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Sensitive High Performance Thin Layer Chromatographic Determination of Lercanidipine Hydrochloride in Pharmaceuticals and in Blood Plasma

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A HPTLC method was developed and validated for determination of Lercanidipine hydrochloride in tablet formulation and in biological sample. The chromatography was performed on pre-coated silica gel 60 F_{254} plates using methanol-toluene 2.5:7.5 (v/*v*) as mobile phase. The optimized chromatographic conditions gave good peak shape with acceptable *R*f value of 0.70 for Lercanidipine. Method was validated according to the ICH guidelines. The calibration plots were linear between 100-600 ng/ band and between 50-500 ng/ band in pure and in biological sample, respectively. Accuracy of the proposed method was evaluated by recovery studies (% recovery= 100.22 % and 99.99 % from tablet sample and biological sample, respectively). The method was also validated for precision, ruggedness, LOD & LOQ and robustness as per ICH guidelines. In stability testing, Lercanidipine was found susceptible to acid hydrolysis, alkali hydrolysis and oxidation (3 % H_2O_2). The method was able to selectively quantitate Lercanidipine hydrochloride in presence of degradation products and components likely to be present in biological matrix.

Keywords: lercanidipine hydrochloride, high performance thin layer chromatography, validation, pharmaceutical formulation, blood plasma

INTRODUCTION

Lercanidipine hydrochloride (Figure 1), chemically is 2-[(3,3diphenylpropyl) (methyl) amino]-1, 1-dimethylethyl methyl- 2, 6-dimethyl-4-(3-nitrophenyl)-1, 4 dihydropyridine-3, 5-dicarboxylate hydrochloride [1]. It is a calcium channel blocker of the dihydropyridine class, used in the treatment of hypertension. It works by relaxing and opening the blood vessels allowing the blood to circulate more freely around the body. Lercanidipine inhibits the reflux of extra cellular calcium

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across the myocardial and vascular smooth muscle cell membrane by deforming the channel, inhibiting ion-control gating mechanisms, and/or interfering with the release of calcium from the sarcoplasmic reticulum.

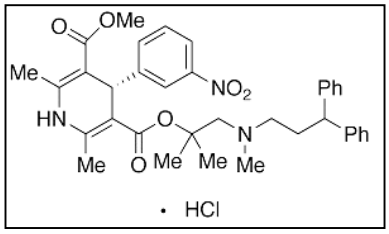

Figure 1. Structure of Lercanidipine hydrochloride

Lercanidipine is not official in any pharmacopoeia. Hence, no official method is available for determination of Lercanidipine hydrochloride. Literature survey revealed few spectrophotometric [2,3], RP-HPLC [4-10] and UPLC-MS [11] methods for determination of Lercanidipine hydrochloride in pharmaceutical formulations and in biological fluids. There is no HPLTC method reported so far for determination of Lercanidipine hydrochloride. In recent time HPTLC has gained importance in pharmaceutical analysis due to its advantages like lower detection limit, no interference from previous analysis- fresh stationery phase and mobile phase for each analysis, low solvent consumption, solvent need no prior treatment like filtration and degassing low cost per sample analysis, less time consuming etc. The present work describes a selective, reliable, economical, rapid and accurate HPTLC method for the determination of lercanidipine hydrochloride in tablets and biological sample. The proposed HPTLC method was validated in accordance with the criteria given by regulatory standards for pharmaceuticals.

MATERIALS AND METHODS

Chemicals and reagents

Analytically pure sample of Lercanidipine hydrochloride was supplied as a gift sample from Glenmark Pharmaceutical Ltd, Mumbai, India and marketed formulation was procured from local market. All chemicals including methanol and toluene were of analytical grade purchased from Merck, Merck Specialties Private Limited. A commercial pharmaceutical preparation, LOTENSYL® 10 (Sun Pharmaceuticals Laboratories Ltd. Ranipool, Sikkim, India) containing labeled amount of 10mg was procured from local market.

Instruments and chromatographic conditions

Chromatographic separation of drugs was performed on pre-coated silica gel 60 F254, (10 cm x 10 cm HPTLC plates purchased from E. Merck, Darmstadt, Germany). The plates were initially prewashed with methanol and dried in oven at for 10 min. Samples were applied on the plate as a band with 6 mm width, by using Camag 100 µl sample syringe (Hamilton, Bonaduz, Switzerland) with a Linomat V sample applicator (Muttenz, Switzerland). A constant application rate of 150 nL s^{-1} was used. The plates were saturated for 15 min in a twin trough glass chamber (for 10 x 10 cm) with the mobile phase of methanol:toluene (2.5:7.5, v/v). The plates were then placed in the mobile phase and ascending development was performed to a distance of 80 mm from the point of application at ambient temperature, and the development time was 12 min. Subsequent to the development, the plates were dried in a current of air with the help of an air drier and a densitometric scanning

was performed at 254 nm using Camag TLC scanner III operated in reflectance-absorbance mode. The scanning speed was 5-100 mm/s. The source of ration used was deuterium lamp, halogen tungsten and mercury vapour emitting continuous UV spectra between 190-800 nm and controlled by WinCATS software (Version 1.4.3.6336).

Preparation of standard stock solution

Standard stock solution of lercanidipine hydrochloride was prepared by dissolving 10.0 mg of drug in 10.0 mL volumetric flask, dissolved and diluted to the mark with methanol to get concentration of 1000 µg/mL.

Selection and optimization of mobile phase

Aliquot portion, 2 µl, of standard stock solution was applied of TLC plate in the form of band. The TLC plate was then chromatographed in different mobile phase systems containing solvents with varying polarity like methanol:ethyl acetate, methanol:chloroform and methanol: toluene were tried to obtain good peak shape with acceptable Rf value (0.2-0.8).

Selection of analytical wavelength

The standard stock solution was appropriately diluted and was applied on the TLC plate using Linomat V automatic sample applicator. After chromatographic development, the band was scanned over the range of 200-700 nm and the spectrum was obtained (Fig. 2). Lercanidipine hydrochloride exhibited significant absorbance at 254 nm and hence it was selected as analytical wavelength.

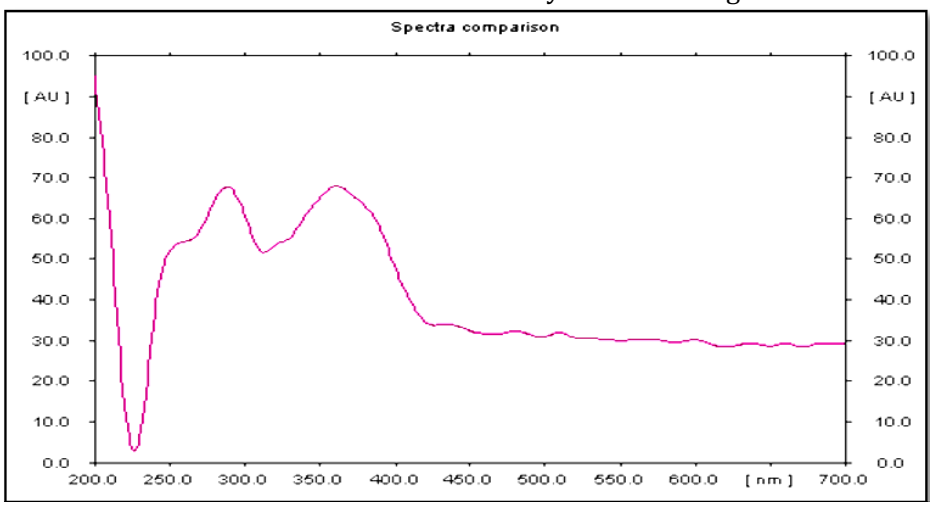

Figure 2. Spectrum of Lercanidipine hydrochloride

Preparation of Tablet solution for Assay

Twenty tablets of Lotensyl were weighed and crushed to obtain fine powder and net content of each tablet was calculated. Tablet powder equivalent to about 10 mg of LER was accurately weighed and transferred to 10.0 mL volumetric flask, 5 mL methanol was added and ultrasonicated for 15 min and volume was made up to the mark with methanol. The solution was mixed and filtered through Whatmann filter paper No. 42, to get a clear solution. The resulting solution was used as sample solution.

Method Validation

Linearity and Range

Working standard solution of Lercanidipine hydrochloride 20, 40, 60, 80, 100 and 120 µg/mL were prepared in methanol and 5µl of each solution was applied and chromatographed under optimum chromatographic conditions. A linear relationship between peak area and concentration was ascertained by making five measurements at six concentration levels over a range of 100-600 ng/band.

Specificity

The ascertain the specificity of the proposed method, blank sample was applied on the TLC plate and chromatographed under optimized chromatographic conditions. The chromatogram was observed for any peak at the Rf value of Lercanidipine hydrochloride.

Limit of detection and limit of quantitation

The limits of detection and quantification of the developed method were calculated using 3a/S and 10a/S phenomena for the limits of detection and quantification, respectively, where a is the standard deviation of the y-intercepts and S is the slope of the calibration curve.

Precision

The system precision was determined by analyzing six replicate injections of standard stock solution. Method precision was ascertained by analyzing six samples of standard stock solutions. The intra-day and inter-day precision was ascertained by analyzing table sample at different time intervals on same day and on three consecutive days, respectively. Precision of method was evaluated by calculating the percent relative standard deviation of peak areas obtained from each spot of sample.

Accuracy

The accuracy of the method was carried out by recovery studies by addition of standard drug solution to pre-analyzed sample solution at three different levels of 80, 100 and 200 %. To the accurately weighed quantity of tablet powder equivalent to 10 mg of Lercanidipine hydrochloride, amount of pure drug 8 mg, 10 mg & 12 mg (80 %, 100 % and 120 % level) was added and analyzed under optimum chromatographic conditions.

Robustness

To evaluate the robustness of the proposed method, small but deliberate variations in the optimized method parameters were done by introducing small changes in the mobile phase composition (± 0.1 mL), mobile phase volume (± 1 mL), duration of chamber saturation with mobile phase (± 5 min), time from spotting to development (5min, 20min and 1hr) and time from development to scanning (10 min, 25 min, 50min and 1 hr). The effects change in method parameters on Rf value and peak area of drugs were examined.

Stress degradation studies

Stress degradation studies were carried out as per ICH guidelines under condition of acid/ base/ neutral hydrolysis, oxidation, dry heat and photolysis. Amount of tablet powder equivalent to about 10 mg Lercanidipine hydrochloride was transferred to 10.0 mL volumetric flasks. 1.0 mL of 0.1M NaOH, 0.1M HCl, 3% H_2O_2 and distilled water was added for alkaline, acid, oxidation and neutral hydrolysis respectively. The flasks were heated in water bath for 2 hrs at 80ºC. Dry heat and photolytic degradation were carried out in solid state. Dry heat studies were performed by keeping tablet sample of Lercanidipine hydrochloride in oven at 50ºC for a period of 24 hrs to study the heat on tablet sample. Photo-degradation studies were also carried out by exposure of tablet powder to UV light up to 254 nm for 24 hrs in UV chamber. After stipulated time interval, all the flasks were cooled to room temperature, dissolved and diluted up to the mark with methanol. The solution was then analyzed under optimum chromatographic conditions.

Application of proposed method for estimation of Lercanidipine hydorchloride in plasma sample

The proposed method was validated as per FDA guideline with respect to selectivity, sensitivity, recovery studies, precision and stability.

Collection of Blood plasma

Fresh blood samples of goat plasma were centrifuged at 10,000 rpm for 20 min for separation of plasma. The separated plasma was employed for the study.

Preparation of Spiking stock solution

10 mg each of LER was weighed and transferred to a separate 100.0 mL volumetric flask. It was dissolved in methanol and then diluted up to 100.0 mL to obtain solution of concentration of100 µg/mL.

Preparation of Quality control samples by spiking of plasma sample

1mL of stock solution of LER (100 ug/mL each) was added to 1.0 mL drug-free plasma in 10 mL centrifuge tubes. The samples were incubated at RT for 2 hrs. Protein precipitation and extraction was carried out using acetonitrile (2mL) and chloroform (4mL) and with vigorous vortex shaking for 2 min and centrifuged at 10,000 rpm for 10 min. The organic phase was recovered and evaporated to dryness at RT. The residual mass was reconstituted with 2.0 mL methanol. Lastly 1.0, 2.0, 3.0, 4.0 µl (Lower Limit of Quantitation LLOQ: 100 ng/band; Low QC sample: 200 ng/band; Middle QC sample: 300 ng/band; High QC sample: 400 ng/band of LER) from the reconstituted solution was applied on TLC plates to obtain the QC samples and developed under the optimized HPTLC conditions.

Optimization of Extraction method

Different solvents were tried for the extraction of LER from plasma. Initially hexane, n-butanol were tried for the precipitation of plasma but the sample recovery was very less. Chloroform (4mL) when employed resulted in significant improvement in sample recovery (80-90%). Acetonitrile was also added to increase the precipitation of plasma proteins. Finally, a mixture of acetonitrile and

chloroform (2:4 mL) was employed as the final solvent for extraction of LER with good sample recovery.

Method Validation

Study of linearity range

Working standard solution of Lercanidipine hydrochloride 10-100 µg/mL were prepared in methanol and 5µl of each solution was applied and chromatographed under optimum chromatographic conditions. A linear relationship between peak area and concentration was ascertained by making five measurements at ten concentration levels over a range of 100-500 ng/band.

Selectivity

The selectivity of the method was investigated by analyzing blank goat plasma. The blank plasma sample was checked for its interference using the proposed LLE procedure under the optimized HPTLC conditions and compared with spiked sample concentration of LER at lower limit of quantification (LLOQ) 100 ng/mL in goat plasma.

Sensitivity

Sensitivity was determined by calculating accuracy and precision at LLOQ (100 ng/band) by analyzing 5 replicates.

Precision and Accuracy

Precision of the method was determined by repeatability (intraday) and intermediate precision (inter-day) and accuracy for set of quality control (QC) samples 100, 200, 300, 400 ng/band (LLOQ, LQC, MQC, HQC) in replicate (n=5).

Extraction efficiency (Recovery)

Absolute recoveries were calculated by comparing peak areas obtained from freshly prepared samples extracted with unextracted standard solutions of same concentration. Recovery data was determined in triplicate at two concentrations (low and high) as recommended by FDA guidelines.

Stability

Analyte stability was determined by short term temperature stability, freeze thaw stability and post preparative stability. Five aliquots of each of the QC samples at low and high concentrations were thawed at room temperature, kept at RT and then analyzed for short term stability. For freeze thaw stability, the samples were stored at -20°C temperature for 24hr and then thawed to RT, three times. Post preparative stability was carried by extracting low and high QC samples kept at -20^0 C for 1 week. The samples were then thawed to RT and then analyzed.

RESULTS AND DISCUSSION

Optimization of mobile phase

After trying several permutations and combinations of solvents with varying polarity, the mobile phase consisting of methanol:toluene (2.5:7.5, v/v) gave good

peak shape for LER with acceptable Rf value (in the range of 0.2-0.8). It gave well defined band when the chamber was saturated for 15 min and hence it was selected for further analysis (Figure 3).

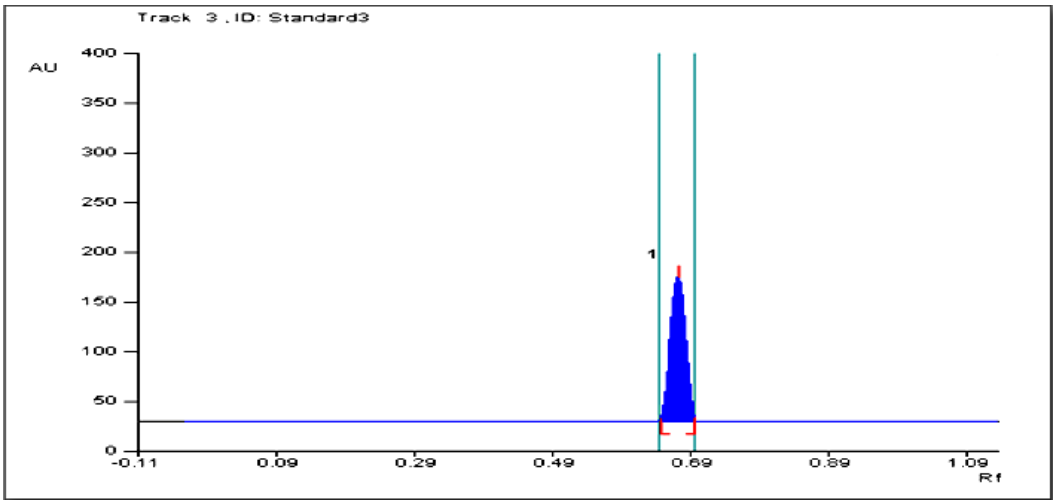

Figure 3: Typical densitogram of Lercanidipine hydrochloride in tablet formulation (Rf 0.69 ± 0.05), at 254 nm.

Analysis of tablet formulation

On the TLC plate two bands of standard stock solution and four bands of sample solution, 2.0 µl each, were applied and the plate was developed and scanned under the optimum chromatographic condition. Amount of drug present in sample was calculated by comparing the mean peak area of sample band with that of the standard band. The result of marketed formulation is given in Table 1.

Table 1. Result of analysis of marketed formulation

Amount of drug estimated (mg/tablet)	% Label Claim*	% R.S.D.
9.904	99.0	0.79

**mean of six determinations*

Method Validation

Linearity and Range

The linearity of detector response for Lercanidipine hydrochloride was observed in the concentration range of 100-600 ng/band (Fig. 4). The regression analysis equation was y= 5.6481 + 245.26 and the correlation coefficient was r^2= 0.9981.

Specificity

Specificity was performed to detect the presence of interfering peak at the retention time of the analyte peak. The chromatogram for blank sample did not show any peak at the retention time of Lercanidipine hydrochloride indicating that the excipients have no interference in the analysis.

Limit of detection and limit of quantitation

LOD and LOQ were separately determined bade on the standard deviation of the response of the calibration curve. LOD and LOQ were calculated by the formula 3.3 σ/s and 10 σ/s, where s is the slope of calibration curve and σ is the standard

deviation. The LOD and LOQ for Lercanidipine hydrochloride were found to be 2.75 ng/band and 9.17 ng/band, respectively. The low LOD and LOQ values indicate the sensitivity of the developed method.

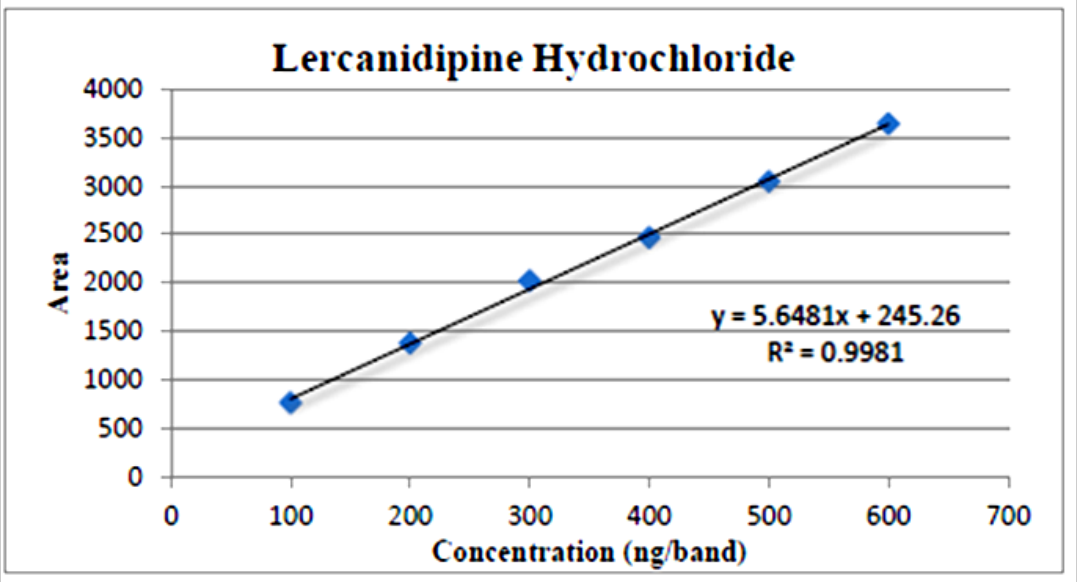

Figure 4. Linearity for Lercanidipine hydrochloride

Precision

The system precision and method precision was determined by analyzing six replication injection of standard stock solution and six samples of standard stock solution, respectively. The intra-day and inter-day precision was evaluated by analyzing table sample at three different time intervals on same day and on three consecutive days, respectively. The percent RSD was found to be less than 2% (Table 2) indicating that the method is precise and reproducible.

Table 2. Results of precision study

Precision	%RSD for peak area*
System precision	0.842
Method precision	0.826
Intra-day precision	0.814
Inter-day precision	0.817

**mean of six determinations*

Accuracy

The accuracy of the method was determined out by preparing recovery samples at the level of 80%, 100% and 120 % of the standard solution. The samples were chromatographed at three different levels and the percentage recovery was calculated. The result of accuracy is shown in Table 3. The percent recovery was found in the range of 99.2 % to 99.8 % indicating that the excipients present in tablet formulation did not interfere in the analysis.

Robustness

Robustness of the method was evaluated by deliberately changing the method parameters. The effect of change in method parameters was not significant as the Rf

value for Lercanidipine hydrochloride was within ± 0.05 Rf units and the percent lable claim obtained under different method parameters was around 100 percent indicating that there was no significant change in peak area. Results of robustness studies are shown in Table 4.

Table 3. Results of accuracy studies

Level of recovery	Amount of drug added	% Recovery*	% R.S.D.
80 %		99.77	0.98
100 %		99.23	0.92
120 %		99.90	0.74

**mean of three determinations*

Table 4. Results of robustness study

	Chromatographic Changes	
Factor	**R_f Value***	**% Label Claim***
Mobile phase composition (± 0.1 mL)	0.74	98.99
Amount of Mobile Phase (± 1.0 mL)	0.69	97.88
Duration for chamber saturation (± 5 %)	0.72	99.19
Time from spotting to development	0.72	98.75
Time from development to scanning	0.69	98.55

**mean of three determinations*

Stress degradation studies

Lercanidipine was found to degrade under acid, alkali and oxide stress conditions. The method was able to selectively quantitate Lercanidipine hydrochloride in the presence of degradation products indicating the stability indicating property of the method. The chromatogram obtained for tablet sample exposed to acid, alkali and oxide stress conditions are shown in Fig. 5, 6 and 7, respectively. The results are summarized in Table 5.

Table 5: Results of stress degradation study

Stress Condition	Temperature and Time	Percent assay of active substance	R_f Value of degraded product
Acid (0.1M HCl)	80 ºC for 2 hrs	73.83	0.65, 0.59, 0.51, 0.49
Alkali (0.1M NaOH)	80 ºC for 2 hrs	80.58	0.74, 0.70, 0.57, 0.55, 0.52
Oxide (3.0 % H_2O_2)	80 ºC for 2 hrs	76.10	0.67, 0.66,.54
Neutral	80 ºC for 2 hrs	97.56	-
Heat	60 ºC for 24 hrs	98.25	-
UV-Exposure	254 nm for 24 hrs	99.47	-

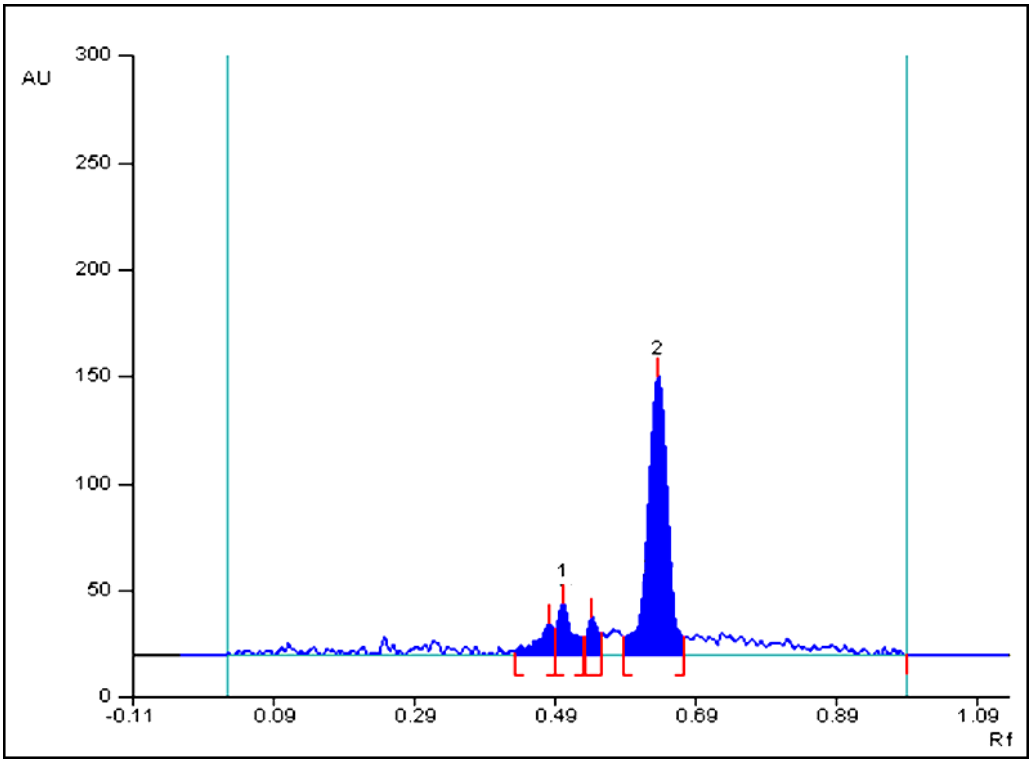

Figure 5. Densitogram of acid (0.1M HCl) treated tablet sample

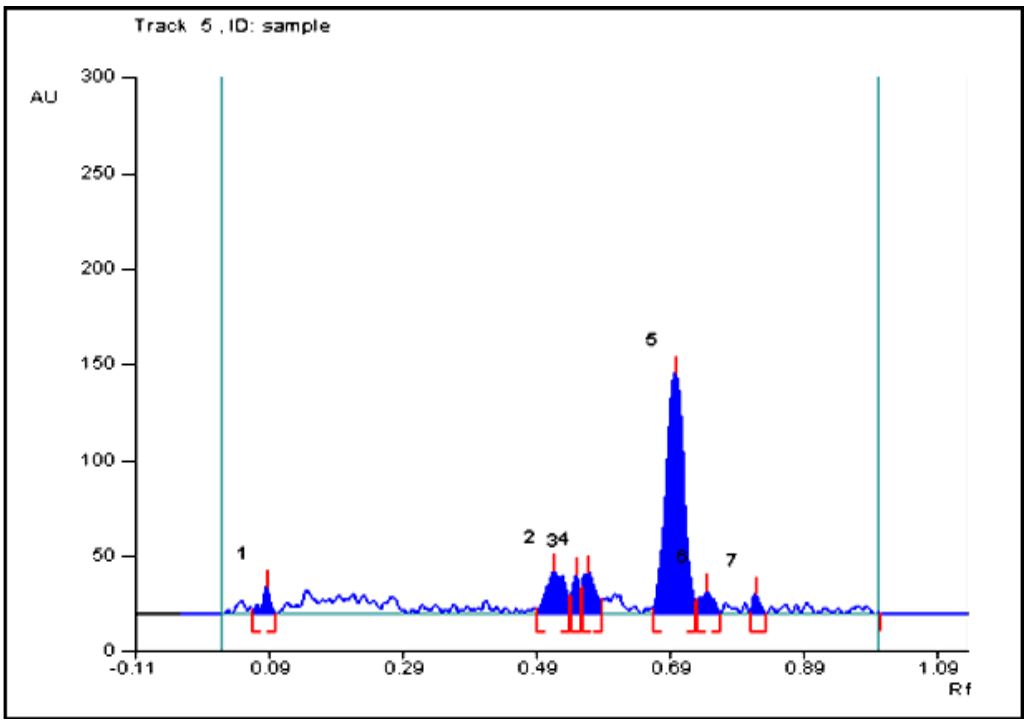

Figure 6. Densitogram of alkali (0.1M NaOH) treated tablet sample

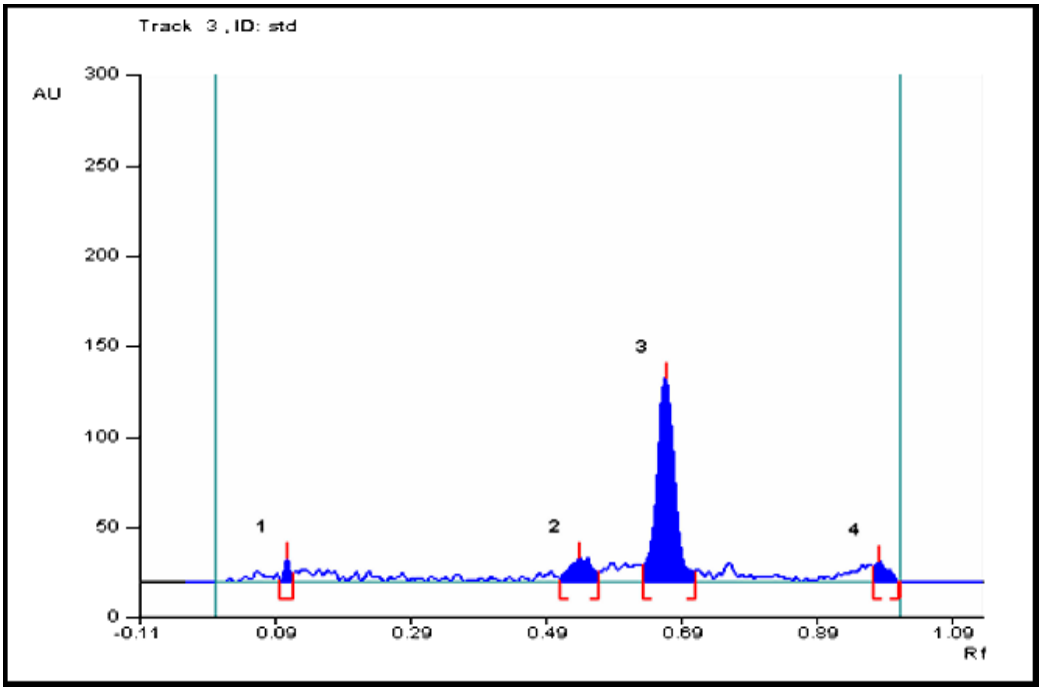

Figure 7. Densitogram of oxide (3.0% H_2O_2) treated tablet sample

Application of proposed method for analysis of Biological sample

Optimization of Extraction method

Different solvents were tried for the extraction of LER from plasma. Finally, a mixture of acetonitrile and chloroform (2:4 mL) was employed as the final solvent for extraction of LER with good sample recovery (Fig. 8).

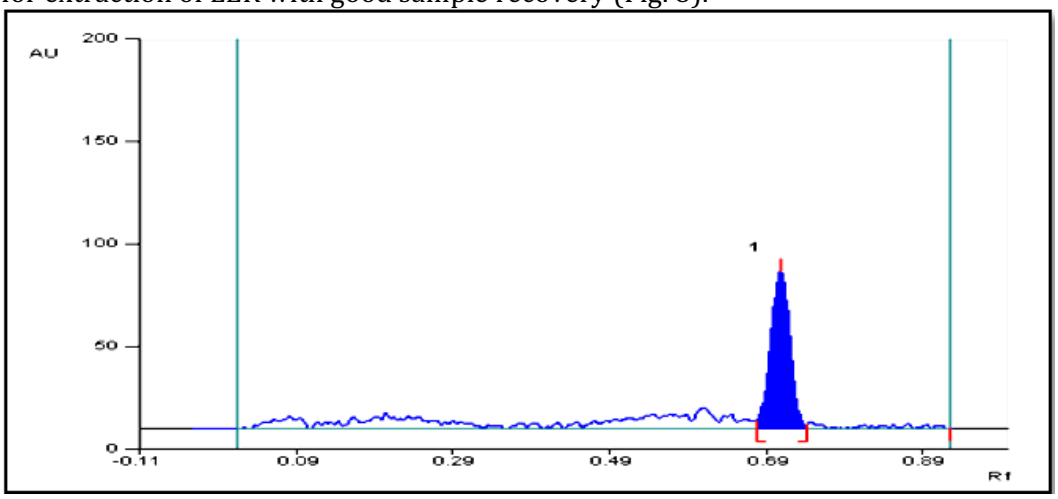

Figure 8. Typical densitogram of goat plasma spiked with LER

Study of Linearity range

Standard stock solution was applied on the TLC plate in the range of 50-500 ng/band. The plate was then developed and scanned under the optimum chromatographic conditions. Peak area was recorded for each drug concentration and the calibration curves of the concentration verses peak area were constructed. The linearity of detector response was observed in the concentration range under

study (Fig. 9). The regression analysis equation was y= 6.7856 -163.31and the correlation coefficient was r^2= 0.9989.

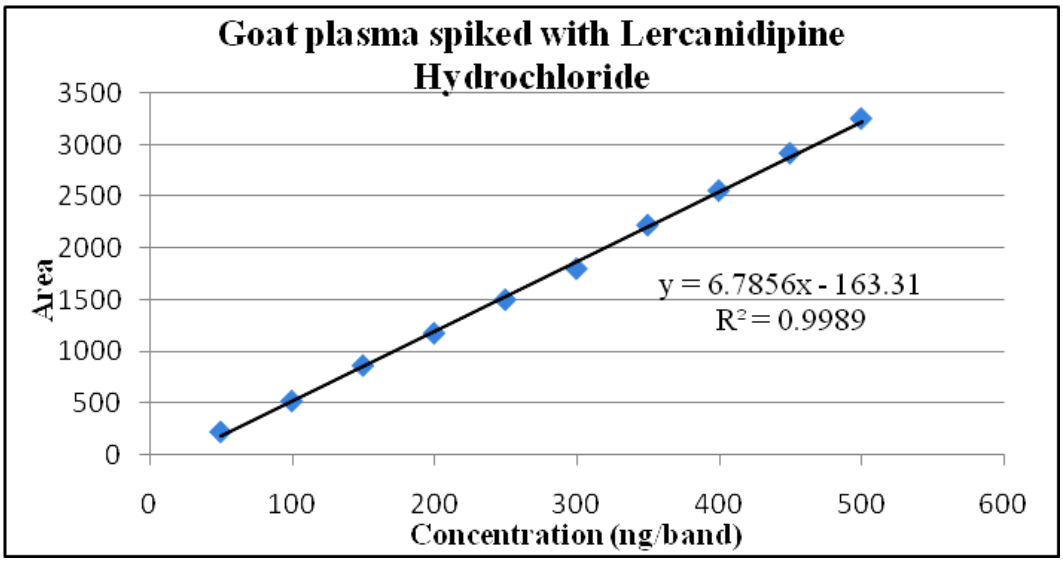

Figure 9. Standard Calibration curve for goat plasma spiked with LER

Selectivity

The selectivity of the method was investigated by analyzing blank goat plasma. The blank plasma sample was checked for its interference by comparing with spiked sample concentration of LER at lower limit of quantification (LLOQ) 100 ng/mL in goat plasma. There was no interference of the component of the biological matrix (plasma) in the quantitation of LER.

Sensitivity

Sensitivity was determined by calculating accuracy and precision at LLOQ (100 ng/band) by analyzing 5 replicates. The lower limit of quantitation was found to be 96 ng/band for LER. The % CV was found to be 1.01% and % Bias was found to be 2.82% for LER, and is within the acceptable limits.

Precision and Accuracy

Precision of the method was determined by repeatability (intraday) and intermediate precision (inter-day) and accuracy for set of quality control (QC) samples 100, 200, 300, 400 ng/band (LLOQ, LQC, MQC, HQC) in replicate (n=5). Intra-day and inter-day precision (% CV) was found to be 2.4%, 1.14%, 1.06%, 0.72% and 3.51%, 0.96% , 1.42%, 0.85% respectively. The accuracy (% BIAS) was found to be 3.37%, 1.91%, 3.61%, 1.4% and 3.8%, 2.1%, 0.35%, 0.97% respectively. The coefficient of variance (% CV) and percent bias (% BIAS) were within the acceptable limits.

Extraction efficiency (Recovery)

Absolute recoveries were calculated by comparing peak areas obtained from freshly prepared samples extracted with unextracted standard solutions of same concentration. Recovery data was determined in triplicate at two concentrations (low and high) as recommended by FDA guidelines. The recovery at two concentrations 50 and 100 ng/band was found to be 87.10%, 94.42% for LER.

Stability

For short term stability the % CV at LQC and HQC was 2.33% and 0.42% respectively. The % BIAS at LQC and HQC was 4.3% and 0.75% for respectively.

For freeze thaw stability the % CV at LQC and HQC was found to be 0.94% and 0.27% respectively. The % BIAS at LQC and HQC was 1.78% and 0.55%, respectively.

For long term stability the % CV at LQC and HQC was 1.30% and 0.24% respectively. The % BIAS at LQC and HQC was found to be 2.79% and 1.24% respectively.

CONCLUSION

Introducing HPTLC in pharmaceutical analysis represents a major step in terms of quality assurance. The proposed HPTLC method gives well resolved peaks for lercanidipine hydrochloride. Based on the results obtained it is concluded that the method is sensitive, accurate, precise, selective, reproducible and less time consuming and can be employed ofr determination of Lercanidipine hydrochloride in bulk, pharmaceutical formulation and biological samples without interference from the excipients. The proposed HPTLC method was also able to selectively quantitate lercanidipine hydrochloride in the presence of degradation products obtained in forced degradation study. Hence, the developed HPTLC method can be used as assay method for pharmaceutical formulation and biological samples containing Lercanidipine hydrochloride.

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