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Amlodipine Bioequivalence Study: Quantification by Liquid Chromatography Coupled to Tandem Mass Spectrometry

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ABSTRACT: *Objective*—To assess the bioequivalence of two amlodipine tablet formulations (Amlodipine® 5 mg tablet from Merck S.A. Indústrias Químicas, Brazil as test formulation and Norvasc® 5 mg tablet from Laboratórios Pfizer Ltd., Brazil as reference formulation) in 24 healthy volunteers of both sexes.

Methods—The study was conducted using an open, randomized two-period crossover design with a 4-week washout interval. Plasma samples were obtained over a 144 h period. Plasma amlodipine concentrations were analyzed by combined liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS) with positive ion electrospray ionization using multiple reaction monitoring (MRM). From the amlodipine plasma concentration vs time curves, the following pharmacokinetic parameters were obtained: AUC_{last}, AUC_{0-inf} and C_{max}. The statistical interval proposed was 80–125% according to the US Food and Drug Administration Agency.

Results—The limit of quantification was 0.1 ng/ml for plasma amlodipine analysis. The geometric mean and the 90% confidence interval (CI) test/reference ratios were 101.2 (92.9–110.2%) for AUC_{last}, 99.6 (91.5–108.4%) for AUC_{0-inf} and 98.5 (89.0–109.1%) for C_{max}.

Conclusion—Since the 90% CI for AUC_{last}, AUC_{0-inf} and C_{max} ratios were within in the 80–125% interval proposed by the US FDA, it was concluded that Amlodipine® 5 mg tablet (test formulation) was bioequivalent to Norvasc® 5 mg tablet, in terms of both rate and extent of absorption.

Key words: pharmacokinetics; desipramine; HPLC; LC-MS-MS

Introduction

Amlodipine, 2-[(2-Aminoethoxy)methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylic acid 3-ethyl 5-methyl ester, a third-generation dihydropyridine calcium antagonist, is used to lower blood pressure in hypertensive patients [1]. It has an average molecular weight of 408.9 g/mol, and is commercially available for clinical use as 5 and 10 mg tablets.

The aim of this study was to assess the bioequivalence in healthy human volunteers of both sexes, of two amlodipine 5 mg tablet formulations. Amlodipine and the internal standard desipramine (IS) (Figure 1) plasma levels were measured using a high performance liquid chromatography assay coupled to tandem mass spectrometry (LC-MS-MS).

Methods

Clinical protocol

Twenty-four healthy volunteers of both sexes, aged between 18 and 45 years old and within the

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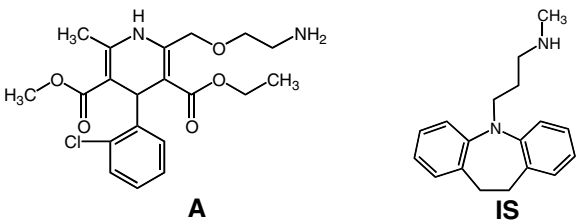

Figure 1. Chemical structure of amlodipine (A) and internal standard desipramine (IS)

15% of the ideal body weight were selected for the study. The volunteers were free from significant cardiac, hepatic, renal, pulmonary, neurological, gastrointestinal and hematological diseases, as assessed by physical examination, ECG, and the following laboratory tests: blood glucose, urea, creatinine, AST, ALT, alkaline phosphatase, γ-GT, total bilirubin, uric acid, total cholesterol, triglycerides, albumin and total protein, hemoglobin, hematocrit, total and differential white cell counts, erythrocyte sedimentation rate and routine urinalysis. All subjects were negative for HIV, HBV (except for serological scar) and HCV.

The male group was composed of 12 volunteers (21.8 ± 2.8, mean $\pm$ s.d.m; range 18–27 years), height between 160 and 186 cm (172.9 ± 7.2 cm), weighing between 55.0 and 90.5 kg (73.0 ± 11.8 kg). The female group was also composed by 12 volunteers (21.8 ± 5.0 year; range: 20–36 years), height between 158 and 173 cm (164.3 ± 5.0 cm), weighing between 50.0 and 73.2 kg (59.6 ± 7.6 kg).

All subjects gave a written informed consent, and the Ceará Federal University Hospital Ethics Committee of Clinical Investigation approved the clinical protocol.

The study was conducted in an open, randomized, two-period crossover fashion with a four-week washout interval between doses. During each period, the volunteers were hospitalized at 10:00 p.m., having already eaten a normal evening meal, and after an overnight fast they received at 7:00 a.m. a single 5 mg dose of the appropriate amlodipine tablet formulation along with 200 ml of tap water. No food was allowed during 3 h following drug administration, after which a standard breakfast was consumed. A lunch and an evening meal were provided 5 and 10 h after dosing, respectively. No other food was permitted during the 'in-house' period. Liquid consumption was permitted *ad libitum* after lunch, but xanthine-containing drinks including tea, coffee, or cola were avoided.

At each blood sampling time, systolic and diastolic arterial pressure (measured non-invasively with a sphygmomanometer) and heart rate were recorded.

Formulations

The following formulations were employed: Amlodipine® from Merck S.A. Indústrias Químicas, Brazil (lot number 99Z047, expiration date 03/2001) as test formulation, and Norvasc® from Laboratórios Pfizer Ltd., Brazil (lot number 804-05034 & 904-05002-B, expiration date 10/2000 & 01/2001, respectively) as reference formulation.

Chemicals and reagents

Amlodipine (hydrochloride form) was provided by Merck Indústria e Comercio Ltda, (Brazil) and desipramine was obtained from Sigma (USA). The following analytical or HPLC grade reagents were used: ammonia solution from Synth (Brazil), diethyl-ether from Jand Química Ind. & Com. Ltd. (Brazil), acetonitrile from Nuclear (Brazil), formic acid, sodium carbonate and sodium bicarbonate from Mallinckrodt (USA), acetic acid glacial from Química Moura (Brazil), hexane from Quimex (Brazil), methyl alcohol from J.T. Baker (Brazil) and water (purified using Milli-Q or Elga UHQ systems). Pools of human plasma for quality control and calibration curve preparations were provided by São Paulo University Hospital, Brazil.

Calibration standards and quality control

Stock solutions of amlodipine and internal standard (desipramine) were prepared in methanol–water (50:50 v/v) at a concentration of 1 mg/ml. Calibrations curves of amlodipine were prepared in blank plasma at concentrations of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 ng/ml and performed in duplicate for each batch. The quality control samples were prepared in blank

plasma at concentrations of 0.5, 2.0 and 10.0 ng/ml (QCA, QCB, and QCC, respectively) independent of the calibration standards.

Drug analysis

Blood samples (10 ml) from a suitable antecubital vein were collected into EDTA-containing tubes before and 1, 2, 4, 6, 8, 10, 12, 14, 24, 48, 96, 120, and 144 h after the administration of each dose of amlodipine. The blood samples were centrifuged at 2500 *g* for 10 min at room temperature and the plasma was decanted and stored at −20°C until assayed for their amlodipine contents.

All frozen human plasma were previously thawed at ambient temperature and centrifuged at 2000 *g* for 5 min at 4°C to precipitate solids. Two hundred µl of IS solution (10 ng/ml desipramine in carbonate buffer pH 9.0) were added to 200 µl aliquot of plasma sample. The tubes were briefly vortex-mixed and the compounds of interest were extracted with 4 ml of diethylether/hexane (80:20 v/v). The mixture was vortex-mixed for approximately 40 s, and the organic phase was evaporated under N_2 at 37°C. The dry residues were reconstituted with 200 µl of mobile phase (60% CH_3CN; 40% H_2O; plus 10 mM formic acid) and transferred to the autoinjector microvials.

Chromatographic conditions

An aliquot (40 µl) of each plasma extract was injected into a Genesis C_{18} 4 µm analytical column (150 mm × 4.6 mm i.d.; Jones Chromatography) operated at a temperature of 40°C. The compounds were eluted by pumping the mobile phase at a flow rate of 0.4 ml/min. Under these conditions, typical standard retention times (RT) were 3.4 min for amlodipine and 3.6 min for desipramine, and back-pressure values of approximately 35–45 bar were observed.

A split of the column eluant of approximately 1:10 was included so that only approximately 40 µl/min entered into the mass spectrometer. The temperature of the autosampler was kept at room temperature (22–25°C) and the total run time was 5.0 min.

Mass-spectrometry conditions

The mass spectrometer (Micromass Quattro LC) equipped with an electrospray source using a crossflow counterelectrode was run in positive mode (ES+), and set up in Multiple Reaction Monitoring (MRM), monitoring the transitions 409.0>238. 1 and 267.1>236.2 for amlodipine and IS, respectively. For both amlodipine and IS, the capillary voltage, the dwelling time, the cone voltage and the gas pressure (argon) were 3.5 kV, 0.8 s, 20 V and 7.4×10^{-4} mbar, respectively. The collision energy was 11 eV for amlodipine and 15 eV for desipramine. The percent of precursor ion attenuation, under the mass spectrometer conditions described above, were 99 and 98% for analyte and IS, respectively. Data acquisition and analysis were performed using the software MassLynx (v 3.2 running under Windows NT (v 4.0) on Pentium PC).

Method development

Full-scan positive mass spectra of amlodipine and IS showed the protonated molecules, $[M-H]^+$, of *m/z* 409 and 267, respectively, according to the previous study [2]. The most abundant ion in the product ion spectra was at *m/z* 238 for amlodipine obtained by an unusual fragmentation mechanism elucidated in the same study [2]. The *m/z* 236 was the most abundant product ion for IS as a result of neutral loss of methylamine (CH_3NH_2). The structures proposed for both product ions are illustrated in Figure 2.

From these results, the mass spectrometer was set as follows: *m/z* 409 for amlodipine and *m/z* 267 for desipramine as the precursor ions and *m/z* 238 and 236 as the respective product ions in the MRM mode. No peak was observed in the mass chromatogram of blank human plasma under the LC-MS-MS conditions described above, as shown in Figure 3A. Also, the mass chromatograms of a sample are shown in Figure 3B, where it can be observed that the retention times of amlodipine and IS were 3.4 and 3.5 min, respectively.

Linearity, precision and accuracy were determined to assess the performance of the method. A linear least-squares regression with a weighting index of $1/x$ was performed on the peak area ratios of amlodipine and IS vs amlodipine

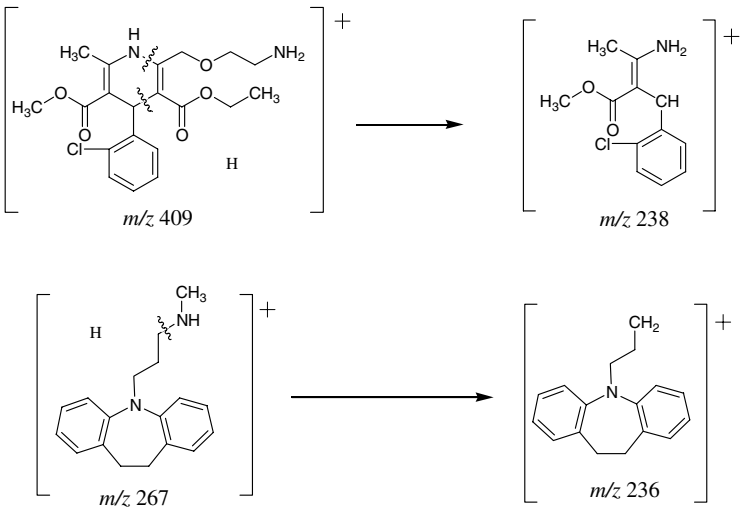

Figure 2. Dissociation route proposed for amlodipine and desipramine

concentrations of the human standards (in duplicate) to generate a calibration curve.

A quality control sample (QCA, QCB or QCC) was analyzed after a sequence of 10 unknown samples. The lowest limit of quantification (LOQ) was defined as the lowest concentration at which both precision and accuracy were less than 20%.

Stability

Quality control samples (1.0 and 10.0 ng/ml) were subject to short-term room temperature (6 h), three freeze/thaw cycles, 24 h-autosampler stability and long-term stability (15 days) tests. Subsequently, the amlodipine concentrations were measured compared with freshly prepared samples. The significance of the obtained results was analyzed by the Student's *t*-test ($p>0.05$).

Pharmacokinetics and statistical analysis

The first-order terminal elimination rate constant (ke) was estimated by linear regression from the points describing the elimination phase on a log-linear plot. Half-life ($t_{1/2}$) was derived from this rate constant [$t_{1/2} = \ln(2)/\text{ke}$]. The maximum observed plasma concentration (C_{max}) and the time taken to achieve this concentration (T_{max}) were obtained directly from the curves. The areas under the amlodipine plasma concentration vs time curves from 0 to the last detectable concentration (AUC_{last}) were calculated by applying the linear trapezoid rule. Extrapolation of these areas to infinity (AUC_{0-inf}) was done by adding the value C_{last}/ke to the calculated AUC_{last} (where C_{last} = the last detectable concentration).

For T_{max} statistical analysis, both the arithmetic mean and the individual T_{max} differences between test and reference formulations were used. Parametric and non-parametric analysis on ln-transformed data were done.

Results and Discussion

Tolerance of both formulations was good. Six volunteers complained of headache and one volunteer of dysmenorrhea. All biochemical parameters monitored presented no clinically relevant alterations.

The calibration curve showed good linearity within 0.1–20 ng/ml of amlodipine ($r^2 > 0.998$) (Figure 4). The limit of quantification was 0.1 ng/ml for amlodipine. The intra-batch precision were 5.7, 4.4, and 5.6% for 0.5, 2.0 and 10.0 ng/ml, respectively. The intra-batch accuracy were −1.0, −4.2, and 1.3%, respectively. The

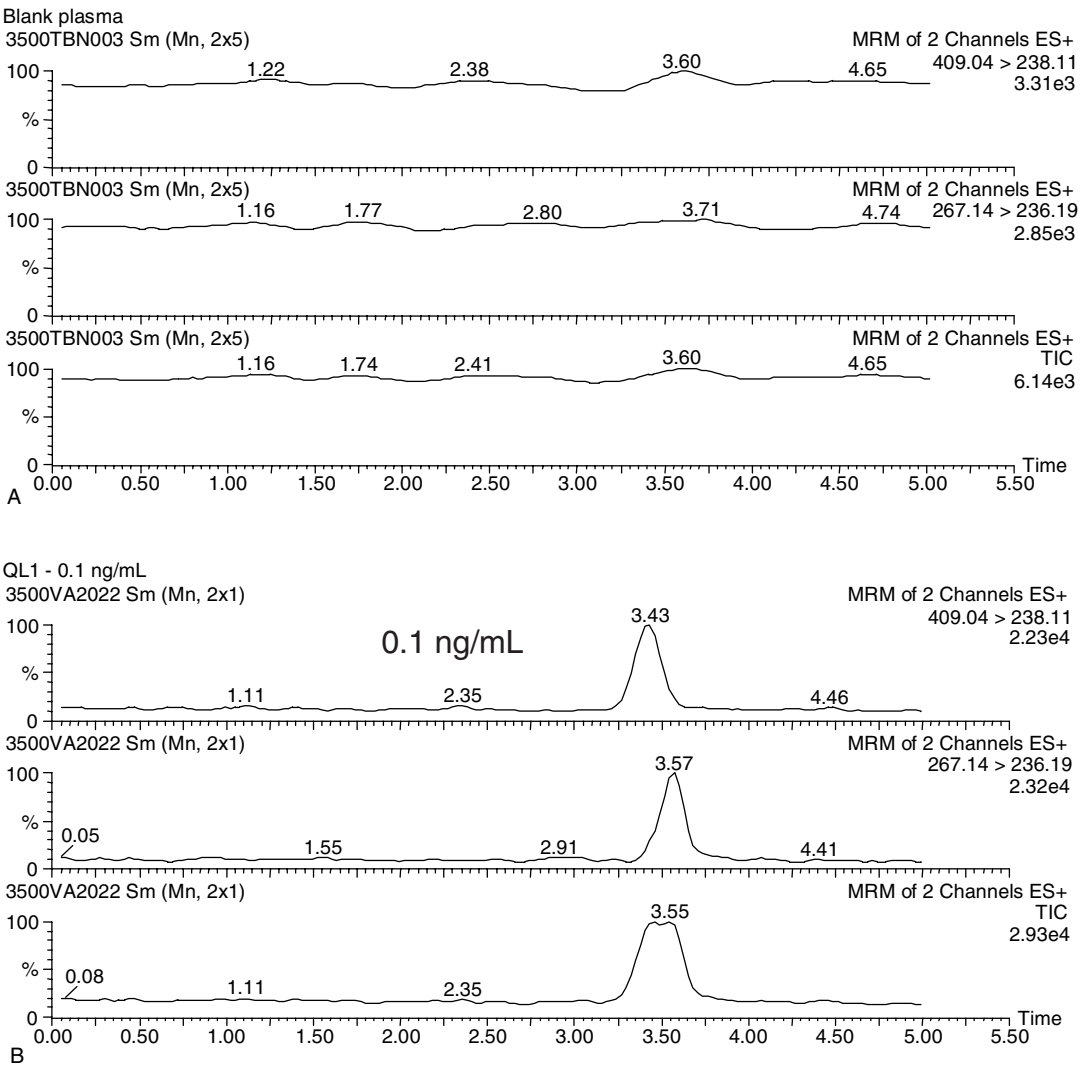

Figure 3. Selected ion chromatograms (MRM) of 2 channels ES+. A: blank human plasma for amlodipine, desipramine and total ion count (TIC). B: 409.0>238.1 for amlodipine and 267.1>236.2 for desipramine. The peaks illustrate the retention time and integrated area

inter-batch precision for the same concentrations were 0.9, 5.1, and 1.0%, respectively, and the inter-batch accuracy were 0.0, −1.5, and 0.2%, respectively (Table 1).

The recovery of amlodipine, based on peak areas are ratios of extracted normal human plasma/mobile phase, both previously spiked at final concentrations of 1.0 and 10.0 ng/ml, were 63.7 and 77.9% ($n = 5$), respectively. For the IS (10 ng/ml), the recovery was 52.7% ($n = 10$). No plasma matrix effect was observed under the described extraction procedure.

Stability analysis was performed with quality control samples (1.0 and 10.0 ng/ml). All samples showed no significant degradation.

The mean amlodipine plasma concentrations vs time profiles after a single oral dose of each 5 mg tablet formulation of amlodipine are shown in Figure 5. Table 2 shows the mean pharmacokinetic parameters obtained from 24 volunteers after the administration of 5 mg amlodipine tablet. Table 3 presents the ratios and the respective 90% confidence intervals for bioequivalence analysis. The geometric mean and

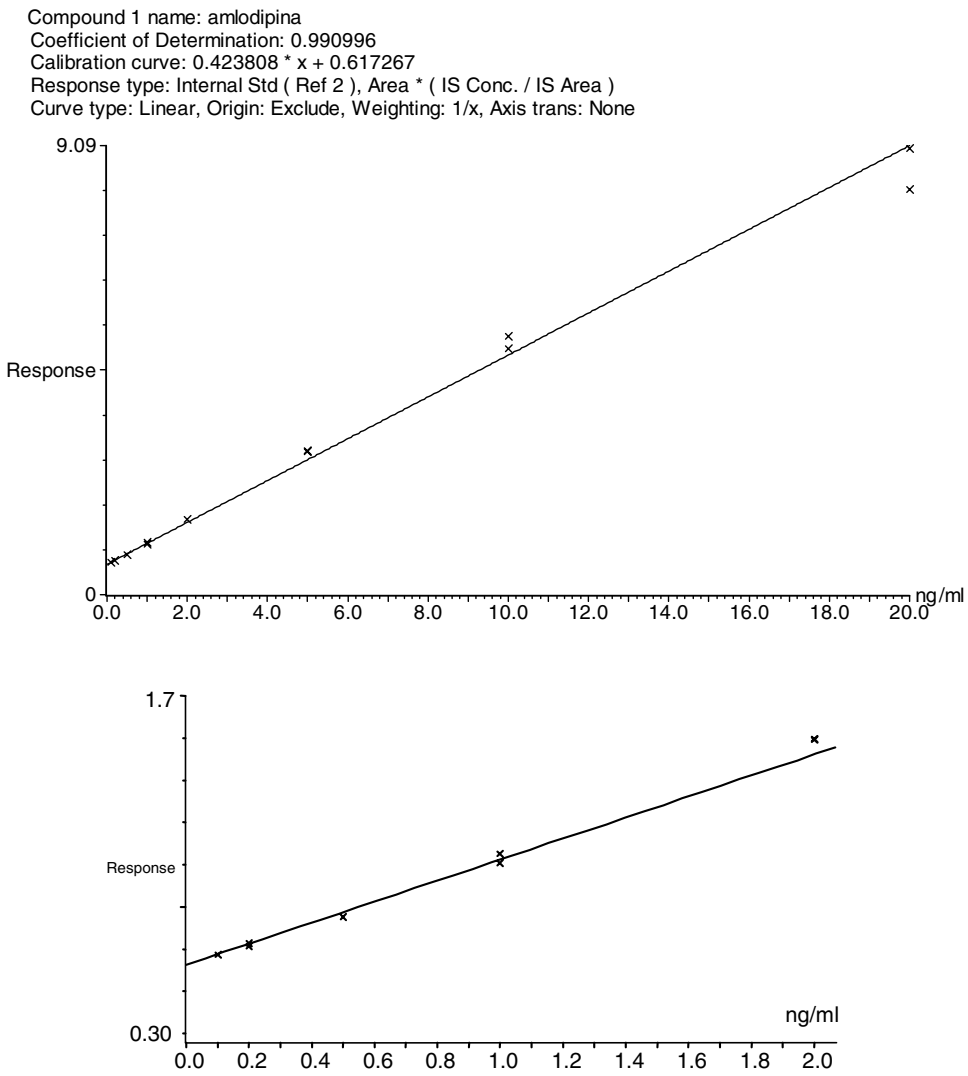

Figure 4. Calibration curve of amlodipine

respective 90% confidence interval (CI) of Amlodipine®/Norvasc® percent ratios were 98.5% (89.0–109.1%) for $C_{max,}$ 101.2 (92.9–110.2%) for AUC_{last}, and 99.6% (91.5–108.4%) for AUC_{0-inf}.

T_{max} was also statistically analyzed and the point estimate for individual differences (Amlodipine® vs Norvasc®) was 0.0 h (90% CI of −1.0 to 2.0) (Table 3).

Amlodipine plasma and serum levels have been detected by different methods such as thin-layer chromatography (LOQ = 2.0 ng/ml) [1], liquid chromatography with amperometric detection (LOQ = 0.2 ng/ml; RT = 8.5 min) [3], and liquid chromatography with ultraviolet detection (LOQ = 0.2 ng/ml; RT>40 min) [4]. Other methods such as capillary electrophoresis (LOQ = 5.0 ng/ml; RT = 9.8 min) [5], and gas chromatography (LOQ = 2.5 ng/ml; RT>9.0 min) [6] were also used to detect amlodipine plasma levels.

Liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS) was recently used to quantify plasma and serum amlodipine

Table 1. Quantified concentration (ng/ml) of individual samples of intra-batch and inter-batch validation

Sample	LOQ	QL2	QCA	QCB	QCC
Intra-batch					
Nominal concentration (ng/ml)	0.1	0.2	0.5	2.0	10.0
Mean	0.1	0.2	0.5	1.9	10.1
S.D.	0.00	0.02	0.03	0.08	0.57
Precision (%)	4.1	7.4	5.7	4.4	5.6
Accuracy (%)	2.3	1.7	−1.0	−4.2	1.3
Inter-batch					
Nominal concentration (ng/ml)	0.1	0.2	0.5	2.0	10.0
Mean	0.1	0.2	0.5	2.0	10.0
S.D.	0.00	0.01	0.00	0.12	0.10
Precision (%)	1.2	4.4	0.9	5.1	1.0
Accuracy (%)	0.9	2.4	0.0	−1.5	0.2

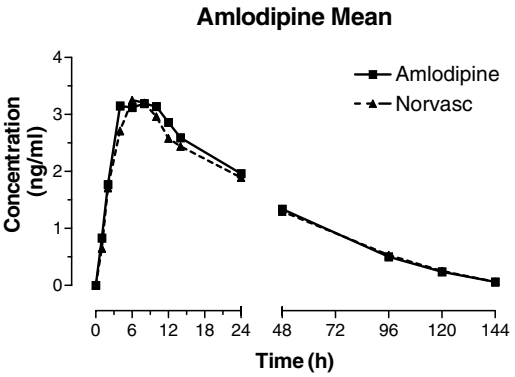

Figure 5. Mean plasma concentrations vs time curve for both amlodipine formulations

Table 2. Mean pharmacokinetic parameters obtained from 24 volunteers after administration of 5 mg amlodipine tablet

Pharmacokinetic parameter	Amlodipine®	Norvasc®
$AUC_{(0-last)}$ (ng h/ml)		
Geom. mean	151.7	147.4
S.D.	78.1	75.1
$AUC_{(0-inf)}$ (ng h/ml)		
Geom. mean	166.9	166.3
S.D.	78.8	76.7
C_{max} (ng/ml)		
Geom. mean	3.9	3.8
S.D.	2.5	2.1
Ke (1/h)		
Median	0.02	0.02
Range	0.01–0.03	0.01–0.04
$T_{1/2}$ (h)		
Median	33.9	37.0
Range	24.3–45.7	18.9–63.4
T_{max} (h)		
Median	6.0	6.0
Range	2.0–14.0	4.0–14.0

concentration [2,7]. These methodologies improved the specificity, accuracy and in shortening the run time. Yasuda *et al*. [2] using an LC-MS-MS with atmospheric pressure chemical ionization (APCI) obtained an LOQ of 0.014 ng/ml. The molecular fragmentation of amlodipine was the same as that in our study (*m/z* 409.0>238.1). Besides the low LOQ, the retention time was longer (4.5 min) than our method and used a deuterated internal standard which is seldom commercially available. Marzo *et al*. [7], developed an LC-MS-MS method using nitrendipine as internal standard. The LOQ was the same (0.1 ng/ml) and the retention time was shorter (2.0 min). However, in the sample extraction step they use higher volume of plasma (1.0 ml) than in our study (200 µl).

The method reported here had a good sensitivity and was used to analyze two amlodipine tablet formulations in a bioequivalence study in healthy volunteers using human plasma for the determination of amlodipine concentrations.

After the oral administration of the amlodipine tablets to the volunteers, the observed amlodipine peak plasma concentration (C_{max}) values

Table 3. Geometric mean of the individual AUC_{last}, AUC_{0-inf}, (test/reference formulation) and the respective 90% CI

Amlodipine®/Norvasc®	Parametric		Non-parametric	
	Geom. mean	90% CI	Geom. mean	90% CI
$AUC_{(0-last)}$% ratio	101.2	92.9–110.2	100.6	92.4–109.7
$AUC_{(0-inf)}$ % ratio	99.6	91.5–108.4	104.2	95.8–113.4
C_{max} % ratio	98.5	89.0–109.1	98.8	89.2–109.4
T_{max} (h) individual differences	−0.2 (Arith. mean)	−1.5–1.2	0.0 (Point estimate)	−1.0–2.0

and the time values taken to be achieved (T_{max}) were similar to those reported [2,8] and equivalent between the formulations. In addition, the calculated 90% CI for mean C_{max}, AUC_{last} and AUC_{0-inf} Amlodipine®/Norvasc® individual ratios were within the 80–125% interval defined by the US Food and Drug Administration [9,10]. Thus, it is concluded that amlodipine test formulation (Amlodipine®) is bioequivalent in terms of both rate and extent of absorption to reference formulation (Norvasc ®).

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