Journal of Chromatography, 490 (1989) 365-375
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4688

DETERMINATION OF DILTIAZEM IN HUMAN WHOLE BLOOD AND PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING A POLYMERIC REVERSED-PHASE COLUMN AND UTILIZING A SALTING-OUT EXTRACTION PROCEDURE

ABU M. RUSTUM[a]

Department of Metabolism and Environmental Fate Chemistry, Hazleton Laboratories America, Inc , 3301 Kinsman Boulevard, Madison, WI 53704 (U.S.A.)

(First received July 15th, 1988; revised manuscript received January 13th, 1989)

SUMMARY

A rapid, simple and sensitive reversed-phase high-performance liquid chromatography method for the separation and measurement of the concentration of diltiazem in human whole blood and plasma has been developed. The method involves a simple one-step solvent extraction of the drug from biological samples followed by salting-out the organic solvent using ammonium sulfate. A 15 cm × 4.1 mm, PRP-1, pH-stable (pH 1–13) polymeric reversed-phase column was used with an isocratic elution of acetonitrile–0.01 *M* tetrabutylammonium hydroxide (60:40, v/v). Diltiazem was monitored at 254 nm and 0.50–0.005 a.u.f.s. The completion time for assay was less than 15 min, and the limit of detection was 10 ng/ml at a signal-to-noise ratio of 3 for a 50-μl injection volume. Using this method, the pharmacokinetic parameters (elimination rate constant, elimination half-life and area under the curve) were calculated from a whole blood concentration versus time profile of diltiazem.

INTRODUCTION

Diltiazem hydrochloride, *cis*-(+)-3-acetoxy-5-(2-dimethylaminoethyl)-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5*H*)-one hydrochloride (Fig. 1), is a relatively new calcium channel blocker extensively used in the treatment of clinical manifestations of variant angina [1–5]. Diltiazem is also

[a]Present address. Analytical Research and Development, Pharmaceutical Product Development, Department No. 417, Abbott Laboratories, 14th Sheridan Road, North Chicago, IL 60064, U.S.A.

0378-4347/89/$03.50 © 1989 Elsevier Science Publishers B.V.

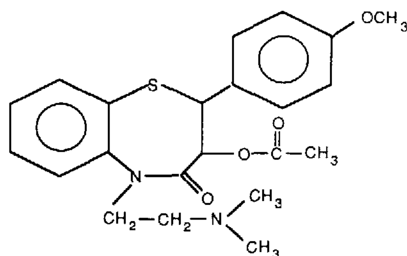


Fig. 1. Chemical structure of diltiazem.

effective in the treatment of hypertension and supraventricular arrhythmias [6]. Diltiazem undergoes presystemic metabolism after an oral dose, which results in only 40% bioavailability [6]. The systemic clearance of the drug is obtained via hepatic metabolism [7]. At least five metabolites of diltiazem have been identified in humans [8] with deacetyldiltiazem reported to be the major metabolite in humans. Recently, it has been shown that the N-monodemethyldiltiazem accumulates in plasma after chronic oral administration of diltiazem to humans [9]. It has also been found that N-monodemethyldiltiazem has measurable pharmacological activity as a coronary vasodilator in anesthetized dogs [10].

The adverse side-effects of diltiazem in humans are relatively minor [11]. However, administration of diltiazem with other commonly prescribed drugs could be extremely dangerous, as has been reported in some situations [12]. The concentration of diltiazem in plasma varies widely from patient to patient. Therefore, monitoring plasma or whole blood diltiazem concentrations in patients who are concurrently taking other prescribed drugs is necessary to obtain optimum therapy with minimum adverse side-effects.

Several methods have been used to determine diltiazem in biological samples. Gas chromatography (GC) using nitrogen-sensitive and electron-capture detection has been used to achieve adequate sensitivity and selectivity for conducting pharmacokinetic studies [13,14]. However, these methods require time-consuming extraction procedures and silylation of the metabolites for detection. Thin-layer chromatography (TLC) has been used to analyze the drug in human biological samples [2]. High-performance liquid chromatography (HPLC) has also been used to determine diltiazem in human biological samples [10,15–20]. The sample preparation of most of the methods is time-consuming, complex, or both. The reproducibility and sensitivity of most of the assays may also not be sufficient for application to single- or multiple-dose pharmacokinetic studies in humans.

The method described here uses a simple and rapid extraction procedure. The evaporation of the extracting organic solvent and derivatization step have been eliminated from the sample preparation procedure. The sensitivity, reproducibility, and selectivity of this method are adequate to monitor diltiazem

concentrations in human plasma or whole blood down to 20 ng/ml. Whole blood and plasma concentrations of the drug were determined for the same samples using this method to determine whether or not the concentrations of diltiazem in whole blood and plasma are similar. Finally, the pharmacokinetics of the drug in healthy subject were followed after a single 60-mg oral dose. Pharmacokinetic parameters such as elimination rate constant (k_{el}), elimination half-life ($t_{1/2}$), time required to reach maximum concentration (t_{max}), maximum concentration of diltiazem in whole blood (C_{max}), and area under the curve (AUC) were calculated from the whole blood time–concentration profile of diltiazem.

EXPERIMENTAL

Equipment

The chromatographic system consisted of a Perkin-Elmer Series Bio-410 solvent-delivery pump (Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with a 100-μl loop Rheodyne 7285 sample injector (Rheodyne, Cotati, CA, U.S.A.). A polymeric reversed-phase 150 mm$\times$4.1 mm column with 10-μm PRP-1 particles was used (Hamilton, Reno, NV, U.S.A.). The ultraviolet (UV)–visible variable-wavelength detector used was a Kratos Spectroflow 757 that was equipped with a flow cell of 8.0 mm path length (ABI Analytical, Kratos Division, Ramsey, NJ, U.S.A.). The UV–visible chromatograms were recorded on a Houston Instrument D5000 strip chart recorder (Houston Instrument, Austin, TX, U.S.A.). A Gilson P-1000 digital pipette was used for all quantitative sampling (Gilson International, Middleton, WI, U.S.A.). A Model 2200 Branson sonicator was used to degas the solvents and mobile phase (Branson Cleaning Equipment, Shelton, CT, U.S.A.). Deionized water was obtained from a Milli-Q® system (Millipore, Milford, MA, U.S.A.). The samples were centrifuged by an IEC centrifuge, Model HN (Damon, IEC Division, Needham Heights, MA, U.S.A.). An analytical balance (Mettler Instrument, Highstown, NJ, U.S.A.), Model AE100, was used for weighing reagents. A vortex mixer (Scientific Industries, Bohemia, NY, U.S.A.) was used. A C-130 Upchurch guard column (Upchurch Scientific, Oak Harbor, WA, U.S.A.) dry packed with 10-μm PRP-1 particles was used throughout the experiment. All other columns that were tested in this experiment were purchased from Alltech Assoc. (Deerfield, IL, U.S.A.). The 10-μm PRP-1 stationary phase for the guard column was purchased from Hamilton.

Materials

Diltiazem was purchased from Sigma (St. Louis, MO, U.S.A.) and was used as received. Methanol and acetonitrile (HPLC grade) were obtained from EM Science (Cherry Hill, NJ, U.S.A.). Dibasic potassium phosphate was obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Spectrograde acetone, isopro-

panol, ethanol, and tetrabutylammonium hydroxide were purchased from Aldrich (Milwaukee, WI, U.S.A.).

The borosilicate glass culture tubes and the disposable glass pipettes were obtained from Curtin Matheson Scientific (Elk Grove, IL, U.S.A.). The aged, pooled plasma and whole blood were obtained from the blood center of southeastern Wisconsin (Milwaukee, WI, U.S.A.).

Chromatographic conditions

The mobile phase of the chromatographic system consisted of 40% acetonitrile in 0.01 *M* dibasic potassium phosphate and 5.0 m*M* tetrabutylammonium hydroxide dissolved in ultrapure deionized water. The nominal pH of the mobile phase mixture was 10.5. The mobile phase was delivered at a flow-rate of 1.0 ml/min. Diltiazem was monitored by a UV–visible absorbance detector at a wavelength of 254 nm and 1.0–0.005 absorbance units full scale (a.u.f.s.).

Preparation of stock solutions

A stock solution of diltiazem was prepared by dissolving 50 mg of diltiazem in 50.0 ml of acetonitrile–water (2:1, v/v). Dilution of this solution was made in order to prepare the plasma, whole blood, and water standards of diltiazem needed to construct the calibration curves. The retention time of diltiazem was determined by injecting an aliquot of the standard solution into the HPLC system.

Cleaning of borosilicate culture tubes

Borosilicate culture tubes were placed in a 2.0-l Pyrex® beaker, and a mixture of spectrograde acetone and acetic acid (1%, v/v) was added. The beaker with the borosilicate tubes and solvent mixture was sonicated on an ultrasonic bath for at least 30 min. The cleaning solvent was decanted and replaced by Milli-Q-filtered water and the above procedure was repeated twice. The tubes were then washed with methanol–isopropanol (1:1, v/v) and the above procedure was repeated. The borosilicate tubes were dried in an oven at 100°C.

Isolation of diltiazem from plasma and whole blood before chromatography

The frozen plasma or whole blood (−25°C) was thawed at a temperature of 25 ± 2°C (room temperature) and 1.0 ml was pipetted into a clean, disposable borosilicate culture tube. An aliquot of the standard solution of diltiazem was added to 1.0 ml of plasma or whole blood and was mixed on a vortex mixer for 30 s. A 500-μl aliquot of acetonitrile was added to the plasma or whole blood diltiazem solution. The solution was mixed on a vortex mixer for 2 min and centrifuged at 2200 *g* for 4 min at room temperature. The supernatant was decanted into a fresh borosilicate culture tube. The decantate was then saturated with anhydrous potassium carbonate and centrifuged for 1 min at 2200 *g*. The salted-out acetonitrile was transferred into a fresh culture tube with a disposable glass pipette. A 50-μl volume of this solution was injected into the HPLC system.

Construction of the calibration curve

The stock solution of diltiazem described in the *Preparation of stock solutions* section was used to prepare the plasma, whole blood, and water standards needed to construct the calibration curves. The stock solution was stored at −25°C and was stable for six months.

A minimum of five solutions of diltiazem was prepared by adding enough stock solution to obtain concentrations of about 0.030, 0.500, 1.00, 5.00, and 10.0 μg/ml. These solutions were prepared in each of three fluids: water, plasma, and whole blood. If the plasma or whole blood samples had diltiazem concentrations higher than 10.0 μg/ml, additional higher-concentration solutions were used to construct a new calibration curve. Under the experimental conditions, the calibration curve was linear up to 25 μg/ml.

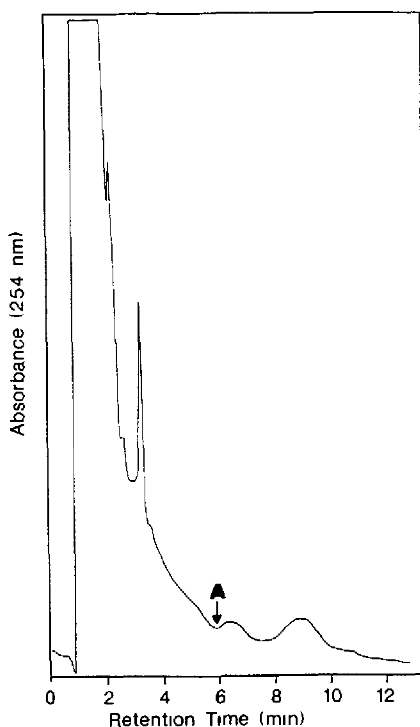
Calculation of the percentage recovery

The percentage recovery of diltiazem from plasma and whole blood samples was calculated by using a procedure described elsewhere [21].

RESULTS AND DISCUSSION

Fig. 2 shows a typical chromatogram of whole blood with no diltiazem. Fig. 3 shows the chromatogram of whole blood of a subject who ingested 60 mg of diltiazem. It is clear from Figs. 2 and 3 that the parent drug is baseline-resolved from endogenous peaks of whole blood and diltiazem metabolites. The chromatogram obtained from human plasma with no diltiazem was similar to that of Fig. 2. Therefore, the diltiazem peak in plasma was also baseline-resolved from the endogenous peaks of plasma. Three calibration curves in three fluids were constructed, one each in water, plasma, and whole blood. The objective of constructing these curves in three different sample fluids was to determine if diltiazem co-precipitates to different extents with the plasma or whole blood endogenous substances, when treated with acetonitrile and potassium carbonate. The parameters of the three calibration curves are given in Table I. Statistical calculation of the three calibration curves at a 98% confidence interval showed that the slopes of all curves were identical. These results indicate that a calibration curve of diltiazem obtained from extraction of water may be used to quantitate the drug in the plasma or whole blood samples. Although the y-intercepts were not identical, the difference in quantitation using three calibration curves was not statistically significant.

The presence of 0.50 ml of acetonitrile in 1.0 ml of plasma or whole blood during the isolation of diltiazem before chromatography precipitated a significant amount of proteins. The addition of potassium carbonate to salt-out acetonitrile may have caused an additional precipitation of endogenous substances that were not affected by acetonitrile. The cleaning of the borosilicate culture test tubes is a critical step of this method in order to achieve reprodu-



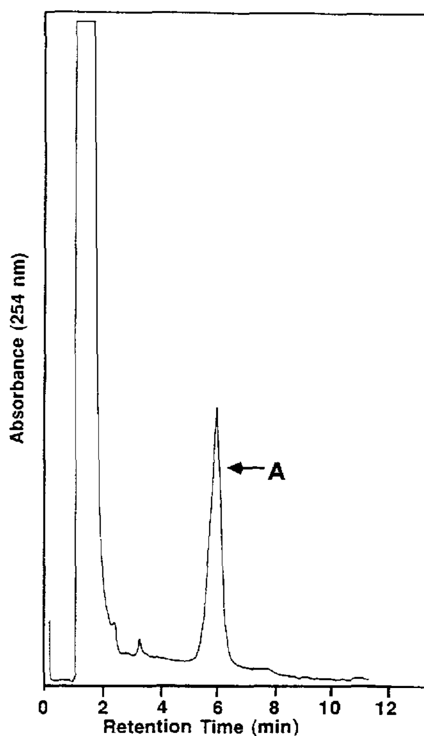


Fig. 2 (left). Chromatogram of whole blood with no diltiazem. A indicates the retention time of diltiazem. Detection at 254 nm and 0.005 a.u.f.s.

Fig. 3 (right). Chromatogram of whole blood from a subject who ingested 60 mg of diltiazem. A indicates the diltiazem peak. Detection at 254 nm and 0.01 a.u.f.s. The concentration of diltiazem detected was 240 ng/ml.

TABLE I

CALIBRATION CURVES OF DILTIAZEM IN THREE FLUIDS

Fluid[a]	Slope	Correlation coefficient	y-Intercept
Acetonitrile	75.9	0.999	−4.10
Plasma	75.1	0.999	3.09
Whole blood	74.7	0.999	2.21

[a]$n=3$ for each fluid.

cible chromatographic results with no interfering peaks. If this step is omitted, large interfering peaks with retention times similar to that of diltiazem appear in the chromatogram. A guard column was used between the injector and the analytical column, which was also packed with 10-μm PRP-1 particles. The

performance of the analytical column remained unchanged up to at least 300 sample injections. After approximately 70–100 injections of plasma or whole blood samples, it was necessary to replace the packing of the guard column to keep the back-pressure of the chromatographic system from exceeding 34.48 MPa; use of the guard column was also needed to achieve a longer life of the analytical column.

The reproducibility and accuracy of the method were determined by repetitive analyses of plasma and whole blood spiked with standard diltiazem solution. The data obtained for same-day and day-to-day analyses are in Tables II and III.

It appears that the reproducibility of the assays (relative standard deviation, R.S.D.) is better at higher concentrations of diltiazem in plasma or whole blood.

TABLE II

ASSAY REPRODUCIBILITY OF DILTIAZEM FOR THE SAME DAY

Actual concentration (μg/ml)	Concentration determined (mean ± S D., $n=7$) (μg/ml)	Relative standard deviation (%)	Deviation from theory (%)
Plasma			
0.10	0.09 ± 0.005	5.6	−10.0
2.0	2.2 ± 0.09	4.0	+10.0
5.0	4.9 ± 0.11	2.3	−2.0
Whole blood			
0.20	0.21 ± 0.009	4.3	+5.0
0.50	0.52 ± 0.019	3.6	+4.0
8.0	7.9 ± 0.26	3.3	−1.3

TABLE III

ASSAY REPRODUCIBILITY OF DIALTIAZEM FOR DAY-TO-DAY ANALYSIS

Samples were analyzed every day for five days.

Actual concentration (μg/ml)	Concentration determined (mean ± S.D., $n=7$) (μg/ml)	Relative standard deviation (%)	Deviation from theory (%)
Plasma			
0.20	0.21 ± 0.014	6.6	+5.0
0.60	0 54 ± 0.027	5.5	−10.0
6.0	6.2 ± 0.21	3.6	+3.3
Whole blood			
0.25	0.26 ± 0.017	6.5	+4 0
4.0	3.8 ± 0 13	3.4	−5.0
8 0	7.8 ± 0.31	3.9	−2.5

An R.S.D. of 10% or less occurred when the concentration of diltiazem in plasma or whole blood was 10 ng/ml.

The use of tetrabutylammonium hydroxide in the mobile phase increased the retention time and selectivity of diltiazem. The peak shape and symmetry of diltiazem also improved significantly when tetrabutylammonium ion was added to the mobile phase. These results indicate that the diltiazem molecule may have formed some type of weak ion pair with the tetrabutylammonium ion while partitioning between the stationary phase and mobile phase of the column and hence was retained longer on the column. This effect may also be related to the ionic strength of the mobile phase since this is increased when tetrabutylammonium hydroxide is added to the mobile phase. The mobile phase ratios used in this experiment were optimal with respect to limit of detection and specificity.

A constant amount of diltiazem was injected into the HPLC system and the percentage of acetonitrile in the mobile phase was varied. From this experiment it appeared that the peak height of diltiazem is linearly related to the percentage of acetonitrile up to at least 70% in the mobile phase when all other experimental conditions are kept constant.

The retention time and capacity factor of diltiazem decreased with the increase of acetonitrile in the mobile phase. The decrease of the capacity factor with increasing percentage of acetonitrile in the mobile phase levels off as the retention time of diltiazem becomes similar to the retention time of the void peak. This retention time alteration may be useful in determining the diltiazem concentration in plasma or whole blood if an interfering peak is observed in a patient's whole blood or plasma sample. The capacity factor of diltiazem should be around 4.0 to achieve resolution of the drug peak from the plasma or whole blood endogenous peaks.

The recoveries of diltiazem from patients' plasma and whole blood samples were determined by using the procedure described in the *Calculation of the percentage recovery* section. The recovery of diltiazem from plasma and whole blood varied from 93 to 108%. The concentrations of diltiazem that were determined for samples of whole blood and plasma taken from the subject at the same sampling time were statistically identical at a 98% confidence interval (plasma, 0.080 μg/ml; whole blood, 0.085 μg/ml). Therefore, either whole blood or plasma may be used to quantitate diltiazem for effective dose adjustment to obtain maximum therapy with minimum adverse side-effects.

Extraction efficiency of diltiazem was studied by adding known amounts of diltiazem to control whole blood or plasma at various concentrations. After extraction, an aliquot of the solution was injected into the chromatographic system. The peak height obtained was compared with the peak height of a standard solution of diltiazem in acetonitrile solution. Extraction efficiency varied from 93 to 101% for acetonitrile. Acetonitrile was used as the extraction solvent because, compared to methanol, ethanol, and isopropanol, it had the

TABLE IV

EFFICIENCY AND SENSITIVITY OF DIFFERENT COLUMNS FOR DILTIAZEM

A 160-ng amount of diltiazem was injected each time. Mobile phase: acetonitrile–0.01 *M* dibasic potassium phosphate (60:40, v/v) and 5.0 m*M* tetrabutylammonium hydroxide.

Colum tested	Theoretical plates per meter	Capacity factor	Mean peak height ($n=2$) (cm)
10 cm×4.6 mm, 5-μm ODS	11870	5.2	17.6
10 cm×4.6 mm, 10-μm Octyl	12340	4.9	18.7
6 cm×4.6 mm, 10-μm PRP-1	9460	4.7	15.8
15 cm×4.6 mm, 10-μm CN	13550	3.6	20.4
15 cm×4.1 mm, 10-μm PRP-1	8670	4.5	14.3

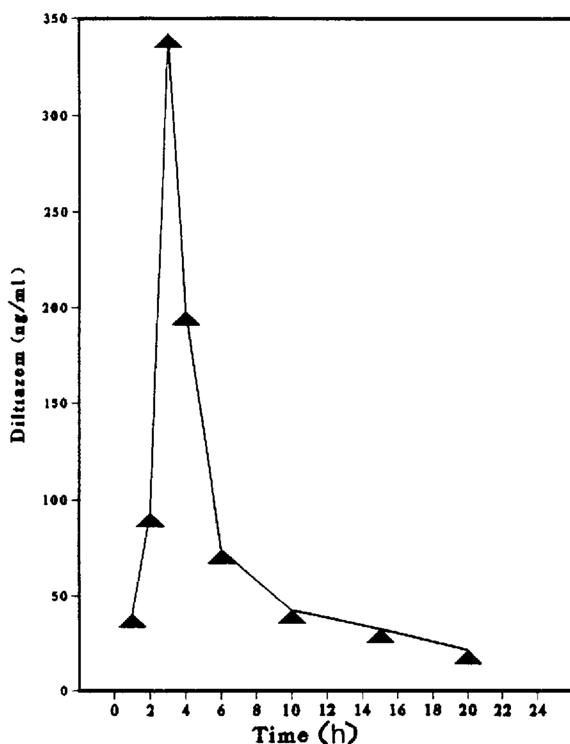


Fig. 4. Whole blood concentration versus time curve following an oral administration of 60 mg of diltiazem to a healthy subject.

best selectivity, sensitivity, and efficiency. The mean extraction efficiencies for acetonitrile, ethanol, methanol, and isopropanol were 95, 83, 87, and 82%, respectively. The percentage of acetonitrile salted-out by anhydrous potassium carbonate was 94±8% ($n=10$) [22]. Several columns packed with different

types of stationary phases were tested in this experiment to obtain the one which had maximum selectivity and sensitivity. The columns tested are listed in Table IV. Except for the column used in this experiment, all other columns showed either poor selectivity, poor sensitivity, or both. Also, silica-based bonded-phase columns are unstable at alkaline mobile phase and cannot be used for this experiment. Therefore, a 15-cm PRP-1 column was used for this experiment.

The in vivo pharmacokinetics of diltiazem in a subject who ingested a single oral dose of 60 mg of diltiazem was studied using the HPLC method developed in this experiment. The pharmacokinetic parameters calculated in this study may not be very accurate due to the fact that the data were obtained from only one subject and samples were collected for just eight time points. Fig. 4 shows the diltiazem concentration in the subject's whole blood with respect to time. The k_{el} value was calculated from an open two-compartment model with first-order absorption and elimination from the central compartment. The data from the elimination portion of Fig. 4 were analyzed by linear regression. The k_{el} value was found to be -0.215 ng·h/ml. The $t_{1/2}$ was calculated from the equation $t_{1/2} = -0.693/k_{el}$ and was found to be 3.22 h. The C_{max} was calculated to be approximately 342 ng/ml. The t_{max} of diltiazem in whole blood was approximately 3.0 h. The $AUC_{0 \to tx}$ was calculated by trapezoidal method and was found to be approximately 1.24 μg·h/ml.

CONCLUSION

The reversed-phase HPLC method described in this paper can be used to determine diltiazem in human plasma or whole blood down to 20 ng/ml. The limit of detection of the method is 10 ng/ml at a signal-to-noise ratio of 3. The sample preparation in this method is unique and simpler than other methods published in the literature because it eliminates the evaporation and derivatization steps. The sensitivity, reproducibility, and accuracy of the method are good. Because of high sensitivity and reproducibility, this method will be suitable for routine analysis of diltiazem in human biological samples and for research studies involving pharmacokinetics, efficacy, and bioavailability. Interferences of caffeine, cimetidine, ranitidine, ampicillin, theophylline, and tetracycline were found to be negative.

REFERENCES

1 P. Theroux, D.D. Waters, G.S. Affaki, J. Crittin, R. Bonan and H.F. Mizgala, Circulation, 60 (1980) 504.
2 K. Kohno, Y. Takeuchi, A. Etoh and K. Noda, Arzneim.-Forsch., 27 (1977) 1, 424.
3 S.J. Rosenthal, R. Ginsburg, I.H. Lamb, D.S. Baim and J.S. Schroeder, Am. J. Cardiol., 46 (1980) 1027.
4 D.D. Waters, J. Szlachic, P. Theroux, F. Dauwe and H.F. Mizgala, Am. J. Cardiol., 47 (1981) 179.

5 V. Rovei, M. Mitchard and P.L. Morselli, J. Chromatogr., 138 (1977) 391.
6 M. Chaffman and R.N. Brogden, Drugs, 29 (1985) 387.
7 E.U. Kölle, H.R. Ochs and K.O. Vollmer, Arzneim.-Forsch., 33 (1983) 972.
8 J. Sugihara, Y. Sugawara, H. Ando, S. Harigaya, A. Etoh and K. Kohono, J. Pharm. Dyn., 7 (1984) 207.
9 H. Yabana, T. Nagao and M. Sato, J. Cardiovasc. Pharmacol., 7 (1985) 152.
10 K. J. Goebel and E.U. Kölle, J. Chromatogr., 345 (1985) 355.
11 B.C. Gilliland and M. Mannik, Harrison's Principles of Internal Medicine, McGraw-Hill, New York, 10th ed., 1983, p. 1382.
12 P.D. Henery, Am. J. Cardiol., 46 (1980) 1047.
13 J.P. Clozel, G. Caille, Y. Taeymans, P. Theroux, P. Biron and J.G. Besner, J. Pharm. Sci , 73 (1984) 207.
14 R. Calaf, P. Marie, Cl. Ghiglione, M. Bory and J. Reynand, J. Chromatogr., 272 (1983) 385.
15 R.E. Wiens, D.J. Runser, J.P. Lacz and D.C. Dimmitt, J. Pharm. Sci., 73 (1984) 688.
16 S.C. Montamat, D.R. Abernethy and J.R. Mitchell, J. Chromatogr., 415 (1987) 203.
17 S.M. Johson and S.K. Whaba Khalil, J. Liq. Chromatogr., 10 (1987) 673.
18 J.P. Clozel, G. Caille, Y. Taeymans, P. Theroux, P. Biron and F. Trudel, J. Pharm. Sci., 73 (1984) 771.
19 C. Verghese, M.S. Smith, L. Aanonsen, E.L.C. Pritchett and D.G. Shand, J. Chromatogr., 272 (1983) 149.
20 D.R. Abernethy, J.B. Schwartz and E Todd, J. Chromatogr., 342 (1985) 216.
21 A.M. Rustum, J. Liq. Chromatogr., 11 (1988) 2315.
22 A.M. Rustum, Ph.D. Dissertation, Marquette University, Milwaukee, WI, 1986, Table 2.8, p 2-32.