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Quantification of carvedilol in human plasma by high-performance liquid chromatography coupled to electrospray tandem mass spectrometry Application to bioequivalence study

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Abstract

A rapid, sensitive and specific method to quantify carvedilol in human plasma using metoprolol as the internal standard (IS) is described. The analyte and the IS were extracted from plasma by liquid–liquid extraction using a diethyl-ether solvent. After removed and dried the organic phase, the extracts were reconstituted with a fixed volume of acetonitrile–water (50/50; v/v). The extracts were analyzed by a high performance liquid chromatography coupled to electrospray tandem mass spectrometry (HPLC–MS/MS). Chromatography was performed isocratically on Alltech Prevail C_{18} 5 μm analytical column, (150 mm × 4.6 mm i.d.). The method had a chromatographic run time of 3.5 min and a linear calibration curve over the range 0.1–200 ng ml^{-1} ($r^2 > 0.997992$). The limit of quantification was 0.1 ng ml^{-1}. This HPLC–MS/MS procedure was used to assess the bioequivalence of two carvedilol 25 mg tablet formulations (carvedilol test formulation from Laboratórios Biosintética Ltda and Coreg® from Roche Químicos e Farmacêuticos S.A standard reference formulation). A single 25 mg dose of each formulation was administered to healthy volunteers. The study was conducted using an open, randomized, two-period crossover design with a 2-week wash-out interval. Since the 90% CI for C_{max} and AUCs ratios were all inside the 80–125% interval proposed by the US Food and Drug Administration Agency, it was concluded that carvedilol formulation elaborated by Laboratórios Biosintética Ltda is bioequivalent to Coreg® formulation for both the rate and the extent of absorption.

Keywords: Healthy volunteer; Plasma; Pharmacokinetics; Carvedilol; LC–MS/MS; Bioequivalence

1. Introduction

Carvedilol is a nonselective β-blocking agent [1,2] and it also has vasodilating properties that are attributed mainly to its blocking activity at receptors. Carvedilol is a racemic compound and the nonselective β-blocking activity resides mainly in the (*S*)-carvedilol, while the α-blocking activity is shared by (*R*)- and (*S*)-enantiomers [3,4], but this drug is used clinically as a racemic mixture of both enantiomers. Carvedilol is used in the treatment of mild to moderate hypertension and angina pectoris [5] and is often used in combination with other drugs. Carvedilol is a anti-hypertensive agent with non-selective β- and α1-adrenergic receptor blocking activities [6] which is also being used in the treatment of congestive heart failure [7,8] and presents antioxidative effects

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in vivo [9]. Carvedilol has been determined in plasma and other biological fluids such as high performance liquid chromatography coupled to fluorometric detection [6,10–18], high performance liquid chromatography coupled to ultraviolet detection [18], capillary electrophoresis coupled to ultra-violet detection [18,19], capillary electrophoresis coupled with laser-induced fluorescence [20], high performance liquid chromatography coupled to electrochemical detection [21], liquid chromatography coupled to tandem mass spectrometry [22,23]. Carvedilol is rapidly and completely absorbed after oral administration, but its absolute bioavailability is rather low due to an extensive first-pass metabolism [24].

Here we present a fast, sensitive and selective method for measuring plasma carvedilol using liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) with positive ion electrospray ionization using multiple reaction monitoring (MRM) mode to quantify carvedilol in human plasma using metoprolol as the internal standard (IS, Fig. 1). This method was employed in a bioequivalence study of two carvedilol 25 mg tablet formulations: carvedilol test formulation from Laboratórios Biosintética Ltda and Coreg® from Roche Químicos e Farmacêuticos S.A standard reference formulation. The bioequivalence study was conducted using a single dose, two-way, open, randomized crossover design with 2-week wash-out period between the doses and 36 healthy volunteers were included.

2. Experimental

2.1. Chemicals and reagents

Carvedilol (99.6%) was provided by Heatwell S.A. Metoprolol (100.2%) was obtained from, Novartis, respectively. Acetonitrile, methanol (HPLC-grade) and ammonium acetate, analytical grade, were purchased from J. T. Baker (Phillipsburg, NJ, USA), diethyl ether (analysis grade) was purchased from Mallinckrodt (Paris, KY, USA), deonized water (analysis grade) was purchased from Millipore (Brazil) and formic acid (86%, analytical- grade) was purchased from Cetus (Brazil). Ultra pure water was obtained from an Elga UHQ system (Elga, UK). Blank human blood was collected from healthy drug-free volunteers. Plasma was obtained by centrifugation of blood treated with the anticoagulant sodium heparin. Pooled plasma was prepared and store at approximately −20 °C until needed.

2.2. Calibration standards and quality control

Stock solutions of carvedilol were prepared methanol–water (70:30, v/v) and internal standard (metoprolol) were prepared in methanol–water (50:50, v/v) at concentrations of 1 mg/ml. Calibration curves of carvedilol were prepared by spiking blank plasma at concentration of 0.1, 0.2, 0.5, 2.0, 10.0, 20.0, 50.0, 100.0 and 200.0 ng/ml. The

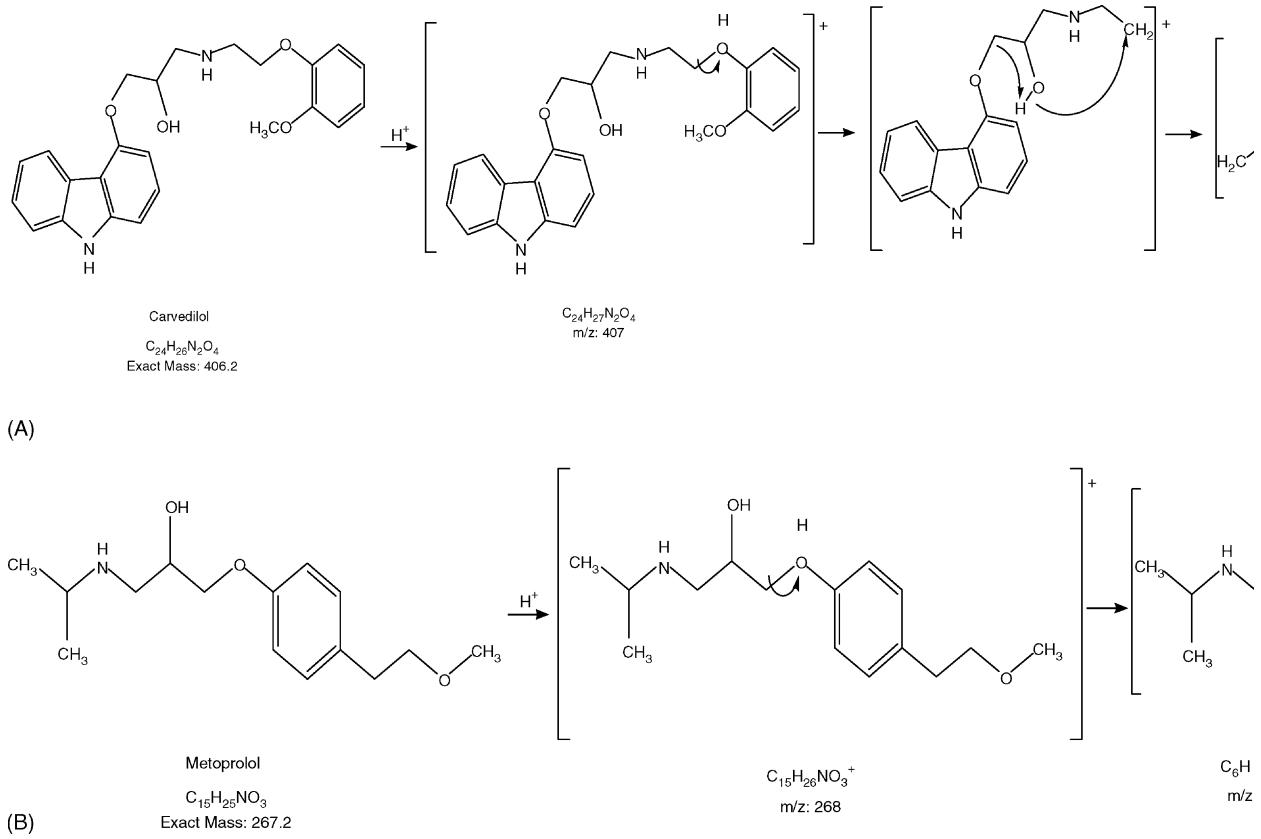

Fig. 1. Proposed fragmentation pathways for the Carvedilol (A) and Metoprolol (B).

analysis was carried out in duplicate for each concentration. The quality control samples were prepared in blank plasma at concentrations of 0.60, 9.00 and 150.0 ng/ml (QCA, QCB and QCC, respectively). The spiked plasma samples (standards and quality controls) were extracted on each analytical batch along with the unknown samples.

2.3. Sample preparation

All frozen human plasma samples were previously thawed at ambient temperature and centrifuged at 2000 × *g* for 4 min at −15 °C to precipitate solids. Two hundred microliters of sample human plasma were introduced into glass tube follow-

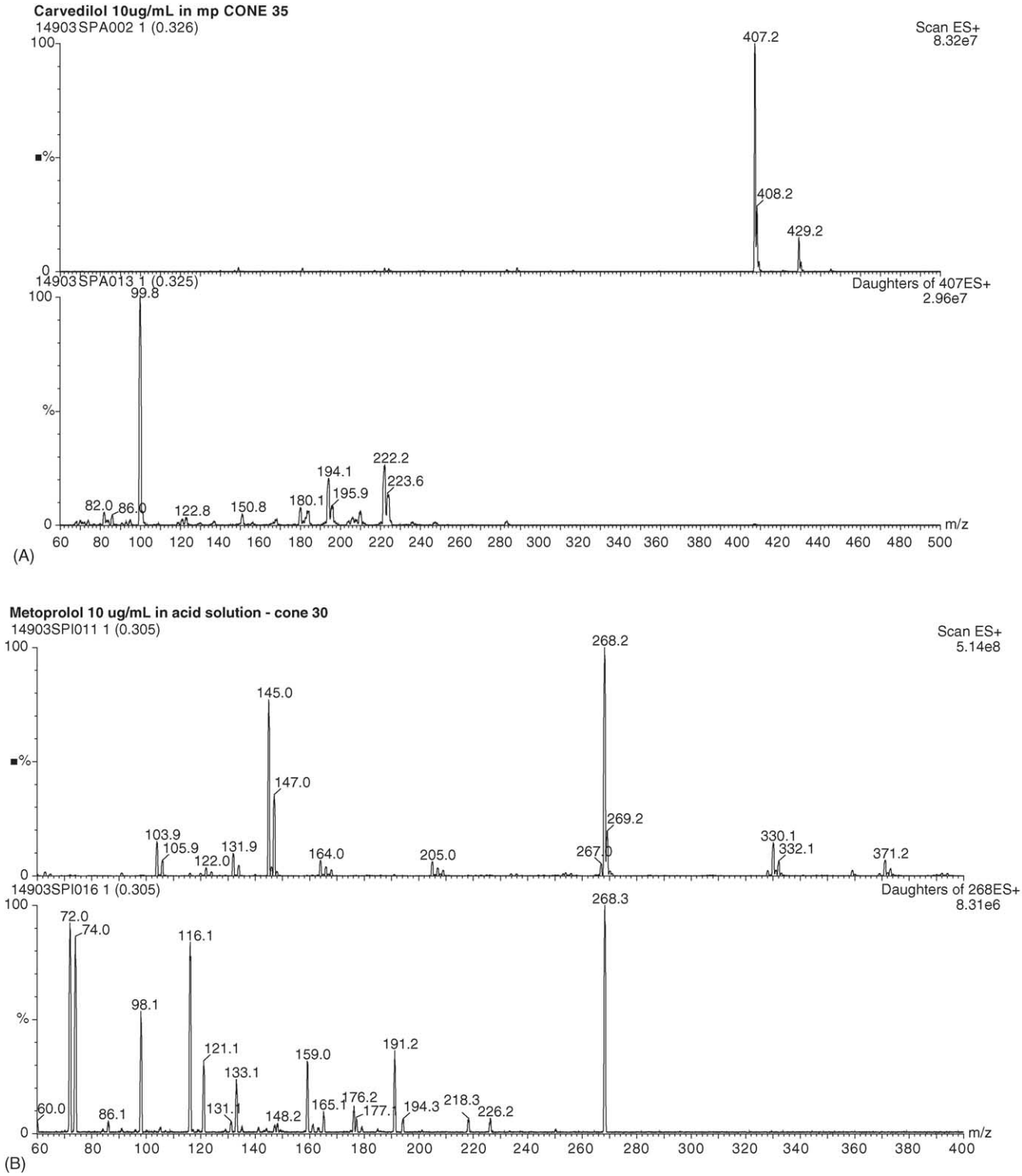

Fig. 2. Full scan mass spectra in upper trace and product ion spectra in lower trace of (panel A) carvedilol and (panel B) metoprolol.

ing by 50 μl of the internal standard solution (300 ng/ml of metoprolol in methanol–water 50/50, v/v solution) and the samples vortex-mixed for approximately 5 s. Diethyl-ether was added (4 ml) to all the tubes and performed the extraction by vortex-mixing during 40 s. The tubes were frozen for 10 min at −70 °C. The upper organic phase was transferred to another set of clean glass tubes and evaporated to dryness under N_2 at 50 °C. The dry residues were dissolved with 0.200 ml of a solution of acetonitrile–water (50/50, v/v), vortex-mixed for 10 s to reconstitute the residues and transferred to 96-well plates using automatic pipettes with disposable plastic tips.

2.4. Chromatographic conditions

An aliquot of each plasma extract was injected into a Alltech Prevail C_{18} 5 um analytical column, (150 mm × 4.6 mm i.d.) and guard column Alltech Prevail C_{18} 5 um (7.5 mm × 4.6 mm i.d.) operating at room temperature. The compounds were eluted by pumping

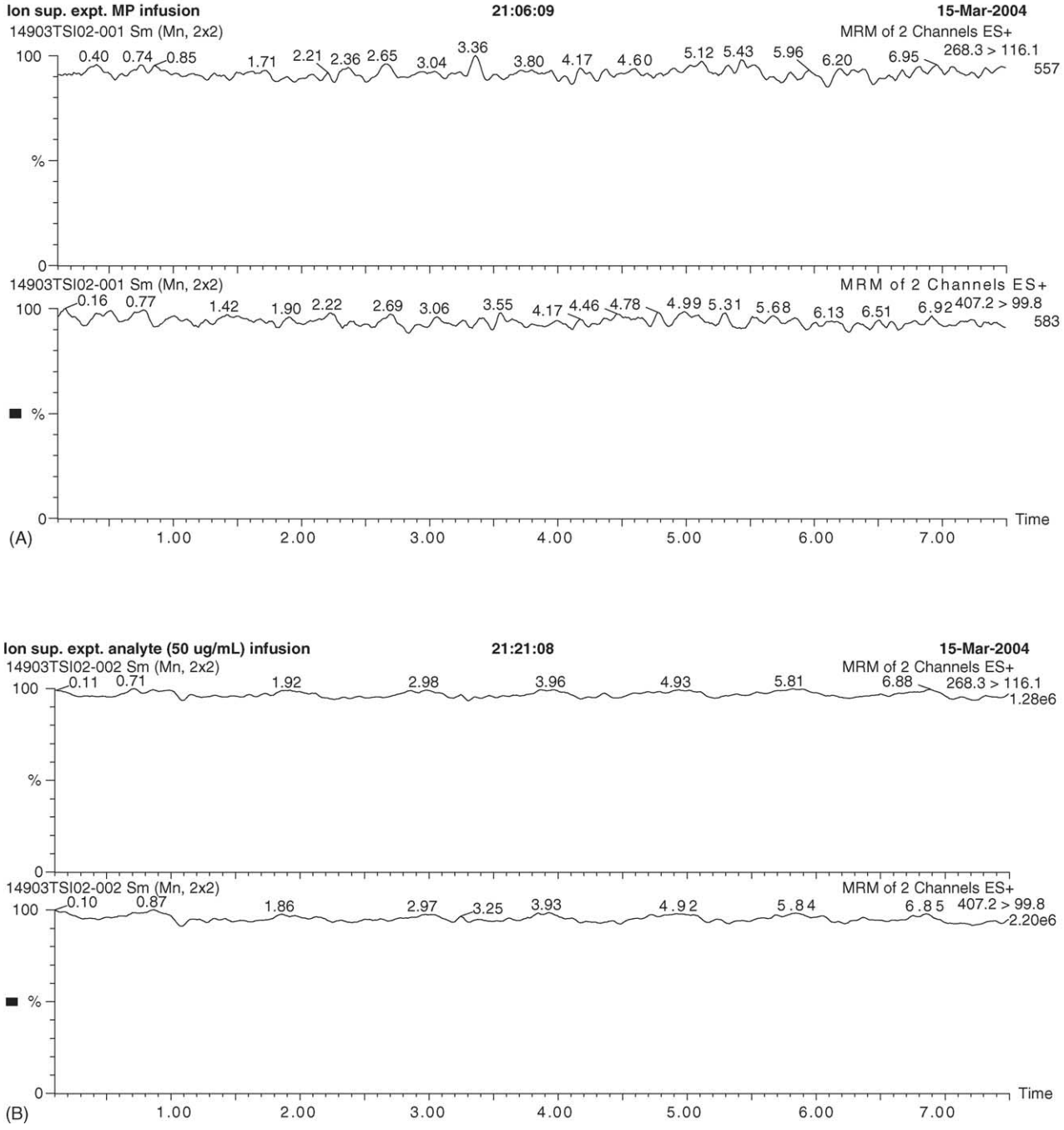

Fig. 3. Ion suppression procedure: (A) Mobile phase infusion, (B) analyte (50 ug/ml) infusion and (C) blank sample injection.

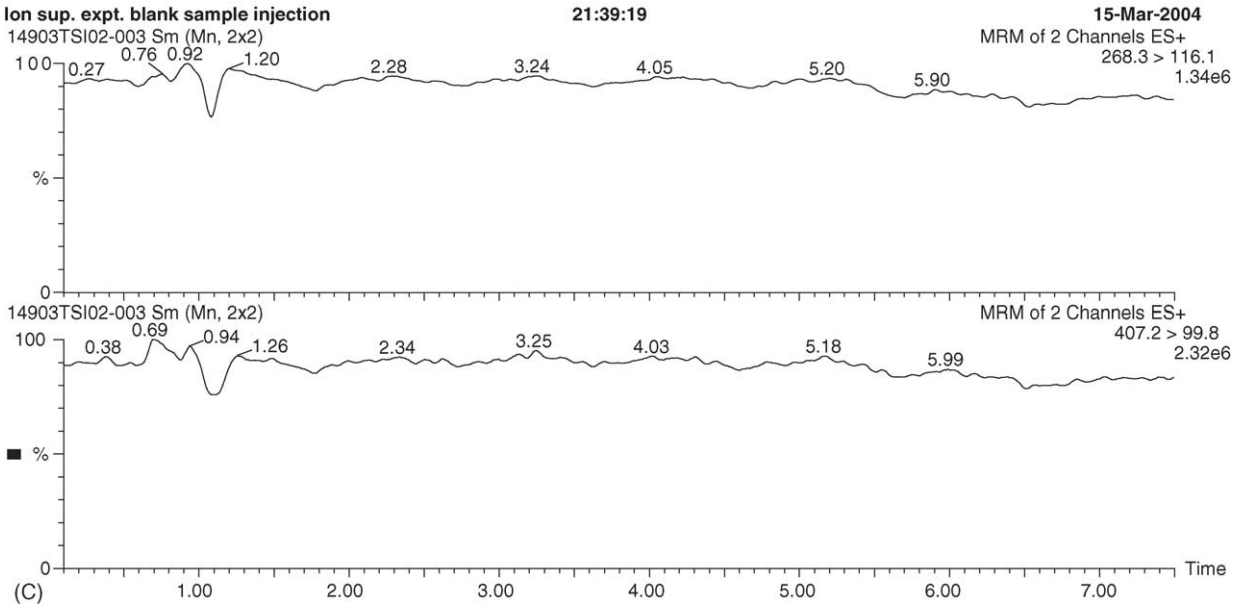

Fig. 3. (*Continued*).

the mobile phase (acetonitrile–water (80/20; v/v) + 12 mM formic acid + 20 mM ammonium acetate at a flow-rate of 1.8 ml/min. Under these conditions, typical standard retention times were 1.6 min ± 0.3 for carvedilol and 1.6 min ± 0.3 for metoprolol and back-pressure values of approximately 90 bar were observed.

A split of the column eluant of approximately 1:10 was included so that only 180 μl/min entered the mass spectrometer. The temperature of the auto-sampler was kept at 13 °C and the run-time was 3.5 min.

2.5. Mass-spectrometric condictions

The mass spectrometer (Micromass,UK, model LC) equipped with an electrospray source using a cross flow counter electrode run in positive mode (ES+), was set up in Multiple Reaction Monitoring (MRM), monitoring the transitions 407.20 > 99.80 and 268.30 > 116.10, for carvedilol and IS, respectively. Fig. 2 shows the full scan spectra (upper trace) and the product ion spectra (lower trace) obtained for carvedilol (panel A) and metoprolol (panel B). The proposed fragmentation pathways for carvedilol and metoprolol (Fig. 1). In order to optimize all the MS parameters, a standard solution of the analyte and I.S. were infused into the mass spectrometer. The following optimized parameters were obtained: the dwell time, the cone voltage and the collision energy were 0.3 s, 35 V and 30 eV and 0.3 s, 30 V and 18 eV for carvedilol and metoprolol, respectively. Data acquisition and analysis were performed using the software MassLynx (v 3.5) running under Windows NT (v 4.0) on Pentium II PC.

2.6. Linearity

Linearity was determined to assess the performance of the method. A linear least-squares regression with a weighting index of $1/x$ and second order was performed on the peak area ratios of carvedilol and IS vs. carvedilol concentrations of the nine plasma standards (0.1, 0.2, 0.5, 2.0, 10.0, 20.0, 50.0, 100.0 and 200.0 ng/ml.) in duplicate to generate a calibration curve.

2.7. Stability

Stability quality control plasma samples (0.60, 9.00 and 150.0 ng/ml) were subjected to short-term (10 h) room temperature, three freeze/thaw (−20 to 25 °C) cycles, 48 h autosampler stability (13 °C) and long-term stability 58 days tests. Subsequently, the carvedilol concentrations were measured compared to freshly prepared samples. The significance of the results obtained was analyzed by Student's *t*-test ($p < 0.05$).

2.8. Recovery

The recovery was evaluated by calculating the mean of the response of each concentration and dividing the extracted sample mean by the unextracted (spiked blank plasma extract) sample mean of the corresponding concentration. Comparison with the unextracted samples, spiked on plasma residues, was done in order to eliminate matrix effect, giving a true recovery. The matrix effect experiments were carried out using the ratio between spiked mobile phase solutions and unextracted samples, spiked on plasma residues.

2.9. Ion supression

A procedure to assess the effect of ion supression on the MS/MS was performed. The experimental set-up consisted

of an infusion pump connected to the system by a "zero volume tee" before the split and the HPLC system pumping the mobile phase„which was the same as that used in the routine analysis of carvedilol, i.e. acetonitrile–water (80/20, v/v) +12 mM formic acid +20 mM ammonium acetate at 1.8 ml/min. The infusion pump was set to transfer (50 μl/min) of a mixture of analyte and internal standard in mobile phase (both 50 μg/ml). A sample of human pooled blank plasma was extracted by the extraction procedure. The reconstituted extract was injected into the HPLC system while the standard mixture was being infused. In this system any ion suppression would be observed as a depression of the MS signal.

2.10. Bioequivalence study

The method was applied to evaluate the bioequivalence of two tablets formulations of Carvedilol (test formulation from Laboratórios Biosintética Ltda, Brazil; lot No. 352/03, expiry October 2005) and Coreg® −25 mg tablet (standard reference formulation from Roche Químicos e Farmacêuticos S.A, lot No. 114933, expiry date April 2005).

The study consisted of an open study of 36 healthy volunteers. After screening and wash-out period (of at least 2 weeks), the individuals who qualified were confined for two periods of approximately 52 h. Each confinement was intervaled by a period of 1 week. Study Schedule Pre-study period: Medical history, General physical examination, Electrocardiogram, Clinical laboratory examination, Confined period: a 4 mL blood sample was collected before dosing and 0:20, 0:40, 1,1:20, 1:40, 2, 2:20, 2:40, 3, 3:30, 4, 4:30, 5, 5:30, 6, 7, 8, 10, 12, 14, 16, 20, 24, 28, 32 and 36 h post-dosing. Post-study period: General physical examination, Electrocardiogram, Clinical laboratory examination.

3. Results

The simplest regression method for the calibration curves of the carvedilol was $y = a + bx$ from 0.1 to 200 ng/ml (calibration curve $0.0335816 \times x + 0.000529367$, $r^2 > 0.997992$). A linear least-squares regression with a weighting index of $1/x$ was carried out on the peak area ratios of carvedilol and I.S. versus carvedilol concentrations of the 9 human plasma standards (in duplicate) to generate a calibration curve. In the case of carvedilol and its internal standard, metoprolol, there was no significant ion suppression in the region where the analyte and internal standard are eluted as shown in Fig. 3.

The recovery of carvedilol was 80.8 (CV 8.2%), 81.4 (CV 8.9%) and 83.9 (CV 2.0%) for the 0.60, 9.00 and 150.0 ng/ml standard concentrations, respectively. For the recovery of I.S. were 82.7 (CV 3.2%), 88.2 (CV 5.9%) and 88.2 (CV 2.0%) for the 0.60, 9.00 and 150.0 ng/ml standard concentrations, respectively. No significant matrix effect was observed. The limit of quantification (LOQ) validated was 0.1 ng/ml defined as the lowest concentration at which both the precision and accuracy were <20%. Stability tests performed indicated that there was no significant degradation under the conditions described (Table 4). Within- and between-run precision and accuracy for the LOQ and QCs are summarized in Table 1.

As shown in Fig. 4, no endogenous peak was observed in the mass chromatogram of blank plasma. The chromatogram for the standard LOQ sample is shown in Fig. 4, in which the retention times for carvedilol and I.S. were 1.6 min ± 0.3, respectively. The mean Carvedilol plasma concentrations versus time profiles after a single oral dose of each 25 mg tablet formulation of Carvedilol is shown in Fig. 5.

Table 2
Mean pharmacokinetic parameters obtained from 36 volunteers after administration of each 25 mg carvedilol tablet formulation

	Carvedilol			
	Coreg®		Carvedilol	
	Mean	S.D.	Mean	S.D.
AUC_{last} ([ng h]/ml)	187.20	101.45	184.67	121.05
AUC_{inf} ([ng h]/ml)	198.59	108.29	194.53	124.61
$AUC_{(0–36\,h)}$ ([ng h]/ml)	188.28	102.22	185.74	121.60
C_{max} (ng/ml)	52.01	34.70	53.84	45.10
$T_{1/2}$(h)	10.16	4.76	9.96	5.38
	Median	(Range)	Median	(Range)
T_{max} (h)–median	1.00	0.33–3.50	0.67	0.67–4.00

Table 1
Accuracy and precision data for carvedilol from the pre-study validation in human plasma

Intra-batch $n = 8$				
Nominal concentration (ng ml^{-1})	0.1	0.60	9.00	150.0
Mean range	0.103 (0.094–0.116)	0.622 (0.582–0.707)	9.74 (8.95–10.2)	153 (144–167)
Accuracy (%)	103.1	103.7	108.2	101.8
Precision (%)	8.2	3.8	4.1	4.2
Inter-batch $n = 24$				
Nominal concentration (ng ml^{-1})	0.1	0.60	9.00	150.0
Mean range	0.105 (0.085–0.126)	0.644 (0.596–0.719)	9.73 (8.96–10.3)	157 (144–167)
Accuracy (%)	105.5	106.6	108.1	104.5
Precision (%)	9.7	4.8	3.0	3.6

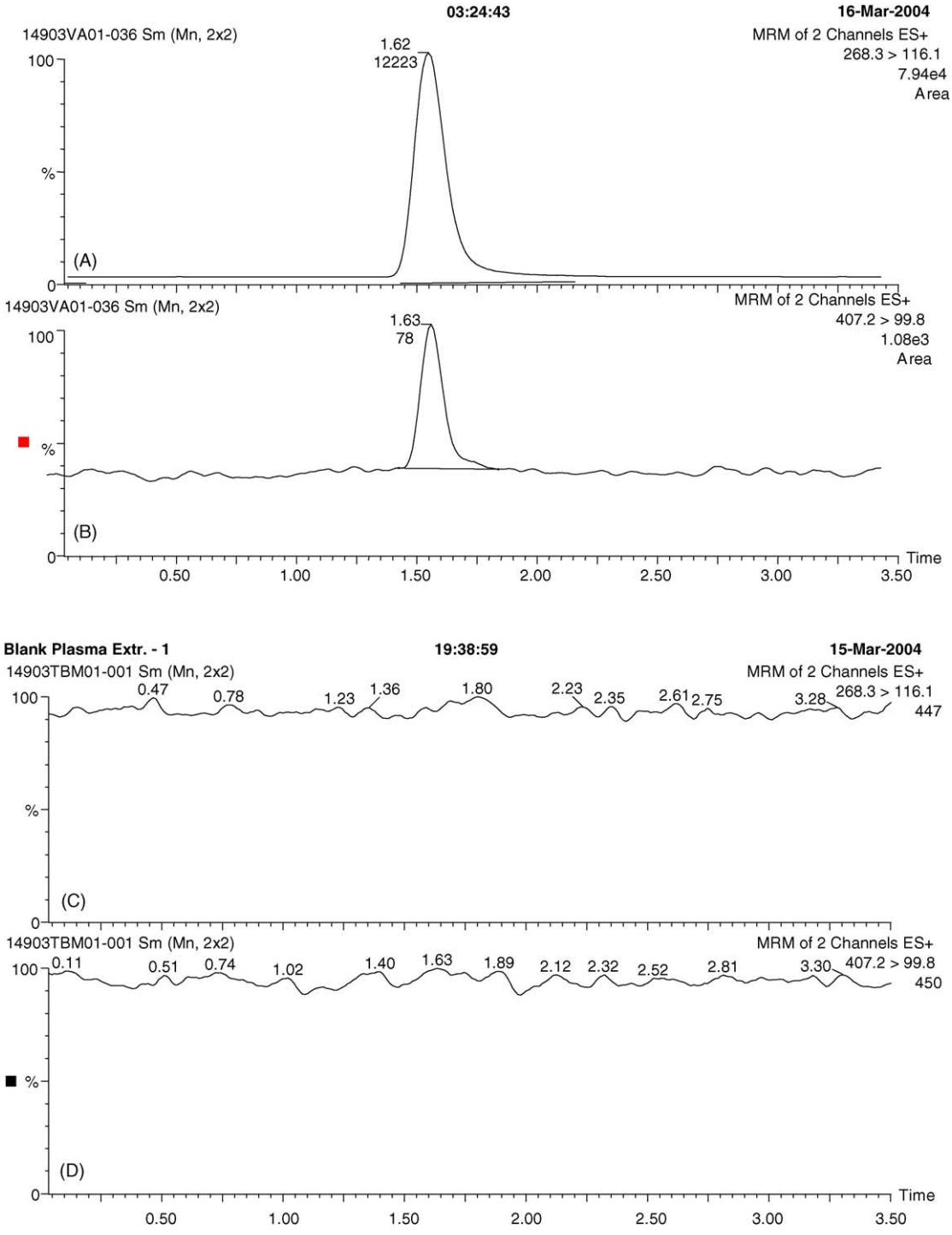

Fig. 4. MRM chromatogram of the LOQ (0.1 ng/mL): (A) metoprolol and carvedilol (B). MRM chromatograms of blank normal human plasma: (C) metoprolol and (D) carvedilol.

4. Discussion

Although it is well known that Carvedilol and Metoprolol are not stable at low pH, no perceivable degradation of the analyte and I.S. was observed under the described liquid chromatographic conditions. The fact that the mobile phase contained a low amount of formic acid did not interfere with the analysis, since the total run time (3.5 min), under these condition of acidity, was not long enough to cause significant degradation. The presence of the acid was necessary in order to improve the detection of the compounds in positive electrospray.

The limit of quantification (LOQ) in published procedures for plasma or serum varies from 0.2 ng/ml to 0.02 mg/L.

Table 3
Geometric mean of the individual AUC_{last}, $AUC_{0-\infty}$ and C_{max} ratios (test/reference formulation), the respective 90% confidence intervals (CI) and power

	Percent geometric mean	90% CI	Power	Percent intra-subject CV
$n = 34$				
AUC_{last}% ratio	94.77	90.58–99.15	1.00	11.01
AUC_{inf}% ratio	94.73	90.38–99.30	1.00	11.45
C_{max}% ratio	97.21	87.93–107.47	0.95	24.42
Male ($n = 16$)				
AUC_{last}% ratio	91.66	86.16–97.51	0.99	9.94
AUC_{inf}% ratio	91.32	85.93–97.05	0.99	9.77
C_{max}% ratio	88.44	75.42–103.72	0.62	25.58
Female ($n = 18$)				
AUC_{last}% ratio	97.62	91.27–104.42	0.99	11.56
AUC_{inf}% ratio	97.88	91.14–105.11	0.99	12.25
C_{max}%*ratio*	105.74	92.66–120.66	0.79	22.68

The LC–MS/MS-CID method described by Gregov et al. [23] shows a poor sensitivity (LOQ of 0.02 mg/L and RT-10 min.), however Varin et al. [10] using HPLC-UV demonstrated in human plasma LOQ of 0.25 ng/ml with RT- 3.8 min. Recently, Yang et al. [26], described in plasma using chiral HPLC–API–MS/MS employed liquid–liquid extraction the LOQ 0.2 ng/mL, the retention time of Carvedilol was shorter than our method (0.5–0.7 min). The method LC–MS/MS,

Table 4
Stability test (post-processing stability test, freeze-and-thaw stability, short-term stability, long-term stability tests)

Post-processing stability test (values in ng/mL)

	Reference values	Values after 24 h	Reference values	Values after 24 h	Reference values	Values after 24 h
	Low sample		Medium sample		High sample	
Mean	0.648	0.669	9.75	10.5	164	170
CV (%)	4.1	3.8	4.6	6.1	2.4	4.2
Variation	3.2		7.8		3.7	
	Reference values	Values after 48 h	Reference values	Values after 48 h	Reference values	Values after 48 h
	Low sample		Medium sample		High sample	
Mean	0.648	0.646	9.75	10.4	164	171
CV (%)	4.1	3.3	4.6	3.8	2.4	2.1
Variation	−0.3		6.5		4.6	

Freeze-and-thaw stability test (values in ng/mL)

	Reference values	Values after 3 cycles	Reference values	Values after 3 cycles	Reference values	Values after 3 cycles
	Low sample		Medium sample		High sample	
Mean	0.638	0.649	9.34	9.49	152	155
CV (%)	3.3	2	3.6	2.1	4	5.2
Variation	1.7		1.6		1.6	

Short-term stability test (values in ng/mL)

	Reference values Low sample	Values after 10 h	Reference values Medium sample	Values after 10 h	Reference values High sample	Values after 10 h
Mean	0.638	0.633	9.34	9.32	152	153
CV (%)	3.3	5.5	3.6	3.1	4	3
Variation	−0.8		−0.2		0.3	

Long-term stability test (values in ng/mL)

	Reference values	Values after 58 days	Reference values	Values after 58 days
	Low sample		Medium sample	
Mean	4.49	4.4	44	41.8
CV (%)	3	2.7	2.8	2.9
Variation	−2		−5	

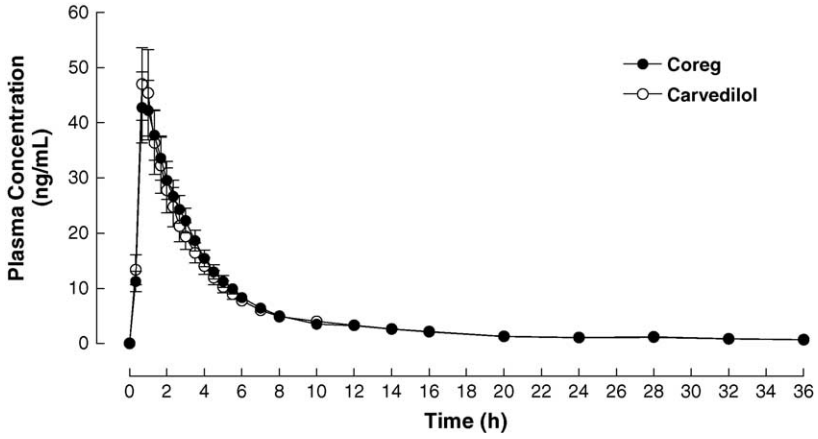

Fig. 5. Carvedilol plasma means concentrations vs. time profile obtained after the single oral administration of 25 mg of carvedilol formulation.

employed liquid–liquid extraction, it was chosen, because is faster, cheaper and has an appropriated recovery with a low variability.

A new method was developed to analytical runs until 48 h, as observed in the post processing stability tests. However, others analitycal runs were made within a maximal time of 13 h (210 samples/day), due to our limited capacity of extraction. Herein is presented a more sensitive assay, compare to many others, it was proved to be rather effective (LOQ of 0.1 ng/ml), thereby, it is simple, straightforward and also shows a good retention time (1.6 min). As demonstrated in this assay, this method is perfectly suitable for a high-throughput routine such as bioequivalence studies.

After the oral administration of the Carvedilol tablets to the volunteers, the observed carvedilol peak plasma concentration (C_{max}) values and the time values taken to be achieved (T_{max}) were similar to those reported in the literature [16] and equivalent between the formulations (Tables 2 and 3). In addition, the calculated 90% CI for mean C_{max}, AUC_{last} and AUC_{0-inf} Carvedilol/Coreg individual ratios were within the 80–125% interval defined by the US Food and Drug Administration [16,25] (Table 4).

Carvedilol is commonly regarded as drug with highly variable pharmacokinetics primarily due to its high first pass metabolism [24,27,28], for this reason, it was required to employ a higher number of volunteers ($n = 36$) in order to assure proper statistical power.

5. Conclusion

The HPLC–MS/MS method described here for carvedilol quantification in human plasma agrees with the concepts of high sensivity, specificity and high samples throughput required for pharmacokinetic assays such as bioequivalence studies.

Since the 90% CI for C_{max} and AUCs ratios were all inside the 80–125% interval proposed by the US Food and Drug Administration Agency, it was concluded that carvedilol formulation elaborated by Laboratórios Biosintética Ltda is bioequivalent to Coreg® formulation for both the rate and the extent of absorption [25].

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