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Felodipine quantification in human plasma by high-performance liquid chromatography coupled to tandem mass spectrometry

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Abstract

A rapid, sensitive, robust and specific method was developed for the determination and quantitation of felodipine, in human blood plasma by liquid chromatography coupled with tandem mass spectrometry using nimodipine as internal standard. Felodipine was extracted from 0.5 mL human plasma by use of a liquid/liquid procedure using diethyl ether/hexane (80/20, v/v) as eluent. The method included a chromatographic run of 5 min using a C_{18} analytical column (100 mm × 4.6 mm i.d.) and the calibration curve was linear over the range from 0.02 to 10 ng mL^{-1} ($r^2 > 0.994$). The between-run precision, determined as relative standard deviation of replicate quality controls, was 5.7% (0.06 ng mL^{-1}), 7.1% (0.6 ng mL^{-1}) and 6.8% (7.5 ng mL^{-1}). The between-run accuracy was ± 0.0, 2.1 and 3.1% for the above-mentioned concentrations, respectively.

Keywords: Felodipine; Antihypertensive; Healthy volunteer; LC–MS/MS; Bioequivalence

1. Introduction

Chemically, calcium channel blockers are classified into three classes, benzothiazepines, dihydropyridines, and phenylalkylamines. These compounds have an important role in the cardiovascular system, such as controlling arterial blood pressure [1].

Felodipine(I), 4-(2,3-dichlorophenyl)-1,4-dihydro-2,6-dimethylpyridine-3,5-pyridinedicarboxylic acid ethyl methyl ester (CAS# 72509-76-3), is a dihydropyridine calcium antagonist widely used as a selective vasodilator in cardiovascular disorders, primarily arterial hypertension [2,3]. The compound is a white crystalline powder with a molecular mass of 384.26 Da and molecular formula $C_{18}H_{19}Cl_2NO_4$.

Several analytical methods based on high resolution gas chromatography (HRGC) mainly with electron capture detector [4–6], high performance liquid chromatography (HPLC) [7–10], HRGC coupled to mass spectrometry (HRGC-MS) [11–13] and HPLC coupled to mass spectrometry (HPLC-MS) [14] and recently by HPLC coupled to tandem mass spectrometry (HPLC-MS–MS) [15] has been used for the felodipine quantitation in plasma. These methods however, not are ideal to pharmacokinetics studies, because are laborious and include time-consuming procedures or long chromatographic run times (>10 min) [14].

Quantification of drugs in biological matrices by liquid chromatography coupled to tandem mass spectrometry

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(LC–MS–MS) is becoming more common; owing to the improved sensitivity and specificity of this technique [16,17]. The objective of this study was to develop a specific, sensitive and rapid LC–MS–MS method for quantifying felodipine in human plasma using nimodipine (CAS# 66085-59-4) as internal standard (IS) for development of pharmacokinetics studies of a formulation containing a reduced concentration (quarter) of felodipine in relation to the marketed formulations (10 mg). Therefore the method required to use in this case need a limit of detection below to the previous validated methods reported [15].

2. Experimental

2.1. Chemicals and reagents

Felodipine and nimodipine were obtained from Cipla (India) and Biosintética (São Paulo, Brazil), respectively, both standards have 99% of purity. Methanol and acetonitrile (HPLC grade) were purchased from J.T. Baker (Phillipsburg, NJ, USA), diethyl ether and hexane from Mallinckrodt, (Paris, KY, USA). Formic acid, analytical grade, was purchased from Merck (Rio de Janeiro, Brazil). Ultrapure water was obtained from an Elga UHQ system (Bucks, UK). Blank blood was collected from healthy, drug-free volunteers. Plasma was obtained by centrifugation of blood treated with anticoagulant sodium heparin. Pooled plasma was prepared and stored at approximately −70 °C until analysis.

2.2. Calibration standards and quality controls

Stock solutions of felodipine and nimodipine (IS) were prepared in methanol–water (50:50, v/v) at concentrations of 1 mg mL^{-1}. The working solution of the IS was prepared in acetonitrile:water (50:50, v/v) at a concentration of 1 ng mL^{-1}. Calibration curves for felodipine were prepared in blank human plasma at concentrations of 0.02, 0.05, 0.10, 0.20, 0.50, 1.00, 2.00, 5.00 and 10.0 ng mL^{-1} and performed in duplicates in each batch. Quality control samples were prepared in blank plasma at concentrations of 0.06, 0.6 and 7.5 ng mL^{-1} (QCA, QCB and QCC, respectively). All the solutions were protected from light using an aluminum foil.

2.3. Sample preparation

Aliquots (0.50 mL) of human plasma were employed for liquid-liquid extraction (LLE) after addition of IS solution (50 μL of the working standard solution). The tubes were vortexed for 20 s and allowed to stand at room temperature for 2 min. Four mL of diethyl ether:hexane (80:20, v/v) were added and the samples were vortexed for 40 s, the upper layer transferred to clean tubes and the solvent evaporated under N_2 (40 °C). The dry residue was redissolved with 200 μL of mobile phase acetonitrile:water (80:20, v/v, with 10 mM of formic acid). The samples were transferred into glass microvials, capped and placed in an autosampler.

2.4. Liquid chromatography and mass spectrometry conditions

An HPLC system (Hewlett-Packard, Model 1100) consisting of a binary pump (G1312A) was used for all analyses. The chromatographic system consisted of a C_8 analytical column (100 mm × 4.6 mm i.d., 3 μm film thickness) and isocratic mobile phase of acetonitrile:water (80:20, v/v, with 10 mM of formic acid) at a flow rate of 0.80 mL min^{-1}. The column was operated at room temperature and present a void time of 1.02 min. The temperature of the autosampler (CTC Analytics, HTS PAL) was maintained at 6.5 °C and was set up to make 40 μL sample injections every 5.0 min. Mass spectrometry was performed in a Sciex API 4000 triple stage quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an API electrospray source operating in positive mode (ES+). The source block temperature was set at 650 °C and the electrospray capillary voltage to 5.5 kV. Nitrogen was used as a collision gas.

The ions monitored in Multiple Reaction Monitoring (MRM) under these conditions were described in the Table 1. MRM *m/z* 383.9 → 352.1 and 419.1 → 343.1, was used for quantitation of felodipine and nimodipine, respectively. The declustering potential were set to at 56 V and 36 V, collision energy were set to at 17 eV and 13 eV and the collision exit potential were set at 16 V and 20 V for felodipine and nimodipine, respectively. Data were acquired by Analyst software (1.3.1, Applied Biosystems) and calibrations curves for the analyte were constructed using the felodipine and IS peak-area ratios via a weighted ($1/x^2$) least-squares linear regression. Unknown sample peak-area ratios were then interpolated from the calibration curve to provide the concentrations of felodipine.

Table 1
Validations with the quality controls (QC) having the results of the accuracy and precision of drug felodipine

	Parameter	Nominal concentration (ng mL^{-1})			
		0.02	0.06	0.60	7.50
Intra-batch	Mean found (n = 8) (ng mL^{-1})	0.0216	0.0565	0.596	7.41
	Precision (%)	20.0	4.9	4.4	3.7
	Accuracy (%)	107.8	94.1	99.4	98.9
Inter-batch	Mean found (n = 3) (ng mL^{-1})	0.0203	0.0559	0.588	7.47
	Precision (%)	17.3	7.4	5.9	4.8
	Accuracy (%)	101.3	93.1	98.0	99.6

2.5. Recovery

The experiments were conducted to evaluate the recovery with the extraction method described above. The percentage recovery was calculated at each standard concentration (0.06, 0.60 and 7.50 ng mL^{-1}) as the ratio of the peak area for extracted blank plasma spiked before extraction relative to peak area of the equivalent blank plasma samples spiked after the extraction.

2.6. Stability

Quality control samples (0.06, 0.60 and 7.50 ng mL^{-1}) were subjected to short-term storage (6 h) at room temperature, three freeze-thaw cycles and 24 h storage in the autosampler (8 °C). Stability was assessed by measuring the felodipine concentrations in processed samples in comparison with freshly prepared samples.

2.7. Precision and accuracy

The within- and between-run precisions were determined as the relative standard deviations, R.S.D. (%) = 100 (S.D./*M*), where *M* is the mean and S.D. is the standard deviation. Accuracy was assessed as the percentage relative error, RE (%) = $(E - T)(100/T)$, where *E* is the experimentally determined concentration and *T* the theoretical concentration.

2.8. Application of the method

The method described before was applied to felodipine plasma samples obtained after multiple dose administration of single 2.5 mg tablets (Felodipin STADA® 2.5 mg retard, STADApharm GmbH, Germany as test and Modip® 2.5 mg, AstraZeneca GmbH, Germany as reference) to healthy human volunteers of both sexes.

A total of 16 non-institutionalized healthy volunteers (eight male and eight female), aged 18–55 years with body-mass index ≥19 kg/m^2 and ≤27 kg/m^2 were enrolled in this pilot crossover study in order to obtain a valid characterization of the usefulness of the analytical procedure for the purpose. Subjects were dosed in fasted state with one 2.5 mg tablet (test or reference) per day for four consecutive days to achieve steady state. After profiling on day 5 (Period 1), treatment was continued for another four days with the alternate medication (reference or test) and ended on day 10 for second profiling (Period 2).

Blood samples (4 mL) were collected in heparin solution containing tubes before as well as 30 min, 1, 1.5, 2, 3, 3.5, 4, 4.5, 5, 6, 8, 10, 12, 16, 20 and 24 h after administration. The blood samples were centrifuged at 2000 × *g* for 10 min at room temperature, decanted and stored at −20 °C until analysis.

$AUC_{0–24h}$, C_{max} and C_{min} were evaluated as pharmacokinetic characteristics. Moreover, point estimates and 90% confidence intervals were calculated for the comparison of both investigational products.

3. Results and discussion

3.1. Method development

Electrospray positive mass spectrum for both compounds (felodipine and nimodipine) showed similar fragmentation (Figs. 1–3) with base peak ions at *m*/*z* 338 for felodipine and *m*/*z* 343 for nimodipine. The MS/MS product ion spectrum of the $[M + H]^+$ for both compounds showed that the major product ions are the same base peak ions observed in the MS (Q1) spectrum (Fig. 3). In both compounds the main fragmentation occurs though the loss of the alcohol parts of carboxyl groups, with formation of substituted ketene ions (Figs. 1 and 2). In the dihydropyridines analyzed the loss of ethyl alcohol is energetically favored in relation to the methyl alcohol and the 2-methoxy-ethyl alcohol is energetically favored in relation the 1-methylethyl alcohol (Figs. 1 and 2).

Due to high intensity of the *m*/*z* 383.9 → 352.1 (felodipine) and *m*/*z* 419.1 → 343.1 (nimodipine) reactions and not interference detectable in plasma samples these transition reactions were used in the present method.

With these reactions we have developed a specific LC–MS–MS assay to determine felodipine from human plasma with a limit of quantification (LOQ) validated of 20 pg mL^{-1} and with a run time of less than 5.0 min. The mass chromatograms of a LOQ sample are shown in Fig. 4, in which the retention times of felodipine and IS were 2.4 and 2.2 min, respectively.

The choice of nimodipine as the IS for felodipine was based on the presence of similar functional groups in both structures and similarity of physical–chemical properties in addition to their similarity concerning molecular weight and chemical behavior. Although generally deuterium-labeled isotopes are more favorable internal standards than structural analogues, they are seldom commercially available and expensive to synthesize. This is also the case for deuterated felodipine.

3.2. Assay performance

Validation results of the analytical procedure are summarized in Table 2. Accuracy and precision of the method was assessed by analyzing of the quality control samples (QCs). Calibration curve was shown to be linear for felodipine from 0.02 to 10 ng mL^{-1} ($r^2 > 0.9970$) $y = 0.417 \pm 0.023x + 0.00182 \pm 0.0025$ using weighting of the $1/X^2$.

Recovery of felodipine, calculated from the peak area ratios of extracted human plasma previously spiked at final concentrations of 0.06, 0.60 and 7.50 ng mL^{-1}, were 107.6%; 103.9% and 99.3%, respectively. For nimodipine (0.06 and 0.60 ng mL^{-1}) the recoveries were 87.0% and 109.0%, respectively. No matrix effect was observed, this was evaluate the ion suppression effect, based on post-column mixing of the analyte of interest with the eluate of a column to which

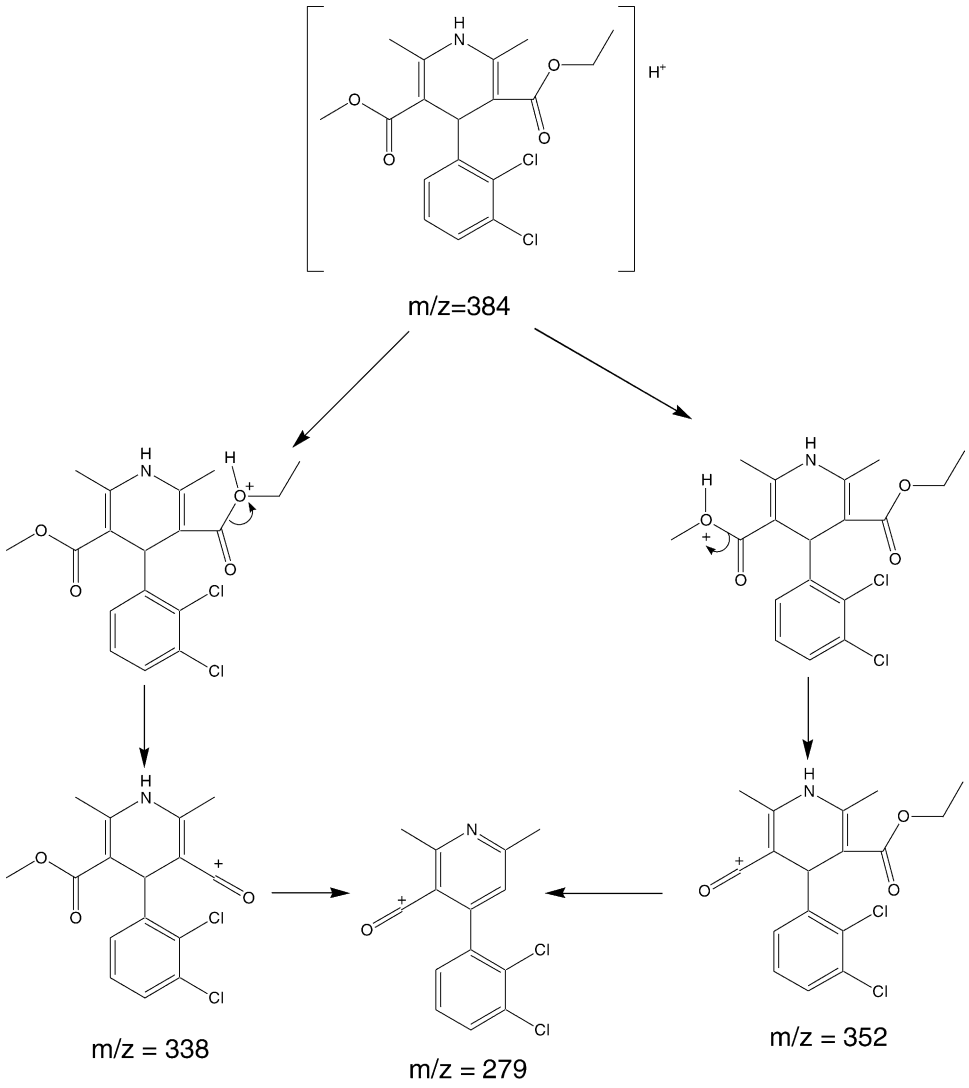

Fig. 1. Proposed mass fragmentation pathways for felodipine.

Table 2
Mean pharmacokinetic parameter for 15 volunteers after the administration of felodipine formulations

Felodipine (in plasma)		Felodipine STADA®		Modip®	
Parameters ($N = 15$)	Units	Test		Reference	
		Geometric mean	(CV%)	Geometric mean	(CV%)
$AUC_{0-\tau}$	H ng/ml	7.51	(27.4)	7.54	(30.4)
C_{max}	ng/ml	0.710	(41.6)	0.643	(34.9)
C_{min}	ng/ml	0.148	(24.4)	0.171	(32.8)
C_{av}	ng/ml	0.313	(27.4)	0.314	(30.4)
PTF	%	176.9	(29.0)	148.0	(20.6)
		Mean	(S.D.)	Mean	(S.D.)
t(max)	H	3.97	(1.83)	5.40	(2.28)
t(1/2)	H	12.37	(9.16)	11.71	(3.40)
MRT	H	9.39	(0.50)	10.24	(0.58)

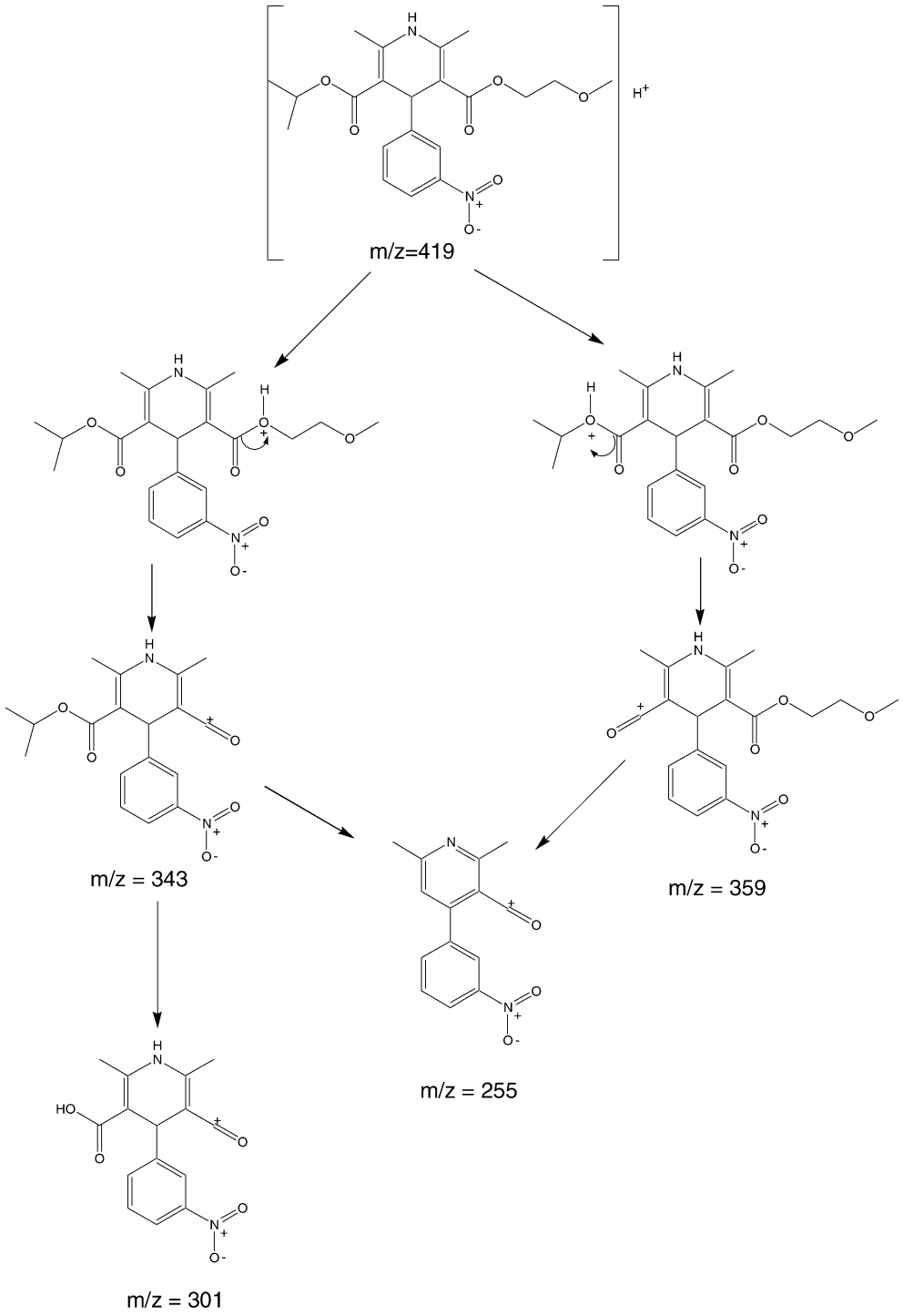

Fig. 2. Proposed mass fragmentation pathways for nemodipine.

a blank sample is injected, has been proposed by Bonfiglio et al. [18].

Between- and within-run accuracy and precision as summarized in Table 2 meet the requirements for bioanalytical procedures as laid down in the international Guidelines [19,20].

Suitability of the newly developed analytical method was tested by measuring felodipine steady state concentrations in plasma samples obtained from healthy volunteers after multiple dosing of 2.5 mg extended release tablets (one tablet daily). The analyte could be quantified with sufficient accuracy and precision in all samples, even at trough values. Mean felodipine plasma concentrations versus time curves measured for both investigational products are shown in Fig. 4. According to the Physician Desk Reference 2001, following the administration of a 10 mg-dose a felodipine

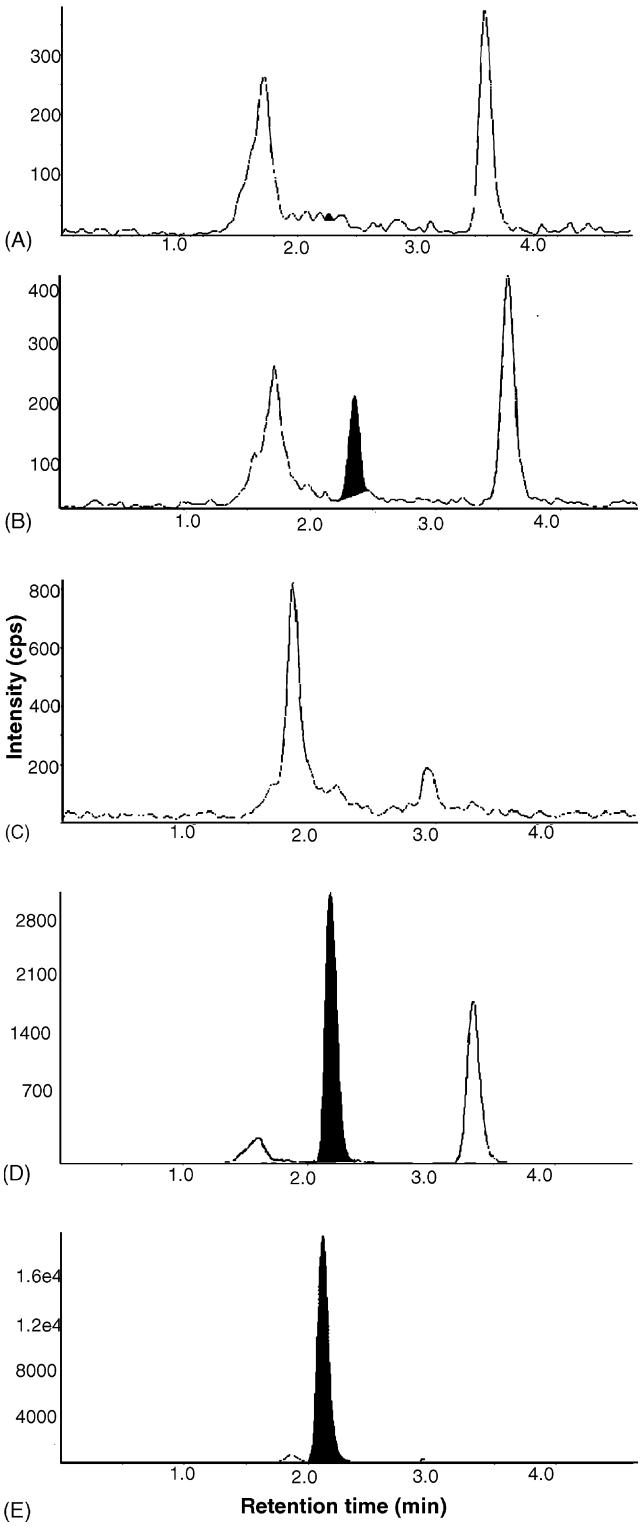

Fig. 3. MRM chromatograms of blank pooled human plasma for felodipine and IS (A and C, respectively) and MRM chromatogram of felodipine spiked in human plasma at a final concentration of 20 pg mL^{-1} (B), representative MRM chromatogram of unknown sample (D) and MRM chromatogram of IS spiked in human plasma (E).

extended-release formulation to young healthy volunteers, mean peak and trough stead-state plasma concentrations were 2.5 and 0.7 ng/mL, respectively, which are very close to our data obtained with 2.5 mg-dose (0.7 and 0.15 ng/mL, respectively). It is important to note that the literature show that peak plasma concentration increases linearly with doses up to 20 mg. Mean peak concentrations following the administration of a felodipine extended-release formulation are reached

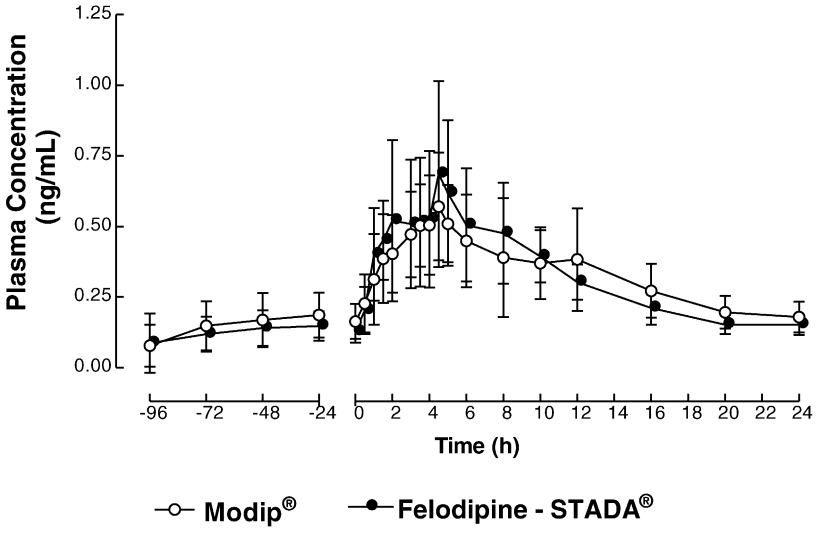

Fig. 4. Mean plasma concentration of felodipine in 15 human volunteers following oral administration of a single 2.5 mg.

within 2.5–5 h (in our study, the mean was 3.97 ± 1.83 h). As in the present bioequivalence study the felodipine dose was a quarter of the normal marketed formulations (10 mg), the analytical methodology needed an improvement of sensibililty in relation to previous validated methods [15]. The present study required method with a limit of detection around 20 pg/ml ($\pm n$ 3% of C_{max}) as recommended by the international Guidelines. The present method has been successfully applied in this case and using only 0.5 ml of plasma sample.

Stability tests indicate no significant degradation under the conditions described above, including long-term investigations (42 days, frozen at $-20\,^{\circ}C$) of human plasma spiked at final concentrations of 0.06 and 0.60 ng mL^{-1}. In the latter case + 13.4% and −5.1%, respectively were determined relative to freshly spiked samples.

4. Conclusions

A LC–MS–MS method for the quantification of felodipine in human plasma was developed and validated according to the requirements laid down in international regulatory guidelines. This method offers advantages over those previously reported, in terms of a simple sample extraction; only need liquid-liquid extraction without clean-up procedures and a faster run time (5 min). The LOQ of 20 pg mL^{-1}. is sufficient for the bioequivalence studies even with the lowest dose strength (2.5 mg/tablet) marketed so far. However, the procedure could be further improved by sample concentration if required. The assay performance results indicate that the method is precise and accurate enough for the routine determination of felodipine in human plasma.

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