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Note

High-performance liquid chromatographic analysis of amiodarone and desethylamiodarone in serum

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Amiodarone, an antiarrhythmic benzofuran derivative has been widely used outside of the United States for as long as ten years [1]. In the U.S.A. it remains an investigational drug which is receiving increasing attention for the treatment of refractory ventricular and supraventricular arrhythmias [2—5]. A high-performance liquid chromatographic (HPLC) assay used to quantitate amiodarone in biological samples was first reported in 1980 [6]. Since then a number of additional methodologies have been reported [7—17]. It was not until 1982 when Storey and Holt [18] published an assay with improved low level sensitivity that it became apparent that the pharmacokinetic parameters of this compound and its metabolite had not been adequately described, particularly with regard to elimination half-life. As has been noted by Latini et al. [19], there exists a paucity of information on amiodarone and its metabolite desethylamiodarone and much work remains to be done to further our understanding of this drug's pharmacology, disposition kinetics, pharmacodynamics, interactions and toxicity. This is especially true in the patient population in which it is used and where major organ system failure (heart, kidney and liver) is often present. Reports of toxicity associated with the use of amiodarone [20] have resulted in the possible need for therapeutic monitoring of this drug.

We report the development of an HPLC assay for amiodarone and desethylamiodarone in serum. The assay has a lower limit of detection for amiodarone and its metabolite than any previously reported method, and the peak resolution is improved relative to the most sensitive published assay. Data are presented to show the utility of this assay for the pharmacokinetic studies of both the parent drug and its metabolite, as well as its usefulness in routine drug level monitoring.

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MATERIALS AND METHODS

Reagents

Amiodarone and desethylamiodarone were provided by Sanofi Research Co. (New York, NY, U.S.A.). Triflupromazine was provided by E.R. Squibb & Sons (Princeton, NJ, U.S.A.). Triethylamine and sodium acetate were purchased from Sigma (St. Louis, MO, U.S.A.). All solvents were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Apparatus

A Beckman Model 100A pump, a Model 420 controller, a Model 504 autosampler equipped with a 20-μl loop, and a Model 160 fixed-wavelength (254 nm) ultraviolet detector (Beckman, Fullerton, CA, U.S.A.) were used. A Hewlett-Packard 3390-A integrator (Hewlett-Packard, Phoenix, AZ, U.S.A.) was used to measure peak heights. Nylon-66 filters (Rainin Instrument Co., Woburn, MA, U.S.A.) and a Millipore filter apparatus (Millipore, Bedford, MA, U.S.A.) were used to filter the mobile phase prior to use. An N-Evap evaporator (Organomation Assoc., Northborough, MA, U.S.A.) and shaker (Eberbach, Ann Arbor, MI, U.S.A.) were used in sample preparation. The analytical column was an Altex Ultrasphere-Si, 5 μm, 25 cm × 4.6 mm normal-phase column (Beckman) preceded by an HC Pellosil silica gel bonded to 30—38 μm glass bead guard column (Whatman, Clifton, NJ, U.S.A.).

Standards and samples

Methanolic 1 g/l (expressed as the free base) solutions of amiodarone or desethylamiodarone were prepared. Standard curves were made by spiking pooled human plasma samples with appropriate dilutions made from the 1 g/l methanolic stock solutions to obtain final concentrations of amiodarone ranging from 2—200 μg/l, and final concentrations of desethylamiodarone ranging from 4—200 μg/l. Control solutions of human plasma spiked with both compounds at low, medium, and high concentrations within the range of the standard curves were made to assess within- and between-day reproducibility.

Procedure

A 1-ml aliquot of the standard or sample is pipetted into a glass culture tube. If amiodarone alone is being quantified, 50 μl of the internal standard, triflupromazine (1.0 μg/l) and 1 ml of 0.2 *M* sodum acetate buffer (pH 5.0) are added. If amiodarone and metabolite or metabolite alone is being quantified, 20 μl of internal standard (1.0 μg/l) is used and acidification with sodium acetate buffer is omitted. An 8-ml volume of hexane is added and tubes are capped with PTFE-lined screw caps. Use of a cardboard-lined screw cap causes subsequent interference in the chromatograms. Samples are placed horizontally in a shaker and extracted at low speed for 30 min, then centrifuged at 1500 *g* for 5 min. The top organic layer is transferred to a disposable glass culture tube and evaporated to dryness at 40° C under a stream of nitrogen. The resulting residue is reconstituted in 40 μl of methanol, transferred to an autosampler vial and capped; 20 μl are injected onto a normal-phase column. All aspects of the assay are conducted at room temperature including injection onto the

column. The mobile phase, which was filtered through 0.45-μm pore-size nylon 66 membranes before use, consisted of methanol—diethyl ether (80:20) to which 12—15 μl of triethylamine per l was added. The flow-rate was 2.2 ml/min. Peak heights are quantified with an integrator, and unknown sample concentrations are calculated from a linear regression fit of the standard curve plotted as peak height ratio versus drug concentration.

Clinical studies

Subject samples containing amiodarone and desethylamiodarone were obtained as part of another study by giving approximately 800 mg (10 mg/kg) of amiodarone hydrochloride intravenously to healthy male volunteers. Blood samples were collected before the start of infusion, at frequent intervals during the first 24 h, and thereafter for a total of approximately 120 days. Serum was separated after collection and stored frozen until assayed.

RESULTS AND DISCUSSION

Our goal was to develop an HPLC assay to simultaneously measure amiodarone and its metabolite desethylamiodarone in human serum samples which would have the precision and sensitivity to allow long term measurement of these two compounds after a single intravenous or oral dose. The resulting method detailed here allows measurement of amiodarone at a concentration of 1 μg/l and desethylamiodarone at a concentration of 3 μg/l. Although the samples may be chromatographed simultaneously, optimal

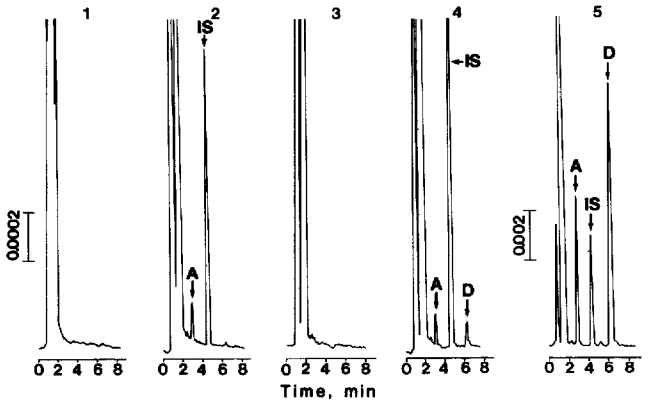

Fig. 1. Representative chromatograms of blank and authentic serum samples. (1) Blank serum extracted according to the procedure for amiodarone. (2) Authentic serum sample extracted according to the procedure for amiodarone. Sample obtained from a subject 86 days after an intravenous dose (10 mg/kg) of amiodarone. The peak corresponds to a concentration of 1.8 μg/l. (3) Blank serum extracted according to the procedure for desethylamiodarone. (4) Authentic serum sample extracted according to the procedure for desethylamiodarone. Sample obtained from the same subject as in (2) 48 days after an intravenous dose (10 mg/kg) of amiodarone. The peaks for amiodarone and desethylamiodarone correspond to concentrations of 6.5 and 3.5 μg/l, respectively. (5) Authentic serum sample extracted according to the procedure for desethylamiodarone. Sample obtained from a subject 60 days after daily dosing with 200 mg amiodarone. The peaks for amiodarone and desethylamiodarone correspond to concentrations of 734 and 1300 μg/l, respectively. Peaks: A = amiodarone; D = desethylamiodarone; IS = internal standard, triflupromazine.

recovery of amiodarone from an aqueous sample occurs at an acidic pH, and optimal recovery of desethylamiodarone occurs at serum pH. Therefore, in practice, only samples containing amiodarone at concentrations greater than 15 μg/l can be measured simultaneously with the metabolite by extracting at serum pH and obtaining incomplete recovery of amiodarone. Our attempt to perform serial extractions, the first at serum pH and the second at pH 5.0, and combining the resulting organic phases was unsuccessful due to unacceptable variability. Fig. 1 shows representative chromatograms of drug-free and authentic serum samples obtained from one subject after an intravenous (10 mg/kg) dose of amiodarone hydrochloride. Complete resolution exists between all three compounds and the retention times are approximately 3.0, 4.5 and 6.0 min. Triethylamine in the mobile phase is essential to the elution of these compounds from a normal-phase column and its concentration affects retention times (increasing concentrations decrease retention time), even at the minute quantities present.

The results of studies to assess the reproducibility of this assay are shown in Table I. Coefficients of variation (C.V.) both within-day and between-day were below 9% when amiodarone or desethylamiodarone are quantified alone and below 15% for both quantified together. Samples having concentrations expected to lie above the range of the standard curve (e.g., those collected during the infusion and for the first three days post-infusion) were diluted with drug-free serum prior to extraction. Using our experimental protocol for dosing, this condition applied only to amiodarone; metabolite concentrations did not at any time exceed 200 μg/l. A standard curve of up to 5000 μg/l amiodarone has been shown to be linear in our system. Repeated assays performed on samples stored frozen at −4°C for a period of five months showed that both drug and metabolite are stable (data not shown).

TABLE I

WITHIN-DAY AND BETWEEN-DAY VARIATION OF THE AMIODARONE AND DESETHYLAMIODARONE ASSAY

Compound	Within-day					Between-day				
	Expected (μg/l)	Observed (μg/l)				Expected (μg/l)	Observed (μg/l)			
		Mean	S.D.	C.V. (%)	*n*		Mean	S.D.	C.V. (%)	*n*
Amiodarone (alone)	8	8.0	0.6	7.5	10	8	7.5	0.6	8.0	6
	30	31.0	1.1	3.6	10	35	37.8	2.0	5.3	6
	100	97.1	8.7	9.0	8	70	67.4	2.8	4.2	4
Desethyl-amiodarone (alone)	12	11.9	0.8	6.7	10	12	12.7	0.6	4.7	5
	30	30.8	2.7	8.8	9	65	65.8	4.3	6.5	4
	150	153.8	6.4	4.2	10	150	148.9	10.7	7.2	4
Amiodarone and desethylamiodarone (together)*										
Amiodarone	25	28.2	4.3	15.3	9	25	26.1	2.3	8.8	5
	70	69.0	4.6	6.7	8	70	68.4	4.1	6.0	5
	100	99.1	7.2	7.3	10	100	98.7	6.1	6.2	4
Desethyl-amiodarone	10	10.1	1.6	15.8	9	10	9.8	0.7	7.1	5
	35	34.9	2.2	6.3	8	35	35.5	1.9	5.4	5
	180	186.3	23.1	12.4	10	180	181.3	10.3	5.7	5

*Samples extracted at plasma pH, a condition which favors the extraction of desethylamiodarone.

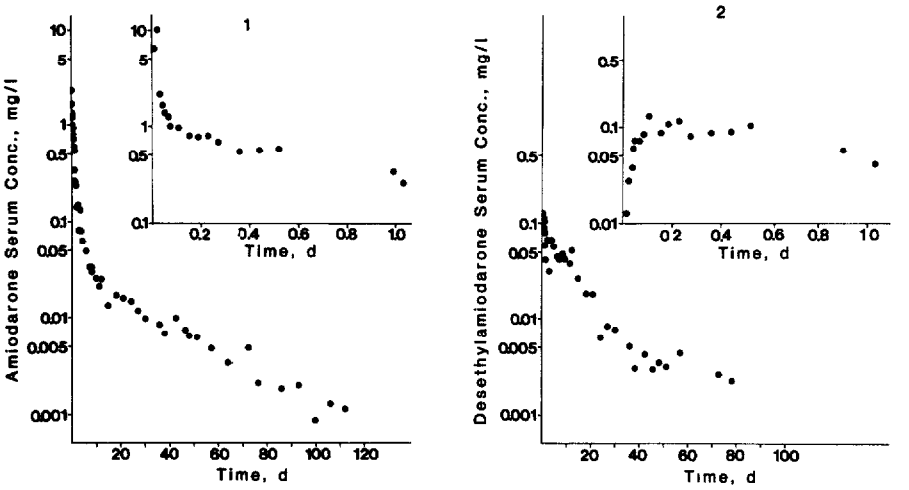

Fig. 2. Amiodarone (1) and desethylamiodarone (2) serum concentrations as a function of time after a 10 mg/kg intravenous dose of amiodarone in one subject. The inset graphs illustrate concentrations achieved during the first day after dosing.

Drugs which have been tested and shown not to interfere with the chromatography of amiodarone are: procainamide, verapamil, furosemide, lidocaine, quinidine, digoxin and hydrocortisone. These compounds were injected directly onto the column and not subjected to our extraction procedures.

Fig. 2 shows typical serum concentration—time profiles of amiodarone (panel 1) and desethylamiodarone (panel 2) obtained from one subject who was given a 30-min infusion of 10 mg/kg amiodarone hydrochloride. The inset graphs depict concentrations achieved during the first 24 h post infusion. The subject was a healthy adult male.

In conclusion, we report the development of a sensitive and precise assay for amiodarone and desethylamiodarone in serum. The assay has proven useful in the acquisition of pharmacokinetic data after single oral and intravenous doses of amiodarone. It is also being used to measure the higher levels attained after chronic dosing of amiodarone aimed at achieving steady-state concentrations. The assay, therefore, is applicable to both single dose studies and therapeutic drug monitoring.

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