the

reproducibility and accuracy of the developed method. Similarly the RSD value for precision was also found to be within the acceptable limit and more important the estimation was performed with in short time.

CONCLUSION

In conclusion a validated RP-HPLC bioanalytical method has been developed for simultaneous estimation of Lercanidipine and Atenolol in human plasma. The results shown that the method was found to be specific, simple, accurate, precise and sensitive. The developed method can be applied for the studies of Pharmacokinetic parameters of Antihypertensive drugs LER and ATN and its metabolites in human plasma. It can also applied in therapeutic drug monitoring Practices and Clinical toxicological assays.

No analytical procedures have been proposed for simultaneous estimation of Lercanidipine and Atenolol in human plasma, so attempt was taken to develop and validate a reversed-phase high performance liquid chromatographic method for simultaneous estimation of Lercanidipine and Atenolol in human plasma with lower solvent consumption along with the short analytical run time that leads to an environmentally friendly chromatographic procedure and will allow the analysis of a large number of samples in a short period of time.

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