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Short communication

Liquid chromatographic determination of carvedilol in human plasma

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Abstract

A high-performance liquid chromatographic method for the quantitation of carvedilol in human plasma is presented. The method is based on protein precipitation with methanol, concentration of the supernatant by evaporation and reversed-phase chromatography with fluorimetric detection. The separation was performed on a Develosil 3 μm ODS 100×4.6 mm I.D. column and the mobile phase consisted of acetonitrile-30 m*M* potassium dihydrogenphosphate buffer, pH 2 (30:70 v/v). With only 250 μl of plasma used for sample preparation, the limit of quantitation 1.3 ng/ml was achieved. Dihydroergocristine mesylate was used as the internal standard. The between-day precision expressed by relative standard deviation was less than 6% and inaccuracy does not exceed 3%. The assay was used for pharmacokinetic studies.

Keywords: Carvedilol

1. Introduction

Carvedilol (Fig. 1) is a multiple action oral antihypertensive drug [1]. Only few methods for determination of carvedilol in plasma or serum can be found in the literature [2–4]. Other papers deal with analyses in urine and various biological material [5,6] or are focused on the separation of enantiomers [7–9], which is usually not necessary for bioequivalence studies. The methods employ either double liquid–liquid extraction or solid-phase extraction, separation on reversed-phase columns using acidic mobile phases and fluorimetric detection. The limit of quantitation (LOQ) in published procedures for plasma or serum varies from 0.25 to 3.6 ng/ml.

We present here a new method with a different sample preparation. Although only 0.25 ml of plasma is needed the low limit of quantitation necessary for pharmacokinetic study with routinely prescribed doses of the drug is maintained.

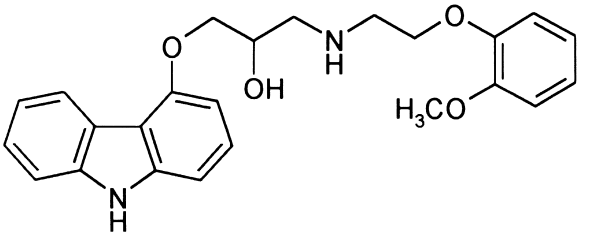

Fig. 1. Structure of carvedilol.

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2. Experimental

2.1. Chemicals

Carvedilol and dihydroergocristine mesylate (internal standard) was obtained from Léčiva, Prague, Czech Republic. Methanol (for chromatography) triethylamine and potassium dihydrogenphosphate (analytical grade) were manufactured by Merck (Darmstadt, Germany). Acetonitrile (HPLC grade) was from Riedel-de Haën (Seelze, Germany) product. *o*-Phosphoric acid and ammonium hydroxide were analytical grade both were obtained from Lachema, Brno, Czech Republic.

2.2. Apparatus

The HPLC system consisted of the following Thermo Separation Products (Riviera Beach, FL) instruments: membrane degasser, pump ConstaMetric 4100, automatic sample injector AS 3000, fluorimetric detector FL2000 and datastation with PC1000 software, version 2.5. The separation was performed on a Develosil 3 μm ODS 100×4.6 mm I.D. column (Watrex, Prague, Czech Republic). A pre-column (4×3 mm I.D.) packed with C-18 reversed-phase (Phenomenex, Torrance, CA) was used.

2.3. Chromatographic conditions

The mobile phase consisted of 30% of acetonitrile and 70% of 30 m*M* potassium dihydrogenphosphate buffer containing 1% triethylamine and 50 mg/l of sodium azide; pH of the buffer was adjusted to 2 with concentrated *o*-phosphoric acid. The flow-rate was 1.5 ml/min at a column temperature 35 °C. The fluorescence detection wavelengths were 238 nm for excitation and 350 nm for emission. The detector time constant was set to 2 s, the lamp rate was 20 Hz, photomultiplier voltage was 600 V and the excitation, emission and PMT slit widths were 20 nm. A system suitability test was performed at the beginning of each working day: a mixture of carvedilol and dihydroergocristine mesylate was chromatographed before the sample analysis. The following conditions had to be fulfilled: (1) the peak asymmetry (measured at 5% of the peak height) should be 0.90–1.5 for both peaks; (2) the column efficiency expressed as the number of theoretical plates should exceed 5000 for both compounds.

A mixture methanol–water–aqueous (26%) ammonium hydroxide (100:20:1 v:v:v) was used as the flushing solvent in the autosampler to avoid sample carryover problems.

2.4. Standards

Stock solutions of carvedilol were made by dissolving of approximately 7 mg in 25 ml of methanol. Separate solutions were prepared for calibration curve and quality control samples. Further solutions were obtained by serial dilutions of stock solutions with methanol. These solutions were added to drug-free plasma in volumes not exceeding 1% of the plasma volume. The methanolic solution of dihydroergocristine mesylate (8 mg in 25 ml) was diluted 1:1 (v:v) with methanol and this solution was used as the internal standard.

All solutions were stored at −18 °C and protected from light. The stability of carvedilol solution is at least 1 month under these conditions.

2.5. Preparation of the sample

The samples were stored in the freezer at −18 °C. The thawing was allowed at room temperature before processing of the sample. Ten μl of internal standard solution (approximately 1600 ng) were added to 0.25 ml of plasma, the tube was briefly shaken and 1 ml of methanol added. The tube was shaken for 30 s at 1500 rpm and centrifuged 5 min at 2600 *g*. The upper organic phase was transferred to another tube and evaporated under nitrogen at 50 °C. Five hundred μl of the mobile phase (see below) was added to the dry residue and the sample was dissolved by shaking for 30 s at 1500 rpm. Finally the solution was centrifuged 5 min at 2600 *g* and the supernatant transferred to the polypropylene autosampler vial. Twenty-five μl were injected into the chromatographic system.

2.6. Calibration curves

The calibration curve was constructed in the range 1.303–142 ng/ml to encompass the expected concentrations in measured samples. The calibration

curves were obtained by weighted linear regression (weighing factor $1/y^2$): the ratio of carvedilol peak height to dihydroergocristine peak height was plotted vs. ratio of carvedilol concentration to that of internal standard in ng/ml.

2.7. Limit of quantitation

Limit of quantitation was defined as the lowest concentration at which the precision expressed by relative standard deviation is better than 20% and inaccuracy (bias) expressed by relative difference of the measured and true value is also lower than 20%. Six identical samples were analyzed for the determination of LOQ.

3. Results and discussion

3.1. Sample preparation and chromatography

Although extraction with a high yield can be easily achieved with toluene or tert-butyl methylether the resulting method accuracy was not satisfactory. Numerous attempts to overcome the problem by changing the extraction conditions (extraction solvent, ratio of plasma to solvent, pH of the extraction) and internal standard (verapamil, naftidrofuryl) were unsuccessful and a different approach to sample preparation had to be selected. Plasma precipitation alone could not be applied due to the resulting unsatisfactory limit of quantitation. However, the problem was overcome by evaporation of the supernatant, reconstitution of the residue in the mobile phase and injection of an aliquot. Usage of the internal standard is necessary, but the overall laboriousness is comparable with a classic liquid–liquid extraction method.

Hitherto published analytical methods for carvedilol determination in plasma used separation on reversed-phases with acidic mobile phases (pH 3–5.5) and fluorimetric detection. A low pH of the mobile phase is convenient especially in the case of back-extraction liquid–liquid procedure but the peak shape is improved by low pH, too. No deterioration of the column performance was observed although a pH 2 mobile phase buffer was used in the present study.

The method selectivity was demonstrated on six blank plasma samples obtained from healthy volunteers: the chromatograms were found to be free of interfering peaks. A typical chromatogram of a blank plasma is shown in Fig. 2. The chromatogram of a plasma sample collected 12 h following administration of 25 mg of carvedilol to a healthy subject is shown in Fig. 3. The measured concentration was 3.209 ng/ml.

3.2. Linearity and limit of quantitation

The calibration curves were linear in the studied range. The mean equation (curve coefficients±standard deviation) of the calibration curve ($N=6$) consisting of six points was $y=72.8\ (\pm 2.0)x-0.0037\ (\pm 0.0015)$ with correlation coefficient $r=0.9998$, where y represents the ratio of carvedilol peak height and the internal standard one and x represents the ratio of carvedilol concentration and that of the internal standard in ng/ml.

The limit of quantitation was 1.303 ng/ml. The

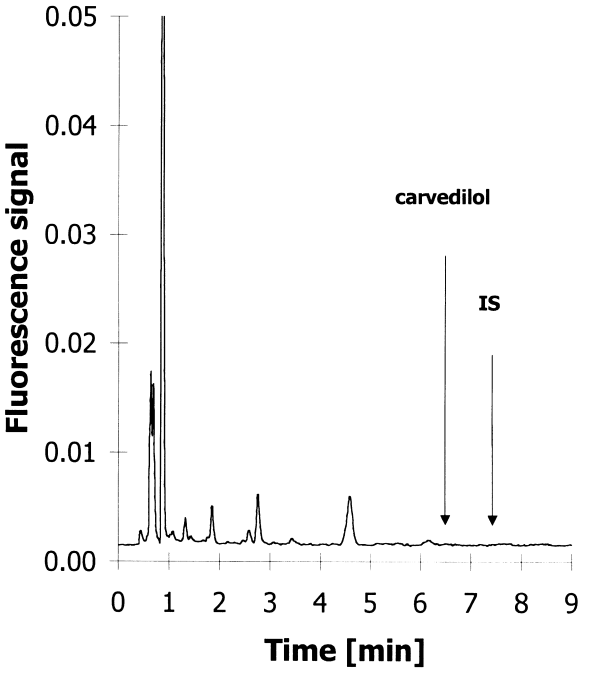

Fig. 2. Typical chromatogram of drug-free human plasma. The arrows indicate the retention time of carvedilol and internal standard.

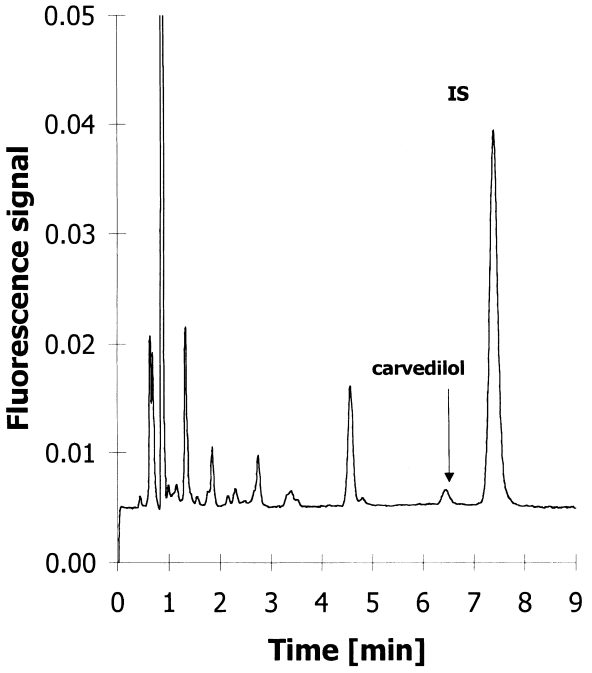

Fig. 3. Chromatogram of a plasma sample from a volunteer 12 h after administration of 25 mg of carvedilol. The respective concentration was 3.209 ng/ml.

precision, characterized by the relative standard deviation, was 5.0% and inaccuracy (bias), defined as the deviation between the true and the measured value expressed in percents, was 1.9% at this concentration ($N=6$). The limit of quantitation can be lowered by increasing the injection volume.

3.2.1. Intra-assay precision

Intra-assay precision of the method is illustrated in Table 1. Six sets of quality control samples (low, medium and high concentration) were analyzed with calibration samples in one batch. The precision was better than 3% and inaccuracy did not exceed 10% at all levels.

3.2.2. Inter-assay precision and accuracy

Inter-assay precision and accuracy was evaluated by processing a set of calibration and quality control samples (three levels analyzed twice, results averaged for statistical evaluation) in six separate batches. The samples were prepared in advance and stored at −18 °C. The respective data are given in Table 2. The precision was better than 6% and the inaccuracy did not exceed 3% at all levels.

3.2.3. Stability study

3.2.3.1. Freeze and thaw stability. Stock solutions of a low and high concentration sample were prepared. The solutions were stored at −18 °C and subjected to three thaw and freeze cycles. During each cycle triplicate 0.25 ml aliquots were processed, analyzed and the results averaged. The results are shown in Table 3. The concentrations found are within ±5% of nominal concentration, indicating no significant substance loss during repeated thawing and freezing.

3.2.3.2. Processed sample stability. Two sets of samples (2.54 ng/ml as a low concentration and 115.1 ng/ml as a high one of carvedilol) were analyzed on 1 day and left in the autosampler at ambient temperature (ca. 25 °C). The samples were analyzed using a freshly prepared calibration samples 5 days later. The results are presented in Table 4. The processed samples are stable at room temperature for at least 5 days.

3.2.3.3. Long term stability. Two sets of samples (low and high concentration of carvedilol) were stored in the freezer at −18 °C for 1 month. The

Table 1
Intra-assay precision and accuracy

N	Concentration (ng/ml)			
	Added	Measured	Bias (%)	RSD (%)
6	2.539	2.321	−9.4	2.3
6	13.86	13.36	−3.7	2.9
6	115.1	112.8	−2.1	2.4

N=number of samples.

Table 2
Inter-assay precision and accuracy

N	Concentration (ng/ml)			
	Added	Measured	Bias (%)	RSD (%)
6	2.539	2.604	2.6	5.9
6	13.86	13.69	−1.2	2.2
6	115.1	113.2	−1.6	1.7

N=number of days.

Table 3
Stability of the samples I

Sample C (ng/ml)	N	Cycle 1		Cycle 2		Cycle 3	
		Measured	Bias (%)	Measured	Bias (%)	Measured	Bias (%)
Freeze and thaw stability							
4.230	3	4.262	0.7	4.417	4.4	4.423	4.6
115.1	3	112.0	−2.7	117.2	1.8	114.9	−0.2

N=number of samples.

Table 4
Stability of the samples II

Sample	C (ng/ml)	N	Conc. found (ng/ml)	RSD (%)	Bias (%)
Processed sample stability					
New	2.540	6	2.321	2.6	−8.6
5 days old	2.540	6	2.548	5.1	0.3
New	115.1	6	112.8	2.4	−2.0
5 days old	115.1	6	115.3	2.5	0.2

N=number of samples.

samples were then analyzed using freshly prepared calibration samples. The results are presented in Table 5. The samples are stable at −18 °C for at least 1 month.

3.2.3.4. Plasma sample stability (room temperature). The stability of plasma spiked with carvediol at ambient temperature was tested for 24 h, too. No decrease of the analyte concentration was observed (see Table 5).

3.3. Application to biological samples

The proposed method was applied to the determination of carvedilol in plasma samples from the bioequivalence study. Plasma samples were periodically collected up to 30 h after oral administration of 25 mg dose to 26 healthy male volunteers. Fig. 4 shows the mean plasma concentration of carvedilol. The plasma level of carvedilol reached a maximum 1.0 h after the administration and thereafter the plasma level declined with an elimination half-time of ca. 7 h. These values agree with previously

Table 5
Stability of the samples III

C (ng/ml)	Storage conditions	N	Conc. found (ng/ml)	RSD (%)	Bias (%)
Plasma sample stability					
2.580	24 h/25 °C	3	2.426	6.3	−6.0
111.5	24 h/25 °C	3	107.9	0.5	−3.3
4.230	1 month/−18 °C	6	4.047	2.2	−4.3
115.1	1 month/−18 °C	6	112.2	2.5	−2.5

N=number of samples.

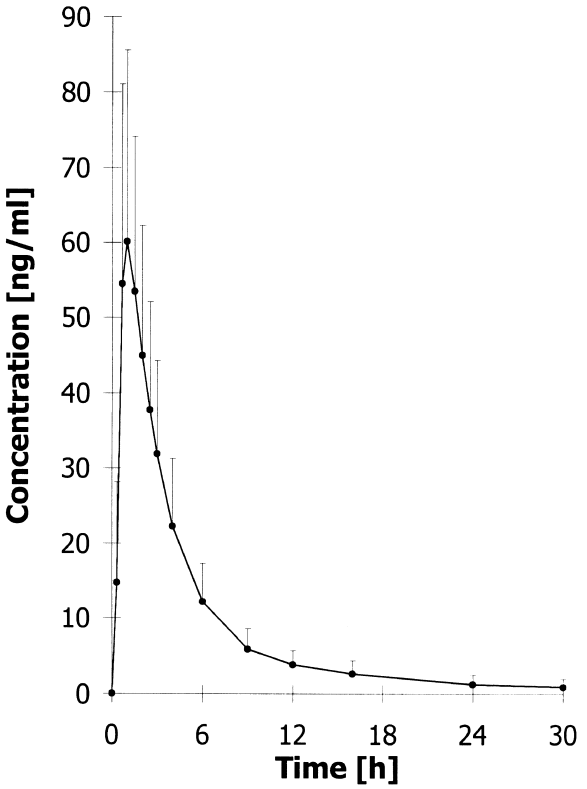

Fig. 4. Mean plasma concentrations (+SD) of carvedilol after 25 mg single oral dose (26 healthy volunteers).

published reports [1]. The AUC measured from 0 to the last sampling point was 93% of the value of AUC extrapolated from 0 to infinity which indicates a suitability of the analytical method for pharmacokinetic studies.

4. Conclusions

The validated method allows determination of carvedilol in the 1.303–142.2 ng/ml range using only 0.25 ml of plasma. The precision and inaccuracy is well within the limits for bioequivalence studies. The method was successfully applied for the pharmacokinetic study.

References

[1] T. Morgan, Clin. Pharmacokinet. 26 (1994) 335.
[2] F. Varin, L.X. Cubeddu, J.R. Powell, J. Pharm. Sci. 75 (1986) 1195.
[3] K. Reiff, J. Chromatogr. 413 (1987) 355.
[4] N. Hokama, N. Hobara, H. Kameya, S. Ohsiro, M. Sakanashi, J. Chromatogr. B 732 (1999) 233.
[5] M. Gergov, J.N. Robson, E. Duchoslav, I. Ojanpera, J. Mass. Spectrom. 35 (2000) 912.
[6] F. Behn, S. Laer, H. Scholz, J. Chromatogr. Sci. 39 (2001) 121.
[7] E.J. Eisenberg, A.M. Patterson, G.C. Kahn, J. Chromatogr. 493 (1989) 105.
[8] G. Neugebauer, W. Akpan, B. Kaufmann, K. Reiff, Eur. J. Clin. Pharmacol. 38 (Suppl. 2) (1990) S108.
[9] H. Spahn, W. Henke, P. Langguth, J. Schloos, E. Mutschler, Arch. Pharm. (Weinheim) 323 (1990) 465.