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DETERMINATION OF AMIODARONE AND ITS N-DEETHYLATED METABOLITE IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

NELSON D. MOSTOW*, DEBORAH L. NOON, CAROLYN M. MYERS, LOUIS RAKITA and JEFFREY L. BLUMER

Department of Medicine, Cleveland Metropolitan General Hospital, Cleveland, OH 44109 (U.S.A.), Department of Pediatrics, Rainbow Babies and Childrens Hospital, Cleveland, OH 44106 (U.S.A.), and the Department of Pharmacology, Case Western Reserve University, Cleveland, OH 44106 (U.S.A.)*

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SUMMARY

A high-performance liquid chromatographic (HPLC) method utilizing hexane extraction and a normal bonded phase column (NH_2-alkylamine) was developed to measure serum concentrations of amiodarone and its N-deethylated metabolite. A single analysis requires 8 min. The one-step extraction efficiencies of amiodarone and the internal standard are greater than 90%. The method is linear between 0.05 and 20.0 μg/ml. The average relative standard deviation of the slope of the standard curve is 4% and the single day coefficient of variation is 3.2%. The use of hexane extraction for sample cleanup and a bonded phase column for chromatography result in a sensitive and reproducible system well suited to laboratories monitoring serum concentrations of multiple drugs by HPLC. A preliminary study has shown the assay to be useful for the investigation of the pharmacokinetics of this agent.

INTRODUCTION

Amiodarone is an antiarrhythmic agent which has demonstrated remarkable efficacy and safety in the treatment of refractory ventricular and supraventricular arrhythmias [1—7]. Using conventional dosage regimens, there is generally a period of 1—2 weeks from the beginning of drug administration until arrhythmia suppression is noted. In patients whose arrhythmias are refractory to all other therapy, such a delay in the onset of a therapeutic effect is life-threatening.

Aggressive therapy with amiodarone intended to shorten or abolish this lag time between the start of therapy and arrhythmia control has been dis-

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couraged by the paucity of information concerning amiodarone's biodisposition. Without knowledge of the adsorption, distribution, metabolism and excretion of the drug and the relationship between serum concentration and therapeutic effect, a rational approach to the initiation of therapy has not been possible. Progress in these areas has been hampered by the lack of a rapid, simple and sensitive procedure for the determination of the concentration of amiodarone in biological fluids.

We report here a method for the determination of amiodarone and its N-deethylated metabolite [8] in serum utilizing high-performance liquid chromatography (HPLC). This method is rapid, clean and durable and therefore well suited to laboratories measuring several compounds by HPLC.

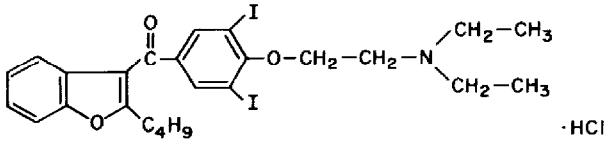
EXPERIMENTAL

Apparatus

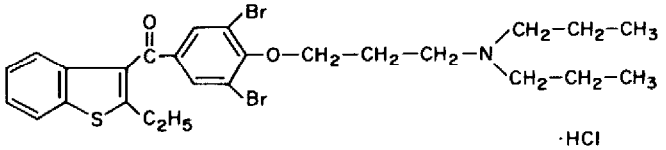
Analyses were performed using a Varian Model 5020 high-performance liquid chromatograph interfaced with a Varichrome variable-wavelength detector and a CDS 111L digital integrator (Varian Instruments, Palo Alto, CA, U.S.A.). Chromatography was performed using a 30 cm × 4 mm Varian NH_2-10-alkylamine column preceded by a 4 cm × 4 mm guard column packed with 40-μm silica particles (Vydac®, Varian Instruments). A heater block maintained the column at 30°C. The flow-rate was 1.5 ml/min which developed a pressure of 44 bars. The detector wavelength was set at 248 nm with an 8-nm bandwidth. Chromatograms were displayed on a strip chart recorder (Linear Instruments, Irvine, CA, U.S.A.).

Chemicals and reagents

The hexane, methylene chloride and methanol used as elution solvents were glass distilled (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). All other solvents and reagents were ACS reagent grade. Amiodarone [2-butyl-3-(3,5-diiodo-4-β-diethylaminoethoxybenzoyl)benzofuran] and the internal standard



AMIODARONE



INTERNAL STANDARD

Fig. 1. Chemical structures of amiodarone and internal standard.

(IS) 2-ethyl-3-(3,5-dibromo-4-β-dipropylaminopropoxybenzoyl)benzothiophene (Fig. 1) were generously supplied by Labaz (Brussels, Belgium). Pooled serum from human volunteers receiving no medication was used in the preparation of standards.

Extraction procedure

A stock solution of IS, 100 μg/ml in methanol, was prepared and kept at 4°C to minimize evaporation. All solutions were handled with glass capillary micropipettes. For serum extraction 10 μl of IS stock solution was placed in the bottom of a 16 × 100 mm borosilicate tube. To each tube 0.2 ml of 3 *M* sodium acetate buffer (pH 4.8) and 0.5 ml serum were added. This was mixed briefly followed by the addition of 5 ml of hexane. Each tube was then vortexed vigorously for 2 min and centrifuged at 7000 *g* for 1 min. The upper hexane layer was removed with a pipette, placed in a 10-ml pear-shaped flask and evaporated to dryness under reduced pressure. The residue was redissolved in 65 μl of mobile phase with vigorous shaking. From this a sufficient quantity (30—40 μl) was recovered to load a 25-μl injector loop.

Mobile phase

The mobile phase consisted of methylene chloride—hexane—methanol—glacial acetic acid—0.117 *N* perchloric acid in methanol (50:39:6:1:1, v/v). This solution was made daily and placed in an ice water bath during use to prevent bubbles from forming in the pump inlet tubing. Column temperature was maintained at 30°C with a heating block.

Calculations

Peak areas of standards and unknowns were normalized by dividing the amiodarone (A) peak area by the IS peak area for each sample. For the pharmacokinetic study sample concentrations were calculated by comparison of their A:IS peak area ratios to that of a single standard serum containing a known amount of amiodarone. The full equation was:

$$C_u = \frac{A_u}{A_{IS}} \times \frac{A_{IS}}{A_{std}} \times C_{std}$$

where C_u and C_{std} are the concentrations of the unknown and the standard serum respectively and A_u, A_{IS} and A_{std} are the peak areas of the unknown, IS and standard serum respectively.

Kinetic parameters were calculated [9] with a non-linear regression computer program (NONLIN [10]) linked with a pharmacokinetic analysis program (AUTOAN [11]) as follows:

$AUC_{0\to\infty}$ = area under the serum concentration vs. time curve extrapolated to infinity (trapezoidal rule)

β = slope of the last linear phase of the log blood concentration vs. time plot

$t_{½\beta}$ = half-life of β-phase = ln 2/β

V_d = volume of distribution = $\frac{\text{Dose}}{AUC_{0\to\infty} \times \beta}$

$$Cl_{bl} = \text{blood clearance} = \frac{\text{Dose}}{AUC_{0\to\infty}}.$$

RESULTS

Sample collection

Blood was collected from normal volunteers and from patients receiving amiodarone as part of their anti-arrhythmic therapy. In collecting these samples, it was discovered that the use of tubes containing a gelatin to separate serum from cells resulted in a significant decrease in the recovery of amiodarone from the sample. When blood from three individuals was divided between serum separator tubes (Corvac®, Sherwood Medical, St. Louis, MO, U.S.A.) and plain glass tubes without the serum separator, the recovery of drug in the former was only 67% of the latter (n=3). In all the studies described herein, only plain glass tubes were employed for sample collection.

Extraction

Methylene chloride, ethyl acetate, chloroform and hexane were investigated as extraction solvents. All but hexane were found to extract a number of serum components which interfered with the detection of amiodarone, its metabolite and/or IS at 248 nm. Hexane extracted no interfering peaks with absorbance at 248 nm. When the extraction solvent was buffered at pH 4.8, nearly quantitative recovery of amiodarone was observed. Amiodarone added to serum at final concentrations of 0.2, 2.0 and 20 μg/ml resulted in one-step extraction efficiencies of 90, 94 and 99%, respectively. These studies were repeated for the internal standard with similar results. No information concerning the recovery of metabolite could be derived owing to the small amount obtainable by the procedure described.

High-performance liquid chromatography of amiodarone

The use of the NH_2-10-alkylamine column permitted the separation of the IS, amiodarone and the N-deethylated metabolite of amiodarone without chromatographic interference. Typical chromatograms of amiodarone, the metabolite and the IS are shown in Fig. 2. The same column has been in use for more than one year, during which period there have been no changes in pressure requirements and no loss of resolution. Droplets of polar solvents from other users of the same chromatograph do not affect retention volumes or peak widths, but do release polar compounds from the column which temporarily interfere with the assay.

The heater block was employed to maintain the column at 30°C in order to avoid minor fluctuations in retention due to changes in ambient air temperature. Finally, a wavelength of 248 nm was chosen for monitoring the column effluent rather than 243 nm, the absorption maximum for amiodarone (unpublished data), because our preliminary studies revealed that methylene chloride, a major component of the elution solvent, absorbs significantly at 243 nm. The 248-nm wavelength resulted in a more favorable signal-to-noise ratio.

The elution solvent, composed of methylene chloride, hexane and methanol,

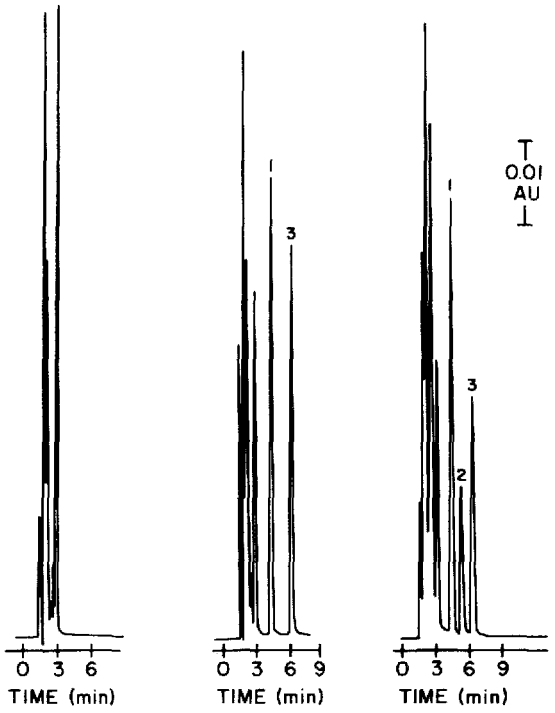


Fig. 2. HPLC of amiodarone and its metabolite. Serum was obtained from a patient prior to (left), during initial (center) and after chronic (right) therapy. Extraction and chromatography were performed as described under Experimental except no IS was added to the prior to therapy sample to demonstrate the absence of interfering peaks in the region of the IS. Peaks: 1 = internal standard, IS; 2 = metabolite; 3 = amiodarone.

was found to separate the compounds of interest from each other and from other serum peaks when acetic acid was added to this mixture to reduce peak tailing and perchloric acid was added as an ion-pairing agent.

Interference studies

Interference studies were performed as follows: A number of commonly used therapeutic agents were dissolved in elution solvent and injected onto the column. The results are shown in Table I. The agents with retention volumes which might have interfered with the assay (furosemide, propranolol, theophylline and caffeine), were then added to pooled human serum and subjected to the extraction and chromatographic procedures described. No peaks were found which would interfere with the determination of amiodarone, its N-deethylated metabolite or the IS.

The lack of endogenous or exogenous interference was confirmed by examining the sera of eight volunteers receiving no medication and multiple cardiac patients receiving a variety of cardiac and non-cardiac drugs including lidocaine, procainamide, quinidine sulfate, furosemide, propranolol, digoxin, hydrochlorothiazide, isosorbide dinitrate, thyroxin and diazepam. No interfering peak was encountered.

TABLE I

RELATIVE RETENTION OF COMMON THERAPEUTIC AGENTS AND AMIODARONE

Compounds were dissolved in HPLC elution solvent and 25 μl were injected onto the chromatographic column. V = Retention volume of compound; V_0 = retention volume of elution solvent.

Compound	Capacity factor (k') $\frac{V - V_0}{V_0}$	Separation factor (α) $\frac{k'_{\text{compound}}}{k'_{\text{amiodarone}}}$
Amiodarone	2.63	1.00
N-Deethylated amiodarone	2.05	0.78
Internal standard (IS)	1.59	0.60
Caffeine	1.84	0.70
Chlorpromazine	4.56	1.73
Digoxin	>10.00	>4.00
Disopyramide	>10.00	>4.00
Furosemide	2.21	0.84
Lidocaine	4.29	1.63
Phenobarbital	0.88	0.33
Phenytoin	0.96	0.37
Prednisolone	5.22	1.98
Prednisone	3.53	1.34
Procainamide	>10.00	>4.00
Propranolol	2.65	1.01
Quinidine	>10.00	>4.00
Theophylline	2.74	1.04

Methodologic verification

In order to determine the stability and sensitivity of the assay, amiodarone solutions at concentrations of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 μg/ml were prepared by adding known quantities of drug in methanol to pooled drug-free human serum. The final methanol concentration never exceeded 1% (v/v) in any sample. At least two of these standards plus a serum blank were run on each analysis day for a total of eleven days over 1.5 months. From these data, a standard curve was constructed each day by plotting the ratio of the amiodarone peak to the IS peak versus amiodarone concentration. The straight line obtained could be described by the equation: $y = 0.84x + 0.07$, where the average relative standard deviation for the slope was 4% and the average regression coefficient (r^2) had a value of 0.9997. The standard deviation of multiple determinations of a single sample performed on the same day was 3.2% (n=14). The assay was found to be linear over a sample concentration range of 0.05—20.0 μg/ml. Serum samples frozen at —20°C remained stable for at least seven months.

Clinical studies

We developed this assay for amiodarone in serum to investigate the pharmacology of this clinically important agent. To determine the utility of our assay for such work, a pilot pharmacokinetic study was performed.

The single-dose kinetics of amiodarone were studied in five healthy male volunteers ages 24—57 years. Amiodarone, 5 mg/kg, was administered as an

TABLE II

SINGLE DOSE PHARMACOKINETIC PARAMETERS IN HEALTHY VOLUNTEERS

Results of a pharmacokinetic study of five healthy male volunteers. A single dose of amiodarone, 5 mg/kg, was infused over 15 min and serum sampled at designated intervals for 24—48 h. β = Slope of the last linear phase of the log blood concentration vs. time plot; $t_{½\beta}$ = half-life of the β phase; V_d = volume of distribution per kg; AUC = area under the serum concentration vs. time plot; Cl_{bl} = blood clearance; $\bar{X}$ = mean; S.D. = standard deviation.

Subject No.	β (h^{-1})	$t_{½\beta}$ (h)	V_d (l/kg)	AUC (μg/ml h)	Cl_{bl} (ml/min kg)
1	0.066	10.46	5.40	14.00	5.94
2	0.046	14.94	4.90	22.26	3.76
3	0.058	11.95	10.80	8.00	10.44
4	0.080	8.66	5.95	10.50	7.94
5	0.066	10.57	4.76	15.93	5.24
$\bar{X}$	0.063	11.32	6.36	14.14	6.66
S.D.	0.012	2.34	2.52	5.50	2.59

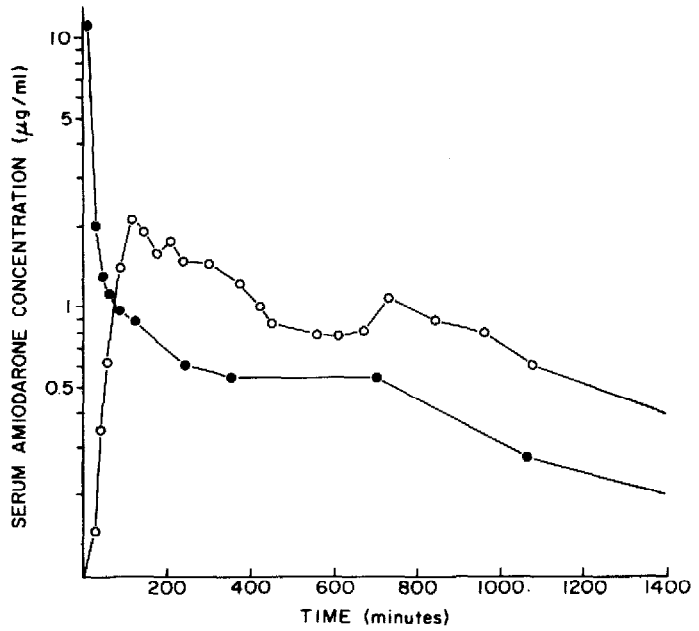


Fig. 3. Single dose intravenous and oral pharmacokinetic study in a normal volunteer. A 34-year-old white male received amiodarone, 5 mg/kg intravenously (●—●) and several months later was redosed with a single 1400-mg oral dose (○—○). Each point represents the average of duplicate determinations of each sample.

intravenous infusion over 15 min. During the next 24—48 h, 15—20 serum samples were obtained at designated intervals from a heparin lock placed in the arm opposite that used for drug infusion. The data fit a two-compartment open pharmacokinetic model [11] with an r value of 0.997. The results of this study are shown in Table II.

The pharmacokinetic evaluation was repeated several months later in one volunteer following a single oral dose of 1400 mg. The elimination half-life ($t_{½\beta}$) was within 1% of that observed after intravenous administration (Fig. 3). The bioavailability of the oral preparation (Cordarone®, Labaz) was 0.48.

DISCUSSION

The method we present here has been used in our laboratory for over 16 months and is rapid, accurate and stable.

Although other methods have been reported [12—16] they have certain disadvantages when compared to the method presented here. Riva et al. [12] and Cervelli et al. [13] reported methods using extraction steps which require more than 30 min. The method reported by Andreasen et al. [14] uses no internal standard. The internal standards used by Riva et al. [12] and Flanagan et al. [15] are chemically dissimilar from amiodarone. Finally, the method reported by Lesko et al. [16] utilizes a silica column which has the chromatographic stability and maintenance problems common among unbounded column materials.

The method reported above has an extraction step which takes only 2 min to complete, utilizes an internal standard very similar to amiodarone and employs a NH_2-alkylamine column. This bonded phase column has proved resistant to the effect of water and other polar solvents which are used in reversed-phase assays performed on the same chromatograph.

For these reasons we feel this method is a valuable alternative to those currently available and should facilitate the pharmacologic investigation of this important agent.

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After initial pre-treatment of a new column with sequential washes of methylene chloride, isopropanol, water, isopropanolol, methylene chloride and hexane, 48 h of continuous mobile phase flow at 0.5 ml/min were required to achieve the chromatographic conditions described.

REFERENCES

1 M.B. Rosenbaum, P.A. Chiale, M.S. Halpern, G.J. Nau, J. Przybylski, R.J. Levi, J.O. Lazzari and M.V. Elizari, Amer. J. Cardiol., 38 (1976) 934.
2 D. Leak and J.N. Eydt, Arch. Intern. Med., 139 (1979) 425.

3 M.B. Rosenbaum, P.A. Chiale, D. Ryba and M.V. Elizari, Amer. J. Cardiol., 34 (1974) 215.
4 A. Jouve, Colloque Sur L'Amiodarone, Labaz Laboratories, Paris, November 21, 1977.
5 K. Nademanee, J.A. Hendrickson, D.S. Cannom, B.N. Goldreyer and B.N. Singh, Amer. Heart J., 101 (1981) 759.
6 J.C. Kaski, L.A. Girotti, H. Messuti, B. Rutitzky and M.B. Rosenbaum, Circulation, 64 (1981) 273.
7 L. Rakita and S.M. Sobol, J. Amer. Med. Assoc., in press.
8 J.L. Blumer, J. Schaffer, D.L. Noon and N.D. Mostow, in preparation.
9 J.G. Wagner, Fundamentals of Clinical Pharmacokinetics, Drug Intelligence Publications, Washington, DC, 1973.
10 C.M. Metzler, G.L. Elfring and A.J. McEwen, A User's Manual for NONLIN and Associated Programs, The Upjohn Company, Ann Arbor, MI, 1980.
11 A.M. Sedman and J.G. Wagner, AUTOAN Manual, Upjohn Center for Clinical Pharmacology, Univ. of Michigan Medical Center, Ann Arbor, MI, 1980.
12 E. Riva, M. Gerna, R. Latini, P. Giani, A. Volpi and A. Maggioni, J. Cardiovasc. Pharmacol., 4 (1982) 264.
13 J.A. Cervelli, J. Kerkay and K.H. Pearson, Anal. Lett., 14 (1981) 137.
14 F. Andreasen, H. Ayerbaek, P. Bjerregaard and H. Gotzsche, Eur. J. Clin. Pharmacol., 19 (1981) 293.
15 R.J. Flanagan, G.C.A. Storey and D.W. Holt, J. Chromatogr., 187 (1980) 391.
16 L.J. Lesko, A. Marion, A.T. Canada and C. Haffajee, J. Pharm. Sci., 70 (1981) 1366.