ABSTRACT

A specific, accurate and precise reversed phase high performance liquid chromatographic method was developed and validated for the quantification of lercanidipine in nanoproliposomes and polymeric nanoparticles. The developed method mobile phase comprised of acetonitrile and potassium dihydrogen phosphate buffer (25 mM; pH 3.5) at the ratio of 70:30% v/v at a flow rate of 1.0 ml/min. The separation of lercanidipine was carried out on a Phenomenex® Gemini C_{18} (250 × 4.6 mm, 5µ) column using UV-visible detector set at 242 nm. The method was found to be specific for the analysis of lercanidipine in the developed novel carrier systems. The calibration curve was linear over concentration range 0.5 to 25.0 µg/ml with coefficient of determination $r^2 > 0.999$. The limit of detection and limit of quantification was found to be 0.05 µg/ml and 0.1 µg/mL, respectively. The method meets validation criteria in accordance with ICH Q2(R1) guidelines indicating its usefulness in quantification of lercanidipine in novel carriers.

Also, a sensitive, accuate and precise high performance liquid chromatographic method was developed for quantification of lercanidipine in rabbit plasma. Protein precipitation method was utilized to extract lercanidipine in rabbit plasma using 0.2% v/v HCl in methanol as protein precipitating agent. Valdecoxib was used as an internal standard. The chromatographic separation was achieved by Hichrom Kromosil 100-5C_{18} (250 × 4.6 mm id) column and the effluent was monitored by an UV-visible detector set at 242 nm. The mobile phase consisted of mixture of acetonitile: 25mM potassium dihydrogen phosphate buffer (pH 3.5) at a ratio of 50:50% v/v at a flow rate of 1 mL/min. The developed method was linear over the range of 25-2000 ng/ml with coefficient of determination of greater than 0.99. The method was validated as per USFDA guidelines. The developed method was successfully used for the preclinical pharmacokinetic studies of novel carrier systems in rabbits.

3.1. INTRODUCTION

The pharmaceutical product analysis is one of the best and obligatory practices followed during product development to secure the quality of the drug product and to meet the stringent regulations established by different regulatory bodies. In this cognizance, development of efficient analytical tools is an important prerequisite in several stages of product development (Hanai, 1999; Kazakevich and Lobrutto, 2007).

In the modern pharmaceutical industry, the product analysis is fuelled by array of sophisticated techniques including, high performance liquid chromatography (HPLC), HPLC-MS (mass spectrometry), HPLC-NMR (nuclear magnetic resonance) and high-throughput purification methods. Among these instruments, HPLC is a major analytical tool applied in almost all stages of formulation development to effectively control and monitor the quality of the prospective drug candidates, excipients and final products (Ohannesian et al., 2002; Kazakevich and Lobrutto, 2007). It is considered as a benchmark analytical technique because of its several advantages like rapid and precise quantitative analysis, sensitivity in detection, automated operation, amenable to variety of samples, etc.

The reliability of HPLC in analytical and bioanalytical method development of pharmaceutical actives is also of great importance. The developed method should be able to analyze the samples both quantitatively and qualitatively. Bioanalytical method is prominently involved in quantification of drugs and their metabolites in biological fluids, which in turn provides interpretation of bioavailability, bioequivalence, pharmacokinetic, and toxicokinetic related information. Initiation of any method development activity demands prior knowledge of the physicochemical parameters of the drug molecule. Moreover, it is also essential to employ fully validated analytical and bioanalytical method to yield reliable results which forms the ideal basis for generated scientific data (Shah, 2007).

The main objective of the present work was to develop novel formulations like, lercanidipine loaded nanoproliposomes and polymeric nanoparticles. Quantitative estimation of lercanidipine in these formulations as well as in pharmacokinetic evaluation was an essential basis to prove the developed deliveries as efficient in achieving desired objectives. In this standpoint, the extensive literature survey did not reveal any method for estimation of lercanidipine in these carriers. Therefore, RP-HPLC

method was developed and validated for determination of lercanidipine in formulations. Similarly, bioanalytical method was also developed for quantification of lercanidipine in rabbit plasma.

3.2. EXPERIMENTAL METHODS

3.2.1. Identification of Lercanidipine Hydrochloride

Lercanidipine Hydrochloride was received as a gift sample from Cipla Ltd., Mumbai, India.

3.2.1.1. UV Spectroscopy

Primary stock solution of 1.0 mg/mL lercanidipine hydrochloride was prepared by dissolving 10.0 mg of the drug in 10.0 mL of methanol. From this, 10.0 µg/mL of the drug solution was prepared by serial dilution which was scanned between the wavelengths of 400-200 nm using UV spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan) to determine the wavelength of maximum absorption.

3.2.1.2. Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR analysis was carried out to understand the physical state of lercanidipine in the formulation. The analysis was carried out using Shimadzu FTIR 8300 Spectrophotometer (Shimadzu, Tokyo, Japan) and the spectrum was recorded in the wavelength region of 4000-400 cm^{-1}. The technique consisted of compressing the sample into a disc using potassium bromide by applying a pressure of 5 tons for 5 min using hydraulic press. The pellet was placed in the light path and the spectrum obtained was recorded (Usha et al., 2008).

3.3. ANALYTICAL METHOD DEVELOPMENT OF LERCANIDIPINE BY HPLC

Lercanidipine is a third generation dihydropyridine calcium antagonist which selectively inhibits influx of calcium ions through L-type calcium channels present in the cardiac and vascular smooth muscle cells (McClellan and Jarvis, 2000; Parmar et al., 2011). The chemical formula of lercanidipine hydrochloride is 3,5-pyridinedicarboxylic acid, 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-2-[(3,3-diphenylpropyl)methylamino]-1,1, dimethylethyl methyl ester hydrochloride. Presently, lercanidipine is administered as immediate release dosage form at a dose of about 10 and 20 mg once or twice daily.

Orally administered lercanidipine is completely and erratically absorbed with peak plasma occurring in 1.5 to 3 h following dosing. It has a plasma half-life of about 2 to 5 h (Sica and Prisant, 2007). Lercanidipine poses absolute bioavailability of 10% due to its extensive and saturable first-pass metabolism. Furthermore, the presence of food increases the absorption of lercanidipine and thereby enhances the bioavailability. Lercanidipine and its salts are virtually insoluble in water and display marginal solubility in acidic media. It has a partition coefficient, pKa of 6.4 (Dedhiya et al., 2007; Pandit and Patil, 2009). Extensive literature survey reveals that there is no suitable method available for determination of lercanidipine in colloidal formulations. In the present study, a rapid, sensitive, specific, accurate and precise reversed-phase high performance liquid chromatographic (RP-HPLC) method with UV detection has been developed and validated in order to determine lercanidipine in novel colloidal formulations.

3.3.1. Preparation of Stock Solutions

Required amount of (10 mg) lercanidipine was weighed and dissolved in 10 mL of methanol to yield primary stock solution of 1.0 mg/mL. Working stock solutions were prepared from primary stock solution by serial dilution method to obtain the required concentrations. Mobile phase was used as diluent. All the prepared solutions were protected from light by storing them in an amber colored glass material at 2-4 °C.

3.3.2. Instrument and Software

The HPLC system, LC-2010CHT (Shimadzu Corporation, Kyoto, Japan) equipped with low pressure quaternary gradient pump along with dual wavelength ultra violet (UV)/ visible detector, column oven and auto sampler has been used for the analysis. The chromatographic data was processed using LC solution, version 1.24 SP1 software.

3.3.3. Selection of Chromatographic Method

Appropriate selection of the method depends upon physicochemical properties of analyte like molecular weight, dissociation constant (pKa) and solubility of the compound. Here, the RP-HPLC method was selected for the initial separations because of its simplicity, suitability, ruggedness and its wide range of utility in quantification of compounds.

3.3.3.1. Initial separation conditions

The mobile phase adopted in the present study was acetonitrile and phosphate buffer solution. The phosphate buffer was chosen as aqueous mobile phase based on available literature to elute lercanidipine (Alvarez-Lueje et al., 2003).

Stationary phase	: Phenomenex® Gemini C_{18} (250 × 4.6 mm, 5µ)
Mobile phase: Solvent A	: Acetonitrile
Solvent B	: Phosphate buffer (25 mM, pH 3.5)
Solvent ratio	: Isocratic run for 20 min using ratio 65:35 % v/v of A:B
Detection wavelength	: 242 nm
Flow rate	: 1 mL/min
Injection volume	: 25 µL
Column oven temperature	: Ambient 25 °C
Auto sampler temperature	: 4 ± 2 °C

25 mM potassium dihydrogen phosphate buffer was prepared by dissolving 3.402 g of potassium dihydrogen phosphate (KH_2PO_4) in 1000 mL of Milli-Q water and pH was adjusted to 3.5 using ortho phosphoric acid. The buffer was filtered and sonicated for 15 min.

The standard solution of lercanidipine 10 µg/mL was prepared, injected to HPLC system and chromatograms were recorded. The retention time of lercanidipine was found to be 9.08 min. In order to fasten the elution of lercanidipine the chromatographic conditions were further optimized.

3.3.3.2. Optimization of chromatographic conditions

The selection of appropriate mobile phase conditions is one of the important and primary steps carried out in the process of chromatographic separation of molecules. Therefore, before carrying out optimization process, a variety of preliminary parameters were assessed to evaluate the retention time, shape and other chromatographic parameters of eluted lercanidipine chromatogram.

- The effect of pH on retention time of lercanidipine was studied.
- The phosphate buffer (pH 3.5) was prepared in various strengths such as 10, 20, 25 and 30 mM and chromatograms were recorded keeping other chromatographic conditions constant.
- The proportion of acetonitrile and phosphate buffer was studied at various ratios for the proper selection of mobile phase.
- The following stationary phases were studied and the chromatograms were recorded,
    - Grace Vydac C_{18} column (250 × 4.6 mm i.d., 5µ)

- Kromasil C_{18} column (250 × 4.6 mm i.d., 5µ)
- Supelco C_{18} (250 × 4.6 mm i.d., 5µ)
- Phenomenex C_{18} column (250 × 4.6 mm i.d., 5µ)
- LiChrospher 100 RP-18e (250 × 4.6 mm i.d., 5µ)

All of the above parameters were assessed with respect to system suitability parameters such as theoretical plates/meter, retention time and asymmetry factor.

3.4. ANALYTICAL METHOD VALIDATION

The analytical method established by the laboratory procedures is required to be validated for demonstrating that a developed method is acceptable for the intended purpose. In the present study, the newly developed method was used for the estimation of lercanidipine in the newer formulations which essentially needs the validation. The developed method was validated as per International Conference on Harmonization guideline, ICH Q2(R1).

3.4.1. Specificity

Specificity is the ability of analytical method to separate the analyte in the presence of impurities or interferences. To evaluate the specificity of the developed method, blank nanoproliposomes and polymeric nanoparticles were processed and evaluated for the interferences at the retention time of the lercanidipine.

3.4.2. Linearity

The linearity of an analytical method is its ability to elicit test results that are directly or by means of well-defined mathematical transformations, proportional to the concentration of analytes in the samples within a given range. The linearity plot was constructed for lercanidipine in the range of 0.5-25.0 µg/mL. The primary stock solution of 1 mg/mL of lercanidipine was prepared separately in methanol. From the primary stock solution, secondary stock solution was prepared to get the concentration of 100 µg/mL. Appropriate dilution of the primary and secondary stock solution was carried out in mobile phase to get concentration of 0.5, 1.0, 2.0, 5.0, 15.0, and 25.0 µg/mL for lercanidipine. Each of the concentration was injected in duplicate and chromatograms were recorded. The calibration curve was plotted as concentration versus the peak area at each level. The coefficient of determination (r^2), slope and intercept values was calculated and statistically evaluated.

3.4.3. Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a true value or a reference value. The accuracy of the method was performed by recovery studies and is expressed as % recovery. The recovery study was conducted by spiking standard drug concentration (80, 100 and 120%) of the assay concentration (i.e. 10 µg/mL) in triplicate to the placebo matrix. The matrix used in the present study was placebo formulations of nanoproliposomes and polymeric nanoparticles. The samples were spiked with 8.0 µg/mL of lercanidipine (80% spiking), 10 µg/mL of lercanidipine (100% spiking), 12 µg/mL of lercanidipine (120% spiking) as standard solution. The recovery limit is 98-102%. The recovery was calculated using the following formula:

$$\text{Recovery (\%)} = \frac{\text{Total Concentration - Preanalyzed Concentration}}{\text{Standard Concentration}} \text{x}100$$

3.4.4. Precision

Precision is a measure of the degree of reproducibility or repeatability of the analytical method under normal operating circumstances. The precision of the method was determined at three different levels covering entire range of linearity. Three different standards were prepared which contained 0.75, 12.0, and 20.0 µg/mL of lercanidipine.

3.4.5. Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. The intra-day precision was determined by injecting known standard for six times and the percentage relative standard deviation (% RSD) was calculated for the resultant peak area of lercanidipine. The acceptance criterion is considered is < 1 % RSD.

3.4.6. Intermediate Precision

Intermediate precision was done by different analysts, equipment's and days. For the present method, intermediate precision was carried out using different liquid chromatographic instrument and the different analyst with freshly prepared standards of lercanidipine. Each standard was injected six times and the relative standard deviation was determined for each level. The acceptance criterion is considered is < 2 % RSD.

3.4.7. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD is the ability of the analytical method to detect the lowest concentration of the analyte. LOQ is lowest concentration of the analyte which can be quantitatively analyzed with acceptable precision and accuracy. LOD and LOQ were calculated using the following formula:

$LOD = 3.3 \times SD/S$

$LOQ = 10 \times SD/S$

Where, SD indicates standard deviation of blank response; S is a slope of regression equation.

3.4.8. Robustness

The robustness is a capacity of any analytical procedure to remain unaffected by small, but deliberate variations. The 10 µg/mL of lercanidipine was used for the determination of robustness of the method. As a part of the assessment of robustness of the present method, the parameters such as, effect of buffer pH in the mobile phase (± 0.2), effect of mobile phase composition (± 2%), effect of flow rate (± 10%) were determined. The acceptance criterion is considered as below 2% RSD.

3.4.9. System Suitability

The purpose of the system suitability test was to ensure that the complete testing system (including instrument, reagents, columns, analysts) was suitable for the intended application.

3.5. BIOANALYTICAL METHOD DEVELOPMENT OF LERCANIDIPINE

The development of proficient bioanalytical method is of utmost important during the process of pharmaceutical product development. These methods fit to support the quantification of drugs and/or their metabolites in the biological matrices such as blood, serum, plasma or urine. The well established and validated bioanalytical method can be of integral part of the pharmacokinetic, pharmacodynamic, bioavailability, bioequivalence and toxicokinetic related studies (Srinivas 2006; Shah 2007; Srinivas 2008).

Basically, the development of suitable method for bioanalysis of sample depends on chemical structure, molecular weight, pKa, solubility and polarity like properties of the analyte. Another major part of the bioanalytical method development is sample

preparation technique. Biological samples are extremely complex matrices comprising of several endogenous components which presents the challenge for extraction of analyte from the biological matrix and separation of endogenous interferences at the retention time of analyte plays crucial role in development of bioanalytical method.

To quantify analytes from these matrices protein precipitation, liquid-liquid extraction (LLE) and solid phase extraction (SPE) methods are routinely employed. In protein precipitation method, generally organic solvents such as, acetonitrile, methanol, acetone and ethanol can be used to remove plasma protein by denaturation or precipitation. Liquid-liquid extraction method involves extraction of biological matter by addition of the water-immiscible organic solvents with or without adjustment of pH of matrix. Solid phase extraction involves partitioning of solutes between two phases such as, solvent with analyte and solid phase of sorbent. Generally, the sample loaded into a solid phase, the undesired components were washed with one solvent and then desired analyte washed with another organic solvent and collected into collection tube (Zwir-Ferenc and Biziuk, 2006).

Another important aspect of bioanalysis is the use of internal standard (IS) during analysis. The IS should be structurally similar analogue added to both calibration standards and samples at known concentration to facilitate quantification of the analyte.

Some of the previous literatures report the development of bioanalytical method for lercanidipine. A rapid ultra-performance liquid chromatography/positive electrospray ionization tandem mass spectroscopy method has been developed for the determination of lercanidipine in human plasma (Kalovidouris et al., 2006). Charde et al., (2007) developed a simple and sensitive RP-HPLC method for the estimation of lercanidipine hydrochloride in rabbit serum using UV detector under isocratic conditions. Another literature reports a liquid-liquid extraction procedure for five different 1,4-dihydropyridines calcium channel antagonists including lercanidipine was developed using high-performance liquid chromatography with diode-array detector from human plasma using experimental design (Baranda et al., 2005). In yet another report, a selective and rapid liquid chromatography-mass spectrometry method was developed for the determination of lercanidipine in human plasma (Salem et al., 2004). However, extensive literature survey did not reveal any method for estimation of lercanidipine in

rabbit plasma. Therefore, in the present study a simple, sensitive and specific RP-HPLC method was developed and validated for determination of lercanidipine in rabbit plasma.

3.5.1. Optimization of Chromatographic Conditions

3.5.1.1. Selection of internal standard

Selection of internal standard is one of the important requirements in the bioanalytical method development. The important criteria for selection of IS was based on chemical structure, solubility and polarity characteristics of the drug. In the present method, various drugs like nisoldipine, nifedipine, irbesartan, telmisartan, nelfinavir, stavudine, pioglitazone, valdecoxib, celecoxib, simvastatin and montelucast etc. were screened and valdecoxib was finally chosen as an IS because it was well resolved from lercanidipine with sharp symmetrical peak shape.

3.5.1.2. Collection of rabbit plasma

The blood collection from rabbits was carried out by inserting 24G BD Neoflon™ straight IV cannula into the central ear artery in the rabbit. Blood sample was collected using heparinized capillaries and directly transferred into vacutainers (containing disodium EDTA as an anticoagulant). The whole blood was immediately centrifuged at 10,000 rpm for 5 min (C24 Centrifuge, Remi Pvt. Ltd., Bangalore, India) and the plasma was collected and stored at -70 $^{\circ}$C until their analysis.

3.5.1.3. Optimization of extraction procedure

In the present work, various experimental trials were conducted to standardize the procedure for extraction of drug from the rabbit plasma. Importantly, liquid-liquid and protein precipitation method were attempted. Different organic solvents such as ethyl acetate, tertiary butyl methyl ether, dichloromethane, diethyl ether etc with/or without mixture of acid were evaluated. Also protein precipitation method using perchloric acid (10% v/v), trichloro acetic acid (10% v/v), chilled acetonitrile, 0.2% HCl in methanol was attempted. Based on the recovery and interferences of analyte and IS, 0.2% v/v HCl in methanol was chosen as a protein precipitating agent for extraction of lercanidipine and IS in rabbit plasma.

3.5.1.4. Sample processing and bioanalysis of lercanidipine by RP-HPLC method

The HPLC system set-up used for the bioanalysis of samples was same as described previously in the section 3.3.2. Briefly, 50.0 µL of valdecoxib (used as an Internal standard; IS) solution (20.0 µg/mL) was spiked into the 500 µL of blank plasma sample.

To this 1000.0 µL of 0.2% v/v HCl in methanol was added as protein precipitating agent for extraction of lercanidipine from the plasma. The mixture was vortex-mixed for 5 min and centrifuged using refrigerated centrifuge unit at 10,000 rpm for 10 min at -4 °C. A 900 µL aliquot of the supernatant was transferred into a 5.0 mL of borosilicate glass tube and evaporated to dryness under a stream of nitrogen in a Turbo-Vap evaporator (Zymark, Hopkinton, MA, USA) at 60 °C. The solid residue at the bottom was reconstituted with 100 µL of chilled acetonitrile and vortexed for 5 min. The reconstituted solution was transferred to 1.5mL of microcentrifuge tube and centrifuged at 10,000 rpm for 10 min at -4 °C. The supernatant was carefully separated and reconstituted with 50.0 µL of MilliQ water and vortex-mixed for 1-2 min. The reconstituted solution was transferred into the 1.5 mL low volume HPLC vials and an aliquot of 100.0 µL was injected into the HPLC system. All the procedures were performed at room temperature. The chromatographic separation was achieved by Hichrom Kromosil 100-5C_{18} (250 × 4.6 mm id) (Hichrom Ltd., Berkshire UK) column. The mobile phase consisted of mixture of acetonitile: 25mM potassium dihydrogen phosphate (KH_2PO_4) buffer (pH 3.5 ± 0.1 adjusted with ortho phosphoric acid) in a ratio of 50:50 (v/v). The LC system was operated isocratically at a flow rate of 1 mL/min and the column temperature maintained at 25 °C (Musmade et al., 2007; Musmade et al., 2010).

3.6. BIOANALYTICAL METHOD VALIDATION

A validation for developed bioanalysis method of lercanidipine in rabbit plasma was performed in accordance with USFDA guidance.

Preparation of stock and standard solutions

Prior to the preparation of stock solutions, corrections to the theoretical concentrations were performed for the selected drugs according to the degree of standard substance impurities, salt and moisture content. Primary stock solutions of lercanidipine were prepared in methanol and further dilutions were made in same solvent. The working stock solutions containing 0.5, 1.0, 2.0, 3.0, 4.0, 10.0, 20.0, 40.0 µg/mL of lercanidipine for calibration curve was prepared from the primary stock solutions. In addition to these, working solutions for quality control standards were prepared in a manner similar to the preparation of calibration standards. Further to these, working solutions for quality

control standards such as Low Quality Control (LQC), Middle Quality Control (MQC), High Quality Control (HQC) containing 1.5 16.0, 36.0 µg/mL of lercanidipine was prepared. The valdecoxib (IS) stock solution of 1.0 mg/mL was prepared in methanol. IS working solution (20.0 µg/mL) was prepared by diluting IS stock solution with methanol. All stock solutions were stored at 2-4 °C.

Calibration standards were prepared by bulk spiking 50.0 µL of each working stock solutions into 950.0 µL of blank rabbit plasma to produce calibration standards equivalent to 25.0, 50.0, 100.0, 150.0, 200.0 500.0, 1000.0 and 2000.0 ng/mL of lercanidipine. Similarly, QCs' (LQC, MQC and HQC) were prepared by bulk spiking 50.0 µL of working quality control standard solutions in 950.0 µL of rabbit plasma to get a concentration of 75.0, 400.0, 1800.0 ng/mL for lercanidipine. During analysis, double blank plasma, zero standard (blank plasma spiked with IS) and a set of calibration standards, QCs' were analyzed. The lower limit of quantification (LLOQ) and upper level of quantitation (ULOQ) of calibration curve standards were used in duplicate. Such validation runs were generated on five consecutive days. Calibration curve requires minimum coefficient of determination (r^2) ≥ 0.98.

3.6.1. Selectivity

Selectivity is the ability of any method to differentiate and quantify the analyte in the presence of other components in the sample. The selectivity in the present methodology was established by analyzing six blank plasma samples from six lots of rabbits without the addition of drug and IS to evaluate presence of interfering peaks. Selectivity was carried out at the lower limit of quantification (LLOQ). In criteria, there should not be any potential interferences (< 5%) at the retention of analytes.

3.6.2. Accuracy and Precision

Intra and inter-day accuracy and precision were assessed by duplicate analysis of six samples at four different levels, i.e. LLOQ (25 ng/mL), LQC (75 ng/mL), MQC (400 ng/mL), HQC (1800 ng/mL) was estimated. These samples were prepared and analyzed on day one and procedure was followed for three consecutive days. For accuracy, the back calculated concentration should be within 15% of nominal concentration and except for LLOQ, where it should be <20%.

3.6.3. Recovery

Recovery refers to the extraction efficiency of an analytical method. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise and reproducible. Recovery of lercanidipine in rabbit plasma was determined at low, medium and high quality control samples (extracted) to the mean peak areas of neat reference solutions of same concentration (unextracted). Recovery of IS was also determined in similar manner.

3.6.4. Stability

Stability of the lercanidipine in rabbit plasma was determined by testing spiked plasma samples at four different stability conditions such as at room temperature (bench top), freezer (freeze-thaw stability), auto-injector stability and long term stability (storing samples at -70 °C for three months). All the analyses were carried out as replicate (n = 6) of low and high QC samples. Bench top stability was carried out by keeping samples approximately for 12 h at ambient conditions in replicate. Freeze-thaw stability analysis was determined by three freeze-thaw cycles. Here the samples were stored at -70 °C and analyzed by thawing them at room temperature and refrozen for 12 to 24 h under the same conditions. Autosampler stability was evaluated by analysis of processed and reconstituted low and high plasma QC samples, which were stored in the auto sampler tray for 24 h. Long-term stability was carried out by storing low and high QC samples at -70 °C for 30 days. Pertaining to all above types of stability analysis, the results were compared with the freshly prepared sets of the QC samples.

3.7. RESULTS AND DISCUSSION

3.7.1. Identification of Lercanidipine Hydrochloride

Lercanidipine HCl (crystalline form) is a yellow powder soluble in methanol and practically insoluble in water.

3.7.2. UV Spectroscopy

The λ_{max} for Lercanidipine was found to be 242 nm as shown in Fig. 3.1.

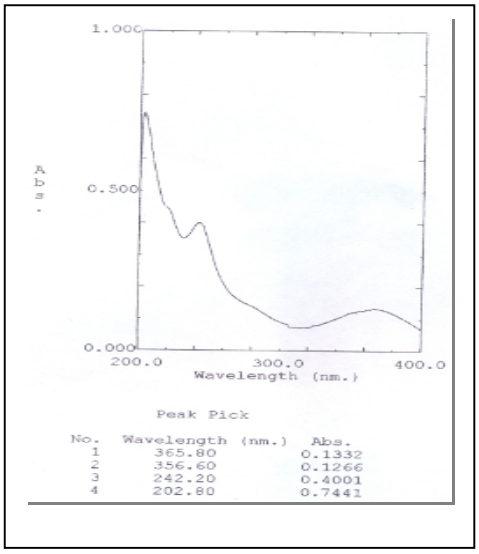

Fig. 3.1. UV spectra of lercanidipine hydrochloride.

3.7.3. FTIR Spectroscopy

The FTIR spectrum of lercanidipine as shown in Fig. 3.2 exhibits a weak absorption band at 3387 cm^{-1} due to the presence of secondary amine functionality. The presence of aromatic system is evident from the characteristic absorption bands at 3063 cm^{-1} due to aromatic C–H stretch and at 1525-1489 cm^{-1} due to the skeletal vibrations involving C-C stretching within the aromatic ring. The spectrum demonstrates the CH_3 aliphatic stretch at 2949 cm^{-1}. Prominent C=O stretch at 1691 cm^{-1} indicates that the ester functionality is associated with a conjugated system. Characteristic peaks at 1220 cm^{-1} and 1114 cm^{-1} correspond to the C-C=O-C stretching of saturated ester. The NO_2 vibrations at 1550 and 1350 cm^{-1} are too extensive and overlap with the aromatic vibrations. A weak shoulder at 777 cm^{-1} can be assigned to the C-N stretching vibration of aromatic nitro group.

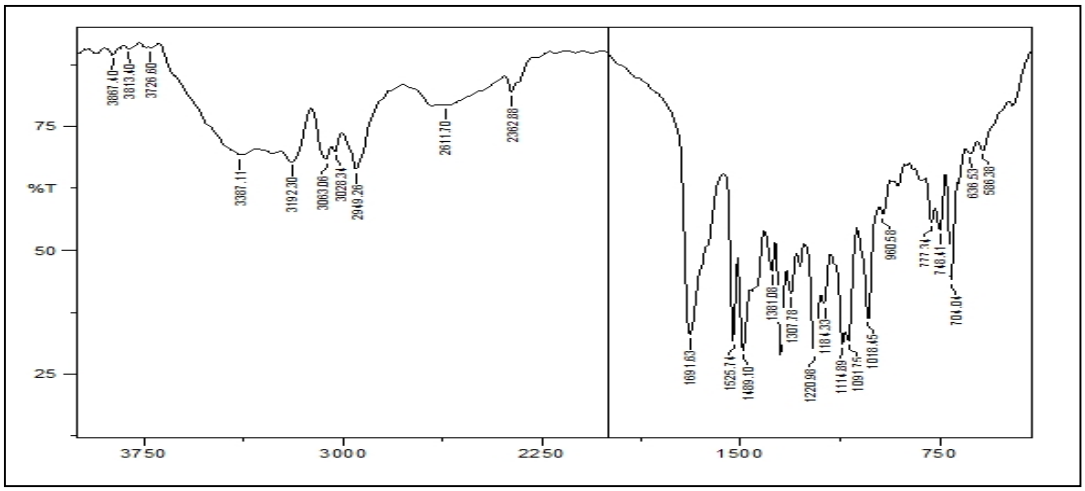

Fig 3.2. FTIR spectra of lercanidipine hydrochloride.

3.7.4. Analytical Method Development of Lercanidipine by HPLC

Assuming at the complexity of the lipids and polymers involved in the formulation, the RP-HPLC method using UV detection was adopted for the determination of lercanidipine in developed carrier systems. The RP-HPLC has a wide acceptance because of its selectivity, sensitivity and simplicity in analyzing variety of drug molecules. The method was developed, optimized and validated as per ICH Q2(R1) guidelines. It was further applied in formulation analysis pertaining to solubility, determination of encapsulation efficiency, *in vitro* drug release studies and *in situ* studies in rat intestine.

3.7.4.1. Optimization of chromatographic conditions

The effect of buffer was studied using phosphate buffer (25 mM) at different pH conditions like, 3.5, 4.0 and 7.0. The retention was found to be 7.46 ± 0.1, 5.24 ± 0.8 and 3.4 ± 0.2 min for pH 3.5, 4.0 and 7.0 respectively. Based on the system suitability parameter like optimum capacity factor, theoretical plates/meter and sharp symmetric peak pH 3.5 was selected as a pH of phosphate buffer. The phosphate buffer was assessed for various buffer strengths. However, there was no much change in the retention time observed when buffer strength was varied, hence, 25 mM phosphate buffer with pH of 3.5 was optimized since elution was rapid and exhibited adequate system suitability parameters. A sharp peak with asymmetric factor < 1.5 was obtained with Grace Vydac C_{18} and Kromasil C_{18} column with retention time of 5.38 ± 0.3 and 4.81 ± 0.1 min respectively. In case of LiChrospher 100 RP-18e and Supelco C_{18} column

retention time was increased to 12.31 ± 0.3 and 9.20 ± 0.8 respectively. The Phenomenex C_{18} column (250 × 4.6 mm i.d., 5m) showed a good peak shape with retention time at 8.03 ± 0.1 min, hence this column was finalized and used for further analysis. The proportion of acetonitrile and phosphate buffer was studied at various ratios like, 80:20, 70:30 and 60:40 % v/v and the retention time of lercanidipine was found to at 6.44 ± 0.3, 7.37 ± 0.2 and 8.62 ± 0.5 min respectively. Here, it can be noticed that as there is increase in acetonitile percentage, drug eluted was faster which might be attributed to the strength of the acetonitrile as well as solubility of lercanidipine in acetonitrile. Based on the experiment, 70:30 (Acetonitrile: Phosphate buffer) was chosen in the present study. The flow rates of 0.8, 1.0 and 1.2 mL/min were tested and it was observed that as the flow rate is increases there was proportionate decrease in the retention time and vice versa. The 1.0 mL/min was selected as optimum flow rate for the present study. All trials of flow rates showed symmetrical peaks with acceptable capacity factor.

Based on the initial optimization of the method following are the chromatographic conditions selected for the validation of lercanidipine.

Stationary phase	: Phenomenex® Gemini C_{18} (250 × 4.6 mm, 5µ)
Mobile phase: Solvent A	: Acetonitrile
Solvent B	: Buffer KH_2PO_4 (25 mM, pH 3.5 ± 0.1)
Solvent ratio	: 70:30 % v/v of A:B
Detection wavelength	: 242 nm
Flow rate	: 1 mL/min
Injection volume	: 25 µL
Column oven temperature	: Ambient 25 °C
Auto sampler temperature	: 4 ± 2 °C
Run time	: 10 min

3.7.4.2. Specificity

In the developed method for estimation of lercanidipine, there was no interference observed at the retention time of the lercanidipine from the placebo formulation of the nanoproliposomes and nanoparticles (Fig. 3.3). The absence in interference clearly signifies the developed method as specific for the estimation of lercanidipine.

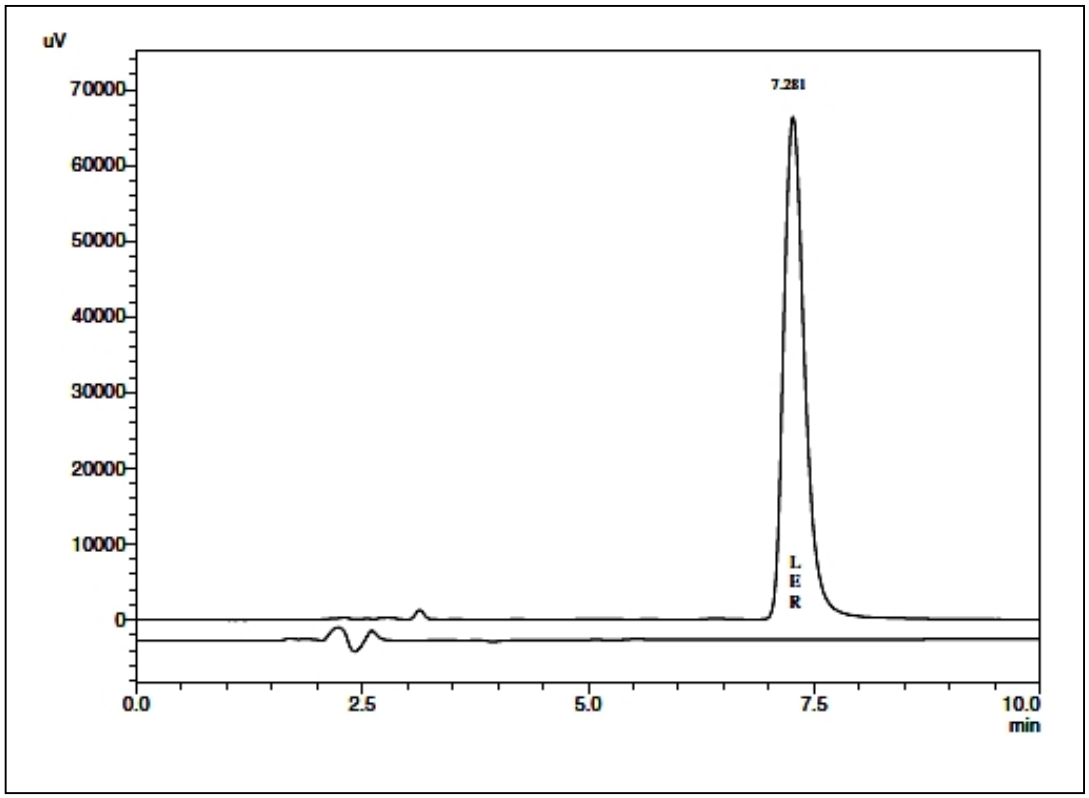

Fig. 3.3. Typical chromatogram of lercanidipine hydrochloride.

3.7.4.3. Linearity

In the present study, the calibration curve of lercanidipine was constructed in the range of 0.5-25.0 µg/mL. The linear regression data for the calibration curve showed good linear relationship in the constructed range. The coefficient of determination (r^2) was found to be >0.999 which indicates that developed method was linear. Slope and intercept were obtained by regression equation (y = mx+c), and these were used for the determination of lercanidipine concentration in the formulation.

3.7.4.4. Accuracy

The mean % recovery at three different concentrations was found to be 98.35 ± 1.78. The recovery experiments fall well within the acceptance limit of 98 to 102% as per ICH guidelines. Hence the developed method was found to be accurate for the estimation of lercanidipine in developed novel formulations.

3.7.4.5. Precision

The developed method had showed repeatability precision of 0.56% RSD and intermediate precision of 0.87% RSD. The values within the acceptance limit indicate that developed method is precise and reproducible.

3.7.4.6. LOD and LOQ

The detection limit of any analytical method is the lowest analyte concentration that produces a response detectable above the noise level of the system. The lower limit of detection was found to be 0.05 µg/mL and limit of quantification was found to be 0.1 µg/ml. These values indicate that the method was sensitive for the quantification of lercanidipine in the developed nanoformulations.

3.7.4.7. Robustness

The robustness of the method was determined by varying different parameters like, buffer pH in the mobile phase (± 0.2), effect of mobile phase composition (± 2%), effect of flow rate (± 10%) etc. By varying these parameters there was no distinct change in the chromatograms observed. Hence the developed method was found to be robust. The %RSD was found to be 0.54, 0.48, 0.25, 0.32, 0.18, 0.20, 0.12, 0.11 which was also well within the acceptable limit of validation procedure.

3.7.4.8. System suitability

The percentage symmetry of lercanidipine was found to be 1.171 which denotes that the peak shape of lercanidipine was symmetrical. The high counts of theoretical plates/meter 56230.1 revealed the column efficiency. The value of capacity factor 2.884 denotes selectivity of the method and lower %RSD values indicates that the developed method was precise.

The results of the analytical method validation along with acceptance criteria are represented in Table 3.1.

3.7.4.9. Application of the developed method

The developed method was successfully applied for the analysis of lercanidipine loaded nanoproliposomes and polymeric nanoparticles. The method was found to be proficient in analyzing encapsulation efficiency, *in vitro* drug release and *in situ* perfusion studies of lercanidipine.

Table 3.1 Summary of the analytical method validation of lercanidipine by RP-HPLC method.

Validation parameters	**Obtained results**			**Acceptance criteria**
Accuracy (% Mean ± SD)	98.35 (± 1.78)			98 - 102
Repeatability precision (% RSD)	0.56%			< 1
Intermediate precision (% RSD)	0.87%			< 2
Linearity (r^2)	≥ 0.9992 (Mean = 0.9996 ± 0.000204) (Slope = 43048.73 ± 289.0387) (Intercept=5399.016 ± 444.0476)			> 0.999
LOD (µg/mL)	0.05			S/N ratio should be 3:1
LOQ (µg/mL)	0.1			S/N ratio should be 10:1
Robustness (% RSD)	Mobile phase pH	3.3	0.54	< 2
		3.7	0.48	
	% buffer	63	0.25	
		67	0.32	
	Wavelength (nm)	237	0.18	
		247	0.20	
	Flow rate (mL/min)	0.9	0.12	
		1.1	0.11	
System suitability	Peak asymmetry (10 %)	1.171		T < 2
	Capacity factor (k^1)	2.884		k' > 2
	Theoretical plates/meter	56230.1		N > 2000
	% RSD of 6 injection	0.15		RSD ≤ 1%

3.7.5. Bioanalytical Method Development

In the present study, valdecoxib was used as an IS, as it was well resolved from lercanidipine with sharp symmetrical peak shape. In the process of extraction of lercanidipine from rabbit plasma, 0.2% v/v HCl in methanol was used as a protein-precipitating agent. This combination agent gave clear baseline with less plasma endogenous interferences at the retention time of lercanidipine and IS. The method was able to extract more than 54.42 ± 1.24% of lercanidipine from rabbit plasma. There was also no interference recorded at retention times of lercanidipine and IS.

3.7.5.1. Optimization of chromatographic conditions

Following are the final chromatographic conditions selected for the determination of lercanidipine from rabbit plasma.

Stationary phase	: Hichrom Kromosil 100-5C_{18} (250 mm × 4.6 mm id)
Mobile phase: Solvent A	: Phosphate buffer (25 mM, pH 3.5)
Solvent B	: Acetonitrile
Solvent ratio	: 50:50 % v/v of A:B
Detection wavelength	: 242 nm
Flow rate	: 1 mL/min
Injection volume	: 100 µL
Column oven temperature	: Ambient 25 °C
Auto sampler temperature	: 4 ± 2 °C
Run time	: 20 min
Rinsing solution	: 30:30:40 (Methanol: Acetonitrile: Water) with 3% IPA

3.7.5.2. Selectivity and sensitivity

The selectivity of the method was assessed by processing six rabbit blank plasma and chromatograms were recorded. There were no chromatographic interferences derived from endogenous substance at the retention time of the drug and IS. The lercanidipine and IS were well separated from the plasma proteins under the described chromatographic conditions. Lercanidipine and IS had retention times of 12.1 ± 0.8 and 8.3 ± 0.6 min respectively as shown in Fig. 3.4. The chromatographic peaks were completely resolved with one another. Therefore, the method was found to be selective for the quantification of lercanidipine in rabbit plasma.

The sensitivity experiment was determined at LLOQ level. In this study, the method could quantify lercanidipine at 25 ng/mL and therefore it was selected as a LLOQ concentration. It also showed acceptable accuracy and precision.

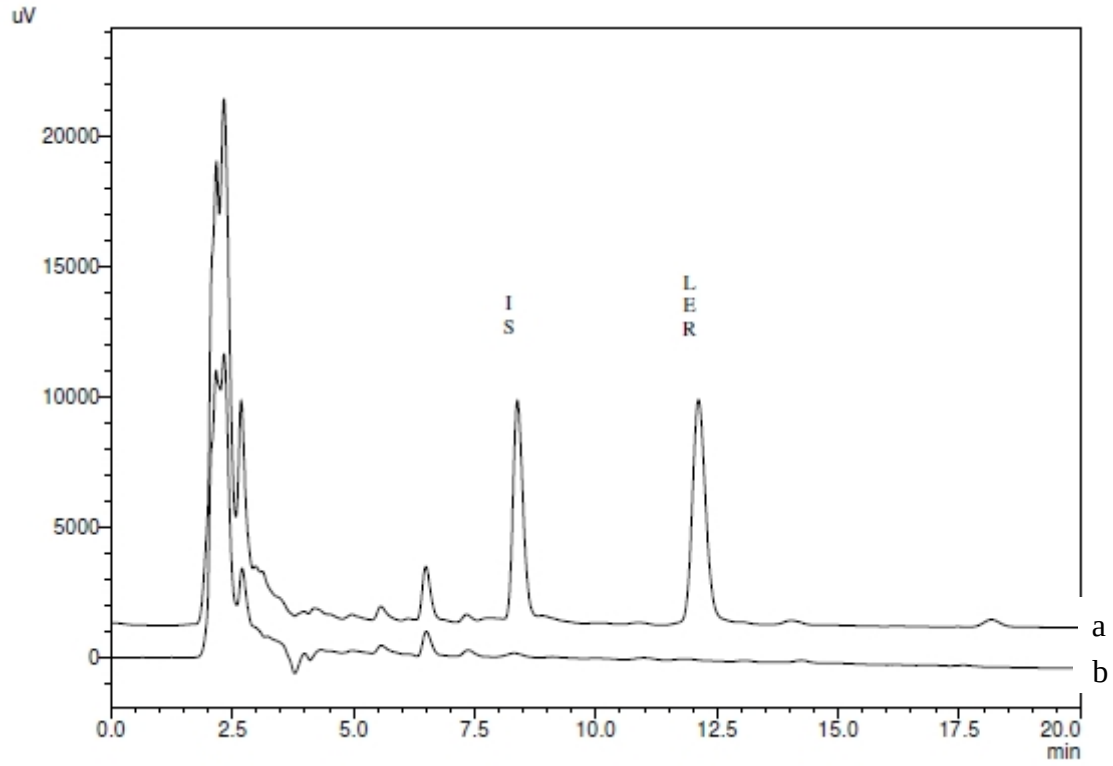

Fig. 3.4. Representative chromatogram of rabbit blank plasma (b) and plasma spiked with standard lercanidipine and IS (a).

3.7.5.3. Linearity

The plasma calibration curve was constructed using seven point calibration standards covering a concentration range from 25.0-2000.0 ng/mL. The calibration model was selected based on the analysis of data by linear regression without intercepts. The calibration curve was prepared by determining the best fit of peak-area ratios (lercanidipine to IS) vs. concentration, and fitted to $y = mx + c$.

3.7.5.4. Accuracy and Precision

The accuracy and precision of all three QC samples, low, medium and high QC samples at intra and inter-day studies were determined by injecting them in a six replicates. All the results were found to be within the acceptance limit. The data demonstrates that the developed method was accurate and precise in quantification of lercanidipine from rabbit plasma.

3.7.5.5. Recovery

The recovery represents the effectiveness of extraction procedure adopted to extract lercanidipine from rabbit plasma in the method. The overall mean recovery of

lercanidipine was found to be 54.42 ± 1.24% whereas, extraction recovery of IS was found to be 70.50 ± 0.70%, indicating the suitability of extraction procedure in quantification of lercanidipine from blank plasma.

3.7.5.6. Stability

The stability of the lercanidipine in rabbit plasma was tested at two levels of QC i.e. LQC and HQC. Lercanidipine was found to be stable in rabbit plasma when stored in the frozen state (-70 °C) for a period of one month. The stability results showed that lercanidipine was stable at all stability conditions. The results of the bioanalytical method validation along with acceptance criteria are summarized in Table 3.2.

3.7.5.7. Application of developed method

The developed bioanalytical method was successfully used for the pharmacokinetic studies of lercanidipine loaded novel carrier systems such as nanoproliposomes and polymeric nanoparticles in rabbits. The developed method was sensitive, selective and accurate to quantify lercanidipine in rabbit plasma.

Table 3.2 Summary results of bioanalytical method validation of lercanidipine in rabbit plasma.

Validation parameters	**Obtained results**			**Acceptance criteria**
Calibration range (Coefficient determination)	25 - 2000 ng/mL $r^2 \geq 0.996$ Mean (0.996 ± 0.0009) Slope (0.0007) Intercept (0.0073 ± 0.00058)			> 0.98 with consistency
System suitability	%CV (Area ratio) ≤ 0.495 %CV (Rt-Analyte) ≤ 0.002 %CV (Rt-IS) ≤ 0.323			%CV ≤ 2.0 for area ratio and Rt of analyte
Sensitivity	%CV = 4.21 Mean % nominal conc. = 99.26			%CV ≤ 20.0 Mean % nominal conc. Should be 80-120%
Intra batch precision (% CV)	LLOQ : 7.53 to 8.4 LQC : 10.71 to 5.4 MQC : 6.09 to 2.3 HQC : 8.43 to 1.5			± 15% deviation from nominal concentration except at LLOQ ± 20% deviation
Intra batch accuracy (%)	LLOQ : 93.65 to 110.56 LQC : 95.80 to 104.98 MQC : 96.75 to 100.42 HQC : 97.11 to 101.34			85 - 115.0% except the LLOQ (80-120%)
Inter batch precision (% CV)	LLOQ:11.54 to -9.76 LQC: 8.81 to -5.46 MQC: 9.21 to -2.3 HQC: 8.63 to -1.5			± 15% deviation from nominal concentration except at LLOQ ± 20% dev.
Inter batch accuracy (%)	LLOQ: 94.45 to 112.34 LQC: 95.67 to 106.26 MQC: 96.07 to 102.45 HQC: 96.63 to 101.34			85 - 115.0% except the LLOQ (80 - 120%)
Recovery analyte Recovery (IS)	LER- 54.42 ± 1.24% IS - 70.50 ± 0.70%			Consistent recovery
Stability	Level	% Nominal	% Change	% Nominal concentration should be within 85-115%, % change should be ± 15%
Freeze thaw (-70 ºC)	LQC HQC	101.74 109.35	4.97 5.35	
Bench top stability (8 h)	LQC HQC	99.14 97.56	-7.26 -0.81	
Auto injector stability (18 h, 4 °C)	LQC HQC	99.63 93.08	-6.31 -9.24	
Long term stability (30 days, -70 °C)	LQC HQC	96.25 94.21	-14.78 -7.64	

3.8. CONCLUSIONS

A rapid, sensitive, specific, accurate and precise RP-HPLC method has been developed and validated for determination of lercanidipine in novel formulations. The method meets validation criteria in accordance with ICH Q2(R1) guidelines. The developed analytical method was successfully applied in analysis of encapsulation efficiency, *in vitro* drug release samples and *in situ* perfusion samples of lercanidipine. Also, the developed bioanalytical method for determination of lercanidipine in rabbit plasma using RP-HPLC was found to be simple, highly sensitive, rapid, specific, accurate and reproducible. The method meets validation criteria in accordance with USFDA guidelines. The simple and less cumbersome protein-precipitation method adopted was efficient in extraction of lercanidipine from the plasma matrix of the rabbit. The developed method was successfully applied for preclinical pharmacokinetic studies for estimation of lercanidipine in rabbits.

3.9. REFERENCES

Alvarez-Lueje, A., Pujol, S., Squella, J.A., Núñez, L.J., 2003. A selective HPLC method for determination of lercanidipine in tablets. J Pharm Biomed Anal. 31, 1-9.

Baranda, A.B., Etxebarria, N., Jiménez, R.M., Alonso, R.M., 2005. Development of a liquid–liquid extraction procedure for five 1,4-dihydropyridines calcium channel antagonists from human plasma using experimental design. Talanta. 67, 933–941.

Charde, S., Kumar, L., Saha, R., 2007. Development and validation of high-performance liquid chromatographic method for estimation of lercanidipine in rabbit serum. Anal Lett. 40, 2128–2140.

Dedhiya, M.G., Chhettry, A., Kapil, R.P., Riccobene, T.A, Abramowitz, W., Rastogi, S. K. 2007. Lercanidipine pH dependent pulsatile release compositions. WO Patent WO/2007/120136.

Hanai, T., (1999). Basic concepts of high performance liquid chromatography. In: Hanai T., (eds) HPLC: A Practical Guide. Cambridge: The Royal Society of Chemistry, pp. 1-10.

http://www.fda.gov/downloads/Drugs/.../Guidances/ucm070107.pdf accessed on 25 August 2012, Guidance for Industry Bioanalytical Method Validation.

http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf accessed on 25 August 2012, Validation of Analytical Procedures: Text And Methodology Q2(R1).

Kalovidouris, M., Michalea, S., Robola, N., Koutsopoulou, M., Panderi, I., 2006. Ultra-performance liquid chromatography/tandem mass spectrometry method for the determination of lercanidipine in human plasma. Rapid Commun Mass Spectrom. 20, 2939–2946.

Kazakevich, Y., Lobrutto, R., (2007). Introduction. In: Kazakevich, Y., Lobrutto, R., (eds) HPLC for Pharmaceutical Scientists. New Jersey: A John Wiley and Sons, Inc., Publication, pp. 3-23.

McClellan, K.J., Jarvis, B., 2000. Lercanidipine: A review of its use in hypertension. Drugs. 60, 1123-1140.

Musmade, P., Deshpande, P. B., Dengle, S.J., Talole, K.B., Pathak, S.M., Bhat, K., Udupa, N., Bairy, K.L., 2010. High performance liquid chromatographic method for determination of clobetasol in rat plasma and its application to skin penetration. J Bioanal Biomed, 2, 001-007.

Musmade, P., Subramanian, G., Srinivasan, K.K., 2007. High-performance liquid chromatography and pharmacokinetics of aceclofenac in rats. Analytica Chimica Acta. 585, 103–109.

Ohnnesian, L., Streeter, A.J., (2002) In: Ohnnesian, L., Streeter, A.J., (eds) Handbook of pharmaceutical Analysis. New York: Marcel Dekker, pp. 585.

Pandit, S.S., Patil, A.T., 2009. Formulation and in vitro evaluation of buoyant controlled release lercanidipine lipospheres . J Microencapsul. 26, 635-641.

Parmar, N., Singla, N., Amin, S., Kohli, K., 2011. Study of cosurfactant effect on nanoemulsifying area and development of lercanidipine loaded (SNEDDS) self nanoemulsifying drug delivery system. Colloids Surf B Biointerfaces. 86, 327–338.

Rozet, E., Ceccato, A., Hubert, C., Ziemons, E., Oprean, R., Rudaz, S., Boulanger, B., Hubert, P., 2007. Analysis of recent pharmaceutical regulatory documents on analytical method validation. J Chromatogr. 1158, 111-25.

Salem II, Idrees J, Al Tamimi JI, Farina P., 2004. Selective and rapid liquid chromatography-mass spectrometry method for the determination of lercanidipine in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci. 803, 201-207.

Shah, V.P., 2007. The history of bioanalytical method validation and regulation: evolution of a guidance document on bioanalytical methods validation. AAPS J. 9, 43-47.

Sica, D.A., Prisant, M.L., 2007. Pharmacologic and Therapeutic Considerations in Hypertension Therapy With Calcium Channel Blockers: Focus on Verapamil. J Clin Hypertens. 9, 1–22.

Srinivas, N.R., 2006. Applicability of bioanalysis of multiple analytes in drug discovery and development: review of select case studies including assay development considerations. Biomed Chromatogr. 20, 383-414.

Srinivas, N.R., 2008. Changing need for bioanalysis during drug development. Biomed Chromatogr. 22, 235-243.

Usha, A.N., Mutalik, S., Reddy. M.S., Ranjith, A.K., Kushtagi, P., Udupa, N., 2008. Preparation and in vitro, preclinical and clinical studies of aceclofenac spherical agglomerates. Eur J Pharm Biopharm. 70, 674–683.

Żwir-Ferenc, A., Biziuk, M., 2006. Solid phase extraction technique - trends, opportunities and applications. Polish J. of Environ. Stud. 15, 677-690.