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Research Article

ESTIMATION OF NIFEDIPINE IN HUMAN PLASMA BY LCMS/MS

SRINIVASA REDDY, IQBAL AHMAD, NIRMALA NAYAK, SARAL THANGAM AND ARINDAM MUKHOPADHYAY*

Norwich clinical services pvt.ltd.147/f, 8th main, 3rd block, koramangala, bangalore– 560034,
Email: arindam.mukhopadhyay@norwichclinical.com

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ABSTRACT

Nifedipine and NifedipineD6, (IS) were extracted from the plasma by precipitation method and then separated on a Reverse phase chromatography using the mobile phase mixture of ammonium acetate and methanol at a flow rate of 1.0 ml/min. The analytes were detected in API 4000 Mass spectrometer in the positive atmospheric pressure chemical Ionization (APCI) mode with multiple reactions monitoring (MRM). The MRM transitions were monitored by following m/z for parent ion 347.2& daughter ion 315.1(Nifedipine), and m/z 353.2& daughter318.2 (Nifedipine D6, IS). A linear calibration plot of Nifedipine was achieved in the concentration ranges of 1.558 ng/mL to 360.561ng/mL. Mean recovery was93.2%. The assay was specific, precise, accurate and reproducible.

Keywords: Nifedipine, LCMS/MS, Human plasma, Validation

INTRODUCTION

Nifedipine, dimethyl-1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)pyridine 3,5-dicarboxylate, is a calcium channel blocker that inhibits the trans-membraneinflux of Ca^{+2} into cardiac muscle cellsand vascular smooth muscle through specific ion channels (1 – 3). It decreases vascular peripheral resistance (4) for which it is widely used in the treatment of hypertension, angina pectoris and various other cardiovascular disorders such as Ray-naud's phenomenon (5).Nifedipine, a highly non-polar compound, is absorbed completely from the gastrointestinal tract, predominantly from the jejunum, but has a very low bioavailability mainly due to pre-systemic metabolism (6, 7). Following absorption, Nifedipine is further metabolized in the small intestine and liver to more polar compounds which are primarily eliminated by the kidney (8-10). Nifedipine is a photo-labile compound, undergoing oxidative biotransformation in human body into pharmacologically inactive metabolites (10, 11).

Several methods for determination of Nifedipine in plasma/biological samples by HPLC or ESI LCMS/MS have been reported. However, most of them are either lacking full validation procedures outlined by regulatory agencies or use cumbersome LLE/SPE procedure (13-16).

We describe here a relatively simple, specific and highly sensitive atmospheric pressure chemical Ionization (APCI) method for estimation of Nifedipine by LCMS/MS which has been validated as per the FDA regulations. This method can also be used for estimation of this drug for pharmacokinetic analysis and other studies.

EXPERIMENTAL

Materials and Reagents

Nifedipine (USP) was purchased from LGC Promochem whereas deuterated Nifedipine, used as an internal standard was fromVivan Life sciences (India). HPLC grade methanol and acetonitrile were obtained fromE -Merck (India). All other chemicals of highest purity grade were locally purchased. Milli Q water(Millipore (USA)) was used throughout the procedure.

K_2 EDTA containing human blood was collected in-house from healthy volunteers. Plasma was separated by centrifuging at 3000 rpm for 10 min at 4^0C.

Preparation of Calibration standard samples

Stock solutions (1mg/ml) of Nifedipine were prepared in methanol. Concentration is then corrected using the potency and actual amount weighed. Working solutions (77.760 ng/ml to 18000.000 ng/ml) were prepared by serial dilution of the stock solution by methanol: water (1:1, v/v). Sodium vapour lamp was used during the whole procedure as a special precaution due to the light sensitivity of Nifedipine.

Similarly for internal standard (IS), stock solution (1mg/ml) of Deuterated Nifedipine (Nifedipine D6) was prepared in methanol and corrected final concentration is obtained using the potency and amount weighed. Working solution (500ng/ml) was then prepared from this stock solution by serial dilution using methanol: water (1:1, v/v) as diluent. All solutions were stored in refrigerator at 2-8ºC with due protection from light until analysis.

Calibration standards of concentration range from 1.555to 360.000ng/ml were prepared by adding 980µl of blank plasma to 20µl of respective working solution and stored at – 70ºC.

Preparation of Quality Control Samples

Working solutions (79.488 ng/ml to 13,800.000 ng/ml) were prepared by serial dilution of the stock solution by methanol: water (1:1, v/v). Quality control samples, marked as LOQQC, LQC, MQC and HQC containing 1.590ng /ml, 4.416ng /ml, 138.000 ng/ml and 276.000ng /ml of Nifedipine respectively, were prepared by adding 980µl of blank plasma to 20µl of respective working solution and stored at -70°C.

Sample extraction

After thawing the sample at room temperature it was vortexed to ensure complete mixing of the contents. 50 µl of IS was added to each of these analyte spiked plasma (100 µl) and vortexed. 1ml of acetonitrile was added to each vial and kept on the vibramax at 2500 rpm for 10 min. After centrifugation at 11000 rpm for 5 min at 4°C, supernatant was then transferred to a vial and kept in an auto-sampler. 10µl was injected to LCMS/MS for analysis.

Chromatography

The drug was separated on a Zorbax SB C8, 100 mm x 4.6 mm column with particle size 3.5µ (Agilent) using the mobile phase [10mM ammonium acetate (pH 4.0±0.2):Methanol::30:70v/v] at a flow rate of 1.0 ml/min in Waters UPLC attached to API 4000 Mass spectrometer (Applied Biosystems, USA) using positive Atmospheric Pressure chemical Ionization mode. The column oven temperature was maintained at 40^0C and the run time was 2.80 min. The analytes were detected on mass spectrometer operating in the multiple reaction monitoring (MRM) modes. The following precursor product ion transition was monitored for MRM transitions: m/z 347.2 315.1(Nifedipine) and m/z 353.2 318.2 (Nifedipine D6) with a dwell time of 200 msec. The APCI source temperature was maintained at 300^0C. Data were acquired and processed with Analyst software 1.5.1.

Matrix Factor

Six replicates of aqueous standard containing analyte at LQC concentration and the intended internal standard concentration were injected. Individual analyte area response and IS area response

of each post extracted sample were compared with the mean analyte area response and mean IS area response of the aqueous standard respectively. Matrix factor is calculated as the ratio of area response in presence and absence of matrix factor.

RESULT AND DISCUSSION

Specificity and Selectivity

For selectivity analysis, eight different lots of plasma including one hemolysed and one lipemic lots were spiked with analytes and internal standard. Interference at the retention times of analytes and IS was evaluated by comparing peak area response with that of blank plasma. Signal to noise ratio for all lots was more than 5.0 indicating the method is selective for Nifedipine. Retention times for Nifedipine and Nifedipine D6were 1.91min and 1.89min, respectively (Fig.1a& b). No interfering peaks were observed in the blank at the retention times corresponding to drug and I.S. indicating that the procedure is specific to Nifedipine.

Similarly, no matrix effect was found while analyzing the human plasma samples, calibration standards and QC samples (Table 1).

Linearity of the Calibration Plot

Calibration plots of Nifedipine showed that the calibrations are linear in the concentration ranges of 1.558ng/mL to 360.561ng/mL with a correlation coefficient (r) of 0.9994.

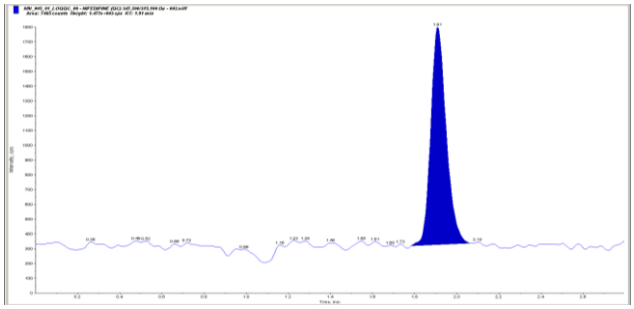

FIG 1A:NIFEDIPINE

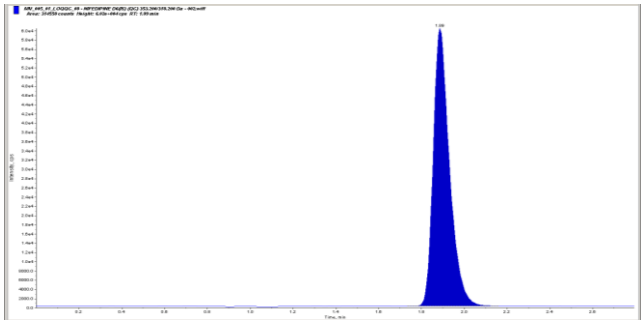

FIG 1 B: NIFEDIPINE D6 (IS)

Table 1: Matrix Effect For Estimation Of Nifedipine

MATRIX ID	ANALYTE AREA IN ABSENCE OF MATRIX	ANALYTE AREA IN PRESENCE OF MATRIX	MATRIX FACTOR FOR ANALYTE	IS AREA IN ABSENCE OF MATRIX	IS AREA IN PRESENCE OF MATRIX	MATRIX FACTOR FOR IS
PL_091	25815	27157	97.13	360644	383125	97.29
PL_103	26953	26957	96.41	377000	375635	95.38
PL_104	28551	25890	92.59	397678	365080	92.70
PL_105	27813	25084	89.71	408633	351172	89.17
PL_106	29329	26162	93.57	406802	371141	94.24
PL_107	29303	27330	97.74	412131	382549	97.14
HPL_099		27001	96.57		378892	96.21
LPL_100		23624	84.49		338019	85.83

Precision & Accuracy

Intra- or inter- day precision and accuracy were determined by six replicate analysis of LOQQC, LQC, MQC and HQC samples. Intraday precisions were ranged from 0.93% to 2.34% for Nifedipine which were within acceptable limit (Table 2). Similarly, for inter-day batch precision %CV ranged from 1.65% to 5.59% for Nifedipine was also within accepted limit (≤ 20% at LOQQC and ≤15% for others).

Table 2: Accuracy And Precision Of Analysis Of Nifedipine In The Quality-Control Samples

		INTRADAY	INTERDAY
LOQQC (n=6)	Actual conc.(ng/ml)	1.566	1.566
	Estimated conc. (ng/ml)	1.520	1.546
	%Accurecy	97.06	98.72
	% CV	0.93	5.59
LQC (n = 6)	Actual conc.(ng/ml)	4.412	4.412
	Estimated conc. (ng/ml)	4.495	4.509
	%Accurecy	100.88	102.19
	% CV	1.92	2.20
MQC (n = 6)	Actual conc.(ng/ml)	117.641	117.641
	Estimated conc. (ng/ml)	117.009	119.464
	%Accuracy	99.46	101.54
	% CV	2.34	2.19
HQC (n = 6)	Actual conc.(ng/ml)	280.097	280.097
	Estimated conc. (ng/ml)	284.693	284.758
	%Accuracy	101.64	101.66
	% CV	1.10	1.65

Mean accuracy for intraday batch ranged from 97.06% to 101.64% for Nifedipine which are within acceptable limit (Table 2). Similarly, for inter-day batch accuracy ranged from 98.72% to 102.19% which were also within accepted limit (≤ 20% at LOQQC and ≤15% for others).

Recovery

Absolute recovery percentage was determined by comparing the mean peak area of Nifedipine obtained by injecting 6 extracted samples of LQC, MQC and HQC with the mean peak area obtained by injection of respective aqueous standard solutions. Mean percentage

recovery was 93.21 with mean % CV of 5.08 (Table 3) which was well within the accepted limits (≤15%).

Table 3: Recovery Of Nifedipine From Biological Matrix

LQC			MQC			HQC		
Unextracted area (n=6)	Extracted area (n=6)	Mean percentage recovery	Unextracted area (n=6)	Extracted area (n=6)	Mean percentage recovery	Unextracted area (n=6)	Extracted area (n=6)	Mean percentage recovery
4.491ng/mL	4.412ng/mL		118.184	117.641ng/mL		281.391ng/mL	280.097ng/mL	
25950	24403	95.72	663517	635025	96.15	1684522	1471260	87.74

Stability

Short – Term/bench - top stability

To check whether the sample is stable during analysis, six aliquots of LQC & HQC samples were thawed and kept at room temperature under yellow monochromatic light for 5 hours, which has been decided based on the time required for analysis. The samples were then processed and analyzed as mentioned above. No significant differences were noticed when these results were compared with those obtained from the freshly spiked samples indicating that Nifedipine was stable at room temperature (Table 4).

Table 4: Stability Of Nifedipine In Biological Matrix

STABILITY CHECK	SAMPLES	NOMINAL CONC.	OBSERVED CONC.	%CV	% STABILITY
Bench Top for (5hrs)	LQC (n=6)	4.412	4.440	3.87	100.63
	HQC(n=6)	280.097	268.961	0.97	96.02
Freeze Thaw (4 cycles)	LQC (n=6)	4.412	4.697	6.92	106.45
	HQC(n=6)	280.097	276.124	1.21	98.58
In-Auto-sampler(19hr)	LQC (n=6)	4.412	4.687	0.92	106.23
	HQC(n=6)	280.097	285.625	1.01	101.97
Wet Extract (3hr)	LQC (n=6)	4.412	4.379	4.06	99.25
	HQC(n=6)	280.097	265.966	2.83	94.95

Auto sampler stability

The stability of the processed samples in the auto sampler during analysis was determined by using six aliquots of LQC, HQC samples. The stability of Nifedipine was assessed for 19 hours, the expected run time for batches of validation samples. The result was then compared with that of freshly spiked samples. The stability of the internal standard was compared in MQC sample by area Ratio (IS/Analyte response) method. The stability was assessed for 19 hours against freshly processed samples. No significant difference in the results indicated that the analytes are stable for at least 19 hour in the auto sampler (Table 4).

Freeze – Thaw stability

Analyte stability was determined after four freeze thaw cycles for six aliquots of each of the LQC and HQC. The samples were stored below – 70^{0}C for 24h and then allowed to thaw at room temperature unassisted. After complete thawing, the samples were again stored at same temperature (– 70^{0}C) for 12h. The freeze thaw cycle was repeated another three times before analyzing the samples. No differences were noticed when the results were compared with the fresh QC samples indicating the stability of Nifedipine in K_2EDTA human plasma for four freeze thaw cycles at about -70°C (Table 4).

Wet Extract stability

To check whether the sample is stable after processing, six aliquots of LQC & HQC samples were processed and kept at room temperature under yellow monochromatic light for 3 hours. The samples were then analyzed as mentioned above. No significant differences were noticed when these results were compared with those obtained from the fresh QC samples indicating that processed samples of Nifedipine was stable at room temperature (Table 4).

Short term stock solution stability

To ensure that analyte is stable in appropriate solution for a short period of time at room temperature, the stability of stock and working solutions of Nifedipine was evaluated at room temperature for 48 hours under yellow monochromatic light. There were no significant changes in stabilities of the stock and working solutions on keeping at room temperature for 48 hours (Table 5).

Long term stock solution stability

To evaluate the stability of analyte in appropriate solution for a long period of time under storage condition (2 – 8^{0}C), the stability of stock solution of Nifedipine was evaluated at 2 – 8^{0}C for 13 days. Table 5 indicates that the stock solution was stable during the storage.

TABLE 5: STABILITY OF AQUEOUS NIFEDIPINE SOLUTIONS

Stability Check	Samples	Fresh Area)	Stock(Avg.	Stability Area)	Stock(Avg.	%cv	% Stability
Short Term Stock Solution Stability (48hr)	Analyte(n=6)	638074		604676		1.93	94.76
	Internal Standard(n=6)	482964		516077		2.03	106.86
Short Term Working solution Stability (48hr)	Analyte(n=6)	638074		585157		2.66	91.70
	Internal Standard(n=6)	348226		325639		2.91	93.51
Long Term Stock Stability (13 Days)	Analyte(n=6)	468033		478609		2.03	102.25
	Internal Standard(n=6)	493510		501772		1.89	101.67

CONCLUSION

A simple, accurate, precise, sensitive and reproducible LCMS/MS method has been developed and validated for the determination of Nifedipine. The sample extraction procedure described here is protein precipitation method which is not only a simpler method compared to other available extraction methods but also produces cleaner samples with no matrix effect.

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