High-Pressure Liquid Chromatography of Amiodarone in Biological Fluids

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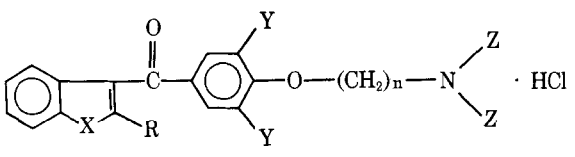
Abstract □ A rapid, sensitive, and specific method of analysis for amiodarone in serum, urine, and tissue by high-pressure liquid chromatography is described. Sample preparation includes acidification with two volumes of 0.2 *M* acetate buffer followed by extraction into five volumes of hexane. The organic solvent is dried, and the residue is reconstituted in mobile phase and injected into the chromatograph. Separation is obtained using a silica column and a chloroform–methanol–ammonia mobile phase. Detection at 254 and 280 nm showed the assay to be specific in the presence of other commonly administered drugs. Levels of 0.05 μg/ml can be measured with a precision of ±15%; but with appropriate modification of the procedure, levels as low as 0.025 μg/ml can be measured with suitable precision. The inter- and intraday coefficient of variation for replicate analysis of spiked serum samples is <5%. This method is useful for single- and multiple-dose pharmacokinetic studies of amiodarone in animals and humans.

Keyphrases □ Amiodarone—high-pressure liquid chromatography in biological fluids □ High-pressure liquid chromatography—amiodarone in biological fluids □ Vasodilator—amiodarone, high-pressure liquid chromatography in biological fluids

Amiodarone[1] (I), a benzofuran derivative, is structurally similar to thyroxine. It is an effective antiarrhythmic agent for the treatment of supraventricular and ventricular tachyarrhythmias (1, 2). The pharmacokinetics of amiodarone have been studied in animals and humans following the administration of radiolabeled amiodarone and the measurement of total radioactivity in serum and urine (3). However, because of the nonspecificity of total radioactivity measurements, a sensitive and specific method of analysis for unlabeled amiodarone is needed. Flanagan *et al.* (4) described a high-pressure liquid chromatographic (HPLC) method for the measurement of amiodarone in plasma or serum with a sensitivity of 0.05 μg/ml, but they did not indicate if the assay was sensitive enough for single-dose pharmacokinetic studies. One disadvantage of their method is the use of a mobile phase containing 15 parts of ether. Its volatility may cause chromatographic variations.



I: X = O, R = C_4H_9, Y = I, n = 2, Z = C_2H_5
II: X = S, R = C_2H_5, Y = Br, n = 3, Z = C_3H_7

[1] Cordarone, Labaz Laboratories, Ambares, France.

The present report describes a rapid, sensitive, and specific HPLC technique for amiodarone in serum, urine, or tissues. Because of a concentrating step during sample preparation, a sensitivity of 0.025 μg/ml with acceptable precision was obtained. This method was used to measure amiodarone in biological fluids of dogs and humans in single- and multidose pharmacokinetic studies and is acceptable for postmortem tissue analysis.

EXPERIMENTAL

Reagents and Chemicals—Amiodarone hydrochloride[2] and the internal standard[2] (II), a benzothiophene derivative of amiodarone, were used as received. Chloroform[3], methanol[3], and hexane were HPLC grade. All other reagents and solvents were analytical grade quality and were used as received.

Amiodarone Standard—A stock solution of amiodarone (1.0 mg/ml) was prepared by dissolving 10.6 mg of amiodarone hydrochloride in 10.0 ml of methanol. Serum standards were prepared by spiking blank control serum with appropriate microliter volumes of the stock solution to obtain five serum standards with the following concentrations of amiodarone: 0.125, 0.25, 0.50, 1.0, and 2.0 μg/ml.

Internal Standard—A stock solution of the internal standard (1.0 mg/ml) was made by dissolving 10.0 mg in 10.0 ml of methanol. An internal standard working solution was prepared by dilution of an aliquot of the stock solution with acetate buffer to contain 0.5 μg/ml.

Acetate Buffer—A 0.2 *M* acetate buffer was prepared by mixing 10.9 parts of Solution A (27.2 g of hydrous sodium acetate/liter) with 89.1 parts of Solution B (11.5 ml of acetic acid/liter). The pH of the buffer was adjusted to 3.8 by dropwise addition of either acetic acid or 1 *N* NaOH.

Instruments and Chromatographic Conditions—A microparticulate, stainless steel, normal-phase column[4], 4.0 mm × 30 cm, at ambient temperature was used for separation. The column was used with a septumless injector[5], a solvent delivery system consisting of a dual-piston pump[6], and a dual fixed-wavelength UV absorbance detector[7] equipped with 254- and 280-nm filters. The eluting solvent was chloroform–methanol–ammonium hydroxide (98.95:1.0:0.05) at a flow rate of 0.8 ml/min. A dual-pen recorder[8] with a range of 0–10 mv was used with a chart speed of 0.5 cm/min. Peak heights were measured to the nearest half-millimeter with a metric ruler.

Prior to analysis, the chromatographic system was stabilized for 10–20 min and 20 μl of a standard test mixture of amiodarone (1 μg/ml) and the internal standard (1 μg/ml) in the mobile phase was injected into the chromatograph. A response factor was calculated from the resulting peak heights to confirm accurate operation.

Sample Preparation—To a disposable screw-capped glass culture tube (16 × 125 mm) were added 0.5 ml of serum or urine and 1.0 ml of the internal standard working solution. The sample was extracted with 5.0 ml of hexane by gentle shaking on a horizontal shaker[9] for 10 min. For tissue analysis, 1.0 ml of tissue homogenate, prepared by homogenizing 1.0 g of blotted tissue in 10.0 ml of distilled water, was mixed with 2.0 ml

[2] Labaz, Centre de Recherche, Brussels, Belgium.
[3] Burdick & Jackson Laboratories, Muskegon, Mich.
[4] μPorasil, Waters Associates, Milford, Mass.
[5] Model U6K, Waters Associates, Milford, Mass.
[6] Model 6000A, Waters Associates, Milford, Mass.
[7] Model 440, Waters Associates, Milford, Mass.
[8] Omniscribe, Houston Instruments, Austin, Tex.
[9] Eberbach Corp., Ann Arbor, Mich.

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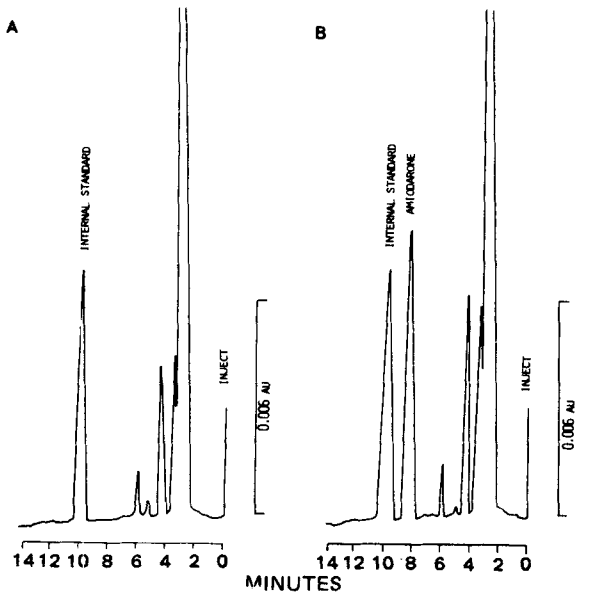

Figure 1—*Representative high-pressure liquid chromatogram of blank human serum (A) and human serum containing amiodarone, 1 μg/ml (B).*

of internal standard working solution and extracted with 10.0 ml of hexane. The organic phase (upper) was separated by centrifugation at 1600 × *g* for 5 min and transferred to a clean, disposable, glass test tube. The hexane was evaporated at 60° under a gentle nitrogen stream. The residue was reconstituted in 50 μl of mobile phase by vortex mixing[10], and 20 μl was injected into the liquid chromatograph. The concentration of amiodarone in the serum was determined from a calibration curve of peak height ratio (amiodarone to internal standard) *versus* amiodarone concentration in serum standards carried through this procedure.

RESULTS AND DISCUSSION

Chromatograms obtained from blank human serum and serum spiked with amiodarone are shown in Fig. 1. The peaks representing amiodarone and the internal standard are symmetrical and well removed from the solvent front and extraneous serum peaks. The retention times of amiodarone and the internal standard are 8.2 and 10.0 min, respectively. Some control of retention times is possible by varying the proportion of ammonium hydroxide in the mobile phase. Increasing amounts of ammonium hydroxide decrease the retention times of both compounds. Reducing the amount of ammonium hydroxide increases retention times and results in notable peak tailing. Although the ratio of methanol in the mobile phase does not significantly alter retention times, a small quantity of methanol is useful in modifying the silica stationary phase and facilitating reproducible separation and peak symmetry.

Calibration curves of peak height ratio *versus* concentration were obtained by analyzing serum standards containing amiodarone in concentrations ranging from 0.05 to 2.0 μg/ml. Calibration curves were prepared using two different wavelengths for detection, 254 and 280 nm. (Detection at two wavelengths is a simple way to validate that there is only one solute under the peak of interest eluting from the column.) The calibration curves at both wavelengths were linear over the concentration range studied. The least-squares linear regression line representing the best fit of the amiodarone data at 254 nm had a slope of 1.69 and a *y* intercept (where *y* = peak height ratio) of −0.04 (r = 0.999). The corresponding line at 280 nm had a slope of 1.01 and a *y* intercept of −0.03 (r = 0.999). Although assay sensitivity is better at 254 nm, the assay is operated routinely at 280 nm. At this wavelength, detector noise is considerably less and solvent transparency to the detector is better, resulting in a more stable baseline and shorter equilibration time.

Assay precision was determined by repeated analysis of spiked serum samples containing low (0.25 μg/ml) and high (1.0 μg/ml) concentrations of amiodarone. The mean concentration, standard deviation, and coefficient of variation for intra- and interday analysis are shown in Table I. The within-day and day-to-day variation at each concentration was <5%.

[10]Vortex-genie, Scientific Industries, Springfield, Mass.

Table I—Precision Data for the Determination of Amiodarone in Serum

Concentration, μg/ml	Intraday			Interday		
	n	Mean ± *SD*, μg/ml	*CV*, %	*n*	Mean ± *SD*, μg/ml	*CV*, %
1.0	5	0.993 ± 0.026	2.62	10	0.951 ± 0.040	4.21
0.25	5	0.230 ± 0.006	2.61	10	0.225 ± 0.011	4.89

The analytical recovery of amiodarone from serum was determined by comparing peak heights of amiodarone obtained by analyzing extracted, spiked serum specimens to the peak heights obtained by direct injection of methanolic solutions of amiodarone containing an amount of amiodarone equivalent to the amount in the spiked serum specimens. The mean ± *SD* recovery of amiodarone was 81.9 ± 5.0% (n = 10) at a concentration of 1.0 μg/ml and 79.7 ± 2.5 (n = 3) at a concentration of 0.25 μg/ml.

The sensitivity of this method is 0.05 μg/ml using 0.2 ml of serum samples and 0.01 aufs. The intraday coefficient of variation for spiked serum samples containing 0.05 μg/ml was ±13.0% (n = 5). Lower concentrations of amiodarone may be detected with an analytical error of <±15% by increasing serum volume, detector attenuation, or injection volume. Detection at 254 nm would also increase sensitivity if necessary. Amiodarone concentrations of 0.025 μg/ml in serum were easily measurable by including such modifications in the procedure.

The described method for amiodarone analysis in serum has been used in single-dose pharmacokinetic studies and for monitoring serum amiodarone concentrations in patients receiving long-term amiodarone therapy. A typical serum amiodarone concentration–time profile obtained in two patients after administration of a single 800-mg oral loading dose is shown in Fig. 2. Amiodarone appears to be absorbed relatively slowly and disappears rapidly from the serum, at least following the first dose. Detailed studies of amiodarone pharmacokinetics in dogs and humans are being conducted.

The present assay uses a silica column and a nonaqueous mobile phase. Although bonded, reversed-phase systems using aqueous mobile phases are preferred by many chromatographers for the analysis of drugs in serum, a normal-phase, liquid–solid chromatographic system is useful for such drugs as amiodarone, cimetidine, trimethoprim, and tricyclic antidepressants. The mobile phase requirements are usually similar: a pure, organic solvent such as chloroform or methylene chloride of intermediate polarity similar to the solute; a small proportion of a miscible, more polar solvent, such as methanol or isopropanol, to modify the adsorbent activity of the stationary silica phase; and a small amount of base to adjust the ionization state of the solute and to minimize peak tailing. The amiodarone assay has been used for over 3 years and has proven to be simple, reliable, and accurate. Column life is often as long as 1 year or over 500 injections. The base in the mobile phase has not caused any apparent deterioration in column efficiency.

A single-step solvent extraction procedure is used to separate amiodarone from serum and to concentrate it. An acidic pH is optimal for the extraction. Organic solvents useful for extraction of amiodarone from serum include ether, methylene chloride, chloroform, and hexane. Hexane provides the cleanest extract and no back-extraction is required. Pretreatment of the serum before injection is rapid, and as many as 20–30 serum samples/day can be analyzed by one technician.

No endogenous substances or drugs have been found to interfere with

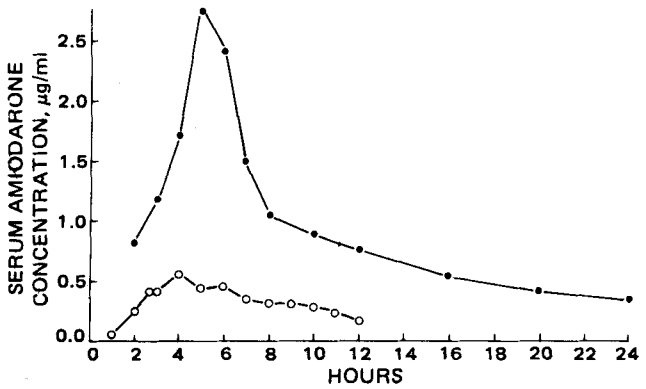

Figure 2—*Serum amiodarone concentrations in two patients after an 800-mg oral loading dose. Key:* ○, *Patient 1; and* ●, *Patient 2.*

amiodarone analysis using this procedure. The assay has been used for patients receiving amiodarone in combination with drugs such as procainamide, quinidine, lidocaine, disopyramide, digoxin, prednisone, furosemide, and other thiazide diuretics.

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Influence of the Method of Application on Pharmacokinetics of Nitroglycerin from Ointment in Humans

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Received April 6, 1981, from the **Bureau of Drug Research, Health Protection Branch, Health and Welfare Canada, Ottawa, Ontario, Canada K1A 0L2*, and the ‡*Drug Consultation Services, Ottawa General Hospital, and Department of Pharmacology, University of Ottawa, Ottawa, Ontario, Canada K1H 8L6.* Accepted for publication June 2, 1981.

Abstract □ A study was designed to test the influence of surface area on the percutaneous absorption of nitroglycerin from a commercial ointment formulation, using a simple crossover design. On separate occasions, three volunteers were given 16 mg of nitroglycerin (2%) over a 25- and 100-cm^2 area. Plasma nitroglycerin concentration was measured at 30, 45, 60, and 90 min using a sensitive capillary GLC–electron-capture detection method capable of quantitating to 150 pg/ml. Plasma concentrations at all times increased at least twofold with the increased surface area; highest observed concentrations were 0.17 and 0.41 ng/ml, respectively. A fourth volunteer received 16 and 32 mg of nitroglycerin over 100 cm^2. Doubling the dose increased the 0–90-min *AUC* by only 76% but caused a 3.5-fold increase in the 90-min plasma concentration. These results suggest that the surface area of application significantly influences the pharmacokinetics of nitroglycerin ointment.

Keyphrases □ Nitroglycerin—ointments, effect of method of application on pharmacokinetics, humans □ Ointments—nitroglycerin, effect of method of application on pharmacokinetics, humans □ Pharmacokinetics—effect of method of application on nitroglycerin ointments, humans □ Vasodilators—nitroglycerin ointments, effect of method of application on pharmacokinetics, humans

Nitroglycerin (glyceryl trinitrate), a vasodilator, is used extensively in the treatment of angina, cardiac infarction, and other circulatory conditions (1). However, because of its rapid elimination (2, 3), sustained-release or percutaneous dosage forms were developed. It was recently shown (4) that the ointment dosage form is more bioavailable than a sustained-action oral capsule formulation, and evidence was presented (5) that blood concentrations obtained after ointment application were dose dependent. However, neither study examined the influence of surface area on percutaneous absorption. It was also demonstrated (6) that the amount of nitroglycerin absorbed through the skin of a rhesus monkey was dependent on the surface area (but not the anatomical area) of application; however, the importance of this finding may not be generally recognized in the everyday use of this dosage form.

The present study investigated the influence of the surface area of application of the ointment on nitroglycerin bioavailability.

EXPERIMENTAL

Three healthy adult male volunteers were each given 650 mg of acetaminophen orally (to control possible side effects), followed by 16 mg of nitroglycerin as a 2% ointment[1]. In the first part of the experiment ("small area"), the ointment was spread on paper[2] cut to 25 cm^2, and the paper was applied over the chest near the sternum and covered with an adhesive bandage. In the second part ("large area"), performed 2 months later on the same volunteers, an identical amount of nitroglycerin ointment was spread over 100 cm^2 in the same region of the chest, outlined on the skin with the help of a template. The ointment was applied as uniformly as possible with a stainless steel spatula and immediately occluded with a sheet of aluminum foil attached with adhesive tape. A fourth volunteer received 16 and 32 mg of nitroglycerin ointment (2%) spread over 100 cm^2 as described for the large area experiment.

Blood (10 ml) was collected by venipuncture, using an all-glass syringe, at the times indicated in Tables I and II, and dispensed at once into a chilled glass tube containing 0.3 ml of heparin sodium (300 U/per tube) and 50 μl of 0.002 *M* $AgNO_3$ (7). The contents were mixed by inversion, centrifuged immediately in the cold (5 min, 2000 rpm, 4°), and the plasma was separated for the nitroglycerin assay.

The assay method[3] consisted of mixing 1 or 2 ml of plasma with 100 μl of 1 *M* $AgNO_3$ (8), extracting with 10 ml of redistilled pentane containing 3 ng of *o*-dinitrobenzene[4], and evaporating the solvent in an ice bath with a gentle nitrogen stream. The residue was redissolved in 25 μl of redistilled hexane, and 4 μl of this solution was chromatographed on a capillary GLC system[5] equipped with an electron-capture detector[6], using a 25-m methyl silicone gum-coated open tubular column[7]. The chromatographic conditions were carrier gas, helium (6-ml/min septum purge, 2-ml/min column flow); makeup gas, argon–methane (95:5, 30 ml/min); injection, splitless, at 200°, 30-sec delay; oven, programmed temperature from 80 to 130° at 30°/min; detector, 150°; and retention

[1] Nitrol, Kramer-Urban, Milwaukee, Wis.
[2] Appli-Ruler, provided by the manufacturer as package insert.
[3] To be described in detail elsewhere.
[4] RotoRack, Fisher Scientific, Ottawa, Canada. Extraction at 15 rpm, 20 min.
[5] Hewlett-Packard model 5730A with model 18740B capillary inlet system.
[6] Hewlett-Packard model 18713A linear electron-capture detector.
[7] Hewlett-Packard model SP 2100.

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