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A NOVEL, SENSITIVE, BIOANALYTICAL METHOD FOR ESTIMATION OF AMLODIPINE BESYLATE IN RAT PLASMA USING FLOURESCENCE DETECTION BY RP-HPLC

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ABSTRACT: The aim of the present work was to develop and validate a novel, rapid, sensitive, accurate and specific RP-HPLC assay with fluorescence detection for the quantification of amlodipine besylate in rat plasma to be applied to preclinical studies. Flourescein was used as internal standard (IS) and the detection was carried out by an online wavelength switching which enabled detection of amlodipine & flourescein in the same method. The plasma proteins were precipitated by a single step protein precipitation extraction procedure using methanol. Mean recovery of amlodipine besylate from rat plasma was more than 85.0 % for 25 - 5000 ng/ml concentrations. The assay exhibited good linear relationship with an r^2 of 0.999. Lower limit of Quantification limit (LLOQ) was 1.84 µg/ml of amlodipine besylate and accuracy and precision were over the concentration range 25 - 5000 ng/ml. Thus, the bionanalytical method for quantification of amlodipine besylate is novel, since use of fluorescence detection & using flourescein as the internal standard has made the method with comparable sensitivity to LC-MS/MS methods. The proposed method is convenient for routine determination of amlodipine besylate in rat plasma and quality control laboratories that require an economic and rapid method.

INTRODUCTION: Amlodipine (as besylate, mesylate or maleate), 2-[(2-aminoethoxy)methyl]-4-(2--chlorophenyl)-3-ethoxycarbonyl-5-methoxy carbonyl-6- -methyl-1,4-dihydropyridine is a long-acting calcium channel blocker of the dihydropyridine (DHP) class used as an antihypertensive and in the treatment of angina pectoris.

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Like other calcium channel blockers, amlodipine acts by relaxing the smooth muscle in the arterial wall, decreasing total peripheral resistance thereby reducing blood pressure; in angina, amlodipine increases blood flow to the heart muscle (although DHP-class calcium channel blockers are more selective for arteries than the muscular tissue of the heart (myocardium), as the cardiac calcium channels are not of the dihydropyridine-type) [1, 2].

Amlodipine is also used to prevent certain types of chest pain (angina). It may help to increase your ability to exercise and decrease the frequency of angina attacks. It should not be used to treat attacks of chest pain when they occur. Several bioanalytical methods for amlodipine besylate by HPLC with UV detection [3], HPLC with flouresence

detection [4, 5, 6] & LC-MS/MS methods [7, 8] have been reported. However, most of the bioanalytical methods have used human plasma as the biological matrix and is applicable to clinical studies. The aim of the present work was to develop a sensitive, economical bioanalytical method to quantify amlodipine besylate in rat plasma.

On-line wavelength switching was adopted to accommodate flourescein as the internal standard. The method could be applied for preclinical pharmacokinetic & drug interaction studies.

MATERIALS AND METHODS:

Chemicals: Amlodipine besylate was a gift sample from IPCA labs ltd. and flourescein was obtained from Sigma-Aldrich chemicals, Mumbai, India. Methanol (HPLC grade), Acetonitrile (HPLC grade) obtained from J.T.Baker and orthophosphoric acid (AR grade) obtained from Fisher scientific. Doubled distilled water was used for analytical purpose.

Biological matrix: Rat plasma was collected from healthy male Wistar rats (using EDTA as the anticoagulant). The experiment was performed as per the guidelines of Institutional Animal Care Committee constituted as per the guidelines of the CPCSEA and the protocol [Protocol no. CPCSEA/IAEC/SPTM/P-55/2013] was duly approved by the Institutional Animal Ethics Committee.

Instrument used: Flourescence measurements were performed using the chromatographic system which consisted of Shimadzu LC-2010CT HPLC equipped with a CBM-20A communication module, RF-20A prominence fluorescence detector and LC Solutions ® software.

Chromatographic conditions: The chromate-graphic separation of amlodipine besylate and internal standard (flourescein) was done using a 250 × 4.6 mm, 5 µm Kromasil C_{18} analytical column. The mobile phase was a gradient of acetonitrile and 0.01 M potassium dihydrogen phosphate (pH adjusted to 3.0 with orthophosphoric acid) **(Table 1).**

TABLE 1: GRADIENT PROGRAM OF MOBILE PHASE

Time (mins)	Acetonitrile	0.01 N potassium dihydrogen phosphate, pH 3.0
0	10	90
8	55	45
12	85	15
15	10	90
17	10	90

Before use, the mobile phase was filtered through a 0.45 µm filter and the filtrate is degassed by using bath sonicator. Emission and excitation wavelengths of amlodipine were 446 & 393 nm [6] & flourescein were 521 & 494 nm [9], respectively. The detection wavelengths in the method was in the gradient mode (**table 2**), which yielded a very stable baseline in rat plasma.

TABLE 2: GRADIENT FLUORESCENCE DETECTOR SETTINGS

Time (mins)	Mode	Wavelength (nm)
0.01	Emission Wavelength	446
0.01	Excitation Wavelength	393
10.50	Emission Wavelength	446
10.50	Excitation Wavelength	393
10.51	Emission Wavelength	521
10.51	Excitation Wavelength	494
12.50	Emission Wavelength	521
12.50	Excitation Wavelength	494
12.51	Emission Wavelength	446
12.51	Excitation Wavelength	393
17.00	Emission Wavelength	446
17.00	Excitation Wavelength	393

Several fluorescent molecules for eg. Dextrorphan, dextromethorphan, quinidine, serotonin etc. were tried. However these molecules did not yield a stable baseline & plasma showed a lot of interferences at their excitation & emission wavelengthd. Flourescein, being an inherently fluorescent molecule, with its excitation & emission wavelengths in the visible.

Range lead to a stable baseline in rat plasma with no interfering peaks. All the procedures were performed at ambient temperature.

Stock solutions: Stock solution of amlodipine besylate was prepared in methanol at a concentration at a concentration of 1000 µg/ml and was kept at 2-8^{o}C. This stock solution was diluted with methanol to obtain concentrations 250, 500, 1000, 2500, 5000, 10000, 25000 & 50000 ng/ml. Amlodipine besylate spiked in rat plasma was in the range of 25 ng/ml to 5000 ng/ml. The internal standard was prepared by dissolving 1 mg of flourescein in 1 ml methanol. Samples for the accuracy, precision and recovery were prepared by spiking standard amlodipine concentrations in rat plasma to yield final concentrations of 25, 50, 500, 2500 and 5000 ng/ml.

Plasma Stability: The stability of Amlodipine besylate in rat plasma was determined by incubating Amlodipine besylate in rat plasma at 37^{0}C for 1 hour. Stability was carried out at a concentration of 100 ng/mL of Amlodipine besylate. The stability was determined by taking aliquots of spiked plasma at 0, 15, 30 and 60 mins. Samples were run in duplicate.
The drug was found to be stable in rat plasma after incubation for 1 hr at 37^{0}C.

Trials of Extraction of drug from rat plasma: Extractions were tried with methanol, acetonitrile and ethyl acetate. Acetonitrile gave best recovery with good peak shape of both amlodipine & flourescein with no interferences.

Final Extraction Procedure: In a microcentrifuge tube, 10 µL flourescein (10 µg/mL), 90 µL blank rat plasma and 10 µL 10X Amlodipine besylate solution were co-spiked and vortexed. The spiked samples were precipitated with 1 mL acetonitrile and vortexed for 5 mins. The resulting solutions were then centrifuged at 4000 rpm for 10 mins. Resulting supernatant (900 µL) was evaporated under a gentle stream of nitrogen at 40^{0}C. The dried samples were reconstituted with 100 µL mobile phase, vortexed, centrifuged and injected on HPLC. All samples were processed in the similar manner as mentioned above.

Method validation: The assay method was evaluated through determination of linearity, accuracy, precision, limit of detection, limit of quantification, recovery & relative matrix effect was evaluated by analyzing different rat plasma from six different rats [10, 11].

Linearity in rat plasma: Standard calibration samples were prepared by making serial dilution from the stock solution of amlodipine besylate (1 mg/ml). Calibration curve of concentration versus peak area ratio was plotted at concentration range 25, 50, 100, 250, 500, 1000, 2500 & 5000 ng/ml.

Limit of detection (LOD) and lower limit of quantification (LLOQ): The limit of detection (LOD) and lower limit of quantification (LLOQ) were measured according to the FDA's guidance for bioanalytical method validation. The limit of detection was defined as the lowest concentration of bupropion resulting in a peak height greater or equal to three times from background noise (S/N ≥ 3.3). The quantification limit was established by assessing the signal to noise ratio level in proportion of 10:1 for each signal. The analyte response at the LLOQ should be at least 5 times the response compared to blank response. Analyte peak (response) should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80-120%.

Precision and Accuracy: The precision and accuracy were determined by analyzing spiked standard and extracted samples at different concentrations ranging from 25, 50, 500, 2500 and 5000 ng/ml. The precision of an HPLC method was determined as the coefficient of variation (%C.V.) of intra- and inter-day.

The intra-day precision was determined by analyzed the spiked standard and extracted samples prepared within a day. The inter-day precision was determined by analyzed the spiked standards and extracted samples on two different days. Each concentration was done six times. After concentrations were calculated by re-fitting peak area ratio obtained with different standards solutions into a derived regression equation from the set of these standard solutions, % C.V. was determined at each concentration of the standard solutions from their average value and SD. The accuracy of the HPLC method was demonstrated by percentage deviation.

The calculated concentrations (or conc. Found) were obtained by re-fitting peak area ratios from standard solutions of known concentrations (or conc. added) into a derived regression equation. The conc. Found and added was then used to determine the absolute percentage deviation at each concentration of the standard solutions.

Recovery: The absolute recovery was calculated by comparing the peak area ratio of extracted and unextracted samples containing amlodipine besylate and phenacetin. Each measurement was made in triplicates. Results of percent recovery from rat plasma.

$$\% \text{ Recovery} = \frac{\text{Mean peak area ratio of extracted samples}}{\text{Mean peak area ratio of unextracted samples}} \times 100$$

RESULTS:

Chromatography: Sensitive, rapid, specific and reproducible HPLC method has been developed and validated for quantitative determination of amlodipine besylate in rat plasma samples. After the pretreatment with a rapid single protein precipitation step, the rat plasma containing amlodipine besylate was separated by reverse phase HPLC with fluorescence detection.

The retention time of amlodipine besylate and fluorescein were 10.10 and 11.2 min., respectively. There was good baseline separation of amlodipine besylate & fluorescein. **Figure 1** shows a representative chromatogram of amlodipine besylate and fluorescein (IS) in rat plasma. The peaks showed very good resolution with a stable baseline separation between amlodipine & fluorescein.

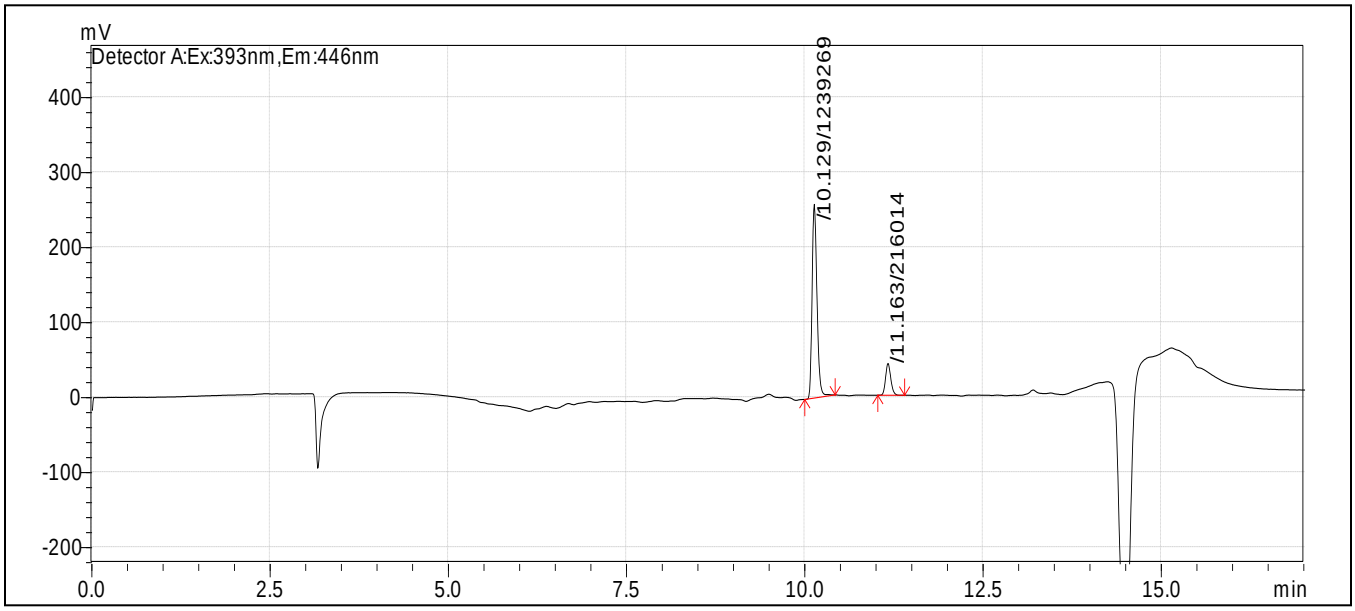

FIGURE 1: REPRESENTATIVE CHROMATOGRAM OF AMLODIPINE BESYLATE AND FLOURESCEIN IN RAT PLASMA

Linearity in rat plasma: Linearity in rat plasma was done at final concentrations of 25, 50, 100, 250, 500, 1000, 2500 & 5000 ng/mL of Amlodipine besylate. Peak area ratio of Amlodipine besylate and fluorescein was calculated. Plot of peak area ratio versus plasma concentration (ng/mL) was plotted on Microsoft Excel 2007. The correlation coefficient of six calibration curves in rats plasma was r^2 = 0.997 ± 0.033. Overlay chromatograms of all the linearity concentrations is shown in **Figure 2.**

The average area ratios for six linearities in rat plasma are summarized in the **Table 3.**

A representative calibration curve (plot of area ratio (Area of amlodipine besylate/area of fluorescein) is shown in **Figure 3.**

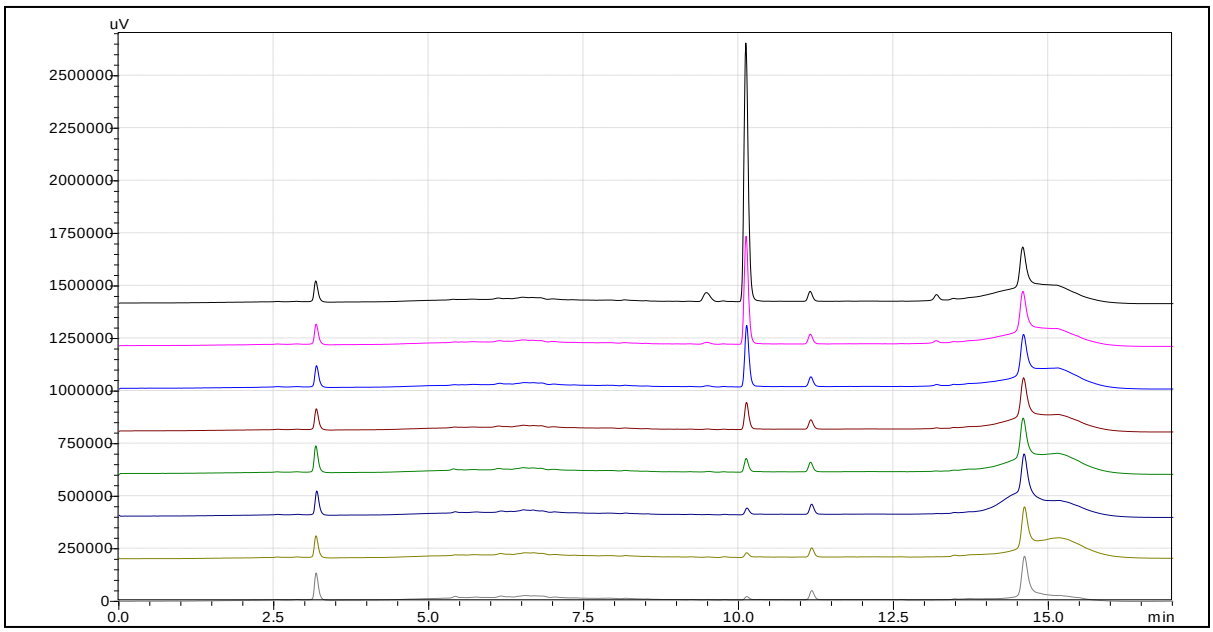

FIGURE 2: OVERLAY CHROMATOGRAMS OF 25, 50, 100, 250, 500, 1000, 2500 & 5000 NG/ML OF AMLODIPINE BESYLATE IN RAT PLASMA

TABLE 3: AVERAGE AREA RATIOS OF SIX LINEARITIES IN RAT PLASMA

Concentration of amlodipine in ng/mL	Average area of Amlodipine	Average area of fluorescein (IS)	Average area ratio of amlodipine/fluorescein (IS)
25	27664.50	76074.18	0.02
50	58505.80	124877.63	0.05
100	109288.20	93238.96	0.10
250	303523.17	209295.59	0.25
500	710203.83	106989.21	0.61
1000	1393273.50	184268.18	1.14
2500	2969356.40	53646.97	2.59
5000	7250993.83	67368.69	5.96

The RP-HPLC method for quantification of amlodipine besylate in rats plasma was linear in the range of 25 – 5000 ng/mL, which makes it a very sensitive method.

Limit of detection (LOD) and Limit of quantification (LOQ): The limit of detection (LOD) and limit of quantification (LOQ) was 10 ng/ml (S/N≥3) and 25 ng/ml. The coefficient of variation (% C.V.) of six replicates of final concentration of 25ng/ml of amlodipine in rat plasma was found to be 15.46 %, which fits in the acceptance criteria as per ICH guidelines.

The LOD & LOQ of amlodipine besylate in rat plasma by the developed method with fluorescence detection is comparable to a reported LC-MS/MS method in human plasma [8].

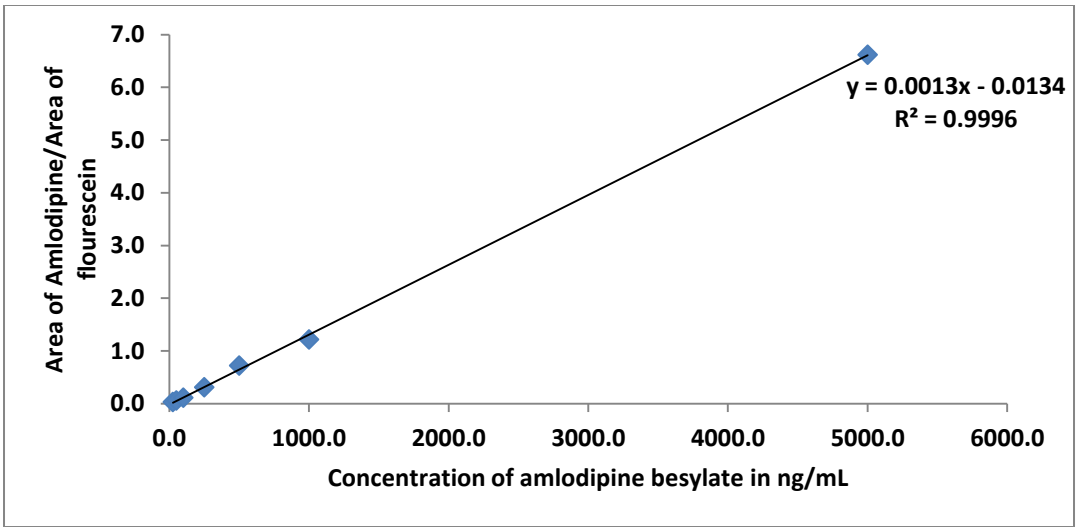

FIGURE 3: REPRESENTATIVE CALIBRATION CURVE OF AMLODIPINE BESYLATE IN RAT PLASMA

Precision and Accuracy: The precision of the assay method was validated by the determination of the intra-day and inter-day coefficient of variation (% C.V.) and percentage deviation. The intra-day and inter-day precision has been done over the concentration range of 25 - 5000 ng/ml. The average % C.V. of intra-day and inter-day precision was 6.82 % and 13.22 %, respectively. All % C.V. were less than 15%. The accuracy of the method was verified by comparing the concentrations measured for amlodipine besylate spiked from extracted sample with actual added concentrations. The intra-day and inter-day accuracy data expressed as percentage deviation of amlodipine besylate assay and the data was shown in **Table 4.** The bioanalytical method was accurate in the range of 25 – 5000 ng/mL in rat plasma.

The accuracy of the method ranged from 85.0 – 99.5 %, which falls in the acceptance criteria. The inter-day & intra-day precision of the method had a % CV less than 20.0% at LLOQ and less than 15 % at the higher concentrations which passes the method as per acceptance criteria.

TABLE 4: RESULTS OF ACCURACY AND PRECISION

Spiked concentration (ng/ml)	Calculated concentration (µg/ml, mean ± *SD, n=3)	**C.V. (%)	Accuracy (%)
	Intra-day (n=3)		
25	25.33 ± 2.37	9.34	98.67
50	50.24 ± 3.22	6.41	99.53
500	626.85 ± 72.93	11.63	85.6
2500	2267.76 ± 109.06	4.81	97.04
5000	5104.91 ± 136.91	2.68	97.9
	Inter-day (n=5)		
25	28.71 ± 5.33	18.56	85.2
50	50.26 ± 6.55	13.04	98.17
500	622.85 ± 61.87	9.94	84.74
2500	2503.89 ± 386.05	15.42	97.65
5000	5724.77 ± 929.19	15.23	87.12

*SD: Standard deviation; ** C.V.: Co-efficient of variation

Recovery: The recovery of amlodipine besylate after protein precipitation procedure was evaluated at five concentrations of 25, 50, 500, 2500 & 5000 ng/ml. Absolute recovery (n=3) was calculated by comparing the peak area ratio for amlodipine besylate and flourescein in methanol with those obtained by acetonitrile extracted plasma samples containing same amount of amlodipine besylate and flourescein. **Table 5** shows the recovery efficiency of amlodipine besylate from rat plasma samples and the average extraction efficiency was found to be 86.00%.

TABLE 5: RECOVERY OF AMLODIPINE BESYLATE FROM RAT PLASMA SAMPLE

Concentration (µg/ml)	Concentration of samples after extraction	Recovery (%)
25	31.00	77.56 ± 11.99
50	51.42	97.18 ± 14.42
500	481.04	83.52 ±11.05
2500	2001.38	77.74 ± 9.50
5000	4599.25	91.98 ± 11.72

Matrix effect: Blank rat plasma was obtained from six different rats and was tested using the proposed extraction procedure for interference. The plasma samples did not show interferences at the retention time of amlodipine besylate. A representative chromatogram is shown in **Figure 4.**

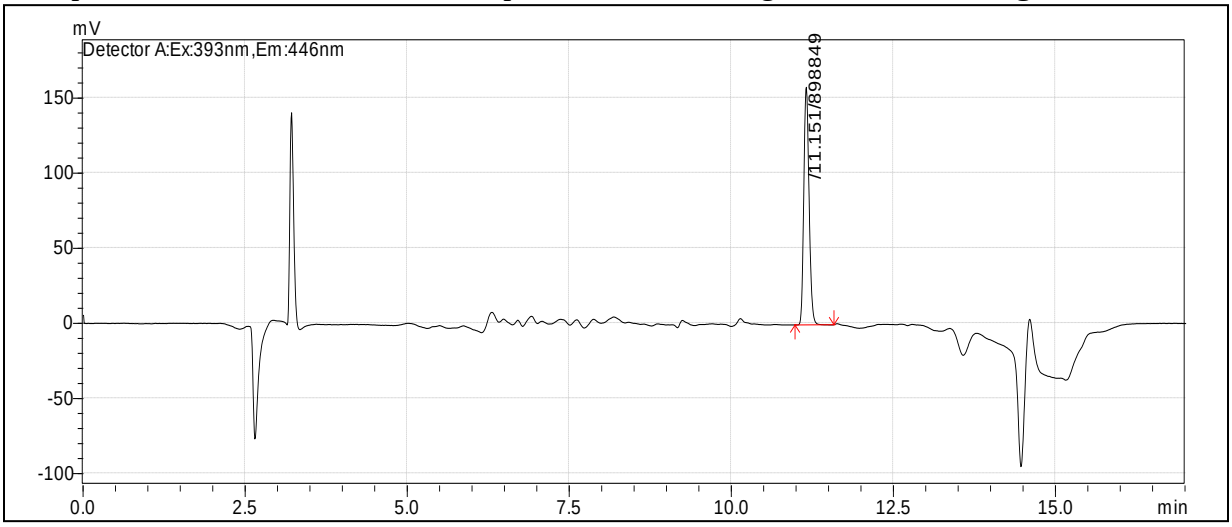

FIGURE 4: REPRESENTATIVE CHROMATOGRAM OF BLANK RAT PLASMA SPIKED WITH FLOURESCEIN (IS)

Several methods have been reported for quantification of amlodipine in human plasma using UV-detection, fluorescence detection & LC-MS/MS detection [3-8]; however few methods have been reported for rat plasma.

Use of the online wavelength switching of excitation & emission wavelengths yielded a stable baseline with the blank rat plasma showing no interfering peaks at retention times of both compounds.

DISCUSSION: Amlodipine besylate is an inhibitor of P-glyco protein (P-gp) & CYP3A2 in rats [12]. Hence amlodipine besylate can be used as substrate for P-gp & CYP3A2 interaction studies for new chemical entities. The present bioanalytical method for the determination of amlodipine besylate in rat plasma samples is novel, sensitive, rapid, specific, accurate and reproducible and can be applied for the above mentioned work at the preclinical level. Extraction procedure is simple protein precipitation which yielded above 85.0 % recovery from rat plasma. Excellent separation was demonstrated between amlodipine besylate and fluorescein (IS).

The calibration curve was linear and the method is suitable for the analysis of plasma samples over the range of 25 to 5000 ng/ml. The accuracy of the method was in compliance with the proposed limits and the precision of the method is satisfactory. This method shows the system suitability parameters are within the limits only.

CONCLUSION: The bionanalytical method for quantification of amlodipine besylate is novel, since use of fluorescence detection & using flourescein as the internal standard has made the method with comparable sensitivity to LC-MS/MS methods. This method can be applied for use in pre-clinical & clinical studies.

Another application of the method to be to evaluate any drug's interaction potential with CYP3A (since amlodipine besylate is a known substrate for CYP3A in rats). The bioanalytical method described here can be successfully applied for pharmacokinetic studies of amlodipine besylate in rats. The proposed method is convenient for routine determination of amlodipine besylate in rat plasma and quality control laboratories that require an economic and rapid method.

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