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# Achiral–chiral LC/LC–FLD coupling for determination of carvedilol in plasma samples for bioequivalence purposes

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**Abstract**

Bioequivalence data for two pharmaceutical formulations (solid oral dosage forms) containing carvedilol is presented for both racemic and enantiomers of the active substance. This was achieved by on-line coupling of two liquid chromatographic separations followed by fluorescence detection. The first LC dimension was used for a fast separation of racemic carvedilol from propranolol (IS) and the endogenous matrix, by means of a reversed phase mechanism. The peak of racemic carvedilol was on-line transferred to the second enantioselective LC dimension, based on a reversed phase separation on cellulose tris(3,5-dimethyl-phenylcarbamate) stationary phase. Both stages were monitored over a single run by means of a fluorescence detector operated at an excitation wavelength of 285 nm and an emission wavelength of 355 nm. Automated shortcutting of the racemic carvedilol peak to the chiral column and simultaneous detection over the two LC dimensions have been obtained by using an experimental set-up based on two six-port rotative switching valves. Linearity was demonstrated on the interval 2–150 ng/mL for racemic carvedilol and on 1–75 ng/mL intervals for enantiomers. LLOQ fits between 0.7 and 1.4 ng/mL. Recoveries of the target compounds are $87 \pm 4$ and $81 \pm 4$% for the IS. Precision ranged from 0.6 to 2.5% and the mean accuracy obtained on quality control samples (measured as % bias) over the whole study falls between −0.8 and 6.3%.


*Keywords:* Carvedilol; Racemate; Enantiomers; Bidimensional LC; Achiral and chiral RPLC separation mechanisms; Cellulose tris(3,5-dimethyl-phenylcarbamate); Simultaneous monitoring; Fluorescence detection; Bioequivalence study; Solid oral dosage forms; Pharmacokinetic parameters

## 1. Introduction

Carvedilol ((±)-1-carbazol-4-yloxy-3-{[2-(*o*-methoxyphenoxy)ethyl]amino}-2-propanol) is a lipophilic $\beta_1$- and $\beta_2$-adrenoreceptor antagonist with antioxidant and antiproliferative effects [1]. Carvedilol also displays $\alpha_1$-adrenergic antagonism, resulting in blood pressure reducing action through vasodilatation [2]. Relative to other β-blockers, carvedilol exhibits minimal inverse agonist activity, consequently producing reduced negative chronotropic and inotropic effects [3]. The metabolic pattern of carvedilol in the bile allows identification of two inactive metabolites (1-hydroxycarvedilol *O*-glucuronide and 8-hydroxycarvedilol *O*-glucuronide, respectively) [4]. The pharmacokinetics and absolute bioavailability of racemic carvedilol were already reported [5,6]. A surprising lack of effect of the physical exercise on carvedilol plasma concentration levels was observed [7,8]. No pharmacokinetic interaction between carvedilol and digitoxin or phenprocoumon was noted [9], while simultaneous administration of nicardipine and nifedipine leads to significantly increased plasma concentration levels [10].

Carvedilol is administrated as a racemate, although enantiomers exhibit different pharmacological effects (both enantiomers exhibit similar $\alpha_1$-blocking activity, but only the (−) *S* enantiomer possesses β-blocking activity) [2]. Studies made on rats under steady state conditions after i.v. administration of carvedilol reveal stereoselective metabolic clearance and distribution [11]. The evidence of stereoselective presystemic metabolism of carvedilol was demonstrated in humans and monkeys [12–15].

Racemic carvedilol was assayed in plasma and tissues samples mostly by RP-HPLC [16–21] although GC was also used

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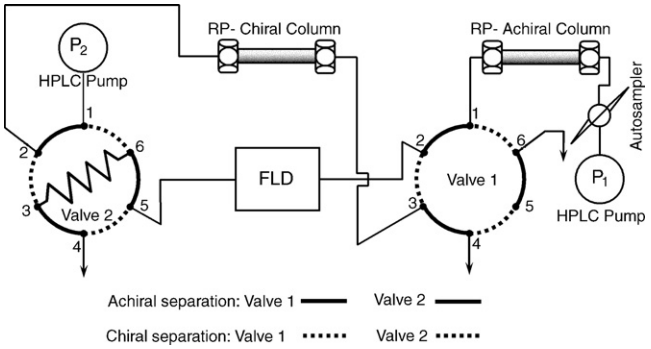


Fig. 1. Experimental set-up for achiral/chiral separation of carvedilol.

after preliminary derivatization with MSTFA and MBTFA [22]. Liquid–liquid extraction [16,19,20,22], protein precipitation with methanol addition followed by solvent evaporation [17] and solid phase extraction [21] were used as sample preparation techniques. The use of MS [22], MS/MS [16], fluorescence [17,19–21] and electrochemical [18] detection modes lead to quantitation limits within 0.1 and 10 ng/mL interval. Major signal suppression effects were observed for carvedilol and its deuterated analogue (used as internal standard) in the MS/MS detection mode [23].

A first approach for the enantioseparation of carvedilol isomers was given by capillary zone electrophoresis, using 2-hydroxypropyl-β-cyclodextrin as a chiral selector [13,24,25]. Pre-column derivatization of enantiomers of carvedilol with 2,3,4,6-tetra-*O*-acetyl-*b*-D-glucopyranosyl isothiocyanate (GITC) [14,15,26] or (+) naproxen [27] followed by RPLC was often used as an alternate analytical feature. Comparison between techniques has been also considered [28,29]. NPLC on enantioselective chemically modified amylose stationary phase has been also used for assaying carvedilol isomers in human whole blood and plasma [30] obtained from 15 patients treated for congestive heart failure. A recent study emphasizes the enantioseparation of β-blockers in NPLC and RPLC modes on cellulose tris(3,5-dimethyl-phenylcarbamate) stationary phases [31].

The goals of the present work relate to the validation of a RPLC/RPLC–FLD method suitable for the assay of racemic carvedilol and related enantiomers within a single chromatographic run, designed for evaluation of the bioequivalence (BE) for two commercially available pharmaceutical formulations.

## 2. Experimental

### 2.1. Instrumentation

The experimental set-up is described in Fig. 1. The first LC dimension was performed on an Agilent 1100 series LC (Agilent Technology, Waldbronn, Germany) system consisting of the following modules: degasser (G1322A), binary pump (G1312A), thermostated autosampler (G1329A/G1330B), column thermostat (G1316A) fitted with an automated switching valve (G1316-68709), fluorescence detector (G1321A). System control and data acquisition were made with the Agilent Chemstation software version B 01.03. The second LC dimension was based on Agilent 1100 series modules (degasser, binary pump) controlled by means of the Controller pad G1323 B. The second six-port switching valve was Vici, Model no. EHMA (obtained from Valco Instruments Co. Inc., U.S.A.) fitted with a 2 mL loop. The first LC system controls the second one and the Vici valve through the external events BCD board G1351-66500. Both systems were operationally qualified before and after the bioequivalence study. Valve functioning program is the following: Valve 1—from 0 to 4.2 min, position ——; from 4.2 to 18 min, position .....; Valve 2—from 0 to 3.7 min, position ——; from 3.7 to 18 min, position .....).

### 2.2. Sample preparation method

The preparation procedure is based upon protein precipitation on addition of a water miscible organic solvent to plasma samples. A plasma aliquot of 0.2 mL was vortexed with 0.4 mL of a solution of the IS (50 ng/mL) in acetonitrile. After centrifugation for 10 min at 7250 × *g*, the supernatant was quantitatively transferred to a vial and diluted with 0.8 mL of an aqueous 50 mM sodium acetate buffer at pH 6. After vortexing, the vial containing the prepared sample was capped and transferred to the LC autosampler rack.

### 2.3. Chromatographic methods

#### 2.3.1. First LC dimension

A single monolithic Chromolith Performance RP-18e column (Merck, Germany), 100 mm length and 4.6 mm internal diameter fitted with a Chromolith Guard Cartridge RP-18e (10 mm × 4.6 mm) was used during the validation stage and entire bioequivalence study. The column was validated before and after study completion, by computing the lowest value corresponding to the height equivalent to the theoretical plate (HETP) in case of the fluoranthene peak (a variation from 8.86 to 9.21 μm was noticed during the whole validation process and BE study, meaning around 1900 injected samples). The column was thermostated at 35 °C.

Separation was based on an isocratic elution, having a duration of 4.2 min, using a mobile phase containing 65% (v/v) aqueous 50 mM sodium acetate buffer at pH 6 and 35% acetonitrile, at a flow rate of 2 mL/min. Injection volume was 20 μL. The following gradient profile of the mobile phase composition was applied only for column washing and re-equilibration (step gradient from minute 4.2 to minute 4.4 up to 90% acetonitrile, composition maintained for the next 7.6 min and step gradient down to 35% acetonitrile in 0.2 min).

#### 2.3.2. Second LC dimension

A Chiralcel® OD-RH (cellulose tris-3,5-dimethylphenylcarbamate coated on silicagel), 150 mm length, 4.6 mm internal diameter and 5 μm particle size fitted with a Guard Cartridge Chiralcel® OD-RH (10 mm × 4 mm) was obtained from Chiral Technologies Europe (France). The column was validated before and after study completion, by computing the reduced plate height ($\bar{h}$) for the peak corresponding to the (−) *S* enan-

tiomer of carvedilol (a variation from 4.4 to 6.1 was noticed during the whole validation process and BE study). The column was thermostated at 35 °C.

Separation was based on an isocratic elution, using a mobile phase containing 10% (v/v) aqueous 50 mM sodium acetate buffer at pH 6 and 90% acetonitrile, at a flow rate of 1 mL/min. The volume of the shortcut loop was 2 mL. Duration of the chiral separation is 15.8 min.

Duration of a chromatographic run (both achiral and chiral separations) is of 18 min.

### 2.4. Detection

Fluorescence detection was applied using an excitation wavelength at 285 nm and an emission wavelength at 355 nm. Detector response time was 4 s and signal amplification (PMT gain) 17. Integration of the chromatographic peaks was made using dedicated automated integration methods for both LC dimensions.

### 2.5. Materials

All solvents were HPLC grade from Merck (Darmstadt, Germany). Reagents were analytical grade from the same producer. Water for chromatography (resistivity minimum 18.2 MΩ and TOC maximum 30 ppb) was obtained within the laboratory by means of a TKA Lab HP 6UV/UF instrument and used during experiments. Propranolol hydrochloride (I.S.) code no. P3500000, batch no. 2 was purchased from European Pharmacopoeia, Council of Europe, Strasbourg, France. Racemic Carvedilol was a secondary reference standard no. 036/24.05.06, batch no. 5CV005 from Cadila Pharmaceuticals Limited (India). (−) *S* Carvedilol was obtained from Synfine Research (Canada).

### 2.6. Methodology and pharmacokinetic parameters

In this open-label, randomized, two-period, two-sequence, crossover study, 30 healthy male volunteers with a mean age of 28.4 (minimum 18; maximum 45) years, mean weight of 77.10 (minimum 61; maximum 99) kg and a mean BMI of 24.4 (minimum 22; maximum 30) kg/m$^2$ received one dose of 25 mg racemic carvedilol of the tested product (Test) and one of the reference product (Ref.), in the sequence determined by randomization, with a 14 days wash-out period between consecutive administrations. It is well known that carvedilol administrated to patients affected by essential hypertension may determine, at the beginning of the treatment, orthostatic hypotension accompanied by dizziness, sometimes even with lipothymia. Considering that women have a lower arterial tension, it was preferred to carry out the study only on a male population of volunteers. In order to avoid adverse reactions, administration was made after a standardized light breakfast, followed by a strict bed rest period of at least 6 h. For the same reason, the selected male subjects were characterized by an increased BMI (not lower than 22) compared to the normal accepted interval (19–25). The protocol of the study was formally accepted by the evaluation department of the Romanian National Drug Agency and received the approval of the Institutional Ethics Committee. Venous blood samples were collected pre-dose (0 h) and the following post-dose intervals of time: 0.33; 0.67; 1; 1.5; 2; 2.5; 3; 3.5; 4; 5; 6; 8; 10; 12; 16; 24; 48; 72 and 120 h. Medical examinations were performed in the screening and at the beginning of each study period (in-house day), in every single blood sampling day and at the end of each study period.

The pharmacokinetic parameters considered for evaluation of the bioequivalence between tested and reference products refer to racemic carvedilol and enantiomers. These parameters were the following: (1) major parameters (according to FDA and EMEA recommendations), $C_{max}$—observed maximum plasma concentration; $AUC_{last}$—area under plasma concentration/time plot until the last quantifiable value; (2) auxiliary parameters, $T_{max}$—sampling time of the maximum plasma concentration; $t_{half}$—terminal elimination half life time; $AUC_{total}$—area under plasma concentration/time plot extrapolated to infinity. Pharmacokinetic parameters were determined by means of the SAS/STAT software (version 9.1). The analysis of variance was performed on the pharmacokinetic parameters. Then, the 90% confidence intervals of the pharmacokinetic parameters characterizing the tested/reference products were determined.

## 3. Results and discussion

### 3.1. Robustness

Robustness on the first LC dimension should be discussed with respect to retention time, efficiency, and symmetry, considering that the peak of the racemic carvedilol should fit in 1 min collecting interval. Separation against the IS is not critical and consequently the chromatographic resolution between target compounds should not represent a major concern.

The influence of the mobile phase composition on the result of the analysis should be considered within the interval commonly accepted as the mixing accuracy of the binary pump. During the operational qualification (OQ) of the module, a bias of ±0.5% from the set value is more often considered as an adequate maximum limit. Consequently, ±1% variation of acetonitrile in the mobile phase composition should be considered as a rationale interval for evaluation. Over this interval, efficiencies calculated for the racemic carvedilol peak are relatively constant (3100 ± 50 theoretical plates) and peak elution intervals (distance between peak end and peak start = 6 × s) are around 0.35 min. Peaks have some post-tailing, the symmetry at 10% from height being 2.4. According to previous data, one easily concludes that the collection interval should not start roughly before $t_R - 0.1$ min and should not end before $t_R + 0.2$ min ($t_R$ is the retention time of carvedilol racemate). The retention time measured for the peak corresponding to racemic carvedilol depends linearly on the content of acetonitrile in the mobile phase, according to the relationship $t_R = 11{,}304 - 0.237 \times$ (% ACN) ($r_{xy} = 0.993$). For a ±0.5% error of the binary pump in mixing components of the mobile phase, retention time of the carvedilol peak moves in the 2.9 and 3.1 min interval, meaning a collection window ranging from 2.8 to 3.4 min, well fitting against set values (2.7–3.7 min). As a confirmation of the above

mentioned considerations, over the 1140 real samples analyzed during the bioequivalence study, the mean retention time of the racemic carvedilol peak was 3.1 min, with a relative standard deviation of 3.4% (minimum value 2.89 min, maximum value 3.45 min).

Influence of the sodium acetate concentration in the aqueous constituent of the mobile phase was studied in the 45–55 mM interval. No major effects on efficiency and peak symmetry were observed. Mean retention time of the racemic carvedilol was 3.1 min. Retention time has a normal variation interval (±2 s) ranging between 2.97 and 3.23 min, requiring a collection interval in the 2.87–3.48 min range. One can conclude the robustness of the method against this operational parameter.

Column temperature was also considered. Although the precision of a Peltier element is lower than ±1 °C, the investigated temperature interval was extended at ±5 °C. No major effects on efficiency and peak asymmetry were observed. Retention time decreases linearly at temperatures in the investigated interval ($t_R = -0.023 \times T + 3.81$; $r_{xy} = -0.992$). For column temperature variations of ±3 °C, retention varies between 2.9 and 3.15 min, imposing a collecting interval ranging from 2.8 to 3.4 min.

pH variation of the aqueous component of the mobile phase was evaluated for the interval 5.5–6.5. Retention time increases on the increase of pH, following a linear relationship ($t_R = 1.049 \times \text{pH} - 3.185$; $r_{xy} = 0.999$). ±0.2 pH units variation lead to a retention interval between 2.9 and 3.3 min, meaning a required collection interval ranging from 2.8 to 3.6 min. Accordingly, we have assumed during experiments that the pH of the aqueous component of the mobile phase should be controlled with ±0.1 unit precision.

Columns belonging to three different production batches have also been tested for retention data reproducibility. Retention of the carvedilol peak varies in the 2.9–3.15 min interval, indicating that column change does not affect retention and consequently there is no need for reevaluation of the shortcut interval.

Robustness on the second LC dimension should be discussed with respect to chromatographic resolution between separated enantiomers.

Variation of the mobile phase composition within ±1% interval does not affect resolution between separated *S* (−) and *R* (+) enantiomers. Same conclusion was obtained when the concentration of the acetate buffer in the aqueous constituent of the mobile phase was varied in the 45–55 mM concentration interval. Column temperature variations in the ±5 °C interval lead to variations in chromatographic resolution ranging from 2 to 2.2. ±0.5 pH units modification in the aqueous component of the mobile phase has practically no influence on resolution between enantiomers. The enantiomeric separation is robust toward the main operational parameter of the method.

### 3.2. Selectivity of the chromatographic method

The first dimension of the chromatographic method separates racemic carvedilol from IS with a resolution of 3.8. Fluorimetric detection adds its own inherent selectivity against the plasma endogenous components still remaining in samples after protein precipitation. During the method validation the selectivity has been proved for six blank plasma samples. Additionally, in all pre-dose collected plasma samples from volunteers participating to the study, no endogenous interference was observed. Residual peak areas in blank samples over the whole study ranged from 9.1 to 15.1% from the peak area of racemic carvedilol corresponding to LLOQ (1.3 ng/mL).

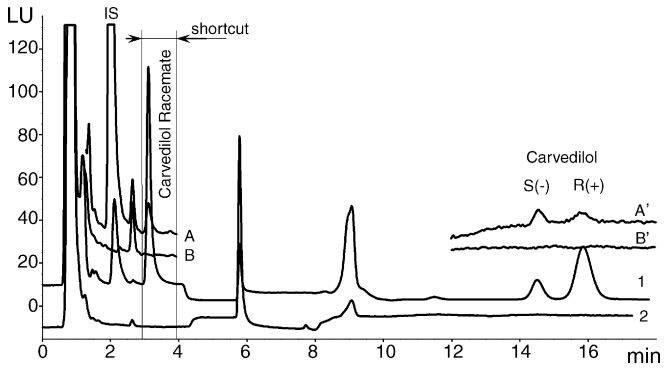


Fig. 2. Typical chromatograms resulting from the application of the method on real or prepared plasma samples (achiral separation from 0 to 4.2 min, chiral separation from 4.2 to 18 min; shortcut in the first LC dimension between 2.7 and 3.7 min; trace 1—real plasma sample at 70 ng/mL level; trace 2—blank plasma sample; details A and B—comparison between a spiked plasma sample at LLOQ and a blank sample on the achiral dimension; details A′ and B′—comparison between a spiked plasma sample at LLOQ and a blank sample on the chiral dimension).

Selectivity on the second LC dimension is preserved by the transfer of the fraction collected over 1 min interval from the first RP separation, corresponding only to racemic carvedilol peak. Residual peak areas in blank samples over the enantioselective dimension are below 15% from areas corresponding to enantiomers spiked at LLOQ level (0.7 ng/mL). Selectivity between enantiomers within the second LC dimension is 2. In Fig. 2 two overlaid chromatograms are given in order to prove the overall selectivity of the chromatographic method. Details A, B and A′, B′, respectively, show chromatographic results on both LC directions for spiked carvedilol in plasma at LLOQ levels (A and A′) and a blank sample (B and B′).

### 3.3. Recoveries

The sample preparation method is based on protein precipitation by addition of a water miscible organic solvent (acetonitrile). Usually, such procedure allows higher recoveries for the target compounds, as the solubility in the supernatant is enhanced by the organic solvent. Racemic carvedilol was spiked to blank plasma samples at 5, 70 and 140 ng/mL levels while IS was added at 100 ng/mL level. Five replicates were processed according to the sample preparation procedure for each concentration level of racemic carvedilol. Same spiking operation was achieved in HPLC grade water. Samples spiked in water were injected as such. All samples were subjected to the LC/LC–FLD method. Recoveries were calculated by rationing peak areas in chromatograms corresponding to spiked plasma samples to corresponding peak areas in chromatograms of the aqueous spiked samples. This approach was preferred to the calculation of the recovery against spikes in protein precipitated

blank plasma bulks, as fluorescence detection which is being used is not affected by signal suppression/enhancement effects as occurring in mass-spectrometric detection. The recoveries corresponding to enantiomers of carvedilol in the second LC dimension should also offer an image on the shortcut transfer from the first column. Mean recovery of racemic carvedilol over the studied concentration interval was 89.2% (experimental values are characterized by a relative standard deviation (R.S.D.%) of 3.1%). The recovery of the IS is 81.6% (R.S.D.% = 4%). Mean recoveries calculated for the (−) *S* and (+) *R* enantiomers of carvedilol in the concentration interval 2.5–70 ng/mL were 86.7 and 87.1%, respectively. Relative standard deviations characterizing experimental data sets are very similar (3.7% for the *S* enantiomer, 3.9% for the *R* enantiomer).

### 3.4. Calibration and quantification limits

Calibration data for target compounds are given in Table 1. LLOQ values were calculated in three different manners (relationships are given in the table footnote). One can observe that evaluation of LLOQ with respect to residual peak areas in blank chromatograms approximates the average between the corresponding values resulting from the other two alternative computational models. Consequently, these results were considered as final values.

During study completion, a calibration was performed for each volunteer. Bulk blank plasma samples were spiked at 2, 6, 8, 15, 50, 80, 100 and 150 ng/mL with racemic carvedilol and 100 ng/mL with IS. 30 aliquots at each concentration level from bulk spiked plasma samples were placed in separate vials and frozen at −40 °C. One set of calibration plasma samples was thawed at the same time as samples from one volunteer, prepared in the same manner and analyzed within the same chromatographic sequence. The normal variation interval of the slopes resulting by computation of the linear regressions was $0.023 \pm 0.002$, while the same interval for intercepts was $-0.001 \pm 0.02$ ($n = 30$) for racemic carvedilol. The slopes of the linear regressions determined for (−) *S* and (+) *R* carvedilol enantiomers were $0.033 \pm 0.012$ and the intercepts fit in the interval $-0.004 \pm 0.016$.

### 3.5. Precision

Precision was checked on spiked plasma samples at three concentration levels of racemic carvedilol (5, 70, and 140 ng/mL). Table 2 enlists experimental results obtained during the evaluation of precision on both chromatographic dimensions, considering as parameters the absolute peak area of racemic/enantiomeric carvedilol, the peak area ratio between racemic/enantiomeric carvedilol and IS, and the corresponding calculated concentration (applying the regression equations obtained under the linearity study). Results for IS peak area values are also given.

During the study completion, for each analytical sequence, quality control (QC) samples were considered for racemic carvedilol at three concentration levels (4, 25 and 120 ng/mL, respectively) injected in duplicate. Intra-sequence precision was

Table 1
Calibration data for target compounds

| Analyte | Dependent variable | Linearity domain (ng/mL) | No. of points ($n$) | Replicates per concentration ($i$) | Slope ($B$) | Intercept ($A$) | Correlation coefficient ($r_{xy}$) | LLOQ[a] (ng/mL) | LLOQ[b] (ng/mL) | LLOQ[c] (ng/mL) |
|---|---|---|---|---|---|---|---|---|---|---|
| Carvedilol racemate | Peak area ratio between carvedilol and IS | 2–150 | 8 | 6 | $0.0221 \pm 0.0003$ | $-0.005 \pm 0.016$ | 0.9999 | 0.80 | 1.8 | 1.3 |
| (−) *S* Carvedilol | | 1–75 | 6 | 6 | $0.0384 \pm 0.0005$ | $0.005 \pm 0.015$ | 0.9999 | 0.45 | 1.0 | 0.7 |
| (+) *R* Carvedilol | | 1–75 | 6 | 6 | $0.0384 \pm 0.0005$ | $0.005 \pm 0.015$ | 0.9998 | 0.45 | 1.0 | 0.7 |
| Internal standard | Peak area | 10–200 | 6 | 1 | $6.47 \pm 0.005$ | $1.6 \pm 5$ | 0.9998 | 1.90 | 4.0 | 3.0 |

[a] Calculation was made according to the relationship $LLOQ = (5 \times s_A)/B$; $s_A$ is the standard deviation of the intercept.
[b] Calculation was made according to the relationship $LLOQ = [t \times (s_A + s_B \times C_m)]/(B + 2 \times t \times s_B)$; $s_A$ and $s_B$ are standard deviations of the intercept A and slope B, $C_m$ is the mean concentration value for the set used for linear regression, $t$ is the Student coefficient considered for $n - 2$ degrees of freedom and a confidence level of 99%.
[c] Evaluation was made according to residual peak areas in blank samples $LLOQ = 5 \times RPA_m$; $RPA_m$ is the mean residual peak area in blank plasma samples.

Table 2
Intra- and inter-day precision for spiked plasma samples

| Analyte | Spiked concentration (ng/mL) | Parameter | Intraday ($n = 10$) | | Interday ($n = 6$) | |
|---|---|---|---|---|---|---|
| | | | Mean ± 2$s$ | R.S.D. (%) | Mean ± 2$s$ | R.S.D. (%) |
| Racemic Carvedilol | 5 | Peak area | 58.2 ± 3.4 | 1.1 | 58.3 ± 4.7 | 4.1 |
| | | Analyte/I.S. peak area ratio | 0.102 ± 0.006 | 2.7 | 0.101 ± 0.006 | 3.1 |
| | | Experimental concentration (ng/mL) | 4.9 ± 0.25 | 2.5 | 4.8 ± 0.3 | 0.3 |
| | 70 | Peak area | 805 ± 22 | 1.4 | 848 ± 63 | 3.7 |
| | | Analyte/I.S. peak area ratio | 1.51 ± 0.04 | 1.4 | 1.53 ± 0.06 | 1.8 |
| | | Experimental concentration (ng/mL) | 68.4 ± 2 | 1.4 | 69.3 ± 2.4 | 1.8 |
| | 140 | Peak area | 1845 ± 6 | 0.2 | 1773 ± 132 | 3.7 |
| | | Analyte/I.S. peak area ratio | 3.21 ± 0.02 | 0.4 | 3.18 ± 0.02 | 0.4 |
| | | Experimental concentration (ng/mL) | 145.4 ± 1.2 | 1.2 | 144 ± 1.2 | 0.4 |
| (−) *S* Carvedilol/(+) *R* Carvedilol | 2.5 | Peak area | 56.5 ± 1.5 | 1.3 | 58.2 ± 2.3 | 2.8 |
| | | | 56.8 ± 2.1 | 1.8 | 58.7 ± 3.3 | 2.8 |
| | | Analyte/I.S. peak area ratio | 0.099 ± 0.002 | 1.5 | 0.101 ± 0.004 | 1.9 |
| | | | 0.100 ± 0.002 | 2.0 | 0.101 ± 0.004 | 2.3 |
| | | Experimental concentration (ng/mL) | 2.47 ± 0.08 | 1.5 | 2.52 ± 0.1 | 2.0 |
| | | | 2.49 ± 0.10 | 2.0 | 2.52 ± 0.12 | 2.5 |
| | 35 | Peak area | 730 ± 5 | 0.5 | 737 ± 28 | 1.9 |
| | | | 727 ± 13 | 0.9 | 734 ± 29 | 2.0 |
| | | Analyte/I.S. peak area ratio | 1.37 ± 0.02 | 0.8 | 1.33 ± 0.02 | 0.6 |
| | | | 1.36 ± 0.04 | 1.1 | 1.32 ± 0.02 | 0.6 |
| | | Experimental concentration (ng/mL) | 35.5 ± 0.6 | 0.8 | 34.5 ± 0.4 | 0.6 |
| | | | 35.4 ± 0.8 | 1.1 | 34.3 ± 0.4 | 0.6 |
| | 70 | Peak area | 1598 ± 17 | 0.5 | 1522 ± 106 | 3.9 |
| | | | 1593 ± 18 | 0.6 | 1515 ± 122 | 4.0 |
| | | Analyte/I.S. peak area ratio | 2.78 ± 0.04 | 0.6 | 2.73 ± 0.04 | 0.6 |
| | | | 2.77 ± 0.04 | 0.7 | 2.70 ± 0.04 | 0.8 |
| | | Experimental concentration (ng/mL) | 72.3 ± 0.9 | 0.6 | 71.1 ± 0.8 | 0.6 |
| | | | 72.1 ± 0.9 | 0.7 | 70.7 ± 1.1 | 0.8 |
| IS | 100 | Peak area | 559 ± 37 | 3.3 | 564 ± 36 | 3.2 |

evaluated in terms of concentration for 30 QC sets (60 samples, respectively). Calculation was made by using the linear regression equation obtained for the calibration corresponding to the sequence. The following results were obtained: for 4 ng/mL level, the mean calculated concentration was 4.2 ± 0.6 ng/mL, with an R.S.D.% of 6.8%; for 25 ng/mL level, the mean calculated concentration was 25.1 ± 2.4 ng/mL, with an R.S.D.% of 4.9%; for 120 ng/mL level, the mean calculated concentration was 119.7 ± 7.2 ng/mL, with an R.S.D.% of 3.2%. For the (−) *S* enantiomer, the mean calculated concentration at 2 ng/mL level was 2.1 ± 0.2 with an R.S.D.% of 6.7%, at 12.5 ng/mL level was 12.4 ± 1.4 with an R.S.D.% of 5.3% and at 60 ng/mL level was 59.6 ± 4.2 with an R.S.D.% of 3.5%. For the (+) *R* enantiomer, the mean calculated concentration at 2 ng/mL level was 2.1 ± 0.2 with an R.S.D.% of 6.1%, at 12.5 ng/mL level was 12.3 ± 1.4 with an R.S.D.% of 5.5% and at 60 ng/mL level was 59.5 ± 4.2 with an R.S.D.% of 3.5%.

In order to illustrate method precision, peak areas integrated for the IS in chromatograms of the samples as well as in calibrations and QC sets have been evaluated for dispersion. From 1620 data, it results in an R.S.D.% of 7.1%.

### 3.6. Accuracy

The accuracy of the method may be evaluated from the QC samples analyzed over the study (30 sets). Inter-sequence accuracy, estimated as the bias (calculated as percentage) of the QC samples against the theoretical concentration values, acts as an accuracy indicator. The set of QC samples corresponding to the low level (3 × LLOQ) should be considered as the most critical one. From all sets of 180 QC values obtained at the low concentration level, only three individual values (two data for the *S* enantiomer and one data for the *R* one) were placed outside the acceptance interval (±15%). No trends of the % bias were observed during the study. This is sustained by the characteristics of the linear regressions: correlation coefficients are close to 0 ($r_{xy}$ for racemic carvedilol is 0.07, for the *S* enantiomer is 0.03 and for the *R* one is 0.13) and slopes are close to 0 (B for racemic carvedilol is −0.0287, for the *S* enantiomer is −0.0125 and for the *R* one is −0.0484), meaning a randomized dispersion of individual values against the average. The mean % bias at the low concentration level over the whole study was 4.6% for racemic carvedilol and 6.3%/5.8% for (−) *S*/(+) *R* enantiomers, respectively.

Table 3
Stability data for carvedilol (racemate, (−) *S* and (+) *R* enantiomers) and IS

| Analyte | Procedure | Parameters | | | | | |
|---|---|---|---|---|---|---|---|
| | | Mean | R.S.D. (%) | Mean | R.S.D. (%) | Mean | R.S.D. (%) |
| Concentration (ng/mL) | | 5 | | 70 | | 140 | |
| Racemic Carvedilol | Freeze and thaw ($n=5$) | 4.9 | 3.3 | 69.9 | 0.5 | 143.6 | 1.3 |
| | Long term ($n=4$) | 4.9 | 3.4 | 70.7 | 1.1 | 145.2 | 0.6 |
| | Short term ($n=4$) | 4.8 | 5.2 | 69.6 | 0.8 | 146.5 | 1.9 |
| | Post preparative ($n=4$) | 4.9 | 4.4 | 70.3 | 1.2 | 147.6 | 1.9 |
| Concentration (ng/mL) | | 2.5 | | 35 | | 70 | |
| (−) *S* Enantiomer | Freeze and thaw ($n=5$) | 2.6 | 2.4 | 35.1 | 0.9 | 71.9 | 0.9 |
| | Long term ($n=4$) | 2.6 | 2.7 | 35.6 | 1.3 | 72.2 | 0.6 |
| | Short term ($n=4$) | 2.6 | 2.4 | 35.0 | 2.2 | 72.3 | 0.8 |
| | Post preparative ($n=4$) | 2.6 | 1.2 | 35.6 | 1.3 | 72.3 | 0.6 |
| (+) *R* Enantiomer | Freeze and thaw ($n=5$) | 2.5 | 1.5 | 34.9 | 1.5 | 71.6 | 0.9 |
| | Long term ($n=4$) | 2.5 | 1.1 | 35.5 | 1.2 | 71.8 | 0.4 |
| | Short term ($n=4$) | 2.6 | 2.6 | 34.9 | 2.7 | 72.0 | 0.8 |
| | Post preparative ($n=4$) | 2.6 | 0.9 | 35.7 | 1.5 | 72.0 | 0.5 |
| Peak area (LU × *s*) | | | | | | | |
| IS | Stock solution ($n=4$) | 607.7 | 7.0 | – | – | – | – |

Another special feature of the proposed method lies with the accuracy of the transfer of the peak corresponding to racemic carvedilol onto the enantioselective column. Representation of the mean concentration values obtained during the study for the racemic carvedilol in samples from volunteers against the addition of mean concentration values in the same samples for (−) *S* and (+) *R* enantiomers should illustrate if the transfer was adequate and quantitative. The above mentioned dependencies are characterized, for the reference and tested products by correlation coefficients of 0.9994 and 0.9983, respectively. Similarity factors $\left(f_2{=}50\ \log\left(100\times\left(1+\left(\sum_{i=1}^{n}(c_{\mathrm{Rac}}-(c_S+c_R))^2\right)/n\right)^{-1/2}\right)\right)$ characterizing the concentration profiles obtained for racemic carvedilol and the addition between values corresponding to (−) *S* and (+) *R* enantiomers, considering both reference and tested drugs, shows differences below 10% ($f_2=97.2$ for reference drug and 96 for the tested one).

### 3.7. Stability of analytes and samples

Stability studies for racemic carvedilol were made on spiked plasma samples having concentrations of 5, 70, and 140 ng/mL. Consequently, in the second LC dimension (−) *S* and (+) *R* enantiomers were observed at 2.5, 35 and 70 ng/mL concentration levels. The stability of the IS stock solution in acetonitrile (50 ng/mL) was also checked over a 30 days period (sampling moments were initial and 5, 15 and 30 days after). Before each analysis, the IS stock solution was spiked to a

Table 4
Statistics of pharmacokinetic parameters

| Analyte | Drug | Statistic | $C_{max}$ (ng/mL)[a] | $AUC_{last}$ (ng/mL h)[a] | $T_{max}$ (h) | $T_{half}$ (h) | $AUC_{tot}$ (ng/mL h) |
|---|---|---|---|---|---|---|---|
| Racemic Carvedilol | Tested (T) | Mean | 49.2 | 214.2 | 1.66 | 7.12 | 232.5 |
| | | R.S.D.% | 42.8 | 56.7 | 52.9 | 76.2 | 56.5 |
| | Reference (R) | Mean | 51.2 | 211.6 | 1.79 | 7.14 | 229.2 |
| | | R.S.D.% | 47.3 | 54.9 | 44.4 | 79.1 | 55.2 |
| | 90% confidence interval for the ratio of the means *T*/*R* | | 85.2–107.3 | 91.8–109.2 | | – | 92.7–109.2 |
| (−) *S* Carvedilol | Tested (T) | Mean | 10.3 | 38.3 | 1.5 | 5.25 | 46.0 |
| | | R.S.D.% | 42.7 | 51.6 | 47.4 | 57.5 | 49.5 |
| | Reference (R) | Mean | 11.6 | 43.4 | 1.5 | 5.78 | 49.3 |
| | | R.S.D.% | 50.7 | 43.2 | 45.2 | 63.1 | 43.9 |
| | 90% confidence interval for the ratio of the means *T*/*R* | | 81.7–106.2 | 89.9–108.2 | | – | 91.0–107.9 |
| (+) *R* Carvedilol | Tested (T) | Mean | 33.6 | 139.4 | 1.5 | 4.05 | 145.5 |
| | | R.S.D.% | 44.7 | 62.5 | 52.3 | 76.8 | 64.6 |
| | Reference (R) | Mean | 34.7 | 120.3 | 2.0 | 4.21 | 124.1 |
| | | R.S.D.% | 46.9 | 61.5 | 42.6 | 66.8 | 62.0 |
| | 90% confidence interval for the ratio of the means *T*/R | | 89.1–118.3 | 94.4–121.5 | | – | 94.7–120.6 |

[a] The statistical power of the test for the main pharmacokinetic parameters $C_{max}$ and $AUC_{last}$ was 1.000.

blank plasma sample at 100 ng/mL level; sample was processed according to the procedure and injected to the chromatographic column.

Freeze and thaw stability was studied for five consecutive cycles, from −40 °C to ambient (thaw process was unassisted).

Long-term stability was studied over 24 days (four samplings, initial and in days 6, 12, and 24, respectively) at −20 °C storage temperature. The interval was considered as a representative for the period assigned to the clinical trial, until samples reached the laboratory. For the analytical study period, the QC samples played the role of stability indicators.

Short-term stability was made over 24 h interval. Frozen spiked plasma samples were thawed unassisted at room temperature and analyzed after 6, 12, and 24 h, respectively.

Post-preparative stability was evaluated by analyzing processed spiked plasma samples after preparation and at 24, 48 and 72 h after preparation, on storage bench top, at room temperature.

Results obtained during stability evaluation study are given in Table 3.

### 3.8. Bioequivalence study

The main pharmacokinetic parameters obtained on study completion are given in Table 4.

Concentration profiles over 15 h interval are given in Fig. 3.

By rationing the $AUC_{last}$ obtained for (+) *R* and (−) *S* enantiomers for both reference and tested products, over the entire population of subjects, the following primary statistics have been obtained: (a) the mean value for the reference drug was 3.31 (R.S.D.% = 35.8%) compared to 3.37 (R.S.D.% = 38.3%) for the tested drug; (b) variations of these ratios over the tested population ranged between 1.66 and 7.24 for the reference product and between 1.75 and 8.42 for the tested product; (c) absolute biases between *R*/*S* ratios calculated for each volunteer after administration of tested and reference products ranged between −1.18 and 0.57. As the $AUC_{last}$ synthetically expresses the bioavailability of the active substance (weighting absorption, metabolization and elimination together), one can conclude that intra-variability is low for the enantiomeric pattern, while the inter-variability is significant. The small intra-subject variability in enantiomer ratios indicates a relatively constant relationship between vasodilatation effects (given by the $\alpha_1$ adrenergic activity common to both enantiomers) and the β-blocking effect sustained only by the (−) *S* enantiomer. As the systemic and/or pre-systemic metabolisms of (+) *R* and (−) *S* carvedilol in the liver are controlled over different expressions of CYP2D6 alleles [33], the inter-individual differences in bioavailability are largely responsible for the pharmacokinetic variability characterizing the drug. Consequently, studies have to be carried out on a large population of subjects, allowing consistent statistical interpretation.

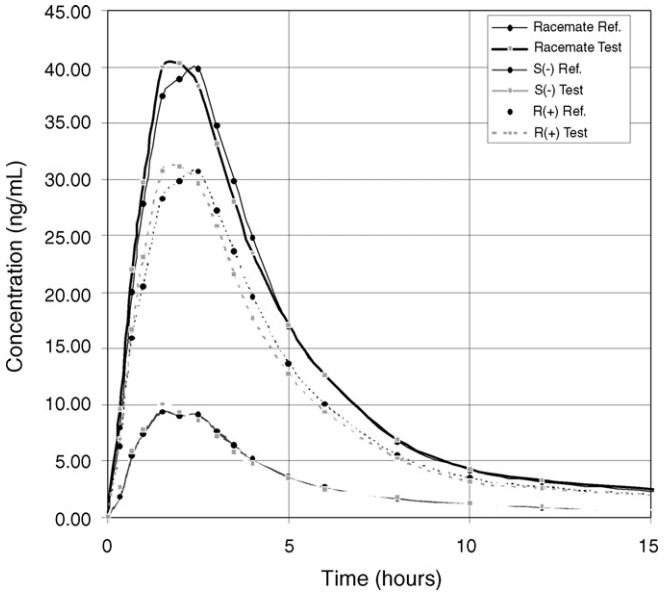


Fig. 3. Mean concentration profiles obtained for racemic carvedilol and its (−) *S* and (+) *R* enantiomers, for reference and tested drug products, over the bioequivalence study carried out on 30 healthy male volunteers.

## 4. Conclusions

An LC/LC/FLD method for achiral/chiral determination of carvedilol in plasma samples is proposed. Both LC separation dimensions are based upon reversed phase mechanisms. Achiral separation is realized on a C18 chemically modified silicagel based stationary phase while the chiral dimension is achieved on cellulose tris(3,5-dimethyl-phenylcarbamate) deposed on silicagel. On both LC dimensions, the chromatographic separations were made in the isocratic mode. The period needed to achieve the chiral separation is used for washing and re-equilibration on the achiral dimension. Transfer of a shortcut from the achiral column to the chiral one and simultaneous fluorescent detection over both LC dimensions during a single chromatographic run were possible by using two high-pressure six-port switching valves. Plasma sample preparation procedure is simple and consists in protein precipitation by addition of a water miscible organic solvent. Propranolol is used as an internal standard. The proposed method was used for the evaluation of the bioequivalence of a carvedilol drug product authorized on the Romanian market against a reference one. Pharmacokinetic parameters are discussed for racemic carvedilol as well as for the (−) *S* and (+) *R* enantiomers. The method was validated according to guidance [32]. Low limits of quantification in the 1 ng/mL range were obtained for the racemate and enantiomers. Precision (calculated on concentration data sets) is characterized by relative standard deviation values below 8%. Accuracy expressed as % bias falls within the accepted interval (±15%) over the whole investigated range of concentration. Robustness of the method is discussed for both LC dimensions. Stability of the target compounds in samples was studied. A good fit was found between concentration profiles of racemic carvedilol and cumulated profiles of (−) *S* and (+) *R* enantiomers in plasma samples taken from volunteers over the study. Pharmacokinetic parameters being obtained are in very good agreement with data from literature. The bioequivalence of the two products was demonstrated.

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