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Original Article

PROTEIN BINDING STUDY OF FELODIPINE USING VALIDATED LIQUID CHROMATOGRAPHIC METHOD

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ABSTRACT

Objective: The aim of present study was to develop and validate a new simple, easy, selective, precise, accurate reverse phase high-performance liquid chromatography for the estimation of felodipine in bulk and pharmaceutical dosage form.

Methods: The separation was carried on HPLC system consisting C_{18} column (150 mm ×4.6 nm, 5 µm) at room temperature coupled with a phenomenixcolumn silica with flow rate 1 ml/min. The mobile phase used was methanol: acetonitrile in the ratio of 50: 50. The drug was detected using UV-visible detector at the wavelength of 230 nm and run time was 10 min.

Results: The retention time was 3.138 min. Linearity was observed in the concentration range of 5-25µg/ml. The accuracy of the method was assessed by percentage recovery studies at three different levels at 80%, 100% and 120% of its working concentration. The percentage recovery of felodipine in the developed method was found to be in the ranges of from 99.81-100.00% that indicates the good accuracy of the method. The percentage % RSD of precision was found to be less than 2%. The method was validated as per ICH guidelines. The developed method was employed in *in vitro* protein binding studies using semi permeable membrane and performed by plotting calibration curve (peak area v_s concentration) the % drug release of felodipine was calculated.

Conclusion: The proposed method was found to be simple, precise, accurate and consistent. The validated parameters are statistically validated for linearity, precision and limit of detection, limit of quantification, robustness, ruggedness were concluded.

Keywords: Felodipine, RP-HPLC, ICH guidelines, %RSD

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INTRODUCTION

Felodipine is a second-generation calcium channel blocker and commonly used antihypertensive agent. Felodipine belongs to the dihydropyridine group of calcium channel blocker (with amlodipine, isradipine, and nifedipine) and is used primarily in the therapy of hypertension. Like other calcium channel blockers, felodipine acts by blocking the influx of calcium ions into the vascular smooth muscle of voltage-gated sensitive channel cells by relaxing L-type calcium channel in their inactive conformation. By preventing the influx of calcium in smooth muscle cells. Felodipine chemically, ethyl methyl-4(2, 3-chlorophenyl)-1, 4-dihydro-2, 6-dimethyl-5methyl ester, is a calcium agonist widely used in the treatment of hypertension, heart failure, and angina pectoris.

The molecular of felodipine is $C_{18}H_{19}C_{12}NO_4$ and molecular weight 384.254g/mol. It is a white or light yellow, crystalline powder, practically insoluble in water, freely soluble in acetone, acetonitrile, ethanol, methanol, with melting point 145 °C [1]. Felodipine is also used to treat mild to moderate critical hypertension [3]. Felodipinestructure was shown in fig. 1.

In literature, very few RP–HPLC [4-8], UV-Spectrophotometric [9] and HPTLC [10], methods have been reported for determination of felodipine in bulk drugs and pharmaceutical formulations and also in combination with other drugs. Hence an attempt has been made to develop a new assay method with less retention time for protein binding study of felodipine.

MATERIALS AND METHODS

Chemicals and reagents

Felodipine gift sample was procured from Madras pharmaceutical private limited. The com mercial tablet dosage form was obtained from local market labelled felogard (5 mg felodipine). HPLC methanol, acetonitrile and water were obtained from MerckspecialtiesPvt. Chennai, India. All the chemicals and reagents were analytical grade.

HPLC instruments and chromatographic conditions

The RP-HPLC method development and partial validation studies are performed using Shimadzu SPD 20ALC20Asystem. The column used for separation of the analyte is a phenomenex C_{18} column (150 mm ×4.6 mm 5 µm) isocratic elution with methanol: acetonitrile as mobile phase (50:50). The sample was injected through Rhenodyne injector mode. The data was analyzed and saved in spinochrome software. The flow rate of the mobile phase is 1.0 ml/min.

Preparation of mobile phase

The mobile phase was a mixture of methanol: acetonitrile, 50:50v/v solution. The mobile phase was filtered by using 0.45 µm membrane filter and sonicated for 15 min prior to use. The mobile phase is also used as diluent.

Preparation of standard stock solution

Weighed accurately about 50 mg of felodipine and transferred into 50 ml volumetric flask and dissolved in 20 ml of diluent. The final volume made up to the mark with diluent to get 1000 µg/ml concentration. From the above stock solution (1000 µg/ml) 1 ml was pipetted out and transferred into a 10 ml clean and dry volumetric flask and diluted with solvent to get 100 µg/ml solution.

Selection of wavelength

From the stock solution (100µg/ml). Further dilutions were made to get 10 µg/ml solution was scanned in UV-visible spectrophotometer in the range of 200-400 nm, where the mixture of methanol (50%) and acetonitrile (50%) was used as a blank. The wavelength of maximum absorbance (λ_{max}) of felodipine was found at 230 nm. The spectrum was shown in fig. 2.

Preparation of sample solution for assay

Twenty tablets of felodipine were weighed and the average weight was calculated. Tablets were finely powdered and the weight equivalent to 50 mg (1.57g) was transferred into a 50 ml volumetric flask 25 ml of diluent was added and sonicated for 20 min. The final volume was made up to the mark with diluent to get 1000 µg/ml solution. The above solution was filtered by using Whatman filter paper 42. From the above solution (1000 µg/ml), 1 ml was transferred into 10 ml volumetric flask and made up to the mark with diluent to get 100 µg/ml solution. Further dilution was made by pipetting out 1 ml of 100 µg/ml solution into 10 ml volumetric flask and made up to the mark with diluent to get 10 µg/ml solution. This solution was injected under chromatographic conditions and the peak area was calculated.

Method development

The HPLC procedure optimized with a view to developing a method for Assay. The column used for separation of analyte was phenomenex C_{18} column (150 mm × 4.6 mm 5 µm) using isocratic elution with methanol and acetonitrile as mobile phase (50:50). The sample was injected through ryanodine injector mode. The data was acquired, stored and analyzed in spinchrome software. The flow rate of mobile was 1.0 ml/min at room temperature. The retention time of both standard and sample felodipine was 3.140 min. The peak shape of felodipine was found to be symmetrical.

Validation of the developed method

The developed method was validated as per ICH guidelines for the following parameters.

Linearity

The linearity of a method is its ability to obtain test results that are directly proportional to the sample concentration over a given range. The linearity was performed in the concentration range of 5 to 25µg/ml (5, 10, 15, 20, and 25). Then the five standard solutions of felodipine were injected into HPLC system at 230 nm by using diluent. The peak area and concentration were plotted to get a standard calibration curve were shown in fig. 3.

Precision

The closeness of agreement between a series of measurements. Multiple sampling of homogeneous samples under a prescribed condition. The precision of the method was demonstrated by method precision, system precision, intraday precision, interday precision.

System precision

The system precision was performed by injecting six replicate injections of the standard solution into HPLC system. The peak area and RSD were calculated, were found to be not more than 2%.

Method precision

The method precision performed by preparing six replicate sample preparations as per testing procedure and injected into HPLC system. The peak area and RSD were calculated, were found to be not more than 2%.

Intraday precision

Intraday precision performed by preparing six replicate sample preparations as per testing procedure and injected into HPLC system. The peak area and RSD were calculated were found to be not more than 2%.

Interday precision

Interday precision performed by preparing six replicate sample preparations as per testing procedure and injected into HPLC system.

Accuracy

The accuracy of an analytical method is the closeness of results obtained by that method to the true value for the sample. It is expressed as recovery (%) and determined by the standard addition method. Samples were spiked with 80%, 100%, and 120% of the standard and analyzed. The experiment performed in triplicate. Recovery (%) and RSD (%) were calculated for each concentration.

Limit of detection (LOD)

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value. The detection limit (LOD) may be expressed as

$LOD = 3.3\ \sigma/S$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

Limit of quantification (LOQ)

LOQ is defined as the lowest concentration of the substance (analyte) in a sample that can be estimated quantitatively with acceptable precision, accuracy and reliability by a given method under stated experimental conditions. Quantification limit (LOQ) may be expressed as

$LOQ = 10\ \sigma/S$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. It is concluded that the method is robust as it is found that the %RSD is less than 2. The results are shown in table 3.

Ruggedness

Ruggedness is defined as the reproducibility of results when the method is performed under actual use conditions. This includes different analysts, laboratories, columns, instruments, the source of reagents, chemicals, solvents etc. Method ruggedness may not be known when a method is first developed, but insight is obtained during subsequent use of that method. The results are shown in table 4.

Protein binding studies

Calibration curve

The calibration curve was performed in the concentration range of 10 to 60µg/ml. Then the six standard solutions of felodipine were injected into HPLC system at 230 nm by using diluent. The peak area and concentration were plotted to get a standard calibration curve was shown in fig. 6.

Preparation of bovine serum albumin

Bovine serum albumin solution was prepared by dissolving the 300 mg of bovine serum albumin in 25 ml of distilled water.

Preparation of stock solution

Weighed accurately about 50 mg of felodipine was transferred into clean and dried 50 ml volumetric flask. To that 25 ml of diluent was added and sonicated for 5 min and the final volume was made up to the mark with diluent to obtain 1000µg/ml. From the above solution, 100µg/ml concentration was prepared by transferring 1 ml into 10 ml volumetric flask and made up to the mark with diluent.

Preparation of semi permeable membrane

An egg shell was kept in concentrated HCL solution for about five minutes. The outer layer gets dissolved leaving membrane. The membrane was removed and washed with distilled water.

Procedure

Both sides open ended cylinder was taken and semi permeable membrane was tied to one end. 20 ml of bovine serum albumin and 20 ml of 100µg/ml felodipine solution was placed inside the semi permeable membrane. The cylinder was kept inside the beaker containing mobile phase.5 ml of solution was collected at different intervals (10-60 min) from the beaker and the same volume was replaced with mobile phase to maintain sink conditions. Then the solution was injected into HPLC system. From the chromatogram peak area was noted and percentage drug release was calculated. Results are shown in table 5.

RESULTS AND DISCUSSION

The current RP-HPLC technique is most accurate, reliable and precise method of analysis for the quantitative estimation of felodipine in bulk drugs and its pharmaceutical dosage form. The current method system suitability was accounted by measuring tailing factor, plate count for the peak of felodipine from standard solution and percentage RSD of the area for the peak of felodipine from six replicate injections of standard solution. The obtained values are 0.9 as tailing factor (should not be more than 2.0), 15500 as plate count (should not be less than 2000), and percentage RSD of the area as 0.5 (should not be not more than 2.0). The typical felodipine retention time in current developed method was about 3.140. All Variations resulted with the very good percentage RSD of assay i.e. less than 2.0%.

Linearity and range

The calibration curve showed good linearity in the range of 5–25µg/ml for felodipine API with correlation coefficient (R^2) value of 0.994 (fig. 1). A typical calibration curve has the regression equation y = 149.9+153.2 for felodipine.

Precision

Method precision

Method precision was determined by performing sample under the test concentration showed % RSD less than 2 concerning % assay of the drug which indicate that the method developed is method precise by the test of repeatability and hence can be understood that the method gives consistently reproducible results shown in table 4.

System precision

Six consecutive injections of the sample at working concentration showed % RSD less than 2 concerning % assay of the drug which indicates that the method developed is system precise by the standard of repeatability and hence can be understood that the method gives consistently reproducible results shown in table 4.

Intraday precision

Intraday precision studies were performed by injecting a standard solution of 10µg/ml at several intervals i.e., 1, 3 and 5 h within a day and the relative Standard deviation was calculated. The results are shown in table 4.

Interday precision

The Interday precision of the proposed method was determined by analysing the corresponding responses on different days for 10 µg/ml felodipine standard solution. The relative standard deviation was calculated. The results are shown in table 4.

Limit of detection and limit of quantification

Method sensitivity was estimated in terms of limit detection and limit of quantification by using formula. The detection of LOD and LOQ is based on the standard response of felodipine by plotting separate calibration curve.

LOD=3.3×150.123/165.7=2.9 µg/ml

LOQ=10×150.123/165.7=9.0 µg/ml

Where s is the slope of calibration plot, σ is standard deviation calculated using values of y-intercept of the regression equation.

Accuracy

The mean recovery of the method determined by standard addition method and found to be 100%. The values of % recovery and % RSD, listed in table 3 indicate that the method is accurate.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters. For the proposed method robustness was checked by making small deliberate changes in wavelength i. e,±0.2 nm. After making these small changes showed thevery slight difference in peak area for estimation of felodipine. The values are given in table 2 point out method is robust.

Ruggedness

It is the reproducibility of test results under operating condition from analyst to analyst, column to column variations. The ruggedness of the method was performed by comparing the results between two analysts, columns in the same laboratory and results given table 3.

Protein binding studies

Protein binding studies were performed based on the calibration curve (concentration v_s peak area) and then calculating the percentage drug release.

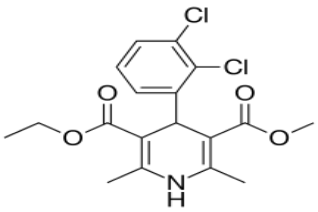

Fig. 1: Structure of felodipine.

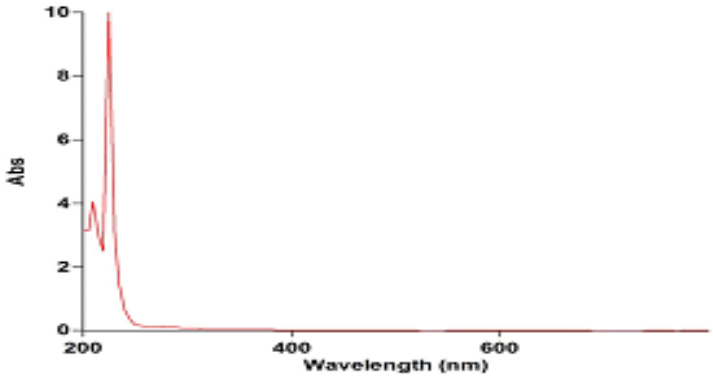

Fig. 2: UV spectrum of felodipine

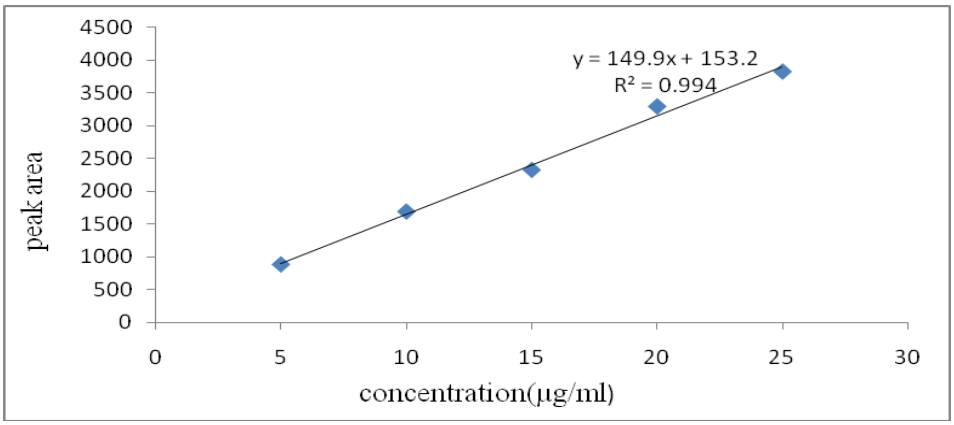

Fig. 3: Calibration curves of felodipine

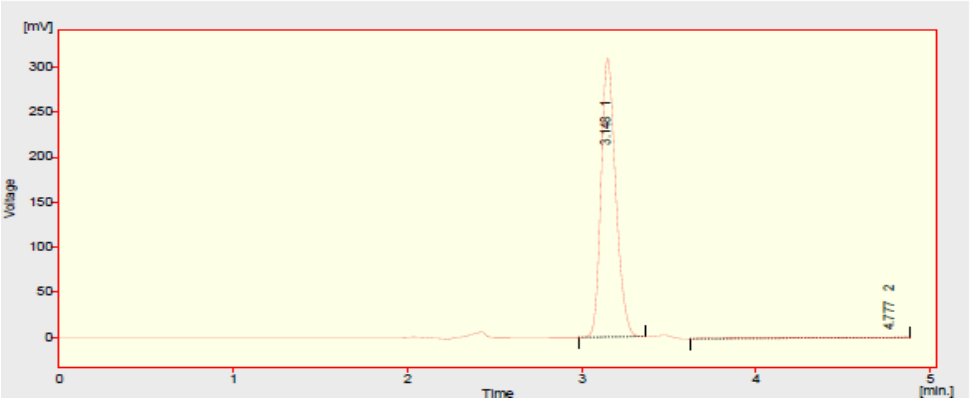

Fig. 4: Typical chromatogram of standard felodipine

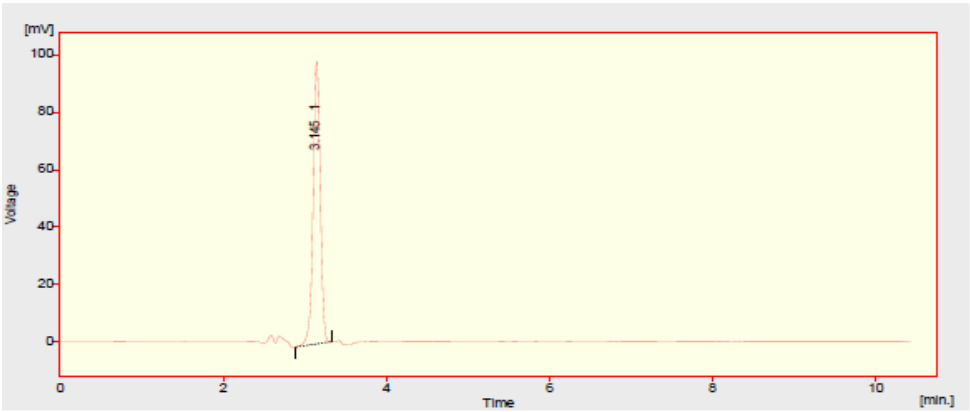

Fig. 5: Typical chromatogram of sample felodipine

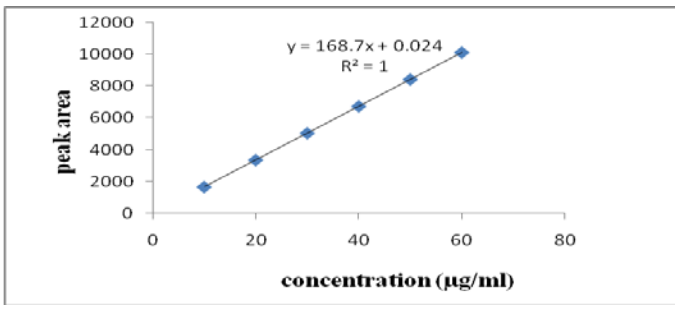

Fig. 6: Standard graph of felodipine

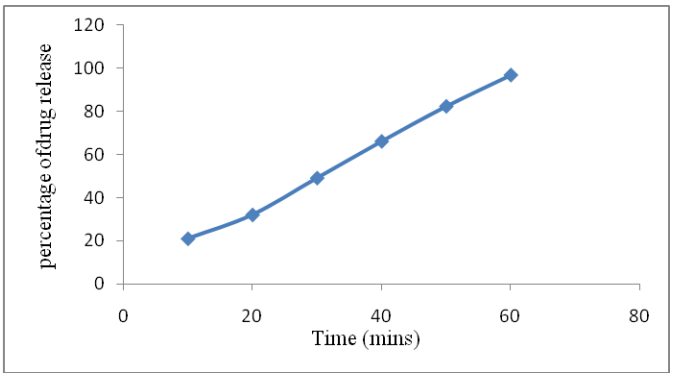

Fig. 7: Drug release kinetics of felodipine

Table 1: Accuracy of felodipine

S. No.	Recovery level	Amount added (µg/ml)	Amount spiked (µg/ml)	% recovery	Statistical analysis of % recovery	
		10	8	99.75	Mean	99.81
1.	80%	10	8	99.3	Std. dev	0.533
		10	8	100.4	%RSD	0.554
		10	10	100.35	Mean	99.91
2.	100 %	10	10	99.9	Std. dev	0.425
		10	10	99.5	%RSD	0.425
		10	12	99.81	Mean	100.006
3.	120%	10	12	100.06	Std. dev	0.176
		10	12	100.15	%RSD	0.175

Table 2: Robustness of felodipine

S. No.	Concentration(µg/ml)	Peak area at different wavelengths	
		228 nm	232 nm
1.	10	3321.038	3595.658
2.		3283.931	3586.846
3.		3318.351	3635.130
		Mean = 3341.106	Mean = 3605.878
		SD = 50.437	SD = 50.437
		%RSD=1.509	%RSD=1.509

Table 3: Ruggedness of felodipine

S. No.	concentration(µg/ml)	Peak area at different analysts		Peak area of different columns	
		Analyst-1	Analyst-2	Column-1	Column-2
1.	10	1270.200	1266.450	1424.208	1266.450
2.		1260.458	1284.904	1454.03	1284.904
3.		1266.449	1281.802	1339.320	1281.802
		Mean = 1275.702	Mean = 1285.012	Mean = 1439.10	Mean = 1285.012
		SD = 6.949	SD = 9.83	SD = 21.075	SD = 9.8
		%RSD=0.5	%RSD=0.7	%RSD=1.4	%RSD=0.7

Table 4: Validation and system suitability parameters

Parameters (units)	Results
Linearity range(µg/ml)	5-25
Correlation coefficient	0.994
Slope	149.9
Intercept	153.2
Recovery (%)	100
Assay	100%
System precision	1.3
Method precision	1.4
Intraday precision	0.7
Interday precision	0.9
Robust	Robust
Retention time	3.140
Tailing factor	1.5

Table 5: Drug *in vitro* protein binding study data

S. No.	Time (min)	% drug release
1.	10	21.038
2.	20	32.076
3.	30	49.114.
4.	40	66.123
5.	50	82.345
6.	60	96.806

CONCLUSION

There is no specific and short retention time analytical method reported in literature for felodipine. The developed method gaveadequate resolution with a short analysis time. The method was validated as per ICH guidelines. All the validation parameters were found to be well within the acceptance criteria. We conclude that the method was accurate, reproducible, repeatable, linear, precise, and reliable. This method is used to perform protein binding study of felodipine with egg membrane and the total drug release after 60 min was found to be 96.8% and it was good agreement with reported pharmacokinetic data and drug release kinetics.

Hence the developed methodis used for routine analysis of felodipine in bulk and pharmaceutical formulations in quality control and pharmacokinetic studies.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest

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