Liquid-Chromatographic Determination of Amiodarone and N-Desethylamiodarone in Serum
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We have developed a stable and simple normal-phase liquid-chromatographic method for simultaneously measuring amiodarone and its metabolite, N-desethylamiodarone, within 8 min. The chromatographic system consists of a 15 cm × 3.9 mm Waters "Resolve" silica column and a mobile phase of ammonium sulfate (17 mmol/L, pH 6.8) and methanol (89/2 by vol), pumped at 1.8 mL/min and monitored at 254 nm. After 250 µL of serum was mixed with 100 µL of 0.36 mol/L NaH₂PO₄, 100 µL of the internal standard solution (L8040, 6 mg/L), and 200 µL of isopropyl ether, the mixture was vortex-mixed and centrifuged. Fifty microliters of the organic layer was injected onto the column. Relative recovery was 100% over the assay range of 0.1 to 20.0 µg/mL for both compounds. Within-run and total (day-to-day) CVs were 3% and 7% for amiodarone and 5% and 8% for N-desethylamiodarone, respectively.

Additional Keyphrases: measurement in tissues - drug assay - monitoring therapy

Amiodarone, a Class III antiarrhythmic agent, is used in the treatment of refractory tachyarrhythmias and premature ventricular contractions. Because its biological half-life is so long (~50 days), the drug can be conveniently administered once daily. There is no defined therapeutic range of its concentration in serum, so dosages of amiodarone have been chosen empirically, which may account for the frequent and numerous side effects reported with this drug (1, 2). For maximal therapeutic effects and minimized undesirable side effects, monitoring the drug concentration may be necessary.

Numerous "high-performance" liquid-chromatographic (HPLC) procedures have been published (3–10) for the measurement of amiodarone and its major metabolite, N-desethylamiodarone. Most require solvent extraction, followed by evaporation and reconstitution before injection into the chromatograph. The recovery is pH dependent, the procedure is tedious and time consuming, and the column life tends to be fairly short. Here we report a stable, simple, and rapid method for simultaneously measuring amiodarone and N-desethylamiodarone within 8 min.

Materials and Methods

Apparatus. We used a Milton Roy minipump (Laboratory Data Control, Riviera Beach, FL 33404), a Model 7125 injector (Rhodeyne, Inc., Cotati, CA 94928), and a Model 440 ultraviolet detector and a 15 cm × 3.9 mm Resolve 5-µm Spherical Silica column (both from Waters Associates, Milford, MA 01757).

Reagents. "HPLC-grade" methanol was from Burdick & Jackson Laboratory, Inc., Muskegon, MI 49442. Isopropyl ether was from Aldrich Chemical Co., Milwaukee, WI 53200. Ammonium sulfate buffer (17 mmol/L) was prepared by dissolving 2.2 g of (NH₄)₂SO₄ in 1 L of doubly distilled water, then filtering it through a 0.45-µm (pore size) Millipore filter (Millipore Corp., Bedford, MA 01730) and adjusting the pH to 6.8 with a 1 mol/L solution of NaOH. The mobile phase, 920 mL of methanol and 80 mL of ammonium sulfate buffer, was degassed with helium for 10 min before use. Phosphate buffer (0.36 mol/L, pH 4.35) for extraction was prepared by dissolving 4.32 g of NaH₂PO₄ in 100 mL of doubly distilled water.

Standards. Amiodarone hydrochloride, N-desethylamiodarone hydrochloride, and the internal standard, L8040 [2-ethyl-3-(3,5-dibromo-4-yl)proplyaminopropoxybenzoyl]-benzothiophene], were kindly provided by Sanofi, Montpelier, France. The working standards of amiodarone and N-desethylamiodarone (1.0 and 2.5 mg/L) were prepared by diluting the stock 1 g/L methanolic solutions of the drugs with plasma. The internal standard was 6 mg of L8040 dissolved in 1 L of methanol.

Assay procedure. Add 250 µL of serum, standard, or control; 100 µL of 0.36 mol/L NaH₂PO₄ buffer; 100 µL of internal standard; and 200 µL of isopropyl ether to a 1.5-mL polypropylene test tube. Vortex-mix for 30 s and centrifuge at 3000 × g for 3 min. Inject 50 µL of the organic (top) layer onto the column and elute with mobile phase at the rate of 1.8 mL/min at ambient temperature. The column effluent is monitored at 254 nm with the detector set at 0.02 A full scale.

Other procedures. For comparison, we measured amiodarone and N-desethylamiodarone by a normal-phase HPLC method as described by Kane and Shaw (9).

The absolute-recovery study was performed by adding amiodarone and N-desethylamiodarone to methanol and to...
drug-free plasma. We then extracted the plasma samples by the regular extraction procedure described above. All extracted (plasma) and unextracted (methanol) samples were injected onto the column in triplicate. Peak heights of the extracted samples were compared with those of the unextracted samples to determine absolute recovery.

For the interference study, we tested flecainide acetate, propranolol, indomethacin, digoxin, and lidocaine. These were dissolved in the appropriate solvent and added to a specimen containing amiodarone and N-desethylamiodarone. Other drugs tested for interference were those contained in Level III TDM control material (Ciba Corning Diagnostic Corp., Irvine, CA 92714), to which amiodarone and N-desethylamiodarone were added.

**Results**

Figure 1 shows a typical chromatogram of a serum blank and serum sample from a patient treated with amiodarone. The retention times were approximately 3.4 min for N-desethylamiodarone, 4.4 min for the internal standard, and 5.7 min for amiodarone.

The absolute recovery is pH dependent. If the pH of the extraction buffer is increased from 4.0 to 7.0, analytical recovery of amiodarone decreases from 103% to 81% and that of N-desethylamiodarone increases from 70% to 92%. Recovery of the internal standard remains constant (∼100%) in the pH range 4.0 to 6.0 and decreases when the pH exceeds 6.0. We use pH 4.35, at which the recovery is consistent and the absolute yields for amiodarone and N-desethylamiodarone are 100% and 73%, respectively.

The assay range of the method is from 0.1 to 20.0 mg/L for both drugs. The day-to-day CV, estimated from 70 daily determinations, was 7% for amiodarone and 8% for N-desethylamiodarone at a concentration of 2.0 mg/L. The within-run CVs were 3% for amiodarone and 5% for N-desethylamiodarone (n = 19, 2 = 2.0 mg/L).

Regression analysis of the results obtained from the comparison method (x) and the current HPLC method (y) yielded the following equations: y = 0.98x + 0 (r = 0.9798, n = 43) for amiodarone and y = 0.96x + 0.1 (r = 0.9487, n = 43) for N-desethylamiodarone. No interference was found from toxic concentrations of any of the drugs in the high-concentration Ciba-Corning Tri Level TDM controls or any of the drug-supplemented samples we tested.

Specimens containing amiodarone and N-desethylamiodarone are stable for at least 90 days if stored at 4–5 °C. However, N-desethylamiodarone tends to increase when specimens are stored at room temperature for longer than five days.

**Discussion**

Conventionally, when a normal-phase chromatographic method is used to separate amiodarone from its metabolite, the amiodarone is eluted from the column earlier than the internal standard and N-desethylamiodarone, as shown in the method of Kane and Shaw (9). By substituting the ammonium sulfate for ammonium phosphate in the mobile phase, the eluting sequence of amiodarone and N-desethylamiodarone is reversed, as shown in Figure 1. That sulfate salt may play an important role in this reversal is evidenced by a different normal-phase procedure (10) in which sulfuric acid was used.

There are several advantages of using these chromatographic conditions. The baseline resolution of each compound is excellent and therefore quantification is accurate. The chromatographic conditions are very stable in that there is little day-to-day change in retention times. The long durability of the column (more than 1500 injections per column) and the ability to recycle the mobile phase make this an economical procedure for use in the clinical laboratory. The same column and pump can be used for flecainide acetate and propranolol analyses by simply equilibrating the column for 15 min in a flecainide mobile phase that is very similar to that of the amiodarone assay (11). Finally, the same chromatographic conditions to measure amiodarone in serum can also be used to measure amiodarone in tissue. Tissue samples obtained from myocardium, lung, adipose, skeletal muscle, etc., were wrapped in plastic wrap, poured into thin layers, frozen, lyophilized, pulverized, and weighed. The powdered tissues were then extracted into a measured amount of isopropyl ether/methanol mixture (50/50 by vol). Amiodarone and N-desethylamiodarone were quantified by mixing the supernate with internal standard and comparing the peak area ratio with that of standards prepared in the same solvent matrix. Tissue samples from a patient receiving no amiodarone showed no peaks with retention times similar to amiodarone, N-desethylamiodarone, or the internal standard.

This method has been used in our laboratory for the last four years for analyses of thousands of serum samples and numerous tissue samples, and we have encountered no significant interferences. The simplicity of the extraction, the durability of the column, and the adaptability of the chromatographic system provide an efficient, cost-effective way of increasing therapeutic drug monitoring services with little additional expense.

**References**

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Effect of Serum Lyophilization on the Rate Constants of Enzymatic Methods for Measuring Cholesterol

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We determined the equilibrium absorbances and rate constants for two enzymatic methods, aca (DuPont) and RA-1000 (Technicon), used in determining cholesterol in reconstituted lyophilized serum. The lyophilized materials included two serum pools, three control materials, a College of American Pathologists' survey material, and Standard Reference Material no. 909. We calibrated the reagents with aca standards for cholesterol (DuPont). The difference in the mean concentrations of cholesterol (aca – RA-1000) was −0.09 g/L overall and was not statistically significant by analysis of variance. The mean rate constant for all materials was 0.23 min⁻¹ for the aca and 1.42 min⁻¹ for the RA-1000, significantly different (P < 0.001). Lyophilization causes lower results for the aca method than for the RA-1000, because the reaction rate for the aca method is slower and has not reached equilibrium when the final absorbance reading is made.

Additional Keyphrases: standardization · variation, source of · bias · discrete analysis · random-access analysis · absorbance at equilibrium

Accurate determination of cholesterol is an important part of the National Cholesterol Education Program's plan to detect, evaluate, and treat high blood cholesterol concentrations (1–3). Maintaining a minimum bias for all cholesterol-measuring methods is essential, because the error in diagnosis is approximately twice the magnitude of the bias (4, 5). Biases do exist between several of the enzymatic methods and the Reference Method for determining cholesterol as performed at the Centers for Disease Control (6, 7). Understanding the cause of bias is crucial to resolving the inaccuracy problem.

Materials and Methods

Apparatus. We used the Lambda 4B High Performance Spectrophotometer (Perkin-Elmer Analytical Instruments, Norwalk, CT 06856).

Reagents. We used two sets of reagents to determine cholesterol: those for the RA-1000 (Technicon Instruments Corp., Tarrytown, NY 10591) and for the aca (DuPont Co., Clinical Systems Division, Wilmington, DE 19898). The aca reagents were cut out of their packs and consolidated on the day of each experiment. To calibrate the reagents at equilibrium, we used aca Standards, Levels 1 through 4 (DuPont).

We tested seven lyophilized materials. Pools A and B were prepared for us and contained no preservatives (8). Three lyophilized materials were from Gilford (Ciba Corning Diagnostics Corp., Irvine, CA 92714): Elevated Lipid Control (lot no. 080603, expiration date August 1989) and two unassayed Normal and Abnormal Controls used in our laboratory. We used two reference materials, Standard Reference Serum (SRM 909) from the National Institute of Standards and Technology (NIST, Gaithersburg, MD 20899) and survey material c-18 (December, 1988) from the...