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Note

New, accurate semi-automatic high-performance liquid chromatographic method for routine monitoring of amiodarone plasma levels

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Amiodarone is a benzofuran derivative used in the treatment of supraventricular and ventricular arrhythmias [1—3]. The relationship between pharmacokinetic parameters and therapeutic use in man has been scarcely investigated, because, for many years, only non-selective and unpractical radioactive methods were available for detecting this drug in human plasma [4].

More recently some selective high-performance liquid chromatographic (HPLC) methods have been proposed for the measurement of amiodarone in plasma and other tissues [5—7]. This work reports a simple and sensitive HPLC procedure, using an original and more suitable internal standard. Optimal conditions for extraction and chromatography were selected in order to have both a satisfactory recovery and a high sensitivity. Furthermore, the use of a computerized HPLC apparatus equipped with an automatic sampler makes this method reliable for routine monitoring of plasma levels.

EXPERIMENTAL

Reagents and chemicals

Diethyl ether for extraction was analytical grade, filtered on ferrous sulphate; methanol for the chromatography was HPLC grade (LiChrosolv); all other reagents were analytical grade products. All reagents were obtained from Merck (Darmstadt, F.R.G.).

Amiodarone hydrochloride was kindly supplied by Sigma-Tau (Pomezia, Italy). The internal standard, 2-ethyl-3-benzofuranyl-[4-(2-(di-methylamino)-propoxy)-3,5-diiodophenyl] methanone*, was synthesized in our laboratory

*The internal standard is available free upon request.

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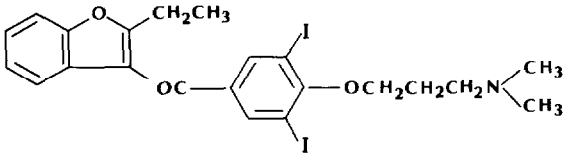
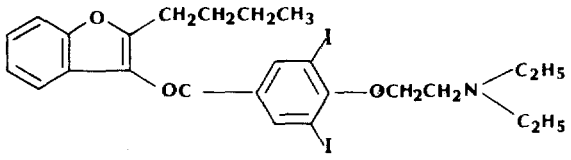
from benziodarone, $NaOCH_3$ and 3-dimethylamino-1-propyl chloride in toluene under reflux for 2 h. Its hydrochloride was crystallized from acetone and showed a melting point of 169—170°C (Fig. 1). The composition of the product $C_{22}H_{24}ClI_2NO_3$ is the following:

	C (%)	H (%)	N (%)	I (%)
Calculated	41.07	3.71	2.00	39.92
Found	41.30	3.62	2.18	39.67

Infrared (IR Spectrophotometer Model Microlab 620 MX, Beckman, Fullerton, CA, U.S.A.) and nuclear magnetic resonance (NMR Model EM 360 L, Varian Instruments Division, Palo Alto, CA, U.S.A.) spectra confirmed the structure.



Internal Standard



Amiodarone

Fig. 1. Structural formulae of amiodarone and internal standard.

Standard solutions

A stock solution of amiodarone hydrochloride was prepared (1.0 mg/ml) in methanol—water (1:1) and stored at 4°C in the dark. Appropriate volumes of methanolic working solutions prepared daily were added to drug-free human plasma to obtain five plasma standard samples at concentrations 0.05, 0.1, 0.5, 1.0 and 2.0 μg/ml.

A stock solution of internal standard (1 mg/ml) was prepared by dissolving 25 mg of the compound in 25 ml of methanol—water (1:1); a working solution was prepared daily by diluting (1:20) the stock solution with methanol.

Sample preparation

A 2-ml volume of 0.2 *M* acetate buffer (pH 3.8) and 20 μl of internal standard working solution were added to 0.5 ml of the plasma sample. The extraction was performed with 4 ml of diethyl ether and carried out in screw-capped

conical glass tubes (16 × 200 mm) using a rotary mixer for 15 min. After centrifugation at 750 *g* for 5 min, the upper organic phase was aspirated and extraction repeated with 4 ml of fresh diethyl ether. After a second centrifugation, the two organic phases were pooled and evaporated under vacuum, at room temperature, using a Vortex evaporator (Buchler Instruments, Fort Lee, NJ, U.S.A.).

The residue was redissolved in 500 μl of the mobile phase for chromatography and transferred in a crimp seal vial (Supelco Inc., Bellefonte, PA, U.S.A.); 50 μl of this solution were automatically sampled and injected for chromatography.

HPLC conditions

A Hewlett-Packard Model 1084-B liquid chromatograph, equipped with an automatic variable-volume injector and a variable-wavelength UV detector, was used for all analyses. The apparatus was controlled by a computer terminal (Hewlett-Packard Model 79850 B LC).

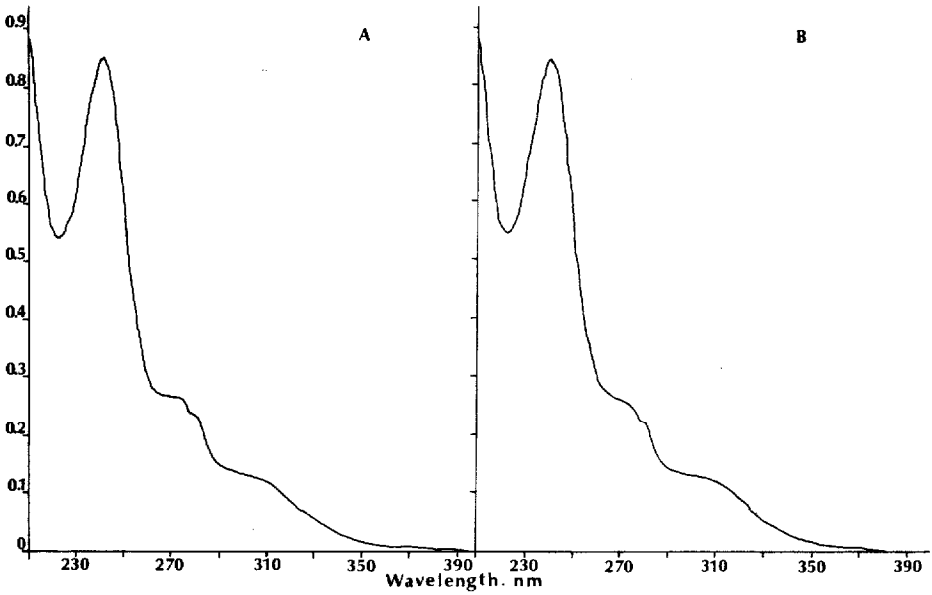


Fig. 2. UV spectra of amiodarone (A) and internal standard (B) dissolved in mobile phase.

The LiChrosorb SI-100 column (HIBAR RT), 5-μm particle size (stainless steel, 250 mm × 4 mm I.D.) was supplied by Merck.

Samples were eluted isocratically at a constant flow-rate of 2.0 ml/min. The mobile phase was methanol—diethyl ether (70:30, v/v) containing 0.02% perchloric acid. The solution was filtered through a 0.5-μm Millipore filter and degassed. The eluent was monitored continuously at 242 nm with a 2^3 attenuation. Printing of chromatograms, integration of peaks and all calculations were performed by the computer terminal of the HPLC apparatus. The peak area ratios of amiodarone to the internal standard were plotted against the

drug concentrations to obtain the standard curve. All glassware was ultrasonicated and carefully cleaned with a sulfuric—dichromate solution and washed with distilled water, methanol and diethyl ether.

UV spectra

To determine suitable detection wavelengths, the absorbance spectra of amiodarone and internal standard were performed preliminarily using a Cary 219 (Varian Instruments Division, Palo Alto, CA, U.S.A.).

The compounds were dissolved in mobile phase at a concentration of about 15 mg/l. Both the amiodarone and the internal standard show the maximum absorbance peak at 242 nm, with an $E^{1\,cm}_{1\%}$, respectively, of 0.562 and 0.581 (Fig. 2).

Furthermore, UV spectra of peaks were checked during the chromatographic procedure by the stop-flow method using the variable-wavelength detector of the HPLC apparatus.

RESULTS AND DISCUSSION

The efficiency and the reproducibility of the extraction were very satisfactory under our conditions (Table I). The diethyl ether was found to be the most suitable solvent for a rapid and complete extraction of amiodarone from the plasma; the steps of decantation and evaporation of the solvent appear to take place more easily. The most critical point of the extraction is the fixing of the pH, which must range between 3.6 and 4.2 for the complete recovery of amiodarone and of the internal standard. We have always used diethyl ether stabilized with 7 ppm of 2,5-di-*tert.*-butyl-4-methylphenol (BHT).

TABLE I

EXTRACTION EFFICIENCY EXPRESSED AS A PERCENTAGE OF PEAK AREA OF ALCOHOLIC STANDARD SOLUTIONS

Amiodarone concentration (μg/ml)	*n*	Mean ± S.D.
0.25	10	96.7 ± 4.6
1.00	10	98.3 ± 3.3

Five plasma samples spiked with amiodarone (0.05, 0.1, 0.5, 1.0 and 2.0 μg/ml) were chromatographed to test the linearity of the method. Amiodarone/internal standard peak area ratios were plotted against drug concentrations and linear regression analysis was performed by the least-squares method, using a Hewlett-Packard 9885 desk-computer. The straight line equation fitting the experimental points was: $Y = 0.523\,X - 0.006$ with a correlation coefficient $r = 0.999$ ($P < 0.01$).

The use of a variable-wavelength UV detector allows the eluate to be monitored at 242 nm with a sensitivity nearly double that of the detector with

a fixed wavelength of 254 nm [6]. Fig. 3 shows the chromatograms of drug-free plasma extracts, and extracts of plasma spiked with amiodarone (1 μg/ml) and internal standard (1 μg/ml), and the extract of a plasma sample of a patient treated with amiodarone.

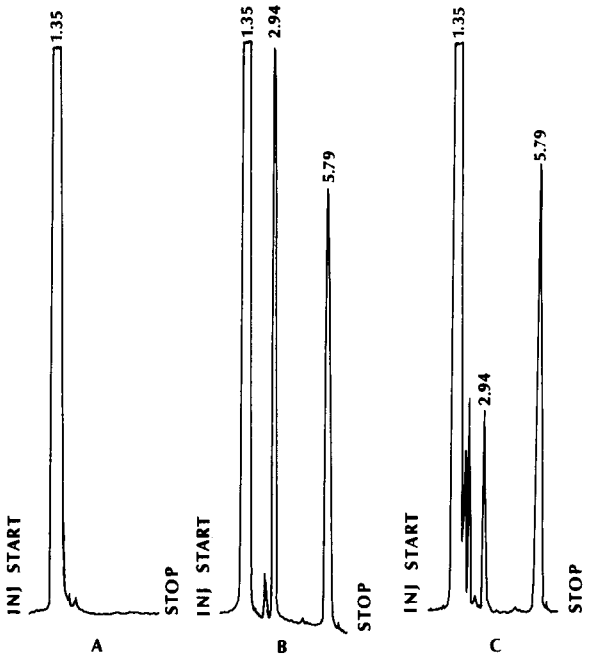


Fig. 3. Chromatograms of a blank plasma (A), a plasma sample spiked with standard amiodarone 1 μg/ml (B), and a patient plasma sample with 0.22 μg/ml amiodarone (C). See text for chromatographic conditions.

Under the chromatographic conditions described above it was possible to achieve a good separation of amiodarone from the internal standard. The retention time was 2.94 min for amiodarone and 5.74 min for the internal standard. The eluent mixture we used allowed symmetric and sharp peaks to be obtained, clearly separated from the solvent front and from a probable amiodarone metabolite, with a retention time of 2.09 min. The percentage of perchloric acid in the eluent mixture is critical for elimination of peak tailing. The retention times are shortened either by a major quantity of perchloric acid or by reduction of the amount of diethyl ether in the mixture. The retention times are negatively influenced by the solvent effect.

Injections of extracts redissolved only in methanol show a drastic reduction in retention times, while the extracts redissolved in diethyl ether or diisopropyl ether show rather longer retention times. Under the analytical conditions indicated, the life of a column is about 800 analyses with only a slightly decreased efficiency.

The UV spectra of the chromatographic peaks were scanned by the stop-flow method, in order to check possible interferences during the analysis of plasma

samples of patients treated with other drugs. The administration of one or more of following drugs is frequently associated with amiodarone treatment: digoxin, quinidine, procainamide, lidocaine, benzodiazepine, dipyridamole, isosorbide dinitrate, theophylline, caffeine, furosemide. In no case have we noted any variation in the UV spectrum or in any ratio other than 3.79 between the absorbance at 242 nm and that at 280 nm.

The sensibility and the linearity of the method (from 0.020 to 5.00 μg/ml) allows suitable monitoring of patients chronically treated with amiodarone, the haematic levels of which ranged between 0.15 and 0.50 μg/ml.

The reproducibility of the method was determined at two different concentrations of amiodarone. The intraday and interday coefficients of variation were calculated for spiked plasma samples containing 1.00 and 0.25 μg/ml amiodarone (Table II).

TABLE II

REPRODUCIBILITY DATA FOR THE DETERMINATION OF AMIODARONE IN HUMAN PLASMA

Amiodarone concentration (μg/ml)	*n*	Intraday		Interday	
		Mean ± S.D. (μg/ml)	C.V. (%)	Mean ± S.D. (μg/ml)	C.V. (%)
1.00	10	0.976 ± 0.0360	3.68	1.011 ± 0.0305	3.02
0.25	10	0.247 ± 0.0106	4.29	0.248 ± 0.0114	4.62

With the method reported above all the critical parameters for accurate measurement of the amiodarone plasma levels were optimized. The method, besides being automated as much as possible with respect to extraction, injection, integration and calculation phases, offers some advantages compared with the methods previously published. The most important advantages are higher sensitivity, higher extraction recovery, more rapid chromatographic separation and optimization of UV detection, compared with the methods of Flanagan et al. [5], Lesko et al. [6] and Covelli et al. [7].

Our chromatograph, suitably programmed for all the parameters, is fit for running automatically sets of 60 samples at a time. We have been using this method for more than a year for monitoring the amiodarone plasma levels. A technician can easily analyse more than 50 samples a day.

The sensitivity of the method is high enough to allow us to perform pharmacokinetics studies which we are now carrying out in our laboratories and will publish as soon as possible. However, the sensitivity of our method could still be improved by the use of unstabilized ethyl ether both for the eluent mixture and for the extraction, and by increasing the initial volume of the plasma to be tested.

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REFERENCES

1 M.B. Rosenbaum, P.A. Chiale and D. Ryba, Amer. J. Cardiol., 34 (1974) 215.
2 J.P. Fauchier, M. Brochier and R. Raynaud, Ann. Cardiol. Angeiol., 22 (1973) 427.
3 M.B. Rosenbaum, P.A. Chiale, M.S. Halpern, G.L. Nau, J. Przybylsky, R.J. Levi, J.O. Lazzari and M.V. Elizari, Amer. J. Cardiol., 38 (1976) 934.
4 J. Broekhuysen, R. Laruel and R. Sion, Arch. Int. Pharmacodyn., 177 (1969) 340.
5 R.J. Flanagan, G.C.A. Storey and D.W. Holt, J. Chromatogr., 187 (1980) 391.
6 L.J. Lesko, A. Marion, A.T. Canada and C. Haffajee, J. Pharm. Sci., 70 (1981) 1366.
7 J.A. Cervelli, J. Kerray and K.H. Pearson, Anal. Lett., 82 (1981) 137.