Determination of Carvedilol in Human Cardiac Tissue by High-Performance Liquid Chromatography

Frederike Behn, Stephanie Läer*, and **Hasso Scholz**
Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Abteilung für Pharmakologie, Universitätsklinikum Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany

Abstract

A new high-performance liquid chromatographic method has been developed for the determination of the β-receptor blocker carvedilol in human cardiac tissue. After homogenizing tissue samples in a microdismembrator, carvedilol and the internal standard naftopidil are extracted with acetone. The extract is evaporated to dryness and reconstituted in a potassium acetate buffer of pH 3.5. Samples are cleaned up with solid-phase extraction columns. Carvedilol and the internal standard show recoveries of 69.8 ± 12.2% and 63.9 ± 9.34%, respectively. The linearity range for carvedilol is 0.01–0.35 ng/mg (parts per billion) tissue (wet weight), and the limit of quantitation is 0.01 ng/mg. The percentage coefficient of variation of the intra-assay varies between 1.45 and 5.38% and the interassay between 4.25 and 6.96%. To use as an application of the assay, the cardiac carvedilol tissue level in a patient on oral carvedilol therapy for congestive heart failure is reported.

Introduction

Carvedilol (a β-receptor blocker) is used for the treatment of hypertension, ischemic heart disease, and congestive heart failure (1,2). Lipophilic β-blockers such as carvedilol are metabolized via cytochrome P450 (CYP) enzymes. The local metabolism of β-receptor blockers may reduce drug concentrations at the target tissue (i.e., the human heart). Because recent studies have shown that cytochrome P450 enzymes are predominantly expressed in the right ventricle (3), varying carvedilol concentrations in distinct regions of the human heart might in part explain differences in its response to carvedilol treatment (4). Presently, no data are available in regards to carvedilol concentrations in the human heart. Taking into account the increasing clinical importance and widespread use of carvedilol, we have developed a sensitive and quantitative high-performance liquid chromatographic (HPLC) method with high accuracy and precision to measure carvedilol (Figure 1A) concentrations in approximately 200 mg of human cardiac tissue samples. We employed an internal standard, naftopidil (Figure 1B), to control the extraction procedure and used solid-phase extraction (SPE) to clean up sample extracts before their analysis on the HPLC system.

* Author to whom correspondence should be addressed: e-mail laeer@uke.uni-hamburg.de.

Experimental

Chemicals and reagents

HPLC-grade acetonitrile was purchased from E. Merck (Darmstadt, Germany). Carvedilol was supplied by Boehringer (Mannheim, Germany) and naftopidil from Research Biochemicals International (Natick, MA). All other chemicals were of analytical reagent grade.

Apparatus

The HPLC system used was a liquid chromatograph (LC) Workstation Class LC10 (Shimadzu, Kyoto, Japan) and consisted of an SIL-10A autoinjector, an LC-10AT LC, an RF-10A spectrofluorometric detector, and software provided by the manufacturer.

Table I. Intra- and Interassay Validation of Carvedilol Concentrations in Human Cardiac Tissue

Amount of carvedilol added (ng/mg)	Carvedilol measured (mean ± SD)	Precision (CV, %)	Accuracy (%)
Intra-assay (*n* = 3)			
0.086	0.082 ± 0.001	1.45	95.0
0.022	0.022 ± 0.001	2.13	100
0.011	0.012 ± 0.001	5.38	108
Interassay (*n* = 3)			
0.086	0.081 ± 0.006	6.96	94.1
0.043	0.043 ± 0.003	6.48	99.1
0.022	0.024 ± 0.001	6.11	109
0.011	0.012 ± 0.001	4.25	113

Reproduction (photocopying) of editorial content of this journal is prohibited without publisher's permission.

Chromatographic conditions

Chromatographic separation was performed on a Spherisorb C6 column (150- × 4.6-mm i.d., 5-µm particle size) (Chromatographie Service, Langerwehe, Germany) with a Spherisorb C6 guard column (17- × 4-mm i.d., 5-µm particle size) at ambient temperature. The mobile phase consisted of 65% acetonitrile (v/v) and 35% (v/v) 0.25M potassium acetate buffer (pH 4). The excitation wavelength was set at 285 nm, and emission was measured at 360 nm. The flow rate was 1 mL/min. Aliquots of the extracts (100 µL) were injected into the HPLC system.

Standards

Stock solutions of 690-ng/mL carvedilol and 1160-ng/mL naftopidil were created in 50% aqueous methanol (v/v). Aliquots of these stock solutions were added to drug-free tissue for obtaining standard curves in the presence of appropriate amounts of the internal standard. Other stock solutions were stored at 4°C until the assay was performed. They remained stable for at least six months.

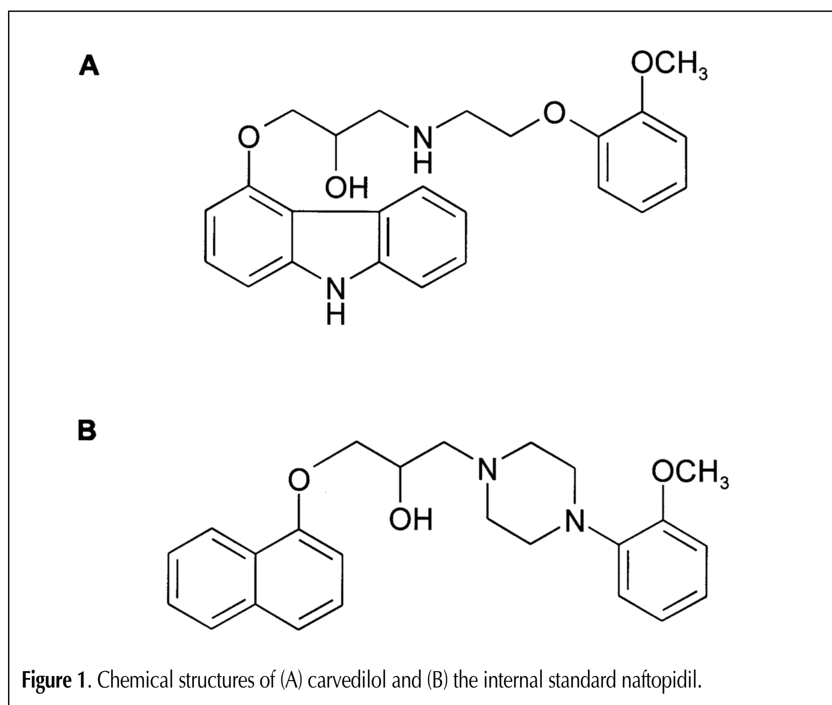

Figure 1. Chemical structures of (A) carvedilol and (B) the internal standard naftopidil.

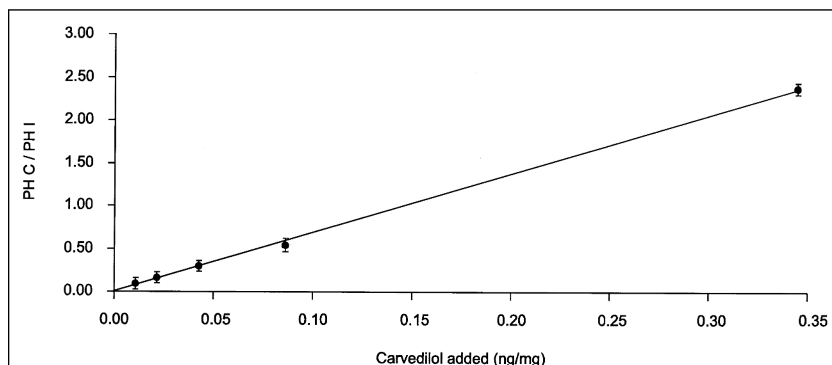

Figure 2. Peak height (PH) ratios of carvedilol (C) and the internal standard (I) at five fortified carvedilol tissue concentrations (0.01–0.35 ng/mg wet weight; means ± SD; n = 3 for each). The linear regression was calculated using the equation $y = 6.84x$ showing a correlation coefficient of $r = 0.99$.

Sample collection

Cardiac ventricle samples were received from explanted human hearts subsequent to orthotopic heart transplantation. The samples were kept at –80°C until analyzed. This procedure has been approved by the local ethics' committee (AZ 532/116/9.7.1991; No. 1228/1997).

Tissue extraction procedure

Approximately 200 mg of frozen tissue was placed into Teflon tubes that were precooled by liquid nitrogen. To each sample, 100 µL of the naftopidil stock solution (1160 ng/mL), 250 µL of double distilled water, and 50 µL of methanol were added. Simultaneously, carvedilol-free cardiac tissue was fortified with 100 µL of a known carvedilol standard (690 ng/mL), 100 µL of the naftopidil stock solution (1160 ng/mL), and 200 µL of double distilled water. Frozen samples were homogenized in a microdismembrator (Braun, Melsungen, Germany) and transferred into Eppendorf micro test tubes. The Teflon tube was rinsed two times—first with 500 µL of double distilled water and then with 500 µL of acetone. The rinsing solutions were added to the test tubes containing the homogenized tissue. The samples were vortexed for 2 min prior to centrifugation at 4°C and 4000 rpm for 20 min. The supernatant was removed and evaporated to dryness under a stream of nitrogen at 40°C. The residues were reconstituted in 1 mL of a 0.50M potassium acetate buffer (pH 3.5) and centrifuged again. The supernatant was then percolated drop-wise through Bond-Elut cartridges packed with 40-µm cyano-bound silica particles (100 mg/mL column volume) (Analytichem International, Harbor City, CA) under vacuum using a Vac-Elut vacuum manifold (Chromatographie Service) and a vacuum pump (KNF Neuberger, Freiburg, Germany). The SPE columns were washed twice with 1 mL of double distilled water. Finally, carvedilol and naftopidil were eluted with two 250-µL aliquots of 80% acetonitrile (v/v) and 20% (v/v) 0.1M potassium acetate buffer (pH 5). A 100-µL aliquot was injected onto the HPLC column.

Recovery rates

The recovery rates were determined by comparing the absolute peak heights of carvedilol and the internal standard naftopidil, respectively, of the analyzed fortified tissue samples (0.01–0.35 ng/mg wet weight of carvedilol) with the absolute peak heights obtained by the direct injection of the corresponding standard solutions onto the HPLC column. Peak heights were measured by the computer system.

Quantitation

Carvedilol was quantitated by relating the peak height ratio of carvedilol and the internal standard naftopidil in the unknown sample to the peak height ratio of a known standard concentration.

Data analysis and statistics

Data are given as the arithmetic means ± standard deviations (SD). For intra-assay and interassay variation, replicate samples at different carvedilol concentrations of 0.01–0.09 ng/mg wet weight were analyzed. The precision was calculated as the percentage coefficient of variation (CV). The accuracy was expressed as the percentage of the carvedilol concentration that was measured in each sample relative to the known amount of carvedilol added.

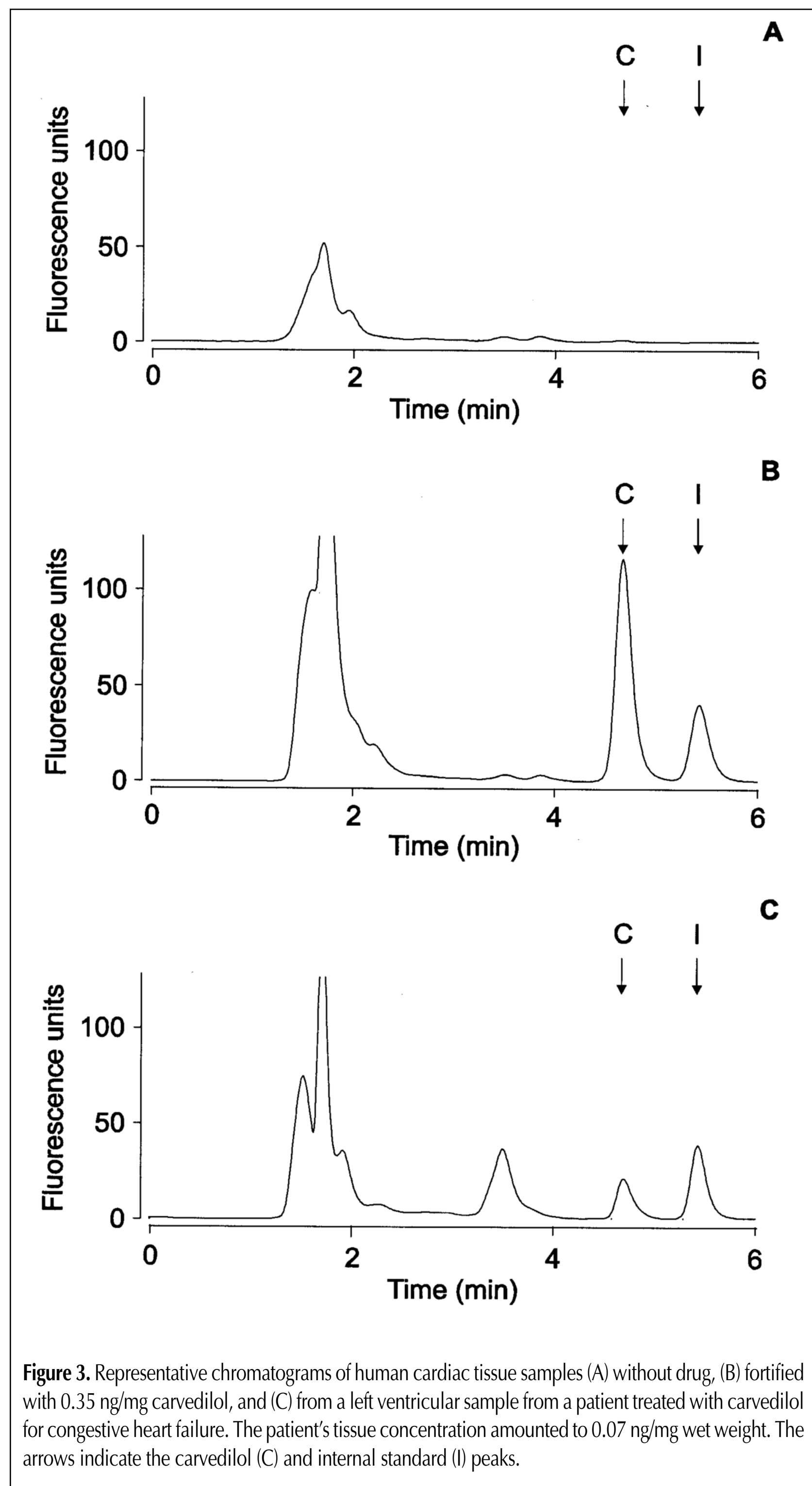

Figure 3. Representative chromatograms of human cardiac tissue samples (A) without drug, (B) fortified with 0.35 ng/mg carvedilol, and (C) from a left ventricular sample from a patient treated with carvedilol for congestive heart failure. The patient's tissue concentration amounted to 0.07 ng/mg wet weight. The arrows indicate the carvedilol (C) and internal standard (I) peaks.

Results and Discussion

The determination of carvedilol in human cardiac tissue showed a linear relation between the concentrations and peak height ratios over the whole range tested (0.01–0.35 ng/mg wet weight, Figure 2). The lower limit of quantitation for carvedilol was 0.01 ng/mg wet weight (signal-to-noise ratio of 10). The recovery rates of carvedilol that were determined at the limit of quantitation were 82.2 ± 9.11%, and those of the internal standard in these samples were 71.3 ± 11.9%. The recovery rates at the carvedilol concentrations of 0.02 and 0.35 ng/mg wet weight were 59.2 ± 9.57% and 60.1 ± 3.88% for carvedilol, respectively, and 56.3 ± 9.36% and 58.2 ± 4.52% for the internal standard, respectively (n = 3 for each, all samples were analyzed in one sequence). Using the internal standard method, the variability in the recoveries of the samples with different carvedilol concentrations was balanced by calculating the ratios between the peak heights of carvedilol and the internal standard.

No interferences were found from substances in the control tissue at the retention times of carvedilol (4.7 min) and the internal standard (5.4 min) (Figure 3A). The precision of the measurement was between 1.45 and 6.96%, and the accuracy was between 94.1 and 113% (Table I). A representative chromatogram of the fortified cardiac tissue is shown in Figure 3B. Figure 3C depicts a typical chromatogram showing the incurred residuals of carvedilol from the ventricular preparation of a patient medicated with carvedilol for congestive heart failure. The value for the patient's carvedilol tissue concentration amounted to 0.07 ng/mg tissue wet weight and was in the linear range of the carvedilol tissue assay.

The HPLC method presented here provides a sensitive and quantitative analysis of human cardiac tissue samples for the β-receptor blocker carvedilol. Previously, no data concerning carvedilol extraction in any human tissue were available. A method used to determine carvedilol concentrations in rat tissue had been developed using a complicated organic solvent extraction (5). We simplified this method by using a solid-phase column extraction and adapted it for human cardiac tissue. For the homogenization of the tissue, potassium dihydrogen phosphate buffers and sodium hydrogen carbonate buffers at pH 4.1 to 9.0 were tested, but optimal extraction conditions were achieved at a neutral pH with double distilled water. Digestion with trypsin was tested, but this did not improve the recovery of carvedilol. Diisopropylether, methanol, ethyl acetate, and acetone were compared for use as lipophilic solvents. Acetone was found to be the most efficient solvent for the extraction of

carvedilol from the homogenized tissue. In order to clean up the samples and minimize extraneous interferences, cyano-packed Bond-Elut SPE columns were used. This procedure allowed for the measurements of carvedilol in human cardiac tissue samples as small as 200 mg (Figure 3C). Interferences from typically co-medicated drugs and metabolites were not detected. This should be checked systematically during the analysis procedure.

With this method, several questions in future work can be addressed by the determination of carvedilol concentrations in the human heart. No data are available as to whether the carvedilol accumulation in human cardiac tissue is different in patients either undergoing short-term or long-term treatment. The influence of a regionally different expression of cytochrome P450 (3) on carvedilol tissue concentrations in distinct regions of the human heart can be determined. Furthermore, polymorphically expressed cytochrome P450 enzymes (such as CYP 2D6 or 2C9) that are potentially involved in carvedilol metabolism (6) might lead even more to interindividually different carvedilol tissue concentrations.

Conclusion

In summary, the HPLC method reported in this study offers a technique to quantitate carvedilol concentrations in human cardiac tissues with good accuracy and precision. This method could possibly be extended to the quantitation of other β-receptor blockers with related molecular structures.

Acknowledgments

We greatly appreciate the help in establishing the HPLC analysis by Dr. Rudolph Thieme. This project was supported by the German Heart Foundation.

References

1. M. Packer, M.R. Bristow, J.N. Cohn, W.S. Colucci, M.B. Fowler, E.M. Gilbert, and N.H. Shusterman. The effect of carvedilol on morbidity and mortality in patients with chronic heart failure. *N. Engl. J. Med.* **334:** 1349–55 (1996).
2. W.H. Frishman. Carvedilol. Drug therapy. *N. Engl. J. Med.* **339:** 1759–65 (1998).
3. T. Thum and J. Borlak. Gene expression in distinct regions of the heart. *Lancet* **355:** 979–83 (2000).
4. M. Fujimura, Y. Yasumura, Y. Ishida, S. Nakatani, K. Komamura, M. Yamagishi, and K. Miyatake. Improvement in left ventricular function in response to carvedilol is accompanied by attenuation of neurohumoral activation in patients with dilated cardiomyopathy. *J. Card. Fail.* **6:** 3–10 (2000).
5. M. Fujimaki. Stereoselective disposition and tissue distribution of carvedilol enantiomers in rats. *Chirality* **4:** 148–54 (1992).
6. H.G. Oldham and S. Clarke. In vitro identification of the human cytochrome P450 enzymes involved in the metabolism of R(+)- and S(–)-carvedilol. *Drug Metab. Dispos.* **25:** 970–77 (1997).

Manuscript accepted November 29, 2000.