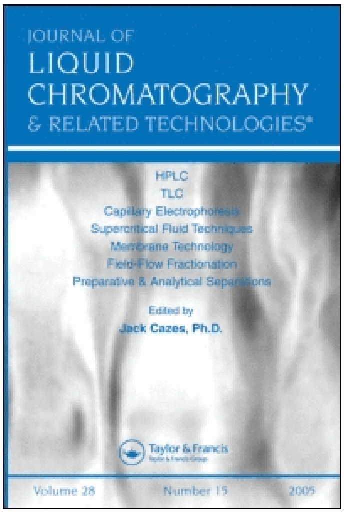

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QUANTITATION OF AMIODARONE AND DESETHYLAMIODARONE FROM BLOOD SERUM AND MYOCARDIUM USING REVERSE PHASE HPLC

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ABSTRACT

A new method for quantitating amiodarone and its metabolite desethylamiodarone from serum and myocardial tissue is described. Serum and tissue components are initially removed before analysis with C_{18} solid phase extraction columns. Quantitation is then achieved using a simple step gradient and a C_{18} reverse phase column. Percent recovery of amiodarone is greater than 90 percent in both serum and myocardial tissue and is linear throughout the therapeutic range with a lower limit sensitivity of .04 μg/ml. No commonly used cardiovascular drugs were found to interfere with the assay.

INTRODUCTION

Amiodarone hydrochloride, a benzofuran derivative, is currently the most potent drug available for the control of life threatening cardiac dysrhythmias. First developed as an anti-anginal agent, amiodarone was found to be a class III antiarrhythmic capable of controlling 80 percent of patients with chronic supraventricular tachycardias resistant to other medications.[1-4,7,8] Recently a variety of side effects have been identified including pulmonary fibrosis and thyrotoxicosis. Because of these dose-related adverse effects, routine monitoring of patient serum concentrations is useful clinically.[4,1,3,6] Amiodarone (Ami) and its major metabolite desethylamiodarone (Dea) are difficult to quantitate by reverse phase High Performance Liquid Chromatography (HPLC) because of their similar structures and lipophilic nature. Several methods for the quantitation of Ami and Dea exist.[9-19] However, most of these methods do not eliminate interfering peaks resulting from extracted endogenous compounds. The presence of these compounds on chromatograms makes the quantitation of Ami and Dea inaccurate. By removing these compounds before analysis, the following reverse phase HPLC method quantitates Ami and Dea with excellent resolution in serum or tissue without interference from extracted endogenous compounds.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

Pure amiodarone HCl, desethylamiodarone HCl, and Internal Standard (L-8040, 2-ethyl-(3,5-dibromo-4-β-dipropylamiopropoxybenzoyl) benzothiopene) were obtained from Sanofi (Labaz Laboratories, France). HPLC grade methanol, hexane, ammonium hydroxide, ammonium phosphate, and acetic acid were obtained from Fisher Scientific (Raleigh, N.C.).

CHROMATOGRAPHIC EQUIPMENT

A reciprocating pump (Model 510, Waters Assoc., Milford MA) running at a flow rate of 1.5 ml/minute in conjunction with a solvent selector (Waters Assoc. Milford MA) comprised the step gradient solvent delivery system. A Waters Intelligent Sample Processor (WISP), (Model 710b, Waters Assoc., Milford MA), was used for sample injection. A Nova Pak-A reverse phase column (C_{18}, 5μm, 8mm, ID, Waters Assoc.) operating at 175 bar made up the separation system. A programmable wavelength detector (Model 490, Waters Assoc.) monitored eluents at 254 nm and a data module (Model 730, Waters Assoc.) integrated the detected responses, providing peak areas and retention times for each sample.

SOLVENT PREPARATION

HPLC grade methanol was used as the first "step" of the solvent system. A solution consisting of methanol/hexane/H_2O/NH_4OH (90.65:8.0:1.2:0.15) and brought to pH 7.7 with glass distilled acetic acid was used as the second "step" of the solvent system (Ami sovent). All solvents were filtered and degassed using a Millipore Solvent Purification Kit with 0.45 μm disposable filters (Millipore Corp., Bedford, MA).

PREPARATION OF SERUM SAMPLES

Internal standard (L-8040), 100 μl of 10μg/ml in methanol, was added to 1.0 ml of blood serum in a 15 ml conical test tube. 3.0 ml of 0.067 M NH_4PO_4 buffer pH 5.4 was added.

PREPARATION OF TISSUE SAMPLES

Myocardial samples weighing 20 to 100 mg were homogenized in 1.0 ml of NH_4PO_4 buffer (0.067 M pH 5.4) which contained 100 µl of 01µg/ml internal standard (L-8040) in methanol. The homogenate was transferred to a 15.0 ml conical test tube using three 1.0 ml aliquots of NH_4PO_4 buffer to complete the transfer.

EXTRACTION OF SAMPLES

Six ml of 2.0 percent isopropanol in hexane were added to each sample and vortexed for 1 minute. Samples were centrifuged at 850g for 10 minutes and the organic layer was removed and placed in another 15 ml conical test tube. The aqueous phase was extracted once more with 6.0 ml of 2.0 percent isopropanol in hexane. The two organic layers were combined and the solvents evaporated using nitrogen gas at 40°C.

SOLID PHASE SAMPLE EXTRACTION

C_{18} Prep-Sep columns were conditioned by flushing with 10 column volumes of HPLC grade methanol. The previously extracted samples were reconstituted with 0.5 ml of methanol and pipetted onto the conditioned columns. The columns were then flushed with 3 column volumes of methanol. Ami, Dea, and internal standard were then eluted off the extraction columns using 2.0 column volumes of Ami solvent. The samples were then evaporated to dryness and reconstituted with 250 µl of methanol of which 100 µl were injected per run.

STANDARD PREPARATION

Standards were prepared by dissolving pure amiodarone, desethylamiodarone and internal standard in HPLC grade methanol.

Concentrations were verified using a UV-Vis spectrophotometer (Perkin-Elmer). These standards were then stored at 0.5° C until needed and were stable up to one year.

COLUMN EQUILIBRATION AND REJUVENATION

Before assays were performed, the HPLC column was flushed with 10 times the column volume of 100 percent methanol and then allowed to equilibrate by running standards using the step gradient solvent system. Reproducible response factors were usually obtained after the second standard run.

STEP GRADIENT FORMATION

Timing for the solvent switching device was designed in the following manner. Methanol was used to elute all serum compounds and other organic agents which might be present even after sample clean-up. Once baseline was again obtained, the system was switched to Ami solvent which elutes amiodarone, desethylamiodarone, and internal standard. After the metabolites were eluted, the system was switched back to methanol to equilibrate the column for the following run. Once the exact timing was determined for the individual steps, the data module was programmed to operate the solvent select valve for automated runs.

RESULTS

Figures 1-3 illustrate the chromatography obtained from serum and tissue extracts. Using this method Dea, Ami, and internal standard

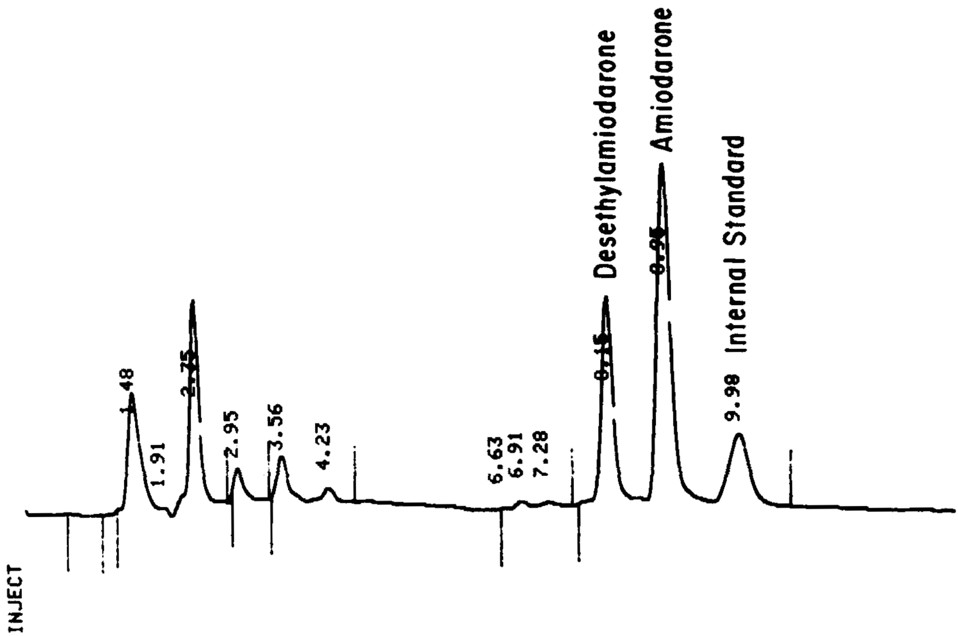

FIGURE 1. Serum Extraction of Amiodarone, Desethylamiodarone and Internal Standard.

have retention times of 8.15, 8.9, and 9.8 min respectively and are free of peaks resulting from extracted endogenous compounds. All retention times remain constant over long automated runs but can be changed by altering the timing of the methanol step gradient.

Ami (10μg/ml), Dea (10μg/ml), and internal standard (10μg/ml) in methanol were added to dog serum to attain serum concentrations of 0.25, 0.5, 1.0, 2.0, and 5.0 μg/ml (n=10 of each concentration). The three compounds were also added to myocardial tissue samples to achieve concentrations of 20.0, 40.0, and 120.0 μg/g. Verification that all of the added standards were absorbed by the myocardium was

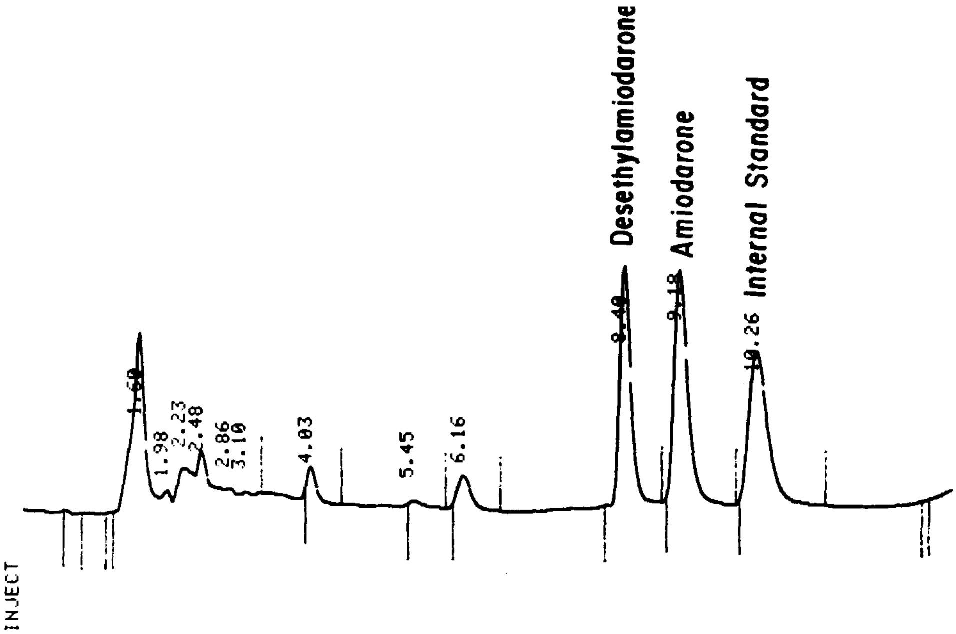

FIGURE 2. Tissue Extraction of Amiodarone, Desethylamiodarone and Internal Standard.

achieved by centrifugation of the homogenate and the testing of the supernatant for Ami, Dea, and internal standard. None of these compounds were detected in the supernatant thus confirming that the compounds had been absorbed by the tissue. All serum and tissue samples were extracted and percent recoveries determined. Recovery was determined by comparing the peak areas of amiodarone and desethylamiodarone to the internal standard peak area.

The mean percent recoveries of Ami and Dea were 95.2±1.2 and 79.4±1.9 in serum and 89.4±1.0 and 86.4±5.8 in tissue extracts with a lower limit of sensitivity of 0.4 μg/ml.

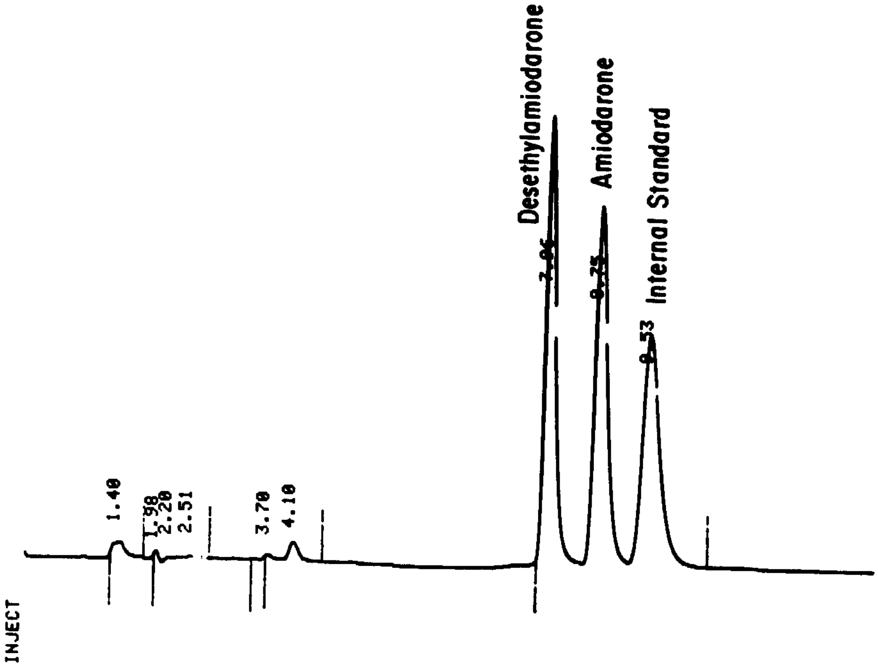

FIGURE 3. Standard Mixture of Amiodarone, Desethylamiodarone and Internal Standard.

TABLE 1

MEAN PERCENT RECOVERY OF AMIODARONE AND DESETHYLAMIODARONE FROM CANINE SERUM AND TISSUE
(n = 10 at each concentration)

SERUM

Concentration (μg/ml)	Recovery (% ± s.e.m.) Ami	Dea
0.25	93.6 ± 2.8	75.2 ± 5.4
0.50	98.6 ± 2.9	85.2 ± 2.7
1.00	92.3 ± 0.9	79.5 ± 3.3
2.00	97.3 ± 2.5	81.4 ± 3.3
5.00	94.1 ± 1.3	75.7 ± 3.7

TISSUE

(μg/g)		
20.0	91.0 ± 6.9	75.5 ± 10.1
40.0	89.5 ± 3.2	88.4 ± 2.4
120.0	87.6 ± 2.4	95.3 ± 2.9

Other commonly used drugs were extracted along with Ami and Dea in order to determine possible interferences. Table 1 lists the drugs and their retention times as found using this method. None of the drugs tested interfered with the quantitation of Ami, Dea, or internal standard.

DISCUSSION

Several methods for the quantitation of amiodarone and desethylamiodarone have been described. The need for a reverse phase HPLC method that would optimize quantitation and be reliable for long automated runs led to the development of this assay. By cleaning the samples of extracted endogenous compounds using solid phase extraction columns and a simple methanol step gradient, this method assures accurate quantitation of Ami and Dea from serum of tissue.

TABLE 2

RETENTION TIMES OF COMMONLY PRESCRIBED DRUGS OF PATIENTS RECEIVING AMIODARONE

Drug	Retention time in min.
Desethylamiodarone	8.20
Amiodarone	8.90
Internal Standard (L-8040)	9.98
Lasix	1.44
Propranolol	6.48
Disopyramide	6.34
Captopril	1.42
Verapamil	6.24
Diltiazem	3.45
Quinidine Sulfate	6.39
Procainamide	6.19
Nifedipine	2.07
Labetalol	6.19
Tocainide	6.16
Coumadin	1.44

Gel-permeation chromatography was first used as a means of solid phase extraction and gave good results as a means of eliminating serum components. However the technique was found to be too time consuming for multiple samples. The use of a C_{18} preparatory column eliminates most extracted serum and tissue components which might interfere with analysis. Pollak, et al[19] developed a method which utilizes a CN packing in which small serum samples are loaded and extracted. However, these columns require a great deal of column equilibration and can only facilitate small sample volumes making them impractical for extracting tissue and serum samples with low Ami and Dea concentrations. Therefore, by using methanol to wash out interfering compounds and amiodarone mobile phase to elute Dea, Ami, and internal standard, a C_{18} preparatory column makes an ideal solid phase extraction system that requires a short equilibration time and sufficiently rids the samples of most endogenous compounds.

The methanol step gradient further ensures exact quantitation of Ami and Dea. Once a sample is injected onto the system, methanol elutes any remaining endogenous compounds while Ami, Dea and internal standard are retained. Only when the amiodarone mobile phase is used are the three compounds separated and eluted. Thus, the step gradient further eliminates the risk of interfering compounds and makes it possible to alter the retention times at which Dea, Ami, and internal standard will be eluted.

An Ammonium phosphate buffer of pH 5.4 is ideal for extracting Dea from blood serum and tissue[12]. This buffer is also instrumental in the homogenization of tissue samples. While other investigators found mechanical homogenization ineffective[19], we found that homogenization of the tissue in pH 5.4 phosphate buffer enables the extraction of Ami and Dea thus eliminating the need for timely enzymatic digestion.

Several extraction solvents were investigated including hexane, cyclohexane, and diethyl ether. Two percent isopropanol in hexane gave the highest extraction percentages and was chosen as the extraction solvent. A single organic extraction was also investigated but was inadequate for completely extracting Ami, Dea, and internal standard. Therefore two organic extractions are utilized.

The use of ammonium hydroxide to elute amiodarone is well documented.[10,17,16,14,12] Yet the pH of these hydroxide-containing mobile phases are either very high, thus jeopardizing column life, or are not adequate for the separation of Dea from Ami. Using a small amount of acidic acid to bring the pH of the mobile phase to pH 7.7 optimizes separation and resolution without jeopardizing column life with excessively high pH.

Utilizing the preparatory extraction columns and the step gradient insures that the samples will be free of any endogenous compounds. This method provides accurate quantitation of amiodarone and desethylamiodarone in both serum and tissue samples by combining the sensitivity of reverse phase HPLC with the selectivity of preparatory columns and step-gradient elution. Furthermore, by reducing column build-up of extraneous compounds, this new method increases column life and is ideally suited for automated determinations of amiodarone and desethylamiodarone in patients undergoing long-term therapy.

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