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Determination of Nifedipine in Human Plasma by Tandem Mass Spectrometry

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An analytical method based on liquid chromatography with positive ion electrospray ionization (ESI) coupled to Tandem mass spectrometry detection (LC-MS/MS) was developed for the determination of nifedipine in human plasma using amlodipine as an internal standard. The analyte and internal standard were extracted from the plasma samples by solid phase extraction. The separation of nifedipine and amlodipine was achieved on a reversed phase (C_{18}) column with a mobile phase consisting of 2 mM ammonium acetate (pH-3 by formic acid) (80:20 v/v). The linearity range was 4.01 to 297.03 ng/mL, inter-, intra-day, accuracy and precision was found 91.65-99.93, 4.47-8.86, 96.07-98.25 and 7.29-11.43, respectively.

Key Words: Tandem mass spectrometry, Nifedipine.

INTRODUCTION

Nifedipine (1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridine dicarboxylic acid dimethyl ester) is a dihydropyridine calcium channel blocker used widely in the management of antihypertensive and antanginal. Although a large number of other usages have been found for this agent, such as Reynaud's phenomenon, premature delivery and painful spasms of the esophagus in cancer and tetanus patients. The empirical formula is $C_{17}H_{18}N_2O_6$. with molecular mass is 346.335 g/mol. The nifedipine has been determined in plasma and other biological fluids using several types of analytical techniques such as a mass spectrometry[6], HPLC[7-9], liquid chromatography coupled to mass spectrometry with electron spray ionization (LC-ESI-MS)[10-12].

The purpose of this work is to develop a rapid and sensitive liquid chromatography-tandem mass spectrometry (LC/MS/MS) method and validated using solid phase extraction for the determination of nifedipine in human plasma. Validation study for the nifedipine was performed with respect to recovery, selectivity, linearity, precision and accuracy. The procedure has been used for the analysis of plasma samples of patients taking nifedipine.

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EXPERIMENTAL

Nifedipine and amlodipine drugs were provided by Raptim Research Ltd. All reagents were of analytical grade. Ammonium acetate (Rankem), methanol (Rankem), formic acid (Merck) and acetonitrile (HPLC, Rankem) sodium hydroxide (Rankem). Milli-Q water, Millipore water was used for the preparation of buffer and other aqueous solutions. Extraction method is solid-phase extraction. Blank human blood was collected from healthy, drug free volunteers. Human plasma was obtained by centrifugation of blood treated with the anticoagulant EDTA. Pooled human plasma was prepared and stored at *ca.* -20 °C until needed.

Chromatographic separation was performed on a Zorbax column (50 mm × 4.6 mm × 5 µm.). The LC-MS/MS equipment comprised of a solvent delivery system (Shimadzu) and Mass detector. The auto ampler (Shimadzu) was used to sample injection. Analyst software was used for data analysis. A lab India pH meter was used to measure the pH of the aqueous mobile phase. The mobile phase was delivered at an isocratic rate of 0.6 mL/min with a pump pressure of *ca.* 750 psi. Total run time for plasma samples was 1.5 min.

Chromatographic conditions: A liquid chromatography Shimadzu Corporation and auto injector are used. An aliquot (10 µL) of each plasma extract was injected into purosphere column C_{18} (150 mm × 4.6 mm) operating at room temperature. The compounds were eluted by pumping the mobile phase 2 mM ammonium acetate (pH-3 by formic acid) (80:20 % v/v) at flow rate 0.6 mL/min. Under these conditions, standard retention times were 1.0 for nifedipine and 0.78 min for amlodipine and back pressure values of *ca.* 780 psi was observed. The temperature of the autosampler was kept at 5 °C, the injection volume was 10 µL and run time was 1.5 min.

The mass spectrometer (Lab India) equipped with electron spray ionization source running in positive mode (ES^+), was set up in multiple reaction monitoring (MRM), monitoring the transitions 347.10 > 315.10, 409.20 > 237.80 for nifedipine and amlodipine, respectively.

The following optimized parameters were obtained: the dwell time and the collision gas 200 pressure were 0.200 s and declustering potential (DP), FP, entrance potential (EP) were 4.0, 400.0, 5.0 and 10.00, 390.00, 6.00 for nifedipine and amlodipine, respectively. Data acquisition and analysis were carried out using software Mass Lynx.

Preparation of stock solution: Stock solution of nifedipine was prepared in methanol (1000 ppm), weigh accurately 25.10 mg of nifedipine transfer to volumetric flask, add 10 mL of methanol sonicate it, adjust the volume with methanol made up to 25 mL. Solutions were stored at 2-8 ºC was used to spike plasma samples. The resulting solution having concentration of 1001690.80 ng/mL of nifedipine.

Stock solution of amlodipine was prepared in methanol (1000 ppm), weigh accurately 25 mg of nifedipine transfer to volumetric flask, add 10 mL of methanol sonicate it, adjust the volume with methanol made up to 25 mL. Solutions were stored at 2-8 ºC was used to spike plasma samples. The resulting solution having concentration of 998700.80 ng/mL of amlodipine.

Preparation of calibration curve standards and quality control samples: Assay performance was determined in accordance with FDA guidance for bioanalytical method of validation for human studies[1]. Linearity was determined to assess the performance of the method. A linear least-squares regression with a weighting index of $1/x^2$ was performed on the peak area ratio of nifedipine and amlodipine *versus* nominal concentrations of the eight plasma standards (4.01, 8.02, 13.37, 22.28, 37.13, 74.26, 148.52 and 297.03) of nifedipine to generate the calibration curve.

The quality control samples were prepared in blank plasma at concentration 12.20, 138.61 and 277.23 ng/mL (LQC, MQC and HQC, respectively). Calibration curves were generated using weighted least squares regression analysis and obtained over the respective standard concentrations in plasma. All standards and QC samples were stored at -20 ºC until analysis.

Preparation of plasma samples: Take 0.5 mL of plasma in ria vial, add 50 µL of Internal Standard (IS) amlodipine of concentration 10 µg/mL, vortex the vials on vortexed, then add 100 µL of 0.1 M NaOH vortex vials for 30 s on vortexes, load sufficient number of Oasis HLB (30 mg/mL) cartridge on SPE manifold. Condition the cartridge with 1 mL methanol followed by 1 mL water. Load the plasma sample; drain out plasma sample at 2 psi pressure. Wash with 1 mL (5 % methanol) followed by 1 mL water. Dry the cartridge at 30 psi pressure for 1 min elutes the sample by passing 0.500 mL of mobile phase. Subject the eluent to injection (10 µL) to LC/MS/MS.

Precision and accuracy: Standards and replicate QC samples of plasma at each concentration were analyzed on three consecutive days, after which inter-and intra-day means, % nominal and coefficients of variation (% CV) were calculated by standard method.

Method validation: Method validation was carried out according to the currently accepted USFDA (food and drug administration) bioanalytical method validation guidelines.

Selectivity: Blank plasma samples of the healthy human used for testing specificity of the method were obtained from six different sources. Each blank sample was tested for the visible interference. Six blank samples and six lower limits of concentration samples were analyzed for specificity.

Linearity: Calibration curves were generated by using the analyte peak area. The sample concentrations were calculated using weighted ($1/x^2$) least squares regression. Acceptance criteria were not more than 20 % deviation at LLOQ and not more than 15 % deviation at above LLOQ levels. The LOD values were calculated by using a signal-to-noise ratio of 3.2, while LOQ value was calculated by using a signal-to-noise ratio of 10.

Precision and accuracy: The intra and inter-day precision accuracy was determined by analyzing six replicates of spiked plasma samples of verapamil at each QC (12.20, 138.61 and 277.23 ng/mL) levels. Inter-day and intra-day precision and accuracy was observed in three consecutive batches. The accuracy was expressed by (mean concentration)/(spiked concentration) × 100 % and the precision by relative

standard deviation (% RSD). The concentration of each sample was calculated using standard curve prepared and analyzed on the same day.

Recovery: Recovery of verapamil was performed at three concentration levels. Recovery was evaluated by comparing detector response for analyte obtained from extracted plasma samples with the true concentration of the analyte (unextracted).

Stability: The stability of nifedipine in the reconstituted solution was assessed by placing quality control samples in auto sampler conditions for 42 h. The freeze-thaw stability of nifedipine was also assessed by analyzing quality control samples undergoing three freeze (-20 ºC)-thaw (room temperature) cycles. Short term and long term stock solution stability (for 8.0 h and 6 d), short term and long term (for 6 and 24 d) stability of drug in matrix was evaluated.

Anticoagulant effect: Anticoagulant effect was performed by comparing high and low quality control samples prepared in EDTA, anticoagulant based human plasma and compared against calibration curve prepared in CPDA anticoagulant based plasma.

Matrix effect: This exercise was done to assess the matrix effect throughout the application of this method. Matrix has a lot of inherent variability and can affect the response of analyte during the method validation and subsequently in subject analysis. The quantification of nifedipine from plasma can be grossly affected by a significant matrix effect.

Ion suppression: Suppression of the MS signal can be caused by contaminants in the LC eluent entering the MS. Thus, a non specific extraction procedure may produce ion suppression that could interfere with the analysis of the samples. The effect of the sample preparation method on the variability of the electron spray ionization response should be determined. To assess the effect of ion suppression on the MS/MS signal of the analyte, nifedipine and amlodipine.

Biological application: To demonstrate the applicability of the procedure for measurement of nifedipine levels in pharmacokinetics studies on healthy male volunteers. We are performing to study the new coating formulation.

RESULTS AND DISCUSSION

Method development: To develop a sensitive, selective and simple method for the extraction and quantification of nifedipine during method development different options were evaluated to optimize detection and chromatography parameters. First we have to find out the mass in tuning mode and also set the following parameters, declustering potential (DP), entrance potential (EP), GS1, GS2 and CXP. Then select the mobile phase varying the composition and pH, optimize it. It is well known that nifedipine is not stable at low pH. Further optimization in chromatography conditions increased the response of analyte. A mobile phase containing 2 mM ammonium acetate (pH-3 by formic acid) (80:20 % v/v) resulted in good response. Use of Zorbax (50 mm × 4.6 mm × 5 µm).column resulted in run time of 1.5 min.

Selectivity: Selectivity of the method was demonstrated by the absence of endogenous interfering peaks at the retention times of drug in six different lots of extracted blank plasma.

Linearity and LLOQ: The peak area of calibration standards was proportional to the concentration of analyte in each assay over the nominal concentration range of 4.01 to 297.03 ng/mL for nifedipine. The calibration curves appeared linear and well described by least squares lines. A weighting factor of $1/x^2$ concentration was chosen to achieve homogeneity of variance. The correlation coefficients were ≥ 0.99 (n = 4) for nifedipine. The mean (± SD) slopes and intercept of the calibration curves (n = 4) for nifedipine were 0.002 (± 0.001) and 0.00007 (± 0.002), respectively. Results of four representative standard curve for HPLC determination of nifedipine are given in Table-1. The lower limit of quantification for nifedipine proved to be 4.01 ng/mL.

TABLE-1
RESULTS OF FOUR CALIBRATION CURVES FOR DETERMINATION OF NIFEDIPINE IN HUMAN PLASMA

Assay/Conc. (ng/mL)	4.01	8.02	13.37	22.28	37.13	74.26	148.52
1	3.96	8.64	12.31	21.90	35.71	81.75	152.57
2	4.25	7.14	16.90	21.73	36.42	72.06	157.23
3	3.94	8.14	17.10	23.14	36.86	66.62	146.75
4	4.00	8.90	11.12	21.65	36.26	83.13	155.02
Average	4.038	8.205	14.358	22.105	36.313	75.890	152.893
SD	0.144	0.777	3.091	0.698	0.475	7.903	4.516
RSD (%)	3.56	9.47	21.53	3.16	1.31	10.41	2.95
Nominal (%)	100.69	102.31	107.39	99.21	97.80	102.19	102.94

Precision and accuracy: Intra-batch and inter-batch precision and accuracy of the method for nifedipine are presented in Tables 2 and 3. The precision deviation values for intra-batch and inter-batch are all within 15 % of the relative standard deviation (RSD) at each quality control level. The accuracy deviation values for intra-batch and inter- batch are all within the (100 ± 15) % of the actual values at each quality control level.

Recovery: Results of extraction efficiency measured for nifedipine was consistent, precise and reproducible. The mean related extraction recovery of nifedipine and amlodipine at each quality control level (12.20, 138.61 and 277.23 ng/mL) were 56.95, 53.32, 49.44 and 43.87, 46.47, 41.25 %, respectively.

TABLE-2
INTRA BATCH PRECISION AND ACCURACY OF THE METHOD FOR DETERMINATION OF NIFEDIPINE IN HUMAN PLASMA

Intra-batch precision and accuracy (n =18)			
Concentration (ng/mL)	Concentration Mean ± SD (ng/mL)	Accuracy (%)	Precision (%)
12.20	11.99 ± 0.94	98.25	7.88
138.61	137.79 ± 15.53	97.97	11.43
277.23	266.33 ± 19.43	96.07	7.29

TABLE-3
INTER BATCH PRECISION AND ACCURACY OF THE METHOD FOR DETERMINATION OF NIFEDIPINE IN HUMAN PLASMA

Intra-batch precision and accuracy (n =6)			
Concentration (ng/mL)	Concentration Mean ± SD (ng/mL)	Accuracy (%)	Precision (%)
12.20	11.18 ± 0.78	91.65	6.97
138.61	138.51 ± 6.20	99.93	4.47
277.23	271.59 ± 24.07	97.96	8.86

Stability: Results of autosampler stability, Freeze-thaw stability and long term stability are given in Table-4. Stability data gives the idea about reliable stability of nifedipine in tested conditions. Nifedipine was found stable during short term and long term stock solution stability. During short term stock solution stability % mean stability of nifedipine was found 98.37 %. Long term stock solution stability was performed for 5 d and % mean stability of nifedipine was found 101.68 %.

TABLE-4
STABILITY OF NIFEDIPINE IN HUMAN PLASMA UNDER TESTED CONDITIONS

Concentration (ng/mL)	% Accuracy (Mean ± SD)	
	12.20 ng/mL	277.23 ng/mL
Autosampler Stability (26 h, 10 ºC)	101.23 ± 1.03	271.71 ± 11.48
Freeze thaw stability (3 cycles, -20 ºC- room temperature)	98.80 ± 0.74	93.73 ± 24.34
Bench top stability (8 h, room temperature)	101.33 ± 0.88	94.57 ± 17.13
Short term stability in matrix (6 d, -20 ºC)	99.22 ± 1.12	97.58 ± 20.16
Long term stability (18 d -20 ºC)	99.34 ± 1.39	103.91 ± 21.19

Anticoagulant effect: As method validation was performed in CPDA base human plasma anticoagulant effect was performed for EDTA base human plasma. Results of anticoagulant effect are summarized in Table-5.

TABLE-5
RESULTS OF ANTICOAGULANT EFFECT

Concentration (ng/mL)	% Accuracy (Mean ± SD)	
	12.20 ng/mL	277.23 ng/mL
Anticoagulant Effect	98.18 ± 1.16	101.47 ± 24.96

REFERENCES

1. FDA Guidance for Industry; Bioanalytical Methods Validation for Human Studies, Center for Drug Evaluation and Research, US Department of Health and Human Services, Rockville, MD (2001).
2. Martindale, The Extra Pharmacopeia, Royal Pharmaceutical Society, London, edn. 31, p. 940 (1996).
3. Goodman and Gilman, The Pharmacological Basis of Therapeutics, McGraw Hill, New York, edn. 10, p. 853 (2001).
4. D. Zendelovska and S. Simeska, *J. Chromatogr. B*, **839**, 85 (2006).

5. Y. Guo and J. Dai, *Int. J. Pharm.*, **341**, 91 (2007).
6. B. Steel and C. Zimmer, *J. Chromatogr. B*, **720**, 119 (1998).
7. D. Zendelovska, S. Simeska, O. Sibinovska, E. Kostova, K. Miloševska, K. Jakovski, E. Jovanovska, I. Kikerkov, J. Trojacanec and D. Zafirov. *J. Chromatogr. B*, **832**, 85 (2006).
8. I. Niopas and A.C. Daftsios, *J. Pharm. Biomed. Anal.*, **32**, 1213 (2003).
9. T. Ohkubo, H. Noro and K. Sugawara, *J. Pharm. Biomed. Anal.,* **10**, 67 (1992).
10. P.A. Soons, J.H.M. Schellens, M.C.M. Roosemalen and D.D. Breimer, *J. Pharm. Biomed. Anal.,* **9**, 475 (1991).
11. X.-D. Wang, J.-L. Li, Y. Lu, X. Chen, M. Huang, B. Chowbay and S.-F. Zhou, *J. Chromatogr. B*, **852**, 534 (2007).
12. Y. Guo, J. Dai, G. Qian, N. Guo, Z. Ma and XingJie Guo, *Inter. J. Pharm.*, **341**, 91 (2007).

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