Quantitation of Diltiazem in Human Cardiac Tissue Using High-Performance Liquid Chromatography

Stephanie Läer*, Hasso Scholz, and Phaslia Uebeler
Abteilung Allgemeine Pharmakologie, Universitäts-Krankenhaus Eppendorf, Martinistrasse 52, D-20246 Hamburg, Germany

Joachim Neumann
Institut für Pharmakologie und Toxikologie, Westfälische Wilhelms-Universität Münster, Domagkstrasse 12, D-48149 Münster, Germany

Norbert Zimmermann
Abteilung für Thorax- und Herzchirurgie der Chirurgischen Universitätsklinik, Heinrich Heine-Universität, Moorenstrasse 5, D-40225 Düsseldorf, Germany

Abstract

A new high-performance liquid chromatographic method has been developed for the determination of diltiazem in human cardiac tissue. Tissue samples are homogenized and digested with trypsin solution. Diltiazem and the internal standard are extracted with acetone. The extract is evaporated to dryness and reconstituted in potassium phosphate buffer. Samples are then cleaned up with solid-phase extraction columns. Diltiazem and the internal standard show recoveries of 59% ± 16 and 52% ± 13. The linearity range is 0.12–2.25 ng/mg wet weight. The limit of quantitation is 0.12 ng/mg (w/w). The percentage coefficient of variation of intra-assay varies between 3.57 and 11.2, and that of interassay varies between 5.42 and 11.7. As an application of the assay, a diltiazem cardiac tissue level in a patient on oral therapy for supraventricular tachycardia is reported.

Introduction

Diltiazem, a calcium channel blocker, is used in chronical atrial fibrillation (1). In addition, it is used for the treatment of coronary ischemia (e.g., angina pectoris) and to prevent arrhythmias after cardiac surgery (2). However, inadequate response to diltiazem treatment is a problem, even if patients receive their recommended dosage (3).

Hence, plasma levels might be a poor predictor of clinical effects, and a knowledge of diltiazem tissue concentrations might in part explain a lack of response and toxic side effects (4,5). Furthermore, one could assume that a strong correlation exists between the therapeutic effect of diltiazem and its concentrations at its site of action, namely the human heart, but no data are available about its concentrations in this organ. Given the clinical importance and widespread use of diltiazem, we developed a sensitive and quantitative high-performance liquid chromatographic (HPLC) method with high accuracy and precision to measure diltiazem (Figure 1A) concentrations in about 200 mg of human cardiac tissue samples. We employed an internal standard, imipramine (Figure 1B), to control the extraction procedure and used solid-phase column extraction to clean up sample extracts before running on the analytical HPLC system.

* Author to whom correspondence should be addressed.

Experimental

Chemicals and reagents

HPLC-grade acetonitrile, methanol, and trypsin were purchased from E. Merck (Darmstadt, Germany). Diltiazem–HCl (diltiazem) was supplied by Gödecke (Berlin, Germany), and imipramine was supplied by Sigma (Deisenhofen, Germany). All other chemicals were analytical reagent grade.

Apparatus

An HPLC system of a LC Workstation Class LC10 (Shimadzu, Kyoto, Japan) consisting of a SIL-10A auto injector, an LC-10AT liquid chromatograph, an SPD-10A ultraviolet–visible (UV–vis) detector, and software provided by the manufacturer was used.

Chromatographic conditions

Chromatographic separation was performed on a Spherisorb C6 column (150 × 4.6-mm i.d., 5-µm film thickness) (Chromatographie Service, Langerwehe, Germany) with a Spherisorb C6 guard column (17 × 4-mm i.d., 5-µm film thickness) at ambient temperature. The mobile phase consisted of 60% (v/v) eluent acetonitrile and 40% (v/v) 0.25M potassium acetate buffer (pH 4). The UV wavelength was set at 237 nm for detection of diltiazem. The flow rate was 1 mL/min. Aliquots of the extracts (80 µL) were injected into the HPLC system.

Standards

Stock solutions of 1.26 µg/mL for diltiazem and 25 µg/mL imipramine were made up in water. Aliquots of these stock solutions were added to drug-free tissue for obtaining standard curves (range indicated in Table I) in the presence of appropriate amounts of internal standard. Other stock standard solutions were divided into aliquots and stored at –80°C until assay. They remained stable for at least six months.

Sample collection

Cardiac atria samples were obtained from patients undergoing coronary bypass surgery. Intraoperatively, right atrial tissue samples of about 200 mg were frozen immediately in liquid nitrogen and kept at –80°C until further analysis. Ventricle samples were received from explanted human hearts subsequent to orthotopic heart transplantation.

Tissue extraction procedure

About 200 mg of frozen tissue was placed into teflon tubes precooled by liquid nitrogen. To each sample, 100 µL of imipramine stock solution (25 µg/mL) and 500 µL of 0.5M potassium acetate buffer (pH 7) were added. Simultaneously, diltiazem-free cardiac tissue was fortified with 100 µL of a known diltiazem standard (1.26 µg/mL). Frozen samples were homogenized in a microdismembrator (Braun, Melsungen, Germany), transferred into glass tubes containing a 1 mL trypsin solution, and incubated at 37°C for 18 h. The sample was extracted with 900 µL acetone. After centrifugation at 2740*g* for 20 min at 20°C, the supernatant was evaporated to dryness under a stream of nitrogen at 40°C. The residues were reconstituted in 1 mL of 0.5M potassium phosphate buffer (pH 5.5) and centrifuged again. The supernatant was percolated slowly through Bond-Elut packed with CN-bound silica particles of 40 µm (100 mg/mL of column volume) (Analytichem International, Harbor City, CA) under vacuum (Vac-Elut vacuum manifold) (Chromatographie Service). Columns were washed once with 1 mL water. Finally, elution of the compounds was performed with 250 µL of 80% (v/v) acetonitrile and 20% (v/v) of 0.1M potassium acetate buffer (pH 5). An 80-µL aliquot was injected into the HPLC column.

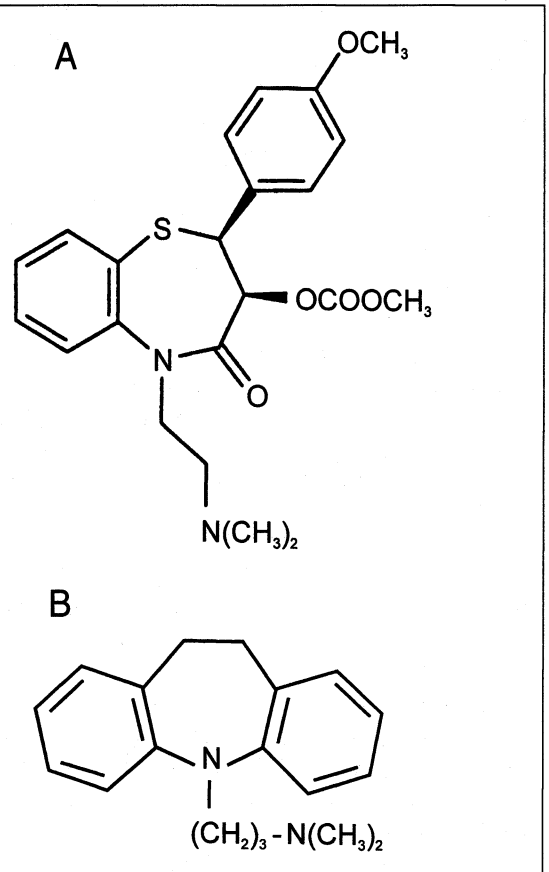

Figure 1. Chemical structures of diltiazem (A) and imipramine (B).

Recovery rates

The recovery rates were determined by comparing the peak heights of diltiazem and the internal standard. Therefore, peak heights of the analyzed fortified tissue samples were compared with the peak heights obtained by direct injection of the standard solution into the HPLC column. This procedure was repeated at different drug concentrations. Peak heights were measured by the computer system (see Table I).

Quantitation

Diltiazem was quantified by relating the peak height ratio of diltiazem and the internal standard imipramine in the unknown sample to the peak height ratio of a known standard concentration.

Data analysis and statistics

Data were given as arithmetic means plus or minus standard deviation (SD). Precision was calculated as percent coefficient of variation (CV). Accuracy was expressed as percent of diltiazem measured in each sample relative to the known amount of diltiazem added.

Results and Discussion

For the fortified tissue samples, there was a linear relationship between concentration and peak height over the total range tested (0.30–2.25 ng/mg w/w). The lower limit of quantitation for diltiazem was 0.3 ng/mg (w/w) (signal-to-noise ratio of 10). The recovery rates for 12 determinations obtained with four different drug concentrations (see Table I) were 59% ± 16 for diltiazem and 52% ± 13 for the internal standard. No interferences were found from substances in the control tissue at the retention times of diltiazem (4.7 min) and the internal standard (6.8 min) (Figure 2A). Representative chromatograms of fortified cardiac tissue in Figures 2B–2F demonstrate good separation of different amounts of diltiazem. Figure 3 depicts a typical chromatogram of an atrial preparation from a patient medicated with 60 mg of diltiazem. The value for the patient's diltiazem tissue concentration amounted to 0.53 ng/mg wet weight and

Table I. Recovery of Diltiazem and the Internal Standard from Cardiac Tissue

Linearity range (ng/mg)	Recovery of diltiazem Mean ± SD (%) (*n* = 3)	CV (%)	Recovery of IS Mean ± SD (%) (*n* = 3)	CV (%)
0.12	65.7 ± 4.0	6.1	55.0 ± 4.2	7.6
0.63	42.3 ± 3.3	7.8	39.7 ± 2.2	5.5
1.13	52.3 ± 2.3	4.4	44.0 ± 5.5	12.6
2.25	86.0 ± 4.2	4.8	78.3 ± 5.8	7.4

was in the linear range of the diltiazem tissue assay. The precision of the assay was assessed by replicate analysis of fortified cardiac tissue containing diltiazem and the internal standard at various concentrations (0.30–2.25 ng/mg, w/w). Intra-assay variation was determined by analyzing four diltiazem concentrations and three replicate samples at concentrations of 0.30–2.25 ng/mg (w/w). The intra- and inter-assay coefficients of variation are presented in Table II. The precision of the measurement, expressed as the coefficients of variation, was between 3 and 12%. The accuracy, expressed as a percent of the concentration of diltiazem measured in each sample relative to the known amount of added diltiazem, was between 91.5 and 108%.

The HPLC method presented here provides a sensitive and quantitative analysis of human cardiac tissue samples for the antiarrhythmic drug diltiazem. Previously, no data concerning diltiazem extraction in any human tissue were available. Several methods have been established to quantitate diltiazem in plasma samples (6). By adopting the published plasma extraction procedure in tissue samples, recovery of diltiazem was only 18%. Extraction of diltiazem from tissue is therefore a crucial point. In order to optimize the extraction procedure and in increase the recovery from tissues, various solvents were tested. The pH values of the extraction buffer were varied from 3.5 to 7.5. Optimal extraction conditions were achieved at a pH of 7.0. In addition, lipophilic solvents like diethylether, chloroform, dichloromethane, and acetone were compared to optimize diltiazem extraction. Acetone was found to be the most efficient for the extraction of diltiazem from tissue. Back extraction into 0.5M potassium acetate buffer was tested at pH values of 2, 3.5, 4.5, 5.5, and 7. The highest diltiazem recovery (59%) was achieved at a pH of 5.5. The recovery of the internal standard without pretreatment with trypsin was significantly lower (30% versus 52%) than that of diltiazem with trypsin pretreatment. The best recoveries of both diltiazem and the internal standard were achieved by digesting the tissue sample in a trypsin solution for 18 h before extraction with acetone. This led to recoveries of 59% ± 16 and 52% ± 13 for diltiazem and the internal standard, respectively, over the range of four different concentrations in three replicate experiments. The coefficients of variation varied from 4.4 to 7.8% for diltiazem and from 5.5 to 12.6% for the internal standard (Table I). Precision was between 3.6 and 11.7%, and the linear range was from 0.12 ng/mg to 2.25 ng/mg (w/w). This procedure allowed measurements of diltiazem in human cardiac tissue samples as small as 200 mg (see Figure 3). Precision and measurement accuracy were determined by the addition of an internal standard. Furthermore, we used solid-phase column extraction to clean up sample extracts to minimize extraneous interferences (Figures 2 and 3) and guaranteed reproducibility of the assay (Table II). Interference from typically comedicated drugs and metabolites was not detected but should be checked systematically during the analysis procedure.

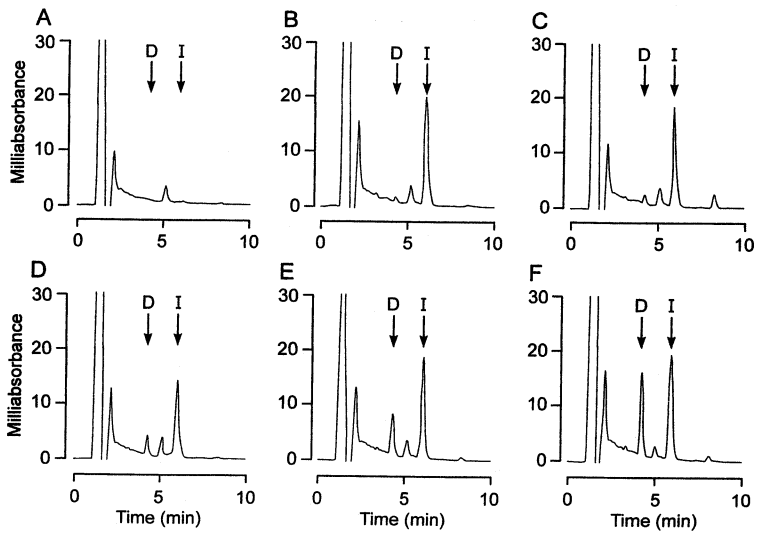

Figure 2. Representative chromatograms of human cardiac tissue samples without drug (A) and with varying amounts of diltiazem fortified human cardiac tissue. (B) 0.30 ng/mg, (C) 0.47 ng/mg, (D) 0.63 ng/mg, (E) 1.13 ng/mg, and (F) 2.25 ng/mg diltiazem. The arrows in Figure 2A indicate the anticipated elution times of diltiazem (D) and the internal standard (I). The arrows in B–F indicate the diltiazem and internal standard peaks.

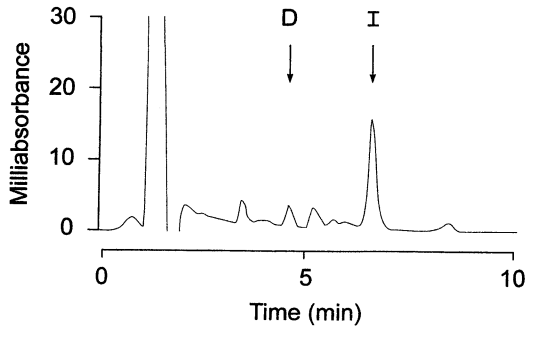

Figure 3. Representative chromatogram of a right atrial sample from a patient treated with 60 mg peroral diltiazem. Tissue concentration amounted to 0.53 ng/mg (w/w). The arrows indicate the diltiazem (D) and the internal standard (I) peaks.

With this method, it will be possible in future work to correlate diltiazem plasma and tissue concentrations from patients who are under antiarrhythmic therapy. We have previously published a method for quantitation of sotalol in human myocardium (7), but unfortunately, that method could not be used for quantitation of diltiazem.

It is unknown whether diltiazem accumulation differs in failing myocardial tissue with various etiologies of cardiovascular diseases (e.g., dilative cardiomyopathy, hypertrophic cardiomyopathy, or coronary heart disease). Furthermore, no data are available concerning whether human cardiac tissue concentrations of diltiazem differ in patients undergoing short- and long-term treatment. As another application of the method, determination of diltiazem concentrations in human cardiac tissue is necessary for validation of in vitro experiments. For

Table II. Precision and Accuracy for Determination of Diltiazem in Human Cardiac Tissue

Amount of diltiazem added (ng/mg tissue)	Diltiazem measured (mean ± SD)	Precision (CV [%])	Accuracy (%)
	Intra-assay (n = 3)		
0.30	0.27 ± 0.03	9.26	91.5
0.63	0.68 ± 0.05	7.35	108
1.13	1.21 ± 0.14	11.2	107
2.25	2.24 ± 0.08	3.57	99.6
	Interassay (n = 3)		
0.30	0.27 ± 0.03	11.7	92.4
0.47	0.47 ± 0.04	8.51	100
0.63	0.61 ± 0.04	6.66	96.3
1.13	1.18 ± 0.06	5.42	104

instance, it is a concern that calcium antagonist concentrations in human cardiac tissue might impair the interpretation of subsequent contraction experiments or other biochemical studies (e.g., radioligand binding studies) (8).

Conclusion

In summary, the HPLC method reported here offers a technique to quantitate diltiazem concentrations in human cardiac tissues with good accuracy and precision. This method might be extended to the quantitation of other calcium antagonists with related molecular structures.

Acknowledgments

Thanks are due to Jochen Rüdiger for his help with the graphics. We greatly appreciate the help of Dr. Rudolph Thieme in establishing the HPLC analysis. We also thank Professor Dr. Peter Kalmár from the Abteilung für Thorax-, Herz-, und Gefässchirurgie, Universitäts-Krankenhaus Eppendorf for providing human tissue.

References

1. T. Lundström and L. Ryden. Ventricular rate control and exercise performance in chronic atrial fibrillation: Effects of diltiazem and verapamil. *J. Am. Coll. Cardiol.* **16:** 86–90 (1990).
2. M. El-Sadek and E. Krause. Postoperative antiarrhythmic effects of diltiazem in patients undergoing coronary bypass grafting. *Cardiology* **85:** 290–97 (1994).
3. F. Chung, P.L. Houston, and D.C.H. Cheng. Calcium channel blockage does not offer adequate protection from perioperative myocardial ischemia. *Anaesthesiology* **69:** 343–47 (1988).
4. J.J. Lehot, P.G. Durand, R. Boulieu, J.L. Bonnefous, C. Flamens, C. Hercule, B. du Grés, J. Villard, S. Ferry, and S. Estanove. Plasma concentrations of diltiazem and its active metabolites in coronary artery surgery: Relationship with preoperative treatment. *Ann Fr. Anaest. Réanim.* **12:** 452–56 (1993).
5. R.P. Mason, D.M. Moisey, and L. Shajenko. Cholesterol alters the binding of Calcium channel blockers to the membrane lipid bilayer. *Mol. Pharmacol.* **41:** 315–321 (1992).
6. R. Boulieu, J.L. Bonnefous, and S. Ferry. Solid phase extraction of diltiazem and its metabolites from plasma prior high performance liquid chromatography. *J. Chromatogr.* **528:** 542–46 (1990).
7. S. Läer, J. Neumann, H. Scholz, P. Übeler, and N. Zimmermann. Determination of sotalol in human cardiac tissue by high performance liquid chromatography. *J. Chromatogr.* **681:** 29–98 (1996).
8. K. Miwa, Y. Miyagi, E. Araie, and S. Sasayama. Effects of diltiazem and verapamil on (+) -PN 200-110 binding kinetics in dog cardiac membranes. *Eur. J. Pharmacol.* **214:** 127–32 (1992).

Manuscript accepted October 21, 1996.