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Note

Determination of amiodarone and its metabolite N-desethylamiodarone in serum by high-performance liquid chromatography – comparison of different extraction procedures

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Amiodarone, 2-butyl-3-(4-diethylaminoethoxy-3,5-diiodobenzoyl) benzofuran hydrochloride (A, Fig. 1), an antiarrhythmic agent of class III, is often used in arrhythmics when other antiarrhythmics are inefficient. The known side-effects of A, such as hyper- and hypothyroidism, pulmonary infiltrations, interstitial fibrosis of the lung, peripheral neuropathy, extrapyramidal symptoms and alterations of skin and cornea, force us to use the lowest therapeutically effective doses of A. Therefore the determination of serum levels of A and its metabolite mono-N-desethylamiodarone (DA, Fig. 1) is vital to the setting of a daily dose of A.

EXPERIMENTAL

Chemicals and reagents

Amiodarone hydrochloride, mono-N-desethylamiodarone hydrochloride and the internal standard, 2-ethyl-3-(3,5-dibromo-4-dipropylaminopropoxybenzoyl)benzothiophene, (L 8040, Fig. 1) were provided by Sanofi (Brussels, Belgium); acetonitrile, methylene chloride, methanol, triethylamine, hexane, ethanol, diethyl ether and hydrochloric acid were obtained from Merck (Darmstadt, F.R.G.); 1-octanesulphonic acid (PIC B-8-reagent) and dibutylamine phosphate (PIC D-4 reagent) from Waters Assoc. (Eschborn, F.R.G.).

Instruments

The high-performance liquid chromatographic (HPLC) system consisted of a Model 721 system controller, a 510 HPLC pump, a WISP 710B injector block and a Lambda-Max Model 481 variable-wavelength detector, all from Waters Assoc.

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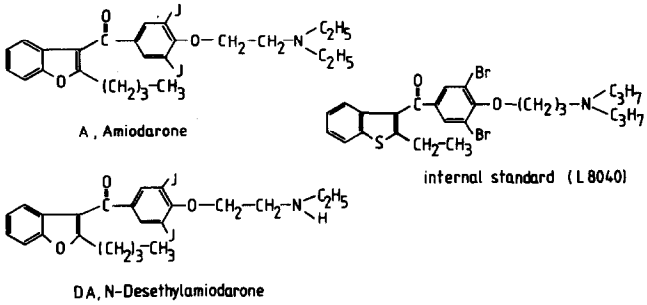

Fig. 1. Chemical structures of amiodarone, N-desethylamiodarone and internal standard.

Chromatographic conditions

The analysis was performed using a Shandon ODS reversed-phase column (125×4.6 mm I.D., particle size 5 μm) (Grom, Ammerbuch, F.R.G.). The mobile phase consisted of 25% R (976.5 ml of water, 10 ml of PIC D-4 reagent, 12.5 ml of PIC B-8 reagent and 1.0 ml of triethylamine solution, pH 4.4) and 75% S (acetonitrile). The flow-rate was 1.0 ml/min. The column effluent was monitored at 242 nm, using a detector range of 0.02 a.u.f.s. and a chart-speed of 0.5 cm/min. The injection volume was 100 μl.

Standards

Three stock standard solutions were prepared, containing 20 μg/ml of A, DA and internal standard (I.S.), respectively, in methanol. The solutions were stable for at least three months if stored at −20°C. A standard working solution was obtained by combining aliquots of the stock solutions and diluting with methanol to concentrations of 2.0 μg/ml for each of the three components A, DA and I.S.

Sample collections

Serum and plasma were obtained by centrifugation at 892 *g* for 10 min. Fourteen serum samples from normal volunteers (eight males, six females) were pooled (normal pool). Twenty serum samples from patients (ten males, ten females) treated with cardiovascular drugs, such as betaxolol, propranolol, metoprolol, furosemide, digoxin, nifedipine, verapamil, propafenon and mexiletine, were pooled (patient pool).

Serum, heparin plasma and EDTA plasma samples from thirteen patients (eleven males, two females) treated with amiodarone (200–400 mg per day) were analysed individually.

Extraction procedure

Spiking of normal pool samples with 100 μl of the standard stock solutions of A, DA and I.S. was used to compare different extraction methods. For each method one blank and seven normal pool samples were used. For the samples (except for the blank), 100 μl of each stock solution A, DA, I.S. were added to the empty tubes and evaporated to dryness. The residue was redissolved in 1.0 ml of the

normal pool and vortexed. The extraction was performed with hexane [1], acetonitrile [2], diethyl ether [3], ethanol [4] or methylene chloride–methanol.

The methylene chloride–methanol procedure was developed in our laboratory: 2.0 ml of methylene chloride, a fixed volume of 1.0 ml of serum, 100 μl of 1 *M* hydrochloric acid and 100 μl of the stock solution of the methanolic I.S. were introduced in a glass centrifuge tube (Kästner, Tübingen, F.R.G.). The stoppered (the stopper from Sarstedt, Nürmbrecht, F.R.G.) tube was vortexed for 60 s and centrifuged at 3570 *g* for 10 min. Subsequently the serum phase was discarded, and 1.0 ml of the methylene chloride phase was transferred to a clean glass tube and evaporated to dryness at room temperature under nitrogen, before being redissolved in 500 μl of methanol.

When using the methylene chloride procedure without methanol, we dissolved the I.S. in 0.01 *M* hydrochloric acid instead of methanol.

Linearity test

The linearity of the chromatographic procedure was tested for A, DA and I.S. by analysing seven methanolic standard solutions with concentrations of 4.0, 3.0, 2.0, 1.0, 0.5, 0.25 and 0.1 μg/ml for each of the components. The linearity test of the entire methylene chloride procedure was tested with the same standard solutions. The amount of each standard required to reach concentrations in the range 0.1–4.0 μg/ml was introduced into a glass centrifuge tube, the methanol was evaporated, and the residue was redissolved in 1.0 ml of the normal pool serum and vortexed. The mixtures were extracted with methylene chloride–methanol and subjected to the chromatographic analysis.

Recovery test

The recovery rates were determined by comparing the peak areas of A, DA and I.S. obtained by analysing a spiked serum sample (100 μl of each stock solution standard plus 1.0 ml of the normal pool) with the peak areas obtained by direct injection of the standard working solution (2 μg/ml A, DA and I.S.).

Quantification

The determination of the concentrations of A and DA in the serum was based on calibration graphs obtained from methanolic standard solutions (0.1, 0.25, 0.5, 1.0, 2.0, 3.0 and 4.0 μg/ml). The lower limit of quantification was 0.1 μg/ml. Since the calibration graph was linear and the calibration was stable for three months, the calculation was performed with the 2 μg/ml standard only, using the following formula:

$$\text{concentration of the substance } (\mu\text{g/ml}) = \frac{\text{peak area of the substance}}{\text{peak area of standard}} \times 2 \times \frac{100}{\text{recovery rate of I.S.}}$$

TABLE I

PERCENTAGE RECOVERIES WITH THE DIFFERENT EXTRACTION PROCEDURES

The technical error of the apparatus was less than 1% for A, DA and I.S. pH values are values after acidification of the serum (basic pH value of the serum 8.8).

Compound	Recovery rate (%)					
	Hexane (pH 4.6)	Acetonitrile (pH 8.8)	Diethyl ether (pH 3.9)	Ethanol (pH 6.0)	Methylene chloride (pH 3.4)	Methylene chloride–methanol (pH 3.4)
A	82.4±9.8	60.0±5.0	71.9±8.4	50.6±4.0	48.6±8.7	92.6±3.1
DA	21.6±3.2	88.2±1.8	98.4±3.6	72.6±13.0	41.1±5.3	94.4±3.6
I.S.	91.6±7.2	52.8±1.1	97.6±5.3	48.8±10.0	39.8±6.8	97.8±2.2

RESULTS

The linearity test showed a linear relation between the concentration and peak area for the standards as well as for the entire methylene chloride–methanol procedure.

The mean recovery rates and the standard deviations obtained using the described extraction procedures are summarized in Table I.

Using the methylene chloride–methanol procedure we did not observe any cardiovascular substances as interfering peaks in the patients pool.

The individual levels of A and DA in thirteen patients, using the methylene chloride–methanol procedure, are shown in Table II.

TABLE II

INDIVIDUAL LEVELS OF AMIODARONE AND DESETHYLAMIODARONE IN PLASMA SAMPLES

Patient	Amiodarone (μg/ml)	Desethylamiodarone (μg/ml)	Dose (mg per day)
1	1.0	0.6	200
2	0.8	0.5	200
3	0.3	0.3	200
4	0.7	0.6	200
5	0.6	0.4	200
6	0.7	0.4	400
7	2.0	0.7	400
8	1.5	0.7	400
9	2.4	1.1	400
10	1.3	0.8	400
11	0.9	0.9	400
12	1.4	0.4	400
13	0.8	0.6	400

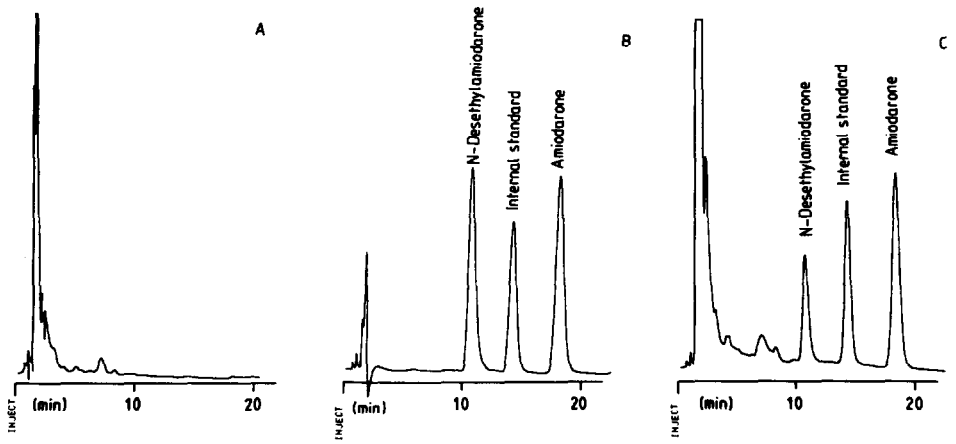

Fig. 2. Chromatograms of (A) blank from the normal pool, (B) a standard working solution and (C) the serum of patient 7 in Table II.

We did not find any difference in the serum levels of A, DA for heparin and EDTA plasma.

DISCUSSION

The hexane procedure described by Mostow et al. [1], Lesko et al. [5] and Latini et al. [6] has a satisfactory recovery for A and I.S. but the recovery of the metabolite DA ($21.5 \pm 3.2\%$) is insufficient. Reliable determinations of the metabolite are important to clarify the proposed relation between metabolite level and side-effects. No improvement of the recovery of the metabolite ($16.8 \pm 1.1\%$) resulted from freezing the inorganic phase of the hexane procedure, and the recoveries of A ($51.4 \pm 9.7\%$) and I.S. ($66.4 \pm 5.4\%$) were reduced.

The acetonitrile procedure (Plomb et al. [2] and Weir and Ueda [7]) showed satisfactory recoveries for the metabolite but the recoveries of A and I.S. were low. In addition an endogenous substance peak with a retention time similar to that of the metabolite was observed using the acetonitrile procedure.

With the diethyl ether procedure (Duranti et al. [3]) good recoveries of the metabolite and I.S. were found. However, the recovery of A was only $71.9 \pm 8.4\%$ and, as in the acetonitrile procedure, an endogenous substance peak appeared with a retention time similar to that of the metabolite. The ethanol procedure (Brian et al. [4]) showed very low recoveries of A, DA and I.S. Again an endogenous substance peak could be demonstrated with a retention time similar to that of the metabolite. Moreover, an additional peak was observed with nearly the same retention time as the I.S.

The drawbacks of the described procedures led us to test another extraction procedure. Methylene chloride applied to serum acidified with 1 *M* hydrochloric acid to pH 3.4 turned out to give quite uniform results for A, DA and I.S. However, the recovery rates were not satisfactory. In order to change the polarities in both phases, methanol was added. The recoveries of this modified methylene chloride

procedure were satisfatory for all three substances (DA, 94.4±3.6%; A, 92.6±3.1%; I.S., 97.8±2.2%).

The separations are demonstrated in Fig. 2. We did not observe the endogenous substance peaks described by Mostow et al. [1] when using methylene chloride, but not hexane. This difference is likely to be caused by differences in methodology (pH value, addition of methanol). In the present study no endogenous substance peak was observed in the blank, either with the hexane procedure or with the methylene chloride–methanol procedure.

Apart from the absence of any interfering peaks, e.g. endogenous substances or other cardiovascular components, further advantages of the methylene chloride–methanol procedure were a better separation of the phases, especially compared with the hexane–methanol method of Muir et al. [8], and a faster extraction procedure because of the low amount of organic solvent (2.0 ml).

It is concluded that the hexane procedure allows a quantitative analysis with good recoveries of A and I.S. The methylene chloride–methanol procedure, however, has good recoveries for A, I.S. and for the metabolite as well. Further studies, including the determination of the levels of the metabolite in serum, are necessary to clarify the proposed relation between the serum metabolite level and the frequency and severity of side-effects.

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