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Research Article

NON-COMPARTMENTAL PHARMACOKINETICS MODELING OF AMLODIPINE IN RATS

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Abstract: In the present study, we did the non –compartmental pharmacokinetics study of amlodipine using high performance liquid chromatography with ultraviolet detector (HPLC-UV) in wistar rats. Rats were allocated to two groups; intravenous group (IV study n=6) and oral group (PO study n=6).In both groups, surgical procedures were carried out under Ketamine HCL (40 mg/kg) and Diazepam (1.5mg/kg) general anesthesia (intramuscular injection).The blood samples were collected at different time interval and were analyzed using HPLC-UV system. Results showed that Amlodipine had a short terminal half-life with relatively high distribution volumes during the steady and terminal phases, and with low plasma clearance. Furthermore, the availability ratio of amlodipine through the intravenous route was higher than that through the oral route, indicating that first pass metabolism and hepatic blood flow are important factor of drug elimination of amlodipine. Bioavailability was estimated to be 78.60 ± 21.33% based on the AUCinf ratios of oral and intravenous administration.
Key**words:** Wistar rats, amlodipine, pharmacokinetics parameters and bioavailability.

Introduction

Rat is an attractive model for many biomedical researches. Availability of various breeds and knockouts that emulate disease states or altered metabolism advocate its importance in pharmacological or pharmacokinetic studies. Being small in size, it requires relatively small quantity of expensive new chemical entities to conduct pharmacokinetic studies [1, 2].

Various methods are available to collect blood from a rat for pharmacokinetic studies. Among these, a timed-sacrifice or tail-bleed methods are widely used. However, the timed-Sacrifice generates inevitable inter-animal variation, whereas tail-bleed limits to fewer samples with low blood volume [3, 4]. Big vessels cannulation generates multiple samples with precision and ease has been successfully applied in rat [5]. The surgical techniques allow low stressed sample collection from a small animal. Direct drug injection to big vessels established pharmacokinetics studies with high precision and accuracy [6].

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 [7, 8]. Experimental data suggest amlodipine binds to both dihydropyridine and nondihydropyridine binding sites. 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 [9] .Amlodipine inhibits calcium ion influx across cell membranes selectively, with a greater effect on vascular smooth muscle cells than on cardiac muscle cells. 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 [10] . 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 [11, 12] .In the present study, we did the non –compartmental pharmacokinetics study of amlodipine using high performance liquid chromatography with ultraviolet detector (HPLC-UV) in wistar rats.

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. Acetronitrile, Potassium dihydrogen phosphate, Orthophosphoric acid were pursed 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, Shimadazu,

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 acetonetrile-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

250 to 350 gm, Rattus Norvegicus (Wistar type) male rats were pursed from Banaspati Bivak, Thapathali, and 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^{o}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.Before conducting study, rats were allocated to two groups; intravenous group (IV study n=6) and oral group (PO study n=6).In both groups, surgical procedures were carried out under Ketamine HCL (40 mg/kg) and Diazepam (1.5mg/kg) general anesthesia (intramuscular injection). The polyethylene catheter CX-8001S (ID; 0.55mm and OD: 0.57 mm, Dehan Ltd, South Korea) was implanted to femoral vein or artery for drug infusion and blood collection. Considering catheter dead volume, amlodipine (5 mg/kg) was infused through a femoral vein in the IV study or through an oral gavage in the oral study. Samples were collected at 0, 15, 30, 60, 120, 240, 360, 480,600, 720, and 1440 minutes in the IV study, and at 0, 15, 30, 60, 90, 120, 240, 360, 480,600, 720, and 1440 minutes in the oral study from femoral artery. Samples were collected with virtually no blood loss, and sample volumes were compensating for with equal volumes of heparinized saline (50units/mL). Plasma was separated by centrifuged at 4000rpm (10 minutes) and stored at -4ºC till analysis begins.

Sample preparation and validation

Sample preparation involved a protein precipitation method with acetronitrile. 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 80µL was spiked with 10 µl standard working solution (desirable concentration of amlodipine standard solution was prepared by dissolving appropriate amount in acetronitrile) and 10 µl internal standard (Hydrochlorothiazide, 1 µg/ml, prepared in acetronitrile), and extracted with 200 µl acetronitrile 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.

Blood samples- analysis

Blood samples were prepared by following way; an aliquot of blood plasma 90µL was spiked with 10 µl internal standard (Hydrochlorothiazide, 1 µg/ml, prepared in acetronitrile), and extracted with 200 µl acetronitrile 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. The effluent was monitored spectrophotometrically at wavelength 227 nm with a mobile phase consisting of acetonetrile-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.

Data Analysis

Non-compartmental pharmacokinetics analysis was performed using WinNonlin^{TM} Professional (Version 2.1, Pharsight, CA, USA) for windows. Results are expressed as mean ± standard deviation (X ± SD) and bioavailability was estimated to be based on AUC_{inf} ratios determined after oral and intravenous administration.

Result 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 acetonetrile alone were found to elute the compounds unresolved.

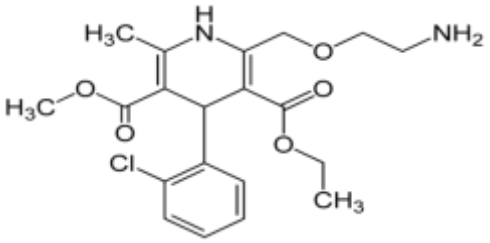

Figure 1: Chemical structure of Amlodipine

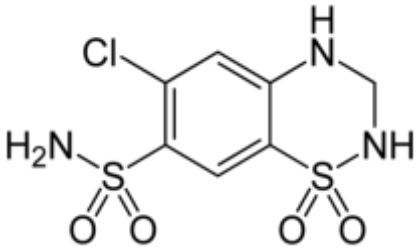

Figure 2: Chemical structure of Hydrochlorothiazide

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, and 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**).

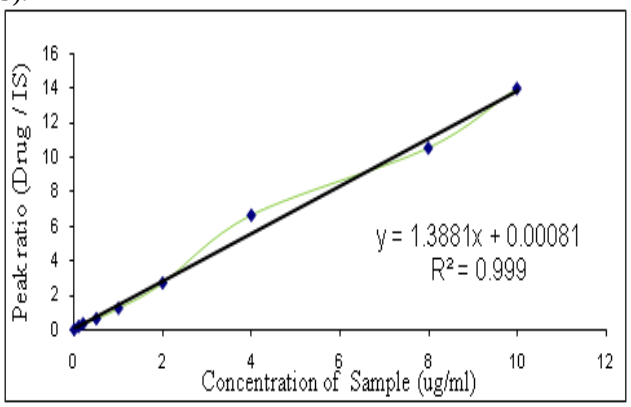

Figure 3: Linear calibration curve of amlodipine in rat plasma

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 if 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
Intarday 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
Intarday 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	
100ng/ml	Accuracy 119 % (CV=11.55%)
4ug/ml	Accuracy 117 % (CV=12.17%)
10ug/ml	Accuracy 101 % (CV=10.27%)

[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-UVconditions described in **Figure 4**.The HPLC-UV chromatogram of Blank + IS was shown in **Figure 5**.

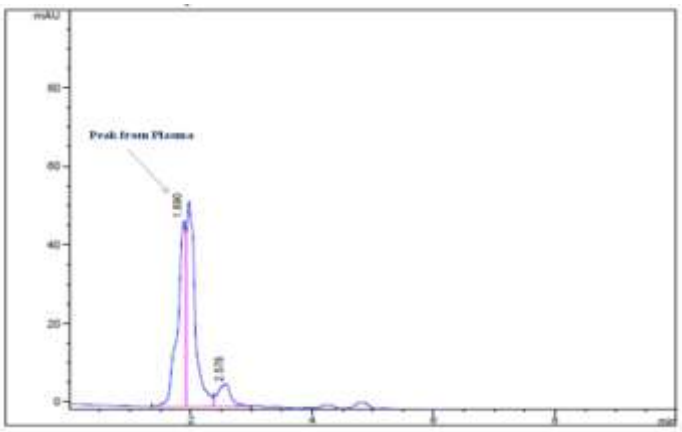

Figure 4: HPLC-UV chromatogram of blank plasma.

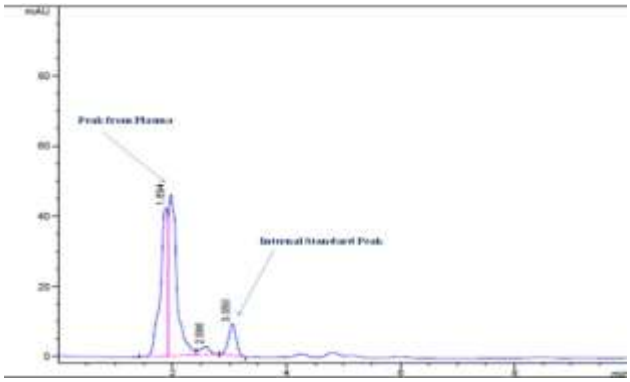

Figure 5: HPLC-UV chromatogram of blank plasma spiked with IS.

The HPLC-UV chromatogram of blood sample at 90 minutes oral study are shown in **Figure 6.**

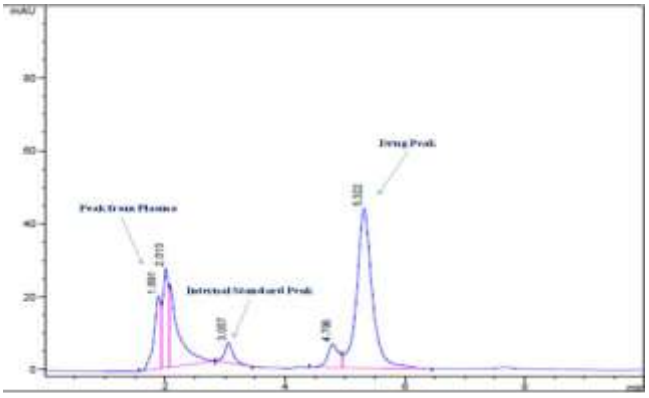

Figure 6: The HPLC-UV chromatogram of blood sample at 90 minutes oral study.

Non-Compartmental pharmacokinetics of Amlodipine

The concentration–time profile of amlodipine following its oral and intravenous administration is shown in **Figure 7** and **Figure 8**. Table 3 summarizes the PK parameters of amlodipine after intravenous and oral administration, respectively.

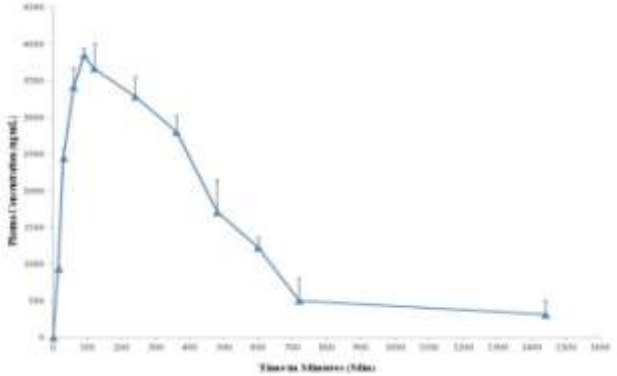

Figure 7: The concentration–time profile of amlodipine following its oral administration.

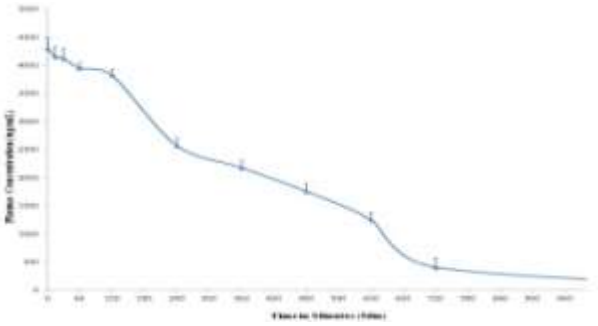

Figure 8: The concentration–time profile of amlodipine following its intervaneous administration.

Table 3: Non-compartmental pharmacokinetics of Amlodipine (5mg/kg) in plasma samples

Parameters	Mean±SD
Oral Administration	
AUCinf(µg.min/ml)	5315.59±308.77
Tmax(min)	90 ±14.27
Cmax (µg/ml)	3849±3.08
T1/2(min)	404.22±22.00
CL(ml/min/kg)	6.97±0.14
Intravenous Administration	
AUCinf(µg.min/ml)	6762.73±123.91
Cmax(µg/ml)	4299.33±11.62
T1/2(min)	133.41±65.92
Vss(ml/kg)	1523.88±308.24
CL(ml/min/kg)	10.83±3.32
Bioavailability	**78.60 ± 21.23%**

Abbreviations;
AUC, area under curve; Tmax, time to reach maximum concentration; Cmax, maximum concentration; T1/2, terminal half life; Vss, distribution volume in the steady state; CL, total clearance.

Areas under curve (AUCinf) values were 5315.59 ± 308.77 and 6762.73 ± 123.91 for oral and intravenous administration, respectively. Amlodipine had a short terminal half-life (404.22 ± 22.00 and 133.41 ± 65.92 minutes in the oral and intravenous studies, respectively) with relatively high distribution volumes during the steady and terminal phases, and with low plasma clearance. This indicates that the absorption of amlodipine is not a limiting factor for plasma clearance and extent of distribution. Volume of distribution (V) is the parameter used to assess the amount of drug in the body from the measurement of a snapshot plasma concentration. The main clinical application of V is to compute a loading dose (e.g. the first dose of a multiple dosage regimen) in order to immediately reach the target therapeutic plasma concentration. Frequently, and often incorrectly, the numerical value of a V is advocated to support claims on the extent of drug distribution. It should be stressed that V was not primarily designed to evaluate drug distribution in the different physiological spaces, and that a V can be much higher than the total body water space . Nevertheless, a physiological interpretation of V is possible but this requires having recourse to models involving drug binding to plasma and tissues [6,9,13].High volume of distribution with relatively short terminal half life indicated that one daily dose of amlodipine is enough to cover 24 hours blood pressure. Similarly, in the oral study, peak concentration was observed at about 90 ± 14.27 minutes after dosing, indicating that amlodipine absorbed rapidly and that its absorption was independent of visit's gastric solubility and pH. Maximum concentration (Cmax) and total clearance (CL) values following oral administration were 3849 ± 3.08 and 6.97 ± 0.14 respectively, and in the intravenous study, these were 4299.33 ± 11.15 and 10.83 ± 3.32, respectively. The availability ratio of amlodipine through the intravenous route was higher than that through the oral route, indicating that first pass metabolism and hepatic blood flow are important factor of drug elimination of amlodipine. Clinically, it has been reported that first-pass metabolism is important when the fraction of the dose administered that escapes metabolism is small and variable. The liver is usually assumed to be the major site of first-pass metabolism of a drug administered orally, but other potential sites are the gastrointestinal tract, blood, vascular endothelium, lungs, and the arm from which venous samples are taken [14,15]. Although we did not study hepatic blood flow during the present study, it has previously been reported that restraint and water immersion stress caused a marked decrease in hepatic blood flow in mice, which most influences the plasma clearances of highly absorbable drugs [16, 17]. Bioavailability was estimated to be 78.60 ± 21.33% based on the AUCinf ratios of oral and intravenous administration.

Conclusion

In conclusion, amlodipine absorbed rapidly and that its absorption was independent of visit's gastric solubility and pH. The availability ratio of amlodipine through the intravenous route was higher than that through the oral route, indicating that first pass metabolism and hepatic blood flow are important factor of drug elimination of amlodipine. Bioavailability was estimated to be 78.60 ± 21.33% based on the AUCinf ratios of oral and intravenous administration.

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