Simplified Liquid-Chromatographic Assay of Amiodarone and Desethylamiodarone after Solid-Phase Extraction

P. Timothy Pollak, S. George Carruthers, and David J. Freeman

We describe a rapid, simplified isocratic "high-performance" liquid-chromatographic method for simultaneous measurement of the antiarrhythmic drug amiodarone and its major metabolite, desethylamiodarone, in small volumes of sera (100 µL). Compared with liquid-liquid extraction, the solid-phase method of extraction saves time and glassware and improves reproducibility for small sample volumes. Amiodarone and desethylamiodarone could be measured at concentrations as low as 250 µg/L. Standard curves for the drug and metabolite are linear over the range of concentrations found in our patients. Within-run CVs (n = 6) ranged from 2.7% to 4.5% for amiodarone and from 4.0% to 5.7% for desethylamiodarone over the range of 250 to 4000 µg/L. Between-run CVs (n = 12) were 8.3% and 5.7% for amiodarone and desethylamiodarone, respectively. Commonly used cardiovascular medications do not interfere with the assay.

Additional Keyphrases: chromatography, reversed-phase, antiarrhythmic drugs

Amiodarone, a relatively new antiarrhythmic agent being considered for release in North America, offers three important advantages over most currently available antiarrhythmics: it has a broad spectrum of activity against both atrial and ventricular arrhythmias; it has a long duration of action, permitting once-daily dosing; and it does not appear to depress left ventricular function (1). Although a broad clinical experience with the drug in Europe has established its efficacy (2-4), there is some concern about its toxicity (5-7). One study has shown a relationship between efficacy and serum concentrations of amiodarone greater than 1 mg/L with toxicity occurring more frequently at concentrations exceeding 2.5 mg/L (8). A better understanding of these relationships would undoubtedly make the use of amiodarone more effective and as a consequence decrease the incidence and severity of toxicity. From our experience the major barrier preventing the collection of such data is the lack of a resource analytical procedure that is accessible to the clinician.

Several liquid-chromatographic assays of amiodarone and desethylamiodarone have been reported. In most, cumbersome solvent extraction techniques are used (9-13), but some involve crude protein precipitation methods (14-17).

Our need to measure amiodarone and desethylamiodarone in small capillary-blood samples from laboratory animals, and the interest shown in therapeutic monitoring of the drug at this medical center, prompted us to develop a liquid-chromatographic method for 100 µL of serum extracted by a convenient solid-phase technique.

Materials and Methods

We developed the assay on a manual chromatographic system consisting of a Model 110A pump (Beckman Instruments Inc., Toronto, Canada M8Z 2G6), a 15 × 0.32 cm stainless-steel column packed (in our laboratory) with Spherisorb-Octyl, a 5-µm particle size reversed-phase packing (PhaseSep, Hauppauge, NY 11787); an Altex fixed-loop injector (Beckman); and a BAS LC6 fixed-wavelength (254 nm) detector (Bioanalytical Systems Inc., W. Lafayette, IN 47906). Peaks of interest were recorded and quantified with an HP 3390A recording integrator (Hewlett-Packard, Mississauga, Canada L4V 1M8). To facilitate automated sample analysis, we later transferred the chromatographic step to a Hewlett-Packard 1051B system with automatic injection.

The solid-phase extraction columns employed were cyanobonded silica (CN) minicolumns of 1-mL capacity (J.T. Baker Chemical Co., Phillipsburg, NJ 08865).

"HPLC"-grade acetonitrile, methanol, and triethylamine (TEA) were obtained from local suppliers. "HPLC"-grade water was prepared by passing distilled water through a Milli-Q system (Millipore Waters Ltd., Mississauga, Canada L4V 1M5). The mobile phase and reconstitution solvent were buffered with an aqueous sodium dihydrogen phosphate solution (10 mmol/L, pH 3.5).

The internal standard used is L8040 (2-ethyl-3,5-di-bromo-4-di-n-propylaminopropoxybenzoylbenzothiophene). The primary (100 µg/L) stock solutions of amiodarone, desethylamiodarone, and internal standard were prepared in methanol and kept refrigerated. All drugs were obtained from Sanofi Recherche, Paris, France. Serum standards containing both amiodarone and desethylamiodarone were prepared by serial dilution of the stock solution over the concentration range of 8000 to 250 µg/L. The working internal standard solution was diluted to 2 µg/L in methanol/water (20/80 by vol).

Chromatographic conditions. The mobile phase consisted...
of acetonitrile/phosphate buffer, 10 mmol/L, pH 3.5 (62/38 by vol), to which we added 0.5 mL of TEA per liter to minimize peak tailing. The mobile phase was filtered and degassed after the final pH was adjusted to 3.5 with phosphoric acid. Mobile phase was pumped at a rate of 0.9 mL/min and was recycled. The back pressure was 7000 kPa with the column at ambient temperature. Although the maximum absorbance of amiodarone and desethylamiodarone is at 240 nm, we found that a fixed-wavelength detector (254 nm) sufficed for the analysis. We integrated the peak areas to quantify the drugs of interest.

**Extraction procedure.** The following extraction techniques were evaluated: protein precipitation with acetonitrile, protein precipitation combined with liquid–liquid extraction, and solid-phase extraction. We chose a simplified single solid-phase extraction procedure with CN minicolumns, which can be re-used several times. To speed the extraction process, we used a reduced-pressure Baker-10 Extraction System manifold (J.T. Baker).

After inserting the minicolumns into the extraction module, we conditioned them by applying 1 mL of methanol followed by three 1-mL washes with water, under reduced pressure. To each column, we then added 0.5 mL of water, 100 μL of standard, control, or unknown serum samples, and 100 μL of internal standard (2 mg/L solution). These mixtures were allowed to pass through the columns by gravity. The columns were washed three times with 1-mL portions of water and then with 1 mL of methanol/water (50/50 by vol). To elute the drugs of interest, we passed 0.5 mL of methanol containing TEA (10 mL/L) through the packing by gravity into 12 × 75 mm glass tubes. The methanolic extract was evaporated under nitrogen at 37 °C, and the residue was reconstituted with 100 μL of acetonitrile/buffer (40/60 by vol). We then injected duplicate 20-μL aliquots of this onto the analytical column.

**Results**

Figure 1 illustrates examples of the chromatograms obtained from extractions of standard sera, pooled blank sera from cardiology patients, and serum from a patient receiving, orally, 400 mg of amiodarone daily for nine months.

Within the range of concentrations studied, the ratios of amiodarone/L8040 and desethylamiodarone/L8040 were linearly related to absorbance at 254 nm (Figure 2). The CVs over the range of concentrations of amiodarone and desethylamiodarone seen in most patients are presented in Table 1. Between-run CVs (n = 12) were 8.5% for amiodarone, 5.7% for desethylamiodarone.

Absolute recovery was determined by comparing the average peak area for six extracted sera at each standard concentration of amiodarone/desethylamiodarone (from 250 to 8000 μg/L) with that for unextracted samples of identical concentrations made up in reconstitution fluid. The mean (± SD) absolute recoveries over this range were 87 ± 11%, 85 ± 9.2%, and 89 ± 2.6% for desethylamiodarone, amiodarone, and L8040, respectively.

We checked for possible interference from 2.5 mg/L solutions of several drugs that could be co-extracted by the solid-phase technique (weak bases) and of agents that might be co-administered with amiodarone, such as beta blockers, calcium antagonists, diuretics, antiarrhythmics, and inhibitors of angiotensin converting enzyme. No peaks were co-eluted with amiodarone or the internal standard (Table 2). Bepridil, an investigational calcium-channel blocking agent, was co-eluted with desethylamiodarone.
We found that at least 1 mL of TEA per liter, in methanol, was required to fully elute each drug, especially the internal standard, from the minicolumns during the extraction procedure.

Elution of undesirable lipid residues from the minicolumns with heptane after every fifth extraction extended their useful lifetime and maintained reproducibility. A decline in the peak area of the internal standard was noted if heptane washes were omitted. We have performed up to 15 extractions on one minicolumn before observing some degradation in performance.

The extraction procedure involves minimal handling of the sample and so saves both time and glassware. Quantities of reagents are minimized with solid-phase extraction. The assay is both rapid and simple, with the retention times less than 12 min under isocratic chromatographic conditions.

The method described here is suitable for simultaneously measuring amiodarone and desethylamiodarone in either serum or plasma. It can be used to measure concentrations of drug accurately and reproducibly in micro-scale samples of blood collected in 250-μL capillary tubes. Commonly used cardiovascular medications do not interfere. Amiodarone is a very effective antiarrhythmic agent, but its adverse effects are potentially dangerous. Therapeutic monitoring to provide information on the relationship between its concentrations in blood and toxicity could help identify patients susceptible to its toxicity.

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References

Table 1. Coefficients of Variation (n = 6) for Amiodarone Analysis

<table>
<thead>
<tr>
<th>Amiodarone, μg/L</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
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<tbody>
<tr>
<td>250</td>
<td>259</td>
<td>6.98</td>
<td>2.7</td>
</tr>
<tr>
<td>1000</td>
<td>1123</td>
<td>42.2</td>
<td>3.8</td>
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<tr>
<td>4000</td>
<td>4427</td>
<td>200.6</td>
<td>4.5</td>
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</tbody>
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Table 2. Retention Time for Drugs Tested for Possible Interference with Amiodarone Assay

<table>
<thead>
<tr>
<th>Drug</th>
<th>Retention time, min</th>
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<tbody>
<tr>
<td>Desethylamiodarone</td>
<td>6.88</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>9.71</td>
</tr>
<tr>
<td>L8040 (ISTD)</td>
<td>11.87</td>
</tr>
<tr>
<td>Verapamil</td>
<td>0.38</td>
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<tr>
<td>Norverapamil</td>
<td>0.48</td>
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<tr>
<td>Nifedipine</td>
<td>1.46</td>
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<td>Diltiazem</td>
<td>2.10</td>
</tr>
<tr>
<td>Propranolol</td>
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<tr>
<td>Labetalol</td>
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</tr>
<tr>
<td>Dilopyramide</td>
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<td>Procainemide</td>
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<td>Quinidine</td>
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<tr>
<td>Tocainide</td>
<td>1.16</td>
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<tr>
<td>Bepridil</td>
<td>6.84</td>
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<td>Furosemide</td>
<td>1.21</td>
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<td>Captopril</td>
<td>0.85</td>
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<tr>
<td>Warfarin</td>
<td>1.56</td>
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</table>

Discussion

We investigated protein precipitation and liquid–liquid extraction techniques, singly and in combination, during development of the present assay procedure. The former did not sufficiently purify the samples, yielding extra peaks in the chromatograms, while analytical recoveries after extractions with hexane were inconsistent, given the small sample volumes we wished to use. We found the solid-phase extraction with CN minicolumns to be faster and more reliable. It requires only two transfer steps: application of the serum to the column and elution of the drug from the column into the glass tube. Centrifugation and liquid-phase separations are obviated. During the solid-phase extractions, water washes remove polar drugs and ionized species whereas the 50/50 methanol/water wash removes intermediate-polarity neutral and acidic drugs before amiodarone and its metabolite are eluted. As a result, the chromatograms are very "clean," the mobile phase can be recycled, and a guard column is unnecessary. We compared reversed-phase C18 minicolumns with the CN type for extraction efficiency, but recovery of the L8040 internal standard was unreliable with the former, presumably because of the greater retention properties of the C18 minicolumns for non-polar drugs.

Decreasing the proportion of acetonitrile in the reconstitution fluid relative to that in the mobile phase improved peak symmetry, as predicted for reversed-phase chromatography. However, we were careful to keep the proportion of acetonitrile in the reconstitution fluid high enough to redissolve the sample residue completely. Thus we chose acetonitrile-buffer (40/60 by vol) for optimal performance.

References
or serum at the concentrations attained following a single 400 mg dose. J Chromatogr 1982;245:377–380.


