

ORIGINAL RESEARCH ARTICLE

Development of Simple Analytical Method to Determine Amlodipine in Rat Plasma; A Preclinical Study Applicable To Bioequivalence TestAshesh Bhandary^{1,2}, Arpana Pradhan Bhandary¹, Gulam Muhammad Khan² and Bijay Aryal^{*3}¹Research and Development Department, Time Pharmaceuticals P.Ltd, Mukundapur, Nawalparasi, Nepal²Department of Pharmacy, School of Health and Allied Sciences, Pokhara University, Kaski, Nepal³Department of Clinical Pharmacology, Chitwan Medical College P.Ltd, Bharatpur-10, Chitwan, Nepal

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ABSTRACT

Amlodipine is a dihydropyridine calcium antagonist that inhibits the movement of calcium ions into vascular smooth muscle cells and cardiac muscle cells. The aim of this work to developed simple analytical method to determine amlodipine in rat plasma using High Performance Liquid Chromatography with Ultraviolet Detector (HPLC-UV). HPLC columns Phenomenex[®] C18 (Phenomenex, CA, USA), 250 mm × 4.6 mm, 5 μm particle size and guard column (C18, 4.0 X 2.0mm, Shimadzu, Japan) were used for analyzing blood samples. The compounds were separated isocratically with a mobile phase consisting of acetonitrile-phosphate buffer (0.05 M) with pH 2.8 ± 0.2 in the proportion of (40/60, v/v) at a flow rate 1.0 mL/min with injection volume 20 μL. The interday/Intraday precisions were expressed as CV% and were below 15% and the accuracy was between 80 % and 120 %, which complies with the FDA regulations [12]. The extraction procedure showed good sensitivity, specificity, precision, accuracy, recovery, and linearity, and hence the method was successfully implemented for the analysis of blood samples. In conclusion, the proposed extraction procedure showed good sensitivity, specificity, precision, accuracy, recovery, and linearity, and hence the method can be implemented for the analysis of blood samples for Pharmacokinetics or Bioequivalence study.

Key words; Amlodipine, HPLC-UV, Pharmacokinetics and Bioequivalence Study.**INTRODUCTION**

Amlodipine is a dihydropyridine calcium antagonist (calcium ion antagonist or slow-channel blocker) that inhibits the movement of calcium ions into vascular smooth muscle cells and cardiac muscle cells [1, 2]. Experimental data suggest amlodipine binds to both dihydropyridine and nondihydropyridine binding sites [3, 4]. The contractile processes of cardiac muscle and vascular smooth muscle are dependent upon the movement of extracellular calcium ions into these cells through specific ion channels [5, 6]. Amlodipine inhibits calcium ion influx across cell membranes selectively, with a greater effect on vascular smooth muscle cells than on cardiac muscle cells [7]. Negative inotropic effects, or decreased heart muscle contractility, can be detected *in vitro*, but such effects have not been seen in intact animals at therapeutic doses. Serum calcium concentration is not affected by amlodipine [8, 9]. Within the physiologic pH range,

amlodipine is an ionized compound (pKa = 8.6), and its interaction with the calcium channel receptor is characterized by a gradual rate of association and dissociation with the receptor binding site, resulting in a gradual onset of effect [10]. Amlodipine is a peripheral arterial vasodilator that acts directly on vascular smooth muscle to cause a reduction in peripheral vascular resistance and reduction in blood pressure. Amlodipine also acts as a functional inhibitor of acid sphingomyelinase. Sphingomyelin is involved in signal transduction and apoptosis, or cell death [11].

There are very few analytical methods to be reported for analysis of amlodipine in plasma sample and reported methods are UV spectrophotometric, Spectrofluorimetric, and Capillary electrophoresis [8, 9, 11]. However the perfect quantitative method has not been developed yet. The aim of this work to developed

simple analytical method to determine amlodipine in rat plasma using High Performance Liquid Chromatography with Ultraviolet Detector (HPLC-UV).

MATERIALS AND METHODS

After getting ethical approval from thesis committee of Department of Pharmacy, School of Health and Allied Sciences, Pokhara University on May 14, 2013, the research work has been conducted at Research and Development Department of Time Pharmaceuticals P.Ltd.

Chemicals and Reagents

Amlodipine (purity 99.96%) and Hydrochlorothiazide (Purity 100.78%) has been received as gift samples from Time Pharmaceuticals P.Ltd. Acetonitrile, Potassium dihydrogen phosphate, Orthophosphoric acid were purchased from Merck, Darmstadt, Germany. Double distilled water was obtained from Fisher Scientific, United Kingdom and all other chemicals used were of HPLC grade.

Apparatus and Chromatographic Conditions

The HPLC-UV system used was an Agilent 1260 series. System control and data analysis were carried out using Agilent HPLC software (Agilent Chemstation V.B.30.01, Germany). HPLC columns Phenomenex® C18 (phenomenex, CA, USA), 250 mm × 4.6 mm, 5 µm particle size and guard column (C18, 4.0 X 2.0mm, Shimadzu, Japan) were used for analyzing blood samples. Chromatographic analysis was carried out at ambient temperature (22°C - 25°C). The compounds were separated isocratically with a mobile phase consisting of acetonitrile-phosphate buffer (0.05 M) with pH 2.8 ± 0.2 in the proportion of (40/60, v/v) at a flow rate 1.0 mL/min with injection volume 20 µL. The effluent was monitored spectrophotometrically at wavelength 227 nm. The mobile phase was filtered by passing through a 0.45 µm membrane filter and then ultrasonicated for 15 min.

Animal handling and surgical procedures for blood collection

250 to 350 gm, *Rattus Norvegicus* (Wistar type) male rats were purchased from Banaspati Bivak, Thapathali, Kathmandu, Nepal. The rats were acclimated for two week before study. Upon arrival, animals were randomized and housed one per cage in strictly controlled environmental condition of 20 to 25°C temperature and 48 to 52% relative humidity (RH). A 12 hour light and dark cycle was used with an intensity of 150 to 300 Lux. The surgical procedures were carried out under Ketamine HCl (40 mg/kg) and Diazepam

(1.5mg/kg) general anesthesia (intramuscular injection). Fresh oxygenated blood was collected from left ventricle by making 5 cm incision at fifth left intercostal space, 1cm from the mid-sternal line using 10 ml syringe (Needle gauge 18G) and blood was mixed with equal volumes of heparinized saline (50units/mL). Plasma was separated by centrifuged at 4000rpm (10 minutes) and stored at -4°C till analysis begins.

Sample preparation and validation

Sample preparation involved a protein precipitation method with acetonitrile. The validation samples were prepared by standard working solution spiking method to access the plasma concentration of amlodipine. For the measurement of amlodipine in plasma sample, the validation samples were prepared by following way; an aliquot of blood plasma 90µL was spiked with 10 µl standard working solution (desirable concentration of amlodipine standard solution was prepared by dissolving appropriate amount in acetonitrile) and 10 µl internal standard (Hydrochlorothiazide, 1 µg/ml, prepared in acetonitrile), and extracted with 200 µl acetonitrile solution with ultrasonicated for 10 minutes. The organic layer was separated by centrifuged at 4000 rpm for 10 minutes and 20 µl was injected to HPLC-UV system.

Lower limit of detection (LLOD) was defined as a peak with signal noise ratio(S/N) more than 10/1, while lower limit of quantification was further narrowed to have percentage coefficient of variation (CV, %) less than 15%. Five sets of validation samples at concentrations of 100 ng/ml, 200ng/ml, 500ng/ml, 1 ug/ml, 2 ug/ml, 4 ug/ml, 8 ug/ml and 10 ug/ml were used to draw calibration curve. Similarly, Inter/ Intra- day validation were assessed to validate the precision and accuracy of the assay. For interday validation, five sets of control samples at different concentrations of 100 ng/ml, 200ng/ml, 500ng/ml, 1 ug/ml, 2 ug/ml, 4 ug/ml, 8 ug/ml and 10 ug/ml were evaluated on five different days. For intraday validation, five sets of control samples at different concentrations of 100 ng/ml, 200ng/ml, 500ng/ml, 1 ug/ml, 2 ug/ml, 4 ug/ml, 8 ug/ml and 10 ug/ml with one standard curve were evaluated on same day. The assay recovery for amlodipine was assessed with five sets of quality control (QC) samples (100ng/ml, 500ng/ml and 10 ug/ml) assayed randomly along with standard samples during the interday and intraday assays.

Data Analysis

The MS Excel version 2007 for windows was used for data analysis. All data were expressed as mean \pm standard deviation ($X \pm SD$).

RESULTS AND DISCUSSION

Selection of Internal Standard and Optimization of Mobile Phase

Our objective of the chromatographic method development was to achieve a peak tailing factor ≤ 2 , retention time in between 2 and 12 min, along with good resolution, hence hydrochlorothiazide (**Figure 2**) was selected as internal standard for analyte amlodipine (**Figure 1**). In order to affect the simultaneous elution of more than one component under isocratic conditions, different chromatographic conditions (organic modifier, flow rate, and pH) have been investigated. Various stationary phases were used like C8 and C18 and phenyl column, poor and distorted peaks were observed with phenyl column while C18 gave satisfactory resolution and free from tailing. Mobile phases containing methanol alone or acetonitrile alone were found to elute the compounds unresolved.

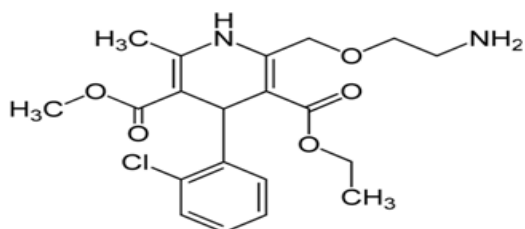


Figure 1: Chemical structure of Amlodipine

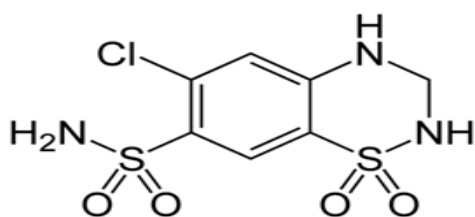


Figure 2: Chemical structure of Hydrochlorothiazide

Increasing the acetonitrile concentration to more than 65% of buffer led to inadequate separation. At a lower acetonitrile concentration ($<40\%$), separation occurred, but with excessive tailing and increased retention time. To avoid multiple peaks of reversed phase columns, the pH must be controlled with buffers, for example potassium dihydrogen phosphate. This objective was obtained using mobile phase consisting of acetonitrile-phosphate buffer (0.05 M) in the proportion of (40/60, v/v) with the pH adjusted to of 2.8 ± 0.2 with orthophosphoric acid. The mobile phase composition was optimized under the described conditions, the analyte peaks were well defined, resolved and free from tailing, the

tailing factors were ≤ 2 for all peaks. The elution orders were hydrochlorothiazide (tR 3.025 min) and amlodipine (tR 5.019 min) at a flow rate of 1.0 ml/min. The optimum wavelength for detection was 227 nm at which much better detector responses for the three drugs were obtained. System suitability tests are used to verify that the column efficiency, selectivity factor (resolution) and reproducibility of the chromatographic system are adequate for the analysis. System suitability tests were carried out on freshly prepared standard stock solutions of amlodipine and hydrochlorothiazide.

Linearity, Detection and Quantitation Limits:

Lower limit of detection (LLOD) and Higher limit of detection (HLOD) were defined as a peak with signal noise ratio(S/N) more than 10/1, while lower limit of quantification was further narrowed to have percentage coefficient of variation (CV, %) less than 15%. LLOD and HLOD were defined at 100ng/ml and 10ug/ml respectively. Five sets of validation samples at concentrations of 100 ng/ml, 200ng/ml, 500ng/ml, 1 ug/ml, 2 ug/ml, 4 ug/ml, 8 ug/ml and 10 ug/ml were used to draw calibration curve. The calibration curve drawn for amlodipine in rat plasma was linear over the concentration range 100ng/ml to 10ug/ml, giving a mean linear regression equation for the calibration curve of $y = 1.3881x + 0.00081$, and the correlation coefficient (r^2) for amlodipine was 0.999 (**Figure 3**).

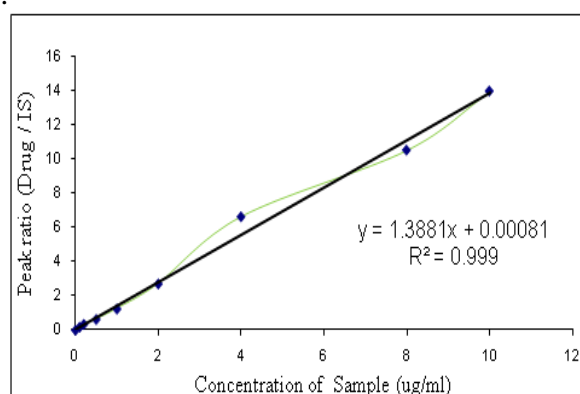


Figure 3: Linear calibration curve of amlodipine in rat plasma

Accuracy, Precision and Specificity:
The interday precisions were expressed as CV% and were below 15% (maximum, 14.242% and minimum, 11.77 % for an LLOD sample), and the accuracy was between 85.42 and 104.79%, which complies with the FDA regulations [12]. The extraction procedure showed good sensitivity, specificity, precision, accuracy, recovery, and linearity, and hence the method was successfully implemented for the analysis of blood samples (**Table 1**). Similarly, the intraday precisions were

also expressed as CV% and were below 15% (maximum, 12.35% and minimum, 9.66 % for an LLOD sample), and the accuracy was between 85.45 and 112.87 %, which complies with the FDA regulations. The recovery percentages of QC samples were between 101% and 119%. (Table 2).

Table 1: Interday validation of the HPLC method for measuring amlodipine in rat plasma

Parameters	Obtained Results (Amlodipine)
Lower limit of detection (ng/ml)	100 ng/ml
Calibration range (ng/ml)	100ng/ml -10ug/ml
Calibration equation	$y = 1.3881x + 0.0008$
Coefficient of regression (r^2)	0.999
Interday Precision (CV %,n=5) ^a	
100 ng/ml	11.77
200 ng/ml	11.022
500 ng/ml	11.65
1ug/ml	11.42
2ug/ml	14.63
4ug/ml	12.011
8ug/ml	13.846
10ug/ml	14.242
Interday Accuracy (% ,n=5) ^b	
100 ng/ml	101.94
200 ng/ml	104.79
500 ng/ml	103.46
1ug/ml	101.95
2ug/ml	82.75
4ug/ml	92.12
8ug/ml	97.45
10ug/ml	85.42

^a %CV=Standard deviation of concentrations determined x 100/ Mean concentration determined

^b Accuracy = Mean concentration determined x100/Concentration expected.

Table 2: Intraday validation of the HPLC method for measuring amlodipine in rat plasma

Parameters	Obtained Results (Amlodipine)
Lower limit of detection (ng/ml)	100 ng/ml
Calibration range (ng/ml)	100ng/ml -10ug/ml
Intaday Precision (CV %,n=5) ^a	
100 ng/ml	9.66
200 ng/ml	11.45
500 ng/ml	11.85
1ug/ml	10.74
2ug/ml	12.35
4ug/ml	11.77
8ug/ml	12.14
10ug/ml	12.45
Intaday Accuracy (% ,n=5) ^b	
100 ng/ml	85.45
200 ng/ml	97.79
500 ng/ml	110.85
1ug/ml	101.95
2ug/ml	105.96
4ug/ml	98.63
8ug/ml	95.87
10ug/ml	112.87
QC Recovery	Accuracy 119 % (CV=11.55%)
100ng/ml	Accuracy 117 % (CV=12.17%)
4ug/ml	Accuracy 101 % (CV=10.27%)
10ug/ml	

^a %CV=Standard deviation of concentrations determined x 100/ Mean concentration determined

^b Accuracy=Mean concentration determined x100/Concentration expected.

The intra- and inter-day precisions expressed as coefficient of variations percent (% CV) should not exceed 15% at any concentration level, with the exception of LLOD, QC samples, where should not exceed ±20% (Bioanalytical Method Validation, FDA guidelines, May 2001).

Chromatographic conditions, especially the composition of the mobile phase, were optimized to achieve good resolution and symmetrical peak shapes for amlodipine and the IS, acceptable retention factors ($k \geq 2$), and a short run time. This objective was obtained using mobile phase consisting of acetonitrile-phosphate buffer (0.05 M) in the proportion of (40/60, v/v). The elution orders were hydrochlorothiazide (tR 3.025 min) and amlodipine (tR 5.019 min) at a flow rate of 1.0 ml/min. System suitability tests showed that the column efficiency, selectivity factor (resolution) and reproducibility of the chromatographic system are adequate for the analysis.

No peaks corresponding to amlodipine or the IS were observed in blank rat plasma using the HPLC-UV conditions described in (Figure 4). A HPLC chromatogram of rat plasma spiked with IS was shown in (Figure 5). The HPLC chromatogram of blood sample (amlodipine 500 ng/ml) was shown in (Figures 6).

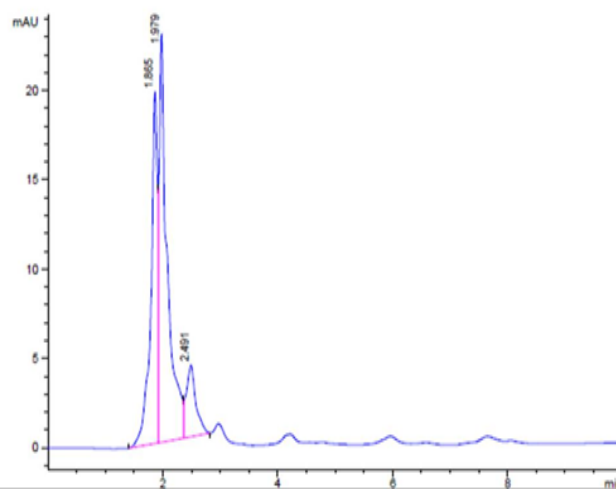


Figure 4: HPLC-UV chromatogram of rat plasma double blank

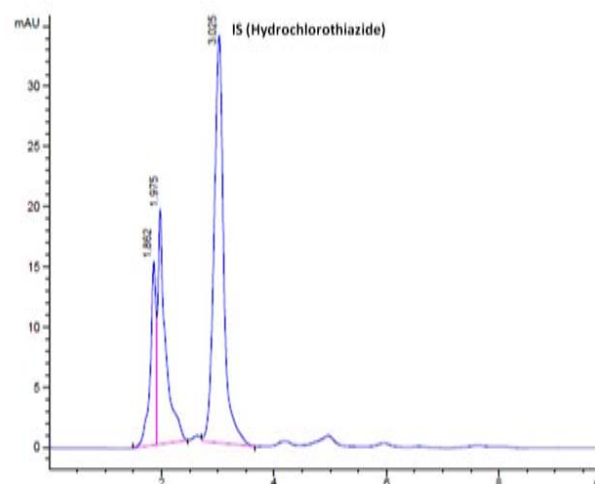


Figure 5: HPLC-UV chromatogram of rat plasma with internal standard hydrochlorothiazide

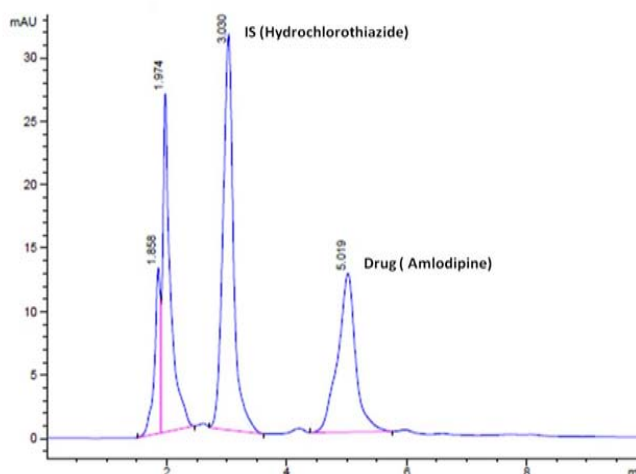


Figure 6: HPLC-UV chromatogram of rat plasma with internal standard hydrochlorothiazide and drug amlodipine (500ng/ml).

CONCLUSION

In conclusion, the proposed extraction procedure showed good sensitivity, specificity, precision, accuracy, recovery, and linearity, and hence the method can be implemented for the analysis of blood samples for Pharmacokinetics or Bioequivalence study.

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