LC-MS Method Development and validation for the estimation of Felodipine in human plasma and Stability studies of freeze thaw analyte.

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Abstract:

Purpose: A simple reverse phase liquid chromatographic and mass spectroscopic analytical method has been developed and validated for estimation of felodipine in plasma. **Methods:** The separation was carried out on Princeton SPHER C18 (150 x 4.6 mm i.d. of 5) as Stationary phase, Mobile Phase: Acetonitrile : 2mM ammonium acetate Elution mode : Isocratic A: B= 80:20% v/v Flow rate: 0.8 ml/min using SPD M-10AVP photo diode array detector at 38.10 nm. **Results**: The described LC MS method was linear over a concentration range of 0.8-13.0ng/ml. Pantaprazole was used as internal standard. The felodipine and pantaprazole showed retention factor of 2.97 respectively. The limit of detection (LOD) and the limit of quantification (LOQ) for felodipine was 0.10 ng/ml, 0.50 ng/ml and for pantaprazole 0.06, 0.21 ng/ml respectively. The stability of the drug spiked human plasma samples during three freeze thaw cycles were stable in plasma for about one month when stored at frozen state. **Conclusions:** The results of the study showed that the proposed LC MS method is simple, rapid, precise and accurate, which is useful for the estimation of felodipine in bulk fluids and biological plasma sample analyte with accuracy and reproducibility.

Keywords: Felodipine, LC MS method, Pantaprazole and Freeze thaw cycles.

Introduction:

Felodipine is slightly yellowish, crystalline powder with melting point 145^0C, Photosensitive and Insoluble in water and is freely soluble in Dichloromethane and ethanol acts as Calcium antagonist-calcium channel blocker. It is O3-ethyl O5-methyl 4-(2,3-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate with Molecular formula $C_{18}H_{19}Cl_2NO_4$, Molecular weight 384.2540 daltons, Log P of 4.36 with 2.5 to 10 mg daily dose. Felodipine Prevents calcium from being released within muscle cells of the small arteries and thereby causes the muscle to relax and the arteries to dilate or expand [1-4].

Pharmacokinetics: Oral bioavailability is 15%, 1% Urinary excretion with 99% Bound in plasma, Clearance 0.8 l/min, Volume of distribution of 10 l/Kg and 11h Half-life. Mean peak concentrations following oral administration of felodipine are reached in 2.5 h. Both peak plasma concentration time curve (AUC) increases linearly with dose up to 20 mg. Felodipine is taken of 5-10 mg once-a-day, maximum 10mg two times a day [5-6].

Literature survey revealed that felodipine is estimated by Felodipine by High-performance Liquid Chromatography-tandem Mass Spectrometry (HPLC-MS/MS), high-performance liquid chromatography coupled to tandem mass spectrometry, Spectrophotometric, Spectrofluorometric, High-performance liquid chromatography with amperometric detection, HPLC and Chemometrically-Assisted Spectrophotometric Estimation, liquid chromatography-tandem mass spectrometry, liquid chromatography/UV diode array detection/atmospheric pressure chemical ionization mass spectrometry, Several methods have been reported for quantification of felodipine in plasma as mentioned above. The present investigation reports a simple, rapid, sensitive, and reproducible LC MS method for analysis of felodipine in plasma, using pantaprazole as internal standard (IS) [7-12].

The Plan of the present study is as follows: **Optimization of chromatographic conditions** were proposed to be developed and optimized like selection of Ionization, selection of initial separation conditions, nature of the stationary phase, nature of the mobile phase (pH, peak modifier, solvent strength, ratio and flow

rate) and Selection of internal standard. The developed method were also proposed to be validated using the various validation parameters such as, Accuracy, Precision, Linearity and Range, Limit of detection (LOD) / Limit of quantitation (LOQ), Selectivity / specificity, Robustness / ruggedness, Stability and System suitability as per ICH guidelines [13]. The Felodipine present in the biological fluid was proposed to be estimated.

Materials and methods:

Chemicals, reagents and Instrumental Conditions: Working Standard of Felodipine was obtained from M/s Saimirra Innopharm Chennai, India and Pantaprazole internal standard was gifted by Dr Reddy's Laboratories, Hyderabad, India. Tablets were procured from the local market. Acetonitrile of HPLC grade by Merck, Ammonium Acetate AR grade obtained from Qualigens fine chemicals and Water HPLC grade from Milli-Q RO system were used. All other reagents used were of HPLC grade. Shimadzu LC2010A HT LCMS system with following configuration was used i.e. LC-10 AD-vp solvent delivery system (pump), SIL 10 AD-vp Auto injector, SPD M-10AVP photo diode array detector, CTO 10 vp column oven, GU 14AM degasser, LC – MS solution data station, Analytical columns of Symmetry (Waters) C18 (150 x 4.6 mm i.d., 5), Shimadzu 160A UV-VIS spectrophotometer. Sartorius single pan digital balance (R200D & 1702) Systronics - pH meter, pH system 361 and Ultra Sonicator were used for investigation.

Ethical approval: The detail of the study was approved by the Institutional Ethical Committee of J.S.S. College of Pharmacy. The volunteers were also instructed to refrain from consuming alcohol, smoking or other stimulant drinks during investigation period.

Chromatographic Conditions:

LC Conditions Stationary phase : Princeton SPHER C18 (150x4.6 mm i.d.,5) Mobile Phase: Acetonitrile:2mM ammonium acetate Elution mode : Isocratic A: B= 80:20% v/v Flow rate : 0.8 ml/min Injection volume : 10µl using Auto injector. **MS Conditions** Interface: ESI Operation mode : SIM Polarity : Negative Probe temperature : Ambient CDL Temperature : 250º C Block Temperature : 200º C Detector voltage : 1.3kv Nebulizer Gas flow: 1.5 l/min Drying gas : 10 L/min Detection : Felodipine – 382.05 Data station : LC-MS solution data station Internal Standard: Pantoprazole - 382.10. The mobile phase was filtered through a 0.22 µ membrane and degassed using ultrasonicator. The experiments were carried out at room temperature of about 20^0C.

Validation of the method Validation is a process which involves confirmation or establishment by laboratory studies that a method / procedure / system / analyst can give the required accuracy, precision, sensitivity, ruggedness, etc. In the most basic form, validation of an analytical procedure demonstrates that the procedure developed is suitable for its intended purpose. Validation of the method was carried out after the development of the HPLC method. This section describes the procedure followed for the validation of the methods developed.

Accuracy The accuracy of the drug was calculated by comparing the concentration obtained from the relative recovery of drug supplemented plasma to the actually added concentration. To drug supplemented plasma, standard Felodipine solution (Three levels) and internal standard solution were added. The resulting sample solution was analysed and the response factor was calculated. The absolute recovery of Felodipine was determined by comparing the response factor of the drug obtained from the plasma with response factor obtained by the direct injection of Felodipine in mobile phase at three different levels. Recovery studies were carried out for three levels at six times and the % recovery, mean, standard deviation and % CV was calculated.

Precision The precision of the method was determined by intraday precision and interday precision. The intraday precision was evaluated by analysis of plasma samples containing Felodipine at three different concentrations containing internal standard using nine replicate determinations for three occasions. The interday precision was similarly evaluated over two week period. Precision studies were carried out for three levels at nine times and three occasions. The mean concentration, standard deviation and % CV were calculated.

Selectivity Method I: The six blank plasma samples obtained from six different volunteers were analysed and the spectrums were recorded. These spectrums were compared with the spectrums obtained from standard solutions. Each spectrum was tested for interference. The combination of the sample preparation procedure and spectrums provided an assay which must be free from significant interfering endogenous plasma components at the retention times of Felodipine and the internal standard. Method II: This method involves the peak purity test method using MS spectrum.

Linearity and Range The different concentrations of standard solutions were prepared to contain 0.8 - 13 ng /ml of Felodipine containing 10.00 µg/ml of internal standard. These solutions were analysed and the peak

areas and response factors were calculated. The calibration curve was plotted using response factor Vs concentration of the standard solutions. The calibration curve was constructed on six different days over a two weeks period to determine the variability of the slopes and intercepts.

Stability Studies The stability studies of plasma samples spiked with Felodipine were subjected to three Freeze thaw cycles, Short term stability at room temperature for 3 hrs and Long term stability at 70^0 C over four weeks. In addition, stability of standard solutions was performed at room temperature for 6 hr and freeze condition for four weeks. The stability of triplicate spiked human plasma samples following three freeze thaw cycles was analysed. The mean concentrations of the stability samples were compared to the theoretical concentrations. The stability of triplicate short term samples spiked with Felodipine was kept at room temperature for 1.00 to 3.00 h before extraction. The plasma samples of the long term stability were stored in the freezer at 70^0 C until the time of analysis. The mean concentrations of the stability samples were compared to the theoretical concentrations. The stability of the Felodipine standard solution at room temperature for 6 h and freezed condition for two weeks were demonstrated by comparing a freshly prepared standard solution. The stability of the internal standard stock solution was also performed by comparing a freshly prepared standard solution containing internal standard.

System Suitability Studies The parameters namely column efficiency, resolution, peak asymmetry factor and capacity factor for the standard solutions was calculated.

Limit of detection Testing method: By HPLC In-house method Based on Signal- to- Noise (3:1) approach Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of Felodipine with those of blank samples (i.e. mobile phase) and establishing the minimum concentration at which the Felodipine can be reliably detected.

Limit of quantitation Testing method**:** By HPLC In-house method Based on Signal to Noise (10: 1) approach Determination of the signal- to- noise ratio is performed by comparing measured signals from samples with known low concentrations of Felodipine with those of blank samples (i.e. mobile phase) and establishing the minimum concentration at which the Felodipine can be reliably quantified.

Ruggedness/robustness The ruggedness of the method was studied by changing the experimental conditions such as, Different operators in the same laboratory, Changing the source of reagents and solvents (different manufacturers like S.D. Fine Chemicals, Ranbaxy, Qualigens Fine Chemicals) and Changing to another column of similar type and estimating the drugs using the assay procedure. The separation factor, resolution time and peak asymmetry factors were then calculated. For demonstrating the robustness of the method, slight variations in the optimised conditions were made and the standard solution was injected. The variation made were, 1 % in the ratio of acetonitrile in the mobile phase, 0.05 ml of the flow rate. Then the separation factor, retention times and peak asymmetry were calculated.

Estimation of felodipine in plasma A Shimadzu LC-MS system was used for the analysis with the following chromatographic conditions. **LC Conditions** Stationary phase: Princeton SPHER C18 (150 x 4.6 mm i.d., 5) Mobile Phase: Acetonitrile: 2mM ammonium acetate Elution mode: Isocratic A: B= 80:20% v/v Flow rate: 0.8 ml/min Injection volume : 10 µl using Auto injector. **MS Conditions** Interface : ESI Operation mode : SIM Polarity : Negative Probe temperature : Ambient CDL Temperature : 250º C Block Temperature : 200º C Detector voltage : 1.3kv Nebulizer Gas flow : 1.5 l/min Drying gas : 10 l/min Detection : Felodipine – 382.05 Data station : LC-MS solution data station Internal Standard : Pantoprazole – 382.10 The mobile phase was filtered through a 0.22 µ membrane and degassed using ultrasonicator. The experiments were carried out at room temperature of about 20^0C.

Preparation of Felodipine standard stock solution Accurately transferred 100 mg of Felodipine working standard into a 100 ml volumetric flask and dissolved in acetonitrile and made the final volume with water and acetonitrile (1:1) to give 1.0 mg/ml solution of Felodipine. Labeled and stored the solution in a refrigerator below 8°C. Preparation of Felodipine standard solution Standard solution for Calibration curve Prepared, 10 ml each of 16, 20, 40, 80, 120, 240, 260 ng /ml of Felodipine standard solutions using the Felodipine standard stock solution and mobile phase and labeled and stored at -2 ± 2^0 C until analysis. Standard solution for QC Prepared, 10 ml each of 16, 120 and 240 ng/ml of Felodipine standard solutions using the Felodipine standard stock solution and mobile phase and stored at -2 ± 2^0C until analyzed.

Preparation of stock and Calibration curve samples (CC) Using 0.5ml of Felodipine standard stock solution, 10.0 ml each of 0.8, 1, 2, 4, 6, 12, 13 ng /ml of Felodipine calibration curve samples was prepared and made up the volume with blank plasma and stored at -7 ± 2^0 C until processing.

Preparation of Quality control (QC) Samples Using 0.5ml of Felodipine standard stock solution, 10.0 ml each of 0.8, 6 and 12 ng/ml of Felodipine calibration curve samples was prepared and made up the volume with blank plasma, transferred in to different 2ml centrifuge tubes and stored at -7 ± 2^0 C until processing.

Preparation of plasma samples At the time of analysis, the samples were removed from the deep freezer and kept in the room temperature and allowed to thaw. A volume of 0.5 ml of sample was pipetted into 2.0 ml centrifuge tube with this 500 µl of internal standard solution (10.0 µg/ml) and 0.5 ml of precipitating agent (10% Perchloric acid) was added. The resulting solution was vortexed for 5 minutes and centrifuged at 4000 r/min for 10 min. Supernatants from the above solutions were separated and used for the analysis.

Results of the investigation of felodipine in plasma by LC MS estimation method:

Figure.1 Typical chromatogram of blank plasma

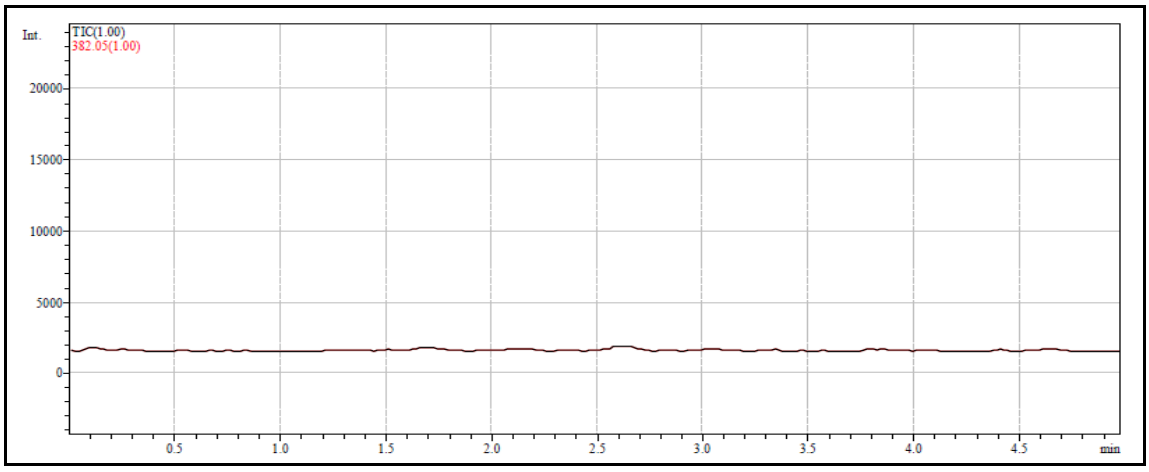


Figure.2 Mass spectrum of felodipine

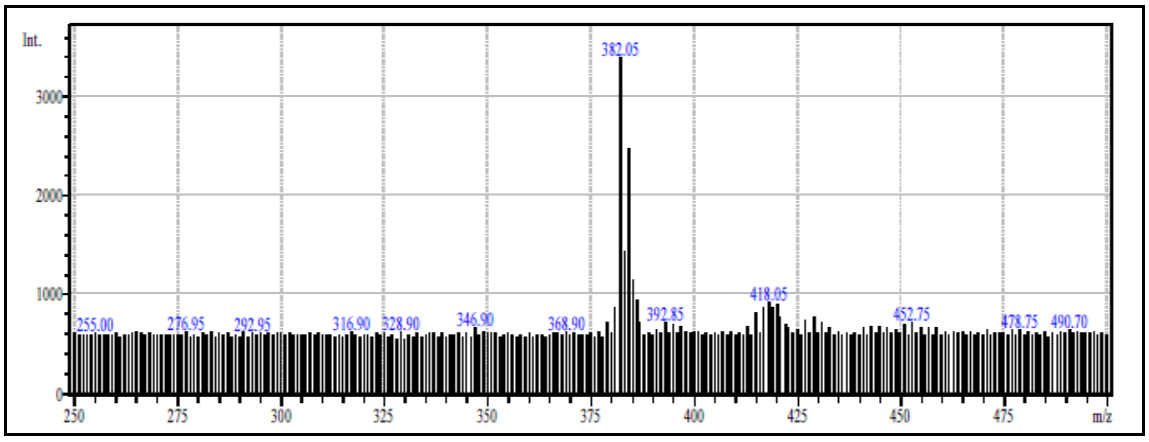


Figure.3 Mass spectrum of internal standard

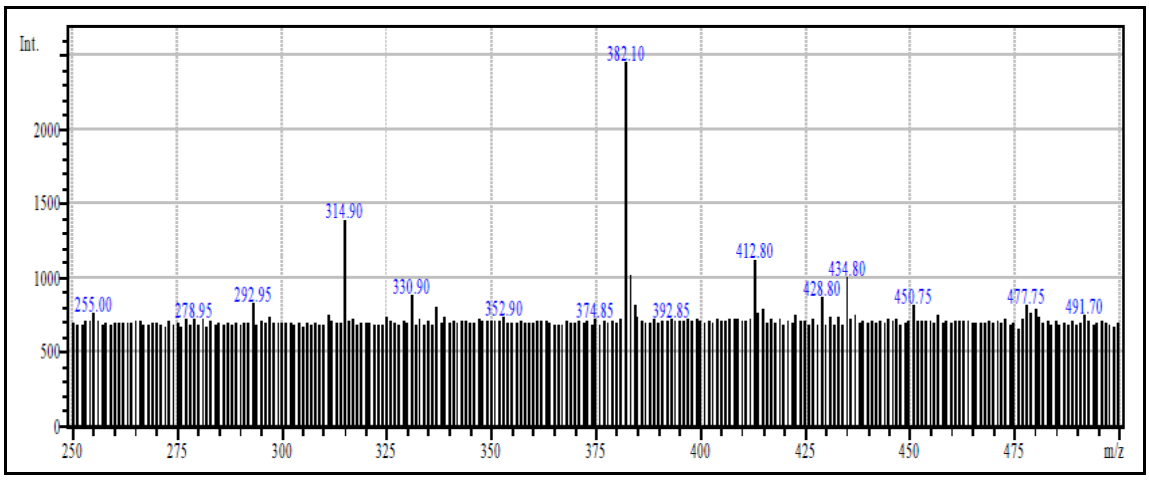


Figure.4 Typical standard chromatogram of felodipine and internal standard

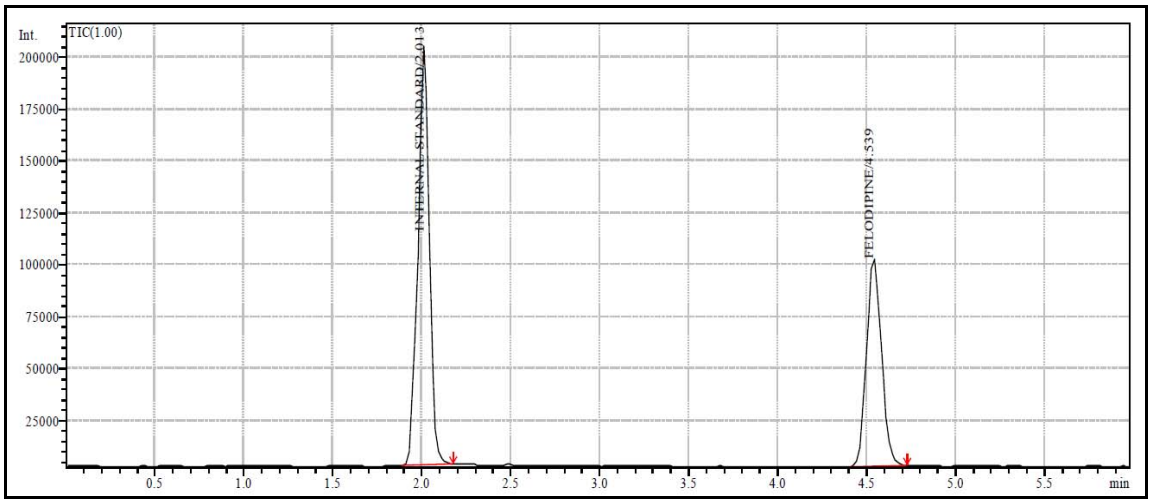


Figure.5 Typical sample chromatogram of felodipine and internal standard

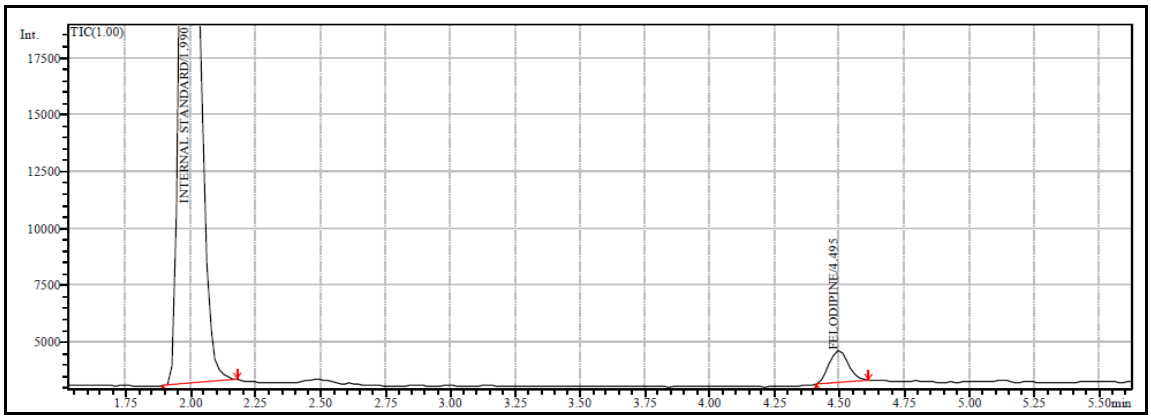
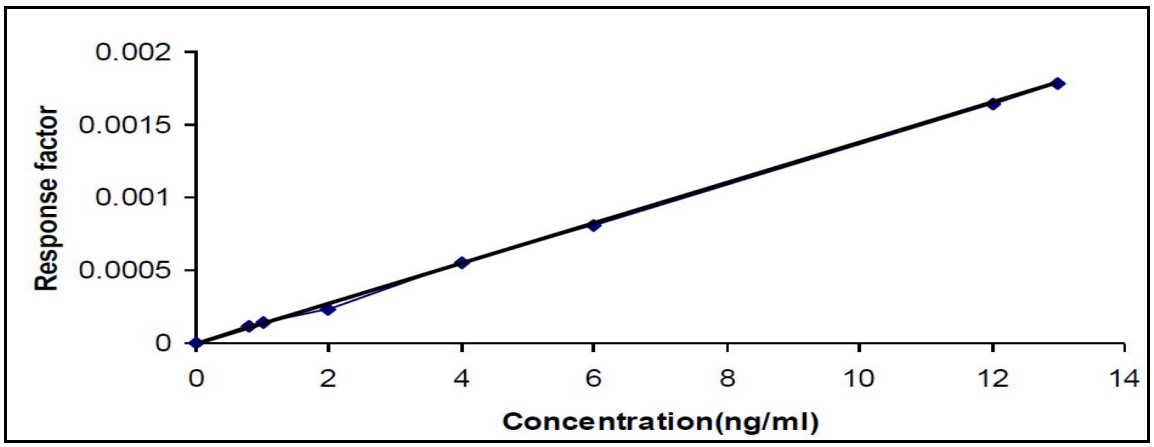


Figure.6 Calibration curve of felodipine



The results observed by LC MS method for recovery studies of felodipine are showed in table no. 1. The Precision studies were indicated in table no.2. Followed by linearity and range results in table no.3. The table no.4 indicates Stability of Felodipine in plasma during storage and sample handling followed by LC-MS System suitability studies for Felodipine in table no.5.

Table 1: Felodipine Accuracy and recovery studies

Level	Concentration of drug added ng/ml	Amount of drug recovered (ng/ml) in plasma sample	Recovery (%)	Amount of Drug recovered (%) in Mobile phase	Relative Recovery (%)
Level I	0.8	0.76±2.51	Mean: 97.57 CV: 2.92 N: 6	Mean: 99.05 CV: 1.04 N: 6	99.36
Level II	6	5.86±1.96	Mean: 98.05 CV: 1.52 N: 6	Mean: 98.96 CV: 1.73 N: 6	98.94
Level III	12	11.84±1.08	Mean: 98.72 CV: 0.91 N: 6	Mean: 99.27 CV: 1.01 N: 6	98.95

Table 2: Felodipine Precision Studies (ng/ml)

Nominal Concentration (ng/ml)			
Sno	**LQC 0.8**	**MQC 6**	**HQC 12**
1	0.7956	5.9865	11.99652
2	0.7523	5.8427	11.7532
3	0.7135	5.7312	11.5984
4	0.7026	5.4023	11.8537
5	0.6938	5.5643	11.3764
Mean	0.73	5.710	11.71
± SD	0.40	0.23	0.23
% CV	5.77	4.02	1.96
% Nominal	91.45	95.09	97.58
n	5	5	5
Nominal Concentration (ng/ml)			
Sno	**LQC 0.8**	**MQC 6**	**HQC 12**
1	0.7853	5.9952	11.8956
2	0.7742	5.6327	11.9534
3	0.7612	5.8348	11.7635
4	0.7398	5.7219	11.7952

5	0.7023	5.5023	11.8234
Mean	0.75	5.74	11.85
± SD	0.03	0.19	0.08
% CV	4.36	3.29	0.65
% Nominal	94.07	95.62	98.72
n	5	5	5
Nominal Concentration (ng/ml)			
Sno	**LQC 0.8**	**MQC 6**	**HQC 12**
1	0.7562	5.9862	11.8695
2	0.7243	5.8324	11.9532
3	0.7395	5.4975	11.7634
4	0.7481	5.7364	11.5132
5	0.7235	5.4231	11.7627
Mean	0.74	5.70	11.77
± SD	0.01	0.23	0.17
% CV	1.95	4.10	1.41
% Nominal	92.29	94.92	98.10
n	5	5	5

Table.3. Linearity and Range of Felodipine

Drug Concentration (ng/ml)	**Internal Standard Concentration (µg/ml)**	**Response Factor (RSD)**
0.8	10	0.00011
1	10	0.00014
2	10	0.00023
4	10	0.00055
6	10	0.00082
12	10	0.00164
13	10	0.00178

Table.4. Stability of Felodipine in plasma during storage and sample handling

Nominal Concentration (ng/ml)			
Freeze and Thaw	**LQC 0.8**	**MQC 6**	**HQC 12**
Cycle 1	0.7523	5.8956	11.9568
Cycle 2	0.7358	5.6327	11.6348
Cycle 3	0.7042	5.4827	11.2672
Mean	**0.73**	**5.67**	**11.62**
S.D (+/-)	0.02	0.21	0.35
C.V. (%)	3.34	3.69	2.97
% Nominal	91.35	94.51	96.83
n	3	3	3
Nominal Concentration (ng/ml)			
Short Term Plasma at RT	**LQC 0.8**	**MQC 6**	**HQC 12**
After 1 h	0.7953	5.8956	11.8965
After 2 h	0.7423	5.5768	11.5324
After 3 h	0.7369	5.6972	11.2746
Mean	**0.76**	**5.72**	**11.57**
S.D (+/-)	0.03	0.16	0.31
C.V. (%)	4.26	2.81	2.70
% Nominal	94.77	95.39	96.40
n	3	3	3
Nominal Concentration (ng/ml)			
Long Term Plasma at 70^0C	**LQC 0.8**	**MQC 6**	**HQC 12**
After 1 week	0.7952	5.8321	11.5632

After 2weeks	0.6835	5.4937	11.1237
After 4 weeks	0.7035	5.1095	11.0743
Mean	**0.73**	**5.48**	**11.25**
S.D (+/-)	0.06	0.36	0.27
C.V. (%)	8.19	6.60	2.39
% Nominal	90.93	91.31	93.78
n	3	3	3
Nominal Concentration (ng/ml)			
Standard stock Solutions	**LQC 0.8**	**MQC 6**	**HQC 12**
After 3 h	0.7952	5.9357	11.8634
After 6 h	0.7632	5.8752	11.6716
After 4 Weeks	0.7452	5.5068	11.6381
Mean	**0.77**	**5.77**	**11.72**
S.D (+/-)	0.03	0.23	0.12
C.V. (%)	3.30	4.02	1.04
% Nominal	95.98	96.21	97.70
n	3	3	3

Table.5. LC-MS System suitability studies for Felodipine

S.No	**Parameters**	**Int Std**	**Drug**
1	Theoretical Plate	176532	22158
2	Resolution factor	2.97	2.97
3	Asymmetric factor	1.25	1.01
4	LOD(ng/ml)	0.06	0.10
5	LOQ(ng/ml)	0.21	0.50

Discussion:

Optimisation of chromatographic conditions are intended to take into account the various goals of the method development and to weigh each goal (resolutions, run time, sensitivity, peak symmetry, etc) accurately, according to the requirements of HPLC can be used for the estimation of Felodipine in plasma samples. The optimised conditions for estimation provided a well defined separation between the drug, internal standard and endogenous components. The blank plasma samples showed no interference at retention time of the drugs and their internal standards. (Fig1,2,3,4 and 5). In the Validation of the developed method the accuracy was determined by relative and absolute recovery experiments. The percentage recovery values for Felodipine were ranged from 97.57 to 98.72 % respectively. Their relative recovery values ranged from 98.94 to 99.36 %. The coefficient of variation (%) of these values was less than 5 %. It is therefore, derived that the developed methods are accurate and reliable.

The optimized methods for the estimation of the drugs were precise as it showed < 10 % coefficient of variation at all concentrations. The six blank plasma samples obtained from six different volunteers were analysed and the chromatograms were recorded. Endogenous interferences were not detected at the retention time of selected drugs and internal standard. The peak purity test method using PDA detector was employed for selectivity studies. Some additional peaks were also observed in the sample chromatograms. These peaks, however, did not interfere with the drugs and internal standards peaks. These observations show that the developed assay method is specific and selective. The linearity range for Felodipine was found to be, 0.8, 1.0, 2.0, 4.0, 6.0, 12.0 and 13.0 ng/ml. The results indicated that no significant inter and intra day variability of slopes and intercepts over the optimised concentration range.

The limit of detection (LOD) value was found to be 0.1 ng/ml for Felodipine and their limit of quantification (LOQ) value was 0.5 ng/ml. This observation showed that the developed methods have adequate sensitivity. These values, however, may be affected by the separation conditions (e.g., column, reagents, and instrumentation and data systems), instrumental changes (e.g., pumping systems and detectors) and use of non HPLC grade solvents and may result in changes in signal to noise ratios. The ruggedness and robustness of the methods were studied by changing the experimental conditions. No significant changes in the chromatographic parameters were observed when changing the experimental conditions (operators, instruments, source of reagents and column of similar type) and optimised conditions (pH, mobile phase ratio and flow rate). System suitability parameters such as column efficiency (theoretical plates), resolution factor and peak asymmetry factor of the optimised methods were found satisfactory.

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

Conclusion:

The developed method for the estimation of Felodipine in plasma is accurate, precise, selective and linear and is therefore, can be employed for estimation of the drug from the spiked samples of plasma with ease of sensitivity and reproducibility in the analysis. Hence the current investigation concluded by representing the significant analytical work which could be used for the felodipine estimation by LC MS method in human plasma for bio availability and pharmacokinetic studies.

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