Journal of Chromatography, 272 (1983) 341—350
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1501

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTITATION OF FLECAINIDE, A NEW ANTIARRHYTHMIC, IN HUMAN PLASMA AND URINE

S.F. CHANG*, T.M. WELSCHER, A.M. MILLER and R.E. OBER

Drug Metabolism Department, Riker Laboratories, A Subsidiary of 3M, 3M Center, Building 270-3S, St. Paul, MN 55144 (U.S.A.)

(First received March 29th, 1982; revised manuscript received September 22nd, 1982)

SUMMARY

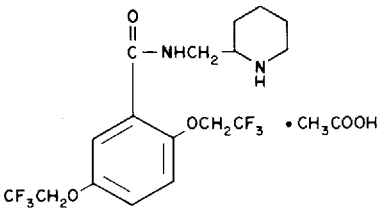
A simple, selective, sensitive, and accurate high-performance liquid chromatographic method for the quantitation of flecainide in human plasma and urine is described. The method is based on initially washing the sample with hexane followed by a single extraction with hexane. The extracted drug and internal standard are chromatographed on a Zorbax TMS column with a mobile phase consisting of acetonitrile—1% acetic acid in 0.01 *M* pentanesulfonate (45:55, v/v). The eluent is monitored at 308 nm.

This method can routinely quantitate plasma or urine flecainide concentrations as low as 22 ng/ml with a 1-ml sample and 11 ng/ml with a 2-ml sample with no interference from endogenous substances and many drugs and their metabolites. The standard curve is linear over a concentration range of 22—1746 ng/ml. The precision and accuracy of the described method are suitable for monitoring flecainide levels in therapeutic, tolerance, and pharmacokinetic studies in humans.

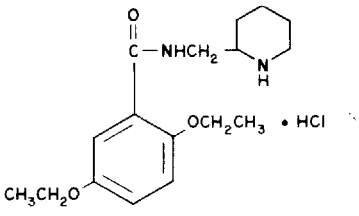
INTRODUCTION

Flecainide acetate, 2,5-bis-(2,2,2-trifluoroethoxy)-N-(2-piperidylmethyl)benz amide acetate (R-818), is a new antiarrhythmic agent. Studies in laboratory animal models indicated the effectiveness in converting experimentally induced auricular and ventricular arrhythmias and demonstrated greater potency than reference agents such as lidocaine, procainamide and quinidine [1—4]. Studies in normal human volunteers indicated a relatively long half-life ($t_{1/2}$ = 14 h) and its potential for sustained therapeutic activity [5—9]. Flecainide acetate is undergoing extensive study in humans worldwide [5—10]. Currently, measurement of flecainide plasma levels is accomplished by first derivatizing flecainide with pentafluorobenzoyl chloride and then analyzing by gas—liquid chromato-

0378-4347/83/0000—0000/$03.00



Flecainide Acetate



Internal Standard

graphy (GLC) with electron-capture detection [11]. Sensitive fluorometric methods have been developed for the monitoring of flecainide in human plasma but suffered from lack of specificity due to interference by other drugs [12, 13]. A high-performance liquid chromatographic (HPLC) procedure with the use of a fluorescence detector has recently been developed [14]. The method has a wide linear range (50—5000 ng/ml), however, it requires mechanically cutting a commercial column and the use of a sophisticated spectrofluorometer. Furthermore, it is not an internal standardization procedure. In this report, we describe a simple, rapid, selective, and accurate HPLC method which requires common LC instrumentation and is capable of routinely monitoring flecainide plasma levels in humans following either single or chronic dosing of flecainide acetate in research settings or for non-research clinical management purposes.

EXPERIMENTAL

Chemicals and reagents

The methanol and acetonitrile were Omnisolv® (MCB Reagents , Cincinnati, OH, U.S.A.) and the hexane used for extraction was Nanograde® (Mallinckrodt, St. Louis, MO, U.S.A.). The 1-pentanesulfonate was purchased from Regis (Morton Grove, IL, U.S.A.) and the trimethylamine hydrochloride from Aldrich (Milwaukee, WI, U.S.A.). All other chemicals were AR grade.

Chromatography system

The analysis was performed on a modular liquid chromatograph equipped with a Model 6000A solvent delivery system, a Model 710 B Intelligent Sample Processor (Waters Assoc., Milford, MA, U.S.A.), and a Model 1203 UV Monitor III with a 308-nm filter (Laboratory Data Control, Riviera Beach, FL, U.S.A.). The separation was achieved on a Zorbax TMS column, 15 cm × 4.6 mm (6 μm particle size, Dupont, Wilmington, DE, U.S.A.), protected by a guard column (Waters Assoc.) packed with Permaphase ETH (35 μm particle size, DuPont). Peak heights were measured by a Model 4100 Computing Integrator (Spectra Physics, Santa Clara, CA, U.S.A.).

The mobile phase was prepared by mixing 450 ml acetonitrile, 5.5 ml glacial acetic acid, and 544.5 ml 0.01 *M* aqueous 1-pentanesulfonate. The mobile phase was filtered through a Millipore membrane (pore size 0.45 μm) and degassed prior to use. The flow-rate was 2.0 ml/min.

Preparation of standard solutions

Standard solutions of flecainide were prepared by diluting a 10 mg/l aqueous stock solution of flecainide acetate. The concentrations used were 22, 44, 87, 262, 524, 873, and 1746 ng flecainide per 0.5 ml. The internal standard solution, 2,5-diethoxy-N-(2-piperidylmethyl)benzamide hydrochloride (S-15277), was prepared by diluting a 6.3 mg per 100 ml aqueous stock solution to 250 ng per 50 μl.

Extraction procedure

Pipet 1 ml plasma or urine into a 150 × 16 mm glass culture tube with a polyethylene-lined cap, add 50 μl internal standard solution (or water for blank), 0.5 ml flecainide standard solution for calibration curve (or water for unknown samples and blank), 1 ml 0.5 *N* aqueous hydrochloric acid, and 10 ml hexane. Cap the tubes, shake on a reciprocal mechanical shaker (tubes in horizontal position) at a speed of 256 cycles/min for 10 min and centrifuge for 5 min at 840 *g*. Aspirate the hexane phase; add another 10 ml of hexane and wash a second time. Aspirate the hexane phase and add the following, in this order, to the aqueous phase: 0.2 ml 0.2 *M* aqueous trimethylamine (TMA) hydrochloride, 10 ml hexane, and 1 ml 1 *N* aqueous sodium hydroxide. Shake for 10 min, centrifuge for 5 min at 840 *g*, and transfer the hexane phase to a 15-ml conical centrifuge tube. Evaporate to approximately 1 ml at 60°C under nitrogen; rinse the tube wall with 1 ml methanol, and evaporate to dryness. Reconstitute the residue with 200 μl of the mobile phase and inject 150 μl into the liquid chromatograph.

Method of calculation

The calculation of flecainide concentration for unknown samples was performed automatically by the Model 4100 Computing Integrator. A least-squares linear regression line was calculated from the concentration of the standards and the peak height ratio of flecainide over the internal standard. For every unknown sample thereafter, the peak height ratio was compared to the linear regression line to determine the unknown concentration. The peak heights can also be measured manually when the response is presented with the use of a 10-mV recorder.

RESULTS

Chromatographic separation

Baseline separation was achieved under the experimental conditions described with retention times of 3.5 and 5.2 min for the internal standard and flecainide, respectively. There was no interference with flecainide and the internal standard by endogenous materials from human plasma. Typical plasma chromatogram tracings are shown in Fig. 1. Similar results were obtained from human urine (Fig. 2).

Extraction recovery

Because of the relatively low concentration of flecainide (a basic molecule) analyzed in this procedure, surface adsorption processes play an important role

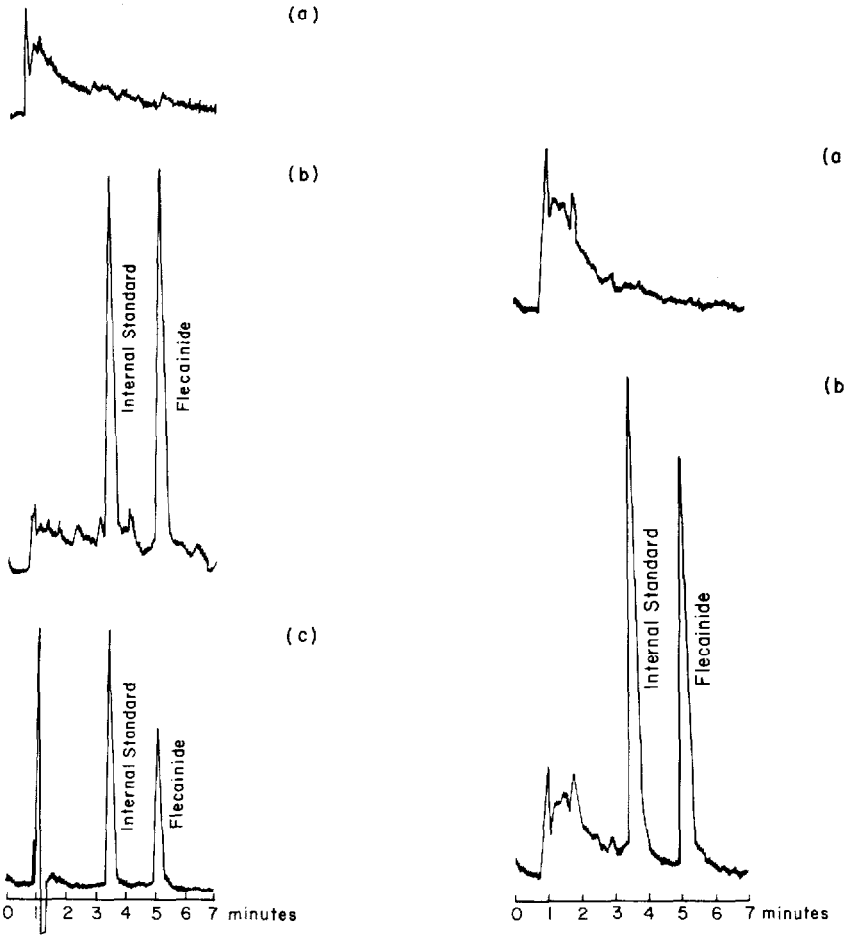


Fig. 1. Typical chromatograms of human plasma extracts for flecainide determination. (a) Blank plasma, (b) plasma of human dosed with flecainide acetate with the internal standard (250 ng) added, and (c) flecainide and internal standard.

Fig. 2. Typical chromatograms of human urine extracts for flecainide determination. (a) Blank urine, (b) urine of human dosed with flecainide acetate with the internal standard (250 ng) added.

in the results of the quantitative determination. The extraction recovery from human plasma was monitored in quadruplicate (triplicate for the 698 ng/ml level) at four concentration levels: 175, 349, 698, and 1310 ng/ml. The extraction recoveries of flecainide were 61.5, 74.5, 70.7, and 73.5% at the concentration levels mentioned above. The relative standard deviations were less than 8% and indicated good reproducibility in extraction recovery between samples. The 175 ng/ml level showed a somewhat lower extraction recovery and thus the use of an internal standard with physical properties similar to flecainide becomes very important. In this assay procedure, the internal standard has been able to compensate for the potential difference in extraction recovery between concentration levels.

Calibration curve

A calibration curve was prepared daily with each batch of unknown samples. The calibration curve consisted of eight single standards: a blank, 22, 44, 87, 262, 524, 873, and 1746 ng/ml. The correlation coefficient for the peak height ratio vs. flecainide concentration (by linear regression) is usually 0.998 or better indicating a high degree of linearity in the calibration curve over the concentration range studied.

Sensitivity

Under the experimental conditions, this procedure can routinely quantitate a flecainide concentration in plasma as low as 22 ng/ml with a 1-ml sample. Concentrations lower than 22 ng/ml can be detected but quantitation was not established. At the 22 ng/ml concentration, the peak height was at least five times the level of the background noise. When a 2-ml sample is used, the minimum concentration that can be quantitated is 11 ng/ml. The urinary concentrations of flecainide are generally much higher than plasma concentrations, therefore sensitivity is not as critical. In fact, in many instances, urine samples have to be diluted before being analyzed.

Selectivity

Many drugs which may be given concomitantly with flecainide acetate were tested for interference in the assay (Table I). The types of drugs tested include β-blockers, analgesics, central nervous system stimulants, cardiac glycosides, anticoagulants, antihypertensives, bronchodilators, vasodilators, and antiarrhythmics. These drugs were tested by direct injection into the liquid chromatograph and their retention times were compared with those of flecainide and internal standard. With two exceptions (quinidine and dipyridamole), the drugs did not show any interference with the flecainide assay, either because of a different retention time or very little UV absorption at 308 nm. A secondary peak (presumably dihydroquinidine) associated with quinidine showed an incomplete separation from flecainide and affected its quantitation. Dipyridamole had a retention time identical to that of the internal standard, but when the drug was added to the plasma, it did not extract under the conditions used here, thus dipyridamole in plasma does not affect the flecainide assay. Although propranolol did not achieve baseline separation from the internal standard, there was enough separation to allow accurate quantitation of flecainide. Some of these drugs: bretylium, procainamide, digoxin, triamterene, mexiletin, thiazide, dilantin, theophylline, propranolol, and quinidine, were also tested by collecting plasma samples from patients who had received these drugs and processing these plasma samples according to the flecainide procedure. Again with one exception (bretylium), the plasma metabolites of these drugs did not interfere with the flecainide assay. In the case of quinidine, the dihydroquinidine-like peak practically disappeared, presumably metabolized in the human body. Thus, this assay procedure can be applied to a variety of clinical situations where a combination of drugs are administered.

TABLE I

DRUGS TESTED FOR INTERFERENCE

Drugs	Amount injected (ng)	Retention time (min)
Flecainide acetate	200	5.17
Internal standard	125	3.50
N-Acetylprocainamide	300	1.88
Acetylsalicylic acid	2000	1.54
Bretylium tosylate	1000	*
Caffeine	1000	*
Diazepam	50	2.97
Digitoxin	1000	*
Digoxin	1000	*
Dilantin	15,000	*
Dipyridamole	100	3.50**
Disopyramide	1000	*
Heparin sodium	1000	*
Hydralazine HCl	100	1.61
Lidocaine HCl	1000	*
Methyldopa	100	1.34
Mexiletin HCl	1000	*
Practolol	5000	1.46
Procainamide HCl	100	1.80
Propranolol HCl	100	3.04
Quinidine sulfate	2500	4.14
Quinine sulfate	2500	4.14
Sulfinpyrazone	1000	2.58
Theophylline	1000	*
Thiazide HCl	10,000	1.26
Tocainide	1000	*
Triamterene	100	1.86

*No peak after 30-min elution.
**When the drug was added to plasma, it did not extract under the flecainide acetate assay procedure.

Precision and accuracy

The intra-day precision and accuracy of the assay were determined by spiking blank human plasma with flecainide at four concentration levels: 175, 349, 698, and 1310 ng/ml, in quadruplicate. These spiked samples were carried through the entire procedure. The results are shown in Table II. The intra-day precisions, expressed as relative standard deviations, were 3.2, 0.7, 6.4, and 0.9% for 175, 349, 698, and 1310 ng/ml, respectively. The accuracies for the above mentioned concentrations, expressed as relative errors, were −0.1, 7.8, 9.8, and −2.4%, respectively. The inter-day precision was established by analyzing samples at three concentration levels: 175, 437, and 1310 ng/ml, for four consecutive days. Excellent day-to-day reproducibility was demonstrated at these concentrations with a relative standard deviation of 5% or less in each case. The precision and accuracy data indicate that this method is more than adequate for the routine measurement of plasma flecainide concentrations for clinical management as well as for research purposes where more precise data are needed.

TABLE II

INTRA-DAY PRECISION AND ACCURACY (n = 4)

Sample concentration (ng/ml)	Mean ± S.D. (ng/ml)	Relative standard deviation (%)	Relative error (%)
175	175 ± 5.5	3.2	−0.13
349	376 ± 2.5	0.7	7.8
698	766 ± 49.0	6.4	9.8
1310	1278 ± 11.8	0.9	−2.4

Correlation of HPLC and GLC methods for plasma flecainide

The GLC method [11] has been used to measure flecainide concentrations in a large number of metabolic, tolerance, and therapeutic studies in humans. To compare the GLC and HPLC methods, about 60 human plasma samples from a clinical study were analyzed by both methods. The results are shown in Fig. 3. Excellent correlation between methods was obtained (correlation coefficient = 0.99). Thus, the HPLC method appears to be capable of providing the same quality of quantitation as the GLC method, yet the HPLC procedure is

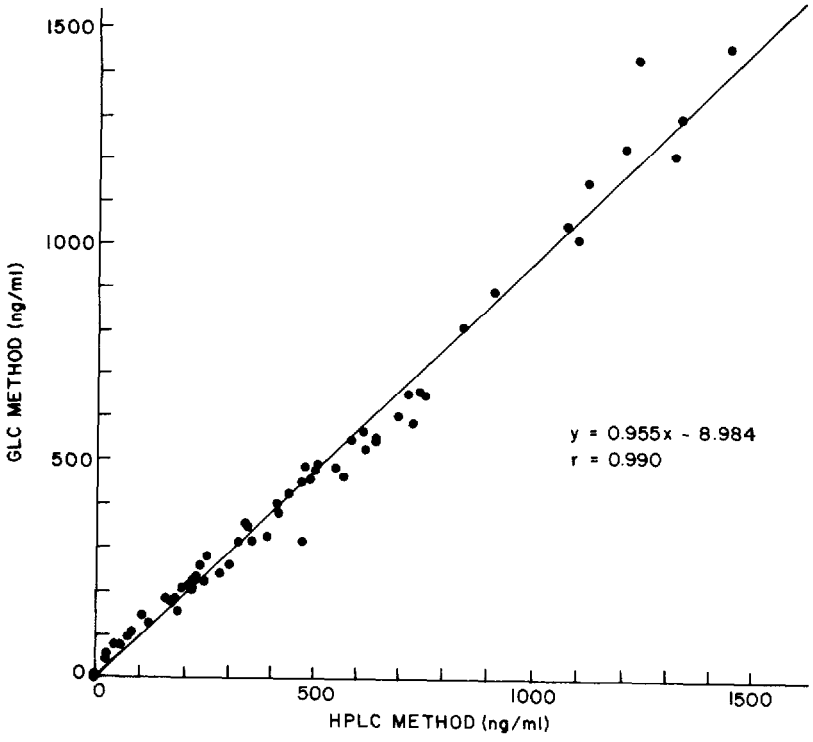


Fig. 3. Comparison of the HPLC method for the determination of flecainide in plasma with an established GLC method.

much simpler in sample preparation, less demanding in laboratory equipment, and easier to reproduce between laboratories. The method is suitable for routine clinical monitoring and pharmacokinetic studies.

DISCUSSION

Extraction

The sample preparation is accomplished by two hexane washings, one hexane extraction, and one evaporation. The entire procedure requires only one transfer; the hexane extract is transferred into a conical centrifuge tube for evaporation. Thus, the need for glassware and manual transfer is kept to a minimum. Furthermore, the initial two hexane washes at an acidic pH remove all the neutral and acidic substances soluble in hexane, thus drastically reducing the potential interference. The final hexane extraction at a strong basic pH (1 ml, 1 *N* sodium hydroxide) selectively extracts flecainide and excludes some of the known urinary metabolites of flecainide in humans [15].

The use of hexane in both the washing and extraction steps resulted from studying the effect of various solvents and solvent combinations on the extent of extraction and cleanliness of the chromatogram. The present sample preparation procedure affords about 70% extraction recovery. The use of diethyl ether in place of hexane substantially improves the extraction recovery, but at the same time, an extra peak is eluted which interferes with the internal standard. Furthermore, a memory peak is adversely affected. The residue resulting from the evaporation of the ether extract cannot be totally dissolved in the mobile phase, thus centrifugation prior to injection into the HPLC system is needed. The use of diethyl ether also requires an explosion-proof centrifuge.

The combinations of hexane and diethyl ether, hexane and chloroform, and hexane and toluene were also tried. Among the combinations, all except hexane–toluene improved the extraction recovery over the pure hexane system. The hexane–diethyl ether still suffers from the same problems as the pure ether system. The hexane–chloroform system affords a slightly better extraction recovery, but chloroform is considered to be a potential health hazard. Upon consideration of all aspects: the extent of extraction, cleanliness of the chromatogram, fewer steps in sample preparation, no need for special equipment (such as explosion-proof centrifuge) and the use of a non-hazardous solvent, hexane was chosen for both the washing and extraction steps.

In the present procedure the plasma is washed twice with hexane. A reduction in the number of hexane washes will result in the appearance of a peak interfering with the internal standard and also an increase in the size of a memory peak.

Trimethylamine (TMA) is known to minimize glass adsorption of low concentrations of amines. The addition of TMA for the extraction of nanogram levels of basic compounds has been a general practice in our laboratory. TMA solution is generally prepared by bubbling TMA gas through benzene and determining the final molarity of the benzene solution by titration. For some laboratories, this titration method might not be available. Thus, an extraction survey was made to find a suitable substitute which can be conveniently

obtained and does not significantly sacrifice the quality of the method. TMA in benzene yields the most consistent extraction recovery of flecainide and the cleanest chromatogram. Triethylamine also meets the requirements, but many extra peaks appeared on the chromatogram. TMA hydrochloride salt is commercially available and converts to the free base during the extraction step. The TMA hydrochloride salt performs well, but with slightly lower recovery than the TMA free base in benzene. For the purpose of providing an extraction procedure which is the least demanding on laboratory facilities the TMA hydrochloride was chosen and has been used for all data reported here. However, for those who have the titration capability, the TMA free base in benzene is recommended (0.2 ml of 0.2 *M* TMA in benzene).

Chromatography

The present chromatographic procedure is a result of the comparison of various chromatographic procedures. The criteria analyzed were the sharpness and symmetry of the flecainide peak with a capacity factor (k') between 3 and 6. An ion suppression technique on two C_{18} reversed-phase columns and one TMS column, an ion-pair chromatographic technique on a TMS column, and the use of a PRP-1 column (Hamilton) were tried. Among the columns tested, none were able to provide a flecainide peak as sharp and symmetrical as the ion-pair technique on the TMS column. During the submission and revision period of the manuscript, it was found that a C_1 column (IBM) also afforded excellent chromatographic characteristics.

There is more than one minute difference in retention times for the flecainide and internal standard peaks. In certain instances, when the absence of other drugs in the unknown sample is assured, the elution process can be speeded up by increasing the acetonitrile content in the mobile phase, thus the throughput of the assay is increased. When the absence of other drugs is not certain, the elution parameters should not be changed. For example, increasing the elution process will deteriorate the resolution between propranolol and the internal standard.

Detection and sensitivity

In the reported procedure, the eluted flecainide and internal standard are monitored with an LDC UV Monitor III detector equipped with a 308-nm filter. The maximum UV absorption of flecainide is at 298 nm. Monitoring the effluent at 298 nm will increase the response by 10% in comparison to that at 308 nm, while at 313 nm (such as with the Waters 440 UV detector) the response decreases by 27%. Thus, with the use of a 313-nm filter, the 22 ng/ml standard can no longer give consistent and reproducible results. However, experimental results indicate that when a 2-ml plasma or urine sample is used, the quantitation of 22 ng/ml at 313 nm can be restored. Similarly, a 11 ng/ml plasma or urine sample can be quantitated with a 2-ml sample at 308 nm. When a 2-ml sample is extracted, additional hexane washings have to be made.

The reported procedure is capable of quantitating 22—1746 ng/ml with a 1-ml sample. This range adequately covers the plasma concentrations in human subjects receiving a single or multiple dose. Although the procedure provides a wide linear range of reference standards, in practice a plasma calibration curve

of 22—873 ng/ml for a single-dose administration, and a 44—1397 ng/ml calibration curve for a multiple dose administration is recommended.

ACKNOWLEDGEMENTS

The authors thank J.M. Fox for the GLC analysis and manuscript review and Dr. R.L. Woosley, Vanderbelt University for providing human plasma samples containing various drugs and their metabolites.

REFERENCES

1 J.R. Schmid, B.D. Seebeck, C.L. Henrie, E.H. Banitt and D.C. Kvam, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 34 (1975) 775.
2 A.N. Welter, J.R. Schmid and D.C. Kvam, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 36 (1977) 1003.
3 E.H. Banitt, W.R. Bronn, W.E. Coyne and J.R. Schmid, J. Med. Chem., 20 (1977) 821.
4 P.D. Verdouw, J.W. Deckers and G.J. Conard, J. Cardiovasc. Pharmacol., 1 (1979) 473.
5 G.J. Conard, G.L. Carlson, J.W. Frost, R.E. Ober, A.S. Leon and D.B. Hunninghake, presented at the 21st National Meeting of the Academy of Pharmaceutical Sciences, Orlando, FL, November 14—18, 1976.
6 G.J. Conard, G.L. Carlson, J.W. Frost and R.E. Ober, Clin. Pharmacol. Ther., 25 (1979) 218.
7 J. Hoback, M. Hodges, G.S. Francis, B. Sharma and R.W. Asinger, Circulation, 58 (1978) 246.
8 P. Somani, Clin. Pharmacol. Ther., 27 (1980) 464.
9 L. Seipel, R.R. Abendroth and G. Breithhardt, Circulation, 62 (1980) 153.
10 J.L. Anderson, J.R. Steward, B.A. Perry, D.D. Van Hamersveld, T.A. Johnson, G.J. Conard, S.F. Chang, D.C. Kvam and B. Pitt, N. Engl. J. Med., 305 (1981) 473.
11 J.D. Johnson, G.L. Carlson, J.M. Fox, A.M. Miller, S.F. Chang and G.J. Conard, in preparation.
12 S.F. Chang, M.J. Jernberg, A.M. Miller, G.J. Conard and R.E. Ober, Arzneim.-Forsch., in press.
13 A. Johnston, personal communication.
14 J.W. de Jong, J.A.J. Hegge, E. Harmsen and P.Ph. de Tombe, J. Chromatogr., 229 (1982) 498.
15 J.D. Johnson, personal communication.