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SIMULTANEOUS DETERMINATION OF AMIODARONE AND ITS MAJOR METABOLITE DESETHYLAMIODARONE IN PLASMA, URINE AND TISSUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple and sensitive high-performance liquid chromatographic method for the simultaneous assay of amiodarone and desethylamiodarone in plasma, urine and tissues has been developed. The method for plasma samples and tissue samples after homogenizing with 50% ethanol, involves deproteinization with acetonitrile containing the internal standard followed by centrifugation and direct injection of the supernatant into the liquid chromatograph. The method for urine specimens includes extraction with a diisopropyl ether—acetonitrile (95:5, v/v) mixture at pH 7.0 using disposable Clin-Elut 1003 columns, followed by evaporation of the eluate, reconstitution of the residue in methanol—acetonitrile (1:2, v/v) mixture and injection into the chromatograph. Separation was obtained using a Radial-Pak C_{18} column operating in combination with a radial compression separation unit and a methanol—25% ammonia (99.3:0.7, v/v) mobile phase. A wavelength of 242 nm was used to monitor amiodarone, desethylamiodarone and the internal standard. The influence of the ammonia concentration in the mobile phase on the capacity factors of amiodarone, desethylamiodarone and two other potential metabolites, monoiodoamiodarone (L6355) and desiodoamiodarone (L3937) were investigated. Endogenous substances or a variety of drugs concomitantly used in amiodarone therapy did not interfere with the assay.

The limit of sensitivity of the assay was 0.025 μg/ml with a precision of ± 17%. The

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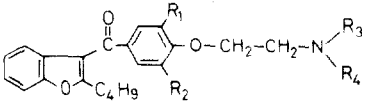
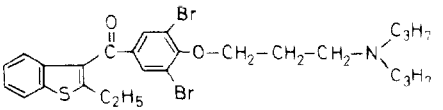
inter- and intra-day coefficient of variation for replicate analyses of spiked plasma samples was less than 6%. This method has been demonstrated to be suitable for pharmacokinetic and metabolism studies of amiodarone in man.

INTRODUCTION

Amiodarone [2-butyl-3-(3,5-diiodo-4-β-diethylaminoethoxybenzoyl)benzofuran, Fig. 1] has antianginal and antiarrhythmic properties [1]. It is used widely in Europe and South America for the treatment of ventricular and supraventricular arrhythmias, especially when resistant to conventional antiarrhythmic agents [2—7]. In addition, it is widely considered the drug of choice for treatment of tachycardias associated with the Wolff-Parkinson-White syndrome [4].



Compound	R_1	R_2	R_3	R_4
Amiodarone L3428	I	I	C_2H_5	C_2H_5
Desethylamiodarone L 328'2	I	I	C_2H_5	H
L 6355	I	H	C_2H_5	C_2H_5
L 3937	H	H	C_2H_5	C_2H_5



Internal standard, L 8040

Fig. 1. Chemical structures of (top) amiodarone, desethylamiodarone, two potential metabolites, and (bottom) internal standard.

Amiodarone is well tolerated and does not usually cause serious untoward effects with maintenance doses of up to 600 mg per day. Adverse effects attributed to amiodarone include photosensitivity, a bluish skin discoloration, formation of corneal micro deposits and disturbances of thyroid function, which appear to be dose- and time-related and reversible upon cessation of treatment [1, 8—12]. From clinical practice it can be suspected that the drug is accumulated in the body and has a long "therapeutic" half-life since optimal antiarrhythmic effects may only be seen days to weeks after initiation of treatment and may persist for weeks after discontinuation of therapy [4]. However, little is known about plasma levels, elimination half-life and body distribution of the drug or the relationship between plasma concentrations and suppression of arrhythmias [13—19]. Amiodarone is metabolised by N-deethylation, and the desethyl metabolite (Fig. 1) is detectable in the plasma of patients on long-term treatment [13, 14]. Data on the pharmacokinetic properties of desethylamiodarone, however, are scarce [16, 18].

In order to assess the above-mentioned pharmacokinetic parameters of amiodarone and desethylamiodarone and to study the correlation between plasma levels and clinical effects, a fast, specific and sensitive method for the assay of amiodarone and desethylamiodarone in biological material is required. Up to now four high-performance liquid chromatographic (HPLC) methods for the determination of amiodarone in plasma or serum have been published [13—15, 19]. However, their applicability for the analysis of both amiodarone and desethylamiodarone in plasma, urine and tissues is questionable. The disadvantages of these methods include lack of use of internal standard [14], relatively complex and time-consuming sample preparation [15], applicability limited to amiodarone assay [13—15, 19] or plasma samples [13, 15] only. Furthermore, only one of the published methods has shown its feasibility for tissue analysis of amiodarone [19].

Therefore, a rapid, simple and sensitive HPLC procedure for the simultaneous determination of amiodarone and its major metabolite desethylamiodarone in plasma, urine and tissues has been developed.

EXPERIMENTAL

Chemicals and reagents

Amiodarone hydrochloride (L3428), desethylamiodarone oxalate (L3812), the internal standard [2-ethyl-3-(3,5-dibromo-4β-dipropylaminopropoxylbenzoyl)benzothiophene, L8040], and the additional potential metabolites L3937 and L6355 (Fig. 1), were obtained from Labaz (Maassluis, The Netherlands). Analytical reagent grade methanol and HPLC grade acetonitrile were purchased from Baker (Deventer, The Netherlands). Ethanol and diisopropyl ether were of analytical grade and were obtained from Merck (Darmstadt, G.F.R.).

Ammonia 25% and ethanol 50% were prepared by dilution with bidistilled water of ammonia 33% (Merck, Art. No. 5426) and ethanol (Merck), respectively. Phosphate buffer, pH 7.0, was prepared by dissolving 7.72 g of disodium hydrogen phosphate·$2H_2O$ (Merck) and 3.18 g of potassium biphosphate in 1 l of bidistilled water. Clin-Elut (CE 1003) extraction columns were obtained from Analytichem International (Lawndale, CA, U.S.A.).

Instruments

The HPLC system consisted of a Model 6000 A solvent delivery pump, a Model U6K injector, a guard column, a radial compression separation unit and a Model 450 variable-wavelength detector, all from Waters Assoc. (Milford, MA, U.S.A.). The radial compression separation unit consisted of a 10 cm × 8 mm I.D. Radial-PAK C_{18} (10 μm RP-18) cartridge and a Model RCM-100 module for compressing the cartridges. The separation unit was preceded by the guard column, packed with Bondapak C_{18}/Corasil (37—50 μm). The output of the detector was displayed on a Varian Model A-25 recorder. The output signal also was fed to a Hewlett-Packard 3353 data system for integration of peak areas.

An all-glass filter apparatus with appropriate 0.45-μm filters (Solvent Clarification Kit, Waters Assoc.) was used to filter the mobile phase before use. Other equipment included a Potter homogenizer (Type E 60, Heidolph Elektro

KG, Kelheim, G.F.R.), a vortex-type mixer, a Büchi Rotavapor and high-speed centrifuge.

Chromatographic conditions

All chromatography was done at ambient temperature. The mobile phase consisted of methanol—ammonia 25% (99.3:0.7, v/v). The flow-rate was 2.0 ml/min, maintained by a pressure of ca. 25 bars. The column effluent was monitored at 242 nm, the absorbance maximum of amiodarone, using a detector range of 0.01 absorbance unit full-scale (a.u.f.s.) and a chart speed of 20 cm/h.

Standards

A stock standard solution in methanol containing 100 μg/ml of amiodarone base and desethylamiodarone base and a stock internal standard solution of 250 μg/ml in acetonitrile were prepared. Standard solutions in methanol, plasma or urine containing amiodarone and desethylamiodarone at concentrations of 0.10, 0.25, 0.50, 1.00, 1.75, 2.50, 3.50 and 5.00 μg/ml were made by appropriate dilution of the stock solution with methanol, plasma or urine, respectively. The plasma and methanolic solutions were stable for one month if stored at 4°C in the absence of light in amber glass containers. An internal standard working solution was prepared by dilution of an aliquot of the stock solution with acetonitrile to contain 5.0 μg/ml.

Procedure for plasma, urine and tissues

Plasma. In a disposable polypropylene-capped glass centrifuge tube (16 × 100 mm) were introduced 1.00 ml of plasma and 2.00 ml of the solution of the internal standard in acetonitrile. The stoppered tube was vortexed for 30 sec, allowed to stand at room temperature for 15 min and centrifuged at 2000 *g* for 15 min. Subsequently the supernatant was transferred to a clean, disposable, glass tube and 50 μl of this solution were injected into the liquid chromatograph.

Urine. To a Clin-Elut 1003 disposable extraction column were successively added 2.00 ml phosphate buffer, pH 7, 40 μl of the stock internal standard solution and 1.00 ml of urine. After waiting for 3 min, 6 ml of a mixture of diisopropyl ether—acetonitrile (95:5) were added to the column. The eluate was collected in a 25-ml conical flask and after at least 3 min the extraction was repeated with one more 6-ml aliquot. After completion of the elution from the column, the collected combined eluates were evaporated to dryness under reduced pressure in a Büchi Rotavapor at room temperature. The sample residue then was reconstituted with 3.0 ml of a mixture of methanol—acetonitrile (1:2, v/v) and 50-μl aliquots were injected into the chromatograph.

Tissue. Tissue was finely minced, dried between Kleenex® tissues, and portions of about 100 mg of the dried minced tissue were homogenized for 10 min with 1.00 ml of 50% ethanol in a glass tube with a homogenizer. The tissue homogenate then was vortexed for 30 sec with 2.0 ml of the internal standard solution and thereafter completely transferred to a clean glass test tube and centrifuged at 2000 *g* for 15 min. From the supernatant 50 μl were injected into the liquid chromatograph. All samples were assayed in duplicate.

Quantitation. The concentration of amiodarone and desethylamiodarone in plasma, urine and tissues was determined from calibration curves of peak area ratios (amiodarone and desethylamiodarone to internal stadard) versus amiodarone and desethylamiodarone concentrations in plasma, urine and tissue standards carried through the described procedures.

RESULTS

Chromatograms from blank human plasma, urine, tissue and those spiked with known concentrations of amiodarone and desethylamiodarone are shown in Fig. 2. Chromatograms of a plasma sample, and a renal and heart tissue sample obtained, respectively, from a patient treated with 200 mg of amiodarone per day for more than a year and from a post-mortem case treated with the same dosage for more than a year are presented in Fig. 3. The peaks representing amiodarone, desethylamiodarone and the internal standard are symmetrical and well removed from the solvent front and interfering peaks from the biological material.

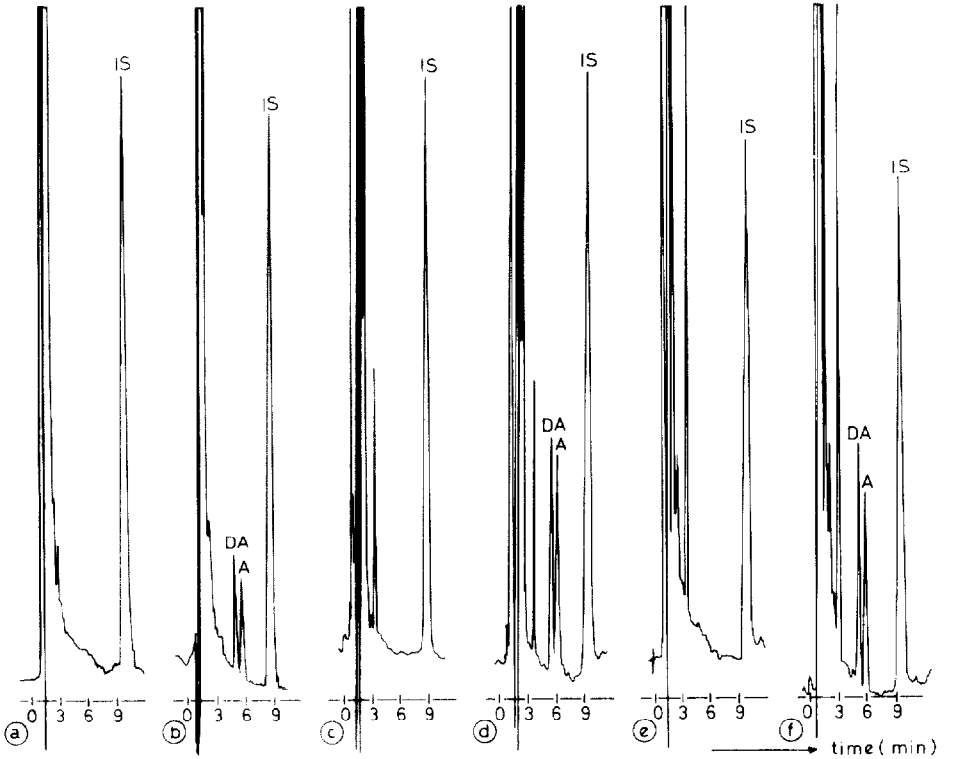


Fig. 2. Chromatograms of (a) blank human plasma, (b) plasma spiked with 1.0 μg/ml amiodarone (A) and desethylamiodarone (DA), (c) blank urine, (d) urine spiked with 2.5 μg/ml A and DA, (e) blank heart tissue, and (f) heart tissue spiked with 25 μg/g A and DA. Internal standard (IS) concentration 3.3 μg/ml. Injection volume: 50 μl. Detector sensitivity: 0.01 a.u.f.s. Recorder chart speed: 20 cm/h.

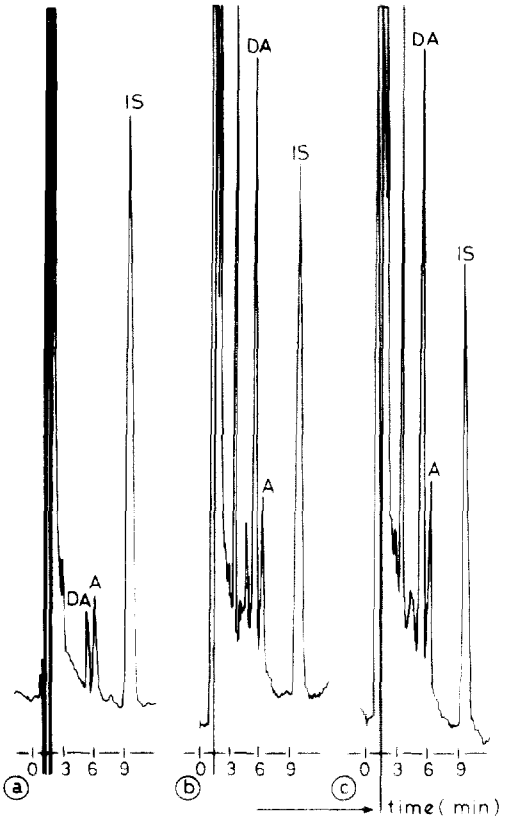


Fig. 3. Chromatograms of (a) plasma of a patient receiving 200 mg amiodarone for more than a year, containing 1.03 μg/ml A and 0.70 μg/ml DA, (b) kidney tissue containing 20.5 μg/g A and 57.4 μg/g DA, and (c) heart tissue containing 22.0 μg/g A and 56.1 μg/g DA from a post-mortem case who had received 200 mg of amiodarone for more than a year. Conditions as in Fig. 2.

The retention times of desethylamiodarone, amiodarone and internal standard were 5.3, 6.3 and 9.4 min, respectively. For the potential metabolites L3937 and L6355 retention times of 4.0 and 5.2 min were observed, respectively. The retention times of the compounds under study were quite stable as demonstrated by a coefficient of variation of less than 3% of the mean of the retention times of these compounds measured each day during a two-month period. A number of drugs were investigated for possible interference with the amiodarone assay. Verapamil, cinnarizine, quinidine and disopyramide were detectable if present at concentrations of about 5 μg/ml and showed in this system retention times of 2.5, 3.2, 4.7 and 4.8 min, respectively. However, they were all completely resolved from the compounds of interest in the chromatographic system. No interfering peaks were observed in the plasma of patients receiving amiodarone in combination with drugs such as acenocoumarol, carbamazepine, carbimazole, chlordiazepoxide, chlorthalidone, diazepam, diclophenac sodium, digoxin, dihydroergotoxine mesylate,

dipyridamole, furosemide, glaphenine, hydrallazine, isosorbide dinitrate, lorazepam, metoprolol, nicotinyl tartrate, nifedipine, phenprocoumon, prazosin, procaine, procainamide, propranolol, theophylline, tolbutamide, triamterene and valproate sodium.

Calibration curves of peak area ratio versus concentration were obtained by analyzing plasma and urine standards containing amiodarone and desethylamiodarone in concentrations ranging from 0.1 to 5.0 μg/ml. The calibration curves in plasma were established every week, and standard plasma samples were analysed during the week to validate the method. The equations of the curves were calculated by least-squares linear regression. For the curves in plasma and urine a good linear relationship was obtained in the concentration range studied for both drugs, with intercepts not significantly different from zero. The linear regression data for the mean calibration curves of amiodarone and desethylamiodarone in plasma and urine are summarized in Table I.

TABLE I

THE LINEAR REGRESSION PARAMETERS FOR THE MEAN CALIBRATION CURVES OF AMIODARONE AND DESETHYLAMIODARONE IN PLASMA AND URINE

Compound	Sample	n*	Linear regression parameters**		
			Slope	y-intercept	Correlation coefficient
Amiodarone	Plasma	8	0.1091	0.0032	0.9998
	Urine	4	0.1237	−0.0008	0.9979
Desethylamiodarone	Plasma	8	0.1100	0.0027	0.9999
	Urine	4	0.1161	0.0000	0.9992

*Number of calibration curves used.

**Peak area ratio of drug to internal standard plotted on the y-axis versus drug concentration in plasma or urine (in μg/ml) on the x-axis.

The plasma and urine curves were the average of eight and four standard curves, respectively, which were run during 2-month and one-month periods, respectively. The mean coefficient of variation of the peak area ratios of both drugs to internal standard for plasma and urine over the studied periods was 1.9% and 3.5% for the 5.0 μg/ml standards, 3.3% and 7.0% for the 2.5 μg/ml standards, 4.7% and 11.8% for the 1.0 μg/ml standards and 19.4% and 29.0% for the 0.10 μg/ml standards, respectively.

The precision of the assay was determined by replicate analyses of spiked plasma samples containing amiodarone and desethylamiodarone at concentrations of 0.75, 2 and 4 μg/ml. The intra-assay and inter-assay coefficients of variation are presented in Table II. The within-day and day-to-day variation at each concentration for both drugs was less than 6%. The sensitivity of the assay was 0.025 μg/ml for both drugs using an injection volume of 100 μl of plasma sample extract. The within-day coefficient of variation for spiked plasma samples containing 0.025 μg/ml was ± 17% (n = 5).

TABLE II

PRECISION DATA FOR THE DETERMINATION OF AMIODARONE AND DESETHYLAMIODARONE IN PLASMA

Concentration (μg/ml)	Coefficient of variation (%)			
	Within-day (n = 7)		Day-to-day* (n = 6)	
	Amiodarone	Desethylamiodarone	Amiodarone	Desethylamiodarone
0.75	3.1	4.6	5.6	5.6
2.00	1.5	3.4	3.5	3.7
4.00	1.2	1.4	2.2	2.8

*Analysis performed on six days during a three-week period.

The analytical recovery of amiodarone and desethylamiodarone from plasma, urine and various tissues was determined by comparison of the peak areas of amiodarone and desethylamiodarone obtained by analysis of 50-μl portions of methanolic standards mixed with internal standard solution (1:2, v/v) to those obtained from freshly prepared sample extracts. The results of the recovery studies are presented in Table III. The recovery of internal standard from plasma and urine was 107% (n = 70) and 76% (n = 40), respectively. For the two potential metabolites L3937 and L6355 (Fig. 1) recoveries in plasma of 103.3% (n = 4) and 103.8% (n = 4), respectively, were observed at a concentration of 1.0 μg/ml. In urine at a concentration of 1 μg/ml recoveries of 85.5% (n = 4) for L3937 and 66.3% (n = 4) for L6355 were found.

TABLE III

RECOVERY OF AMIODARONE (A) AND DESETHYLAMIODARONE (DA) FROM PLASMA, URINE AND TISSUES

Concentration (μg/ml or μg/g*)	Recovery (%)					
	Plasma (n** = 8)		Urine (n = 5)		Tissues*** (n = 12)	
	A	DA	A	DA	A	DA
0.10	99.7	104.0	84.2	76.2	—	—
0.25	102.3	105.6	85.3	86.4	—	—
0.50	100.7	105.5	84.4	74.8	—	—
1.00	104.8	106.7	90.2	85.2	86.1	89.3
1.75	102.2	104.3	90.9	77.7	—	—
2.50	104.2	100.8	81.0	72.4	97.0	94.7
3.50	103.5	101.5	89.2	81.6	—	—
5.00	98.9	102.0	90.5	75.6	99.6	98.2
Mean	102.0	103.8	87.0	78.7	94.2	94.1
S.D.	2.1	2.2	3.7	5.1	7.2	4.5

*Tissue concentrations in μg/g.
**Number of determinations at each concentration.
***Mean recovery data from aortic arch, esophagus, lung, heart, liver and renal tissue.

The described method was used to determine the plasma levels of amiodarone and desethylamiodarone after single oral and intravenous administration and after repeated oral administration of the drug. The plasma concentration—time curves of amiodarone obtained after single oral and intravenous administration of 400 mg to a healthy volunteer are shown in Fig. 4. The mean steady-state plasma levels of amiodarone and desethylamiodarone in seventeen patients receiving 200 mg a day for at least one month were 1.11 ± 0.33 μg/ml and 1.02 ± 0.31 μg/ml, respectively. After 400 mg a day, a mean level in seventeen patients of 1.70 ± 0.57 μg/ml and 1.40 ± 0.36 μg/ml for amiodarone and desethylamiodarone, respectively, was observed.

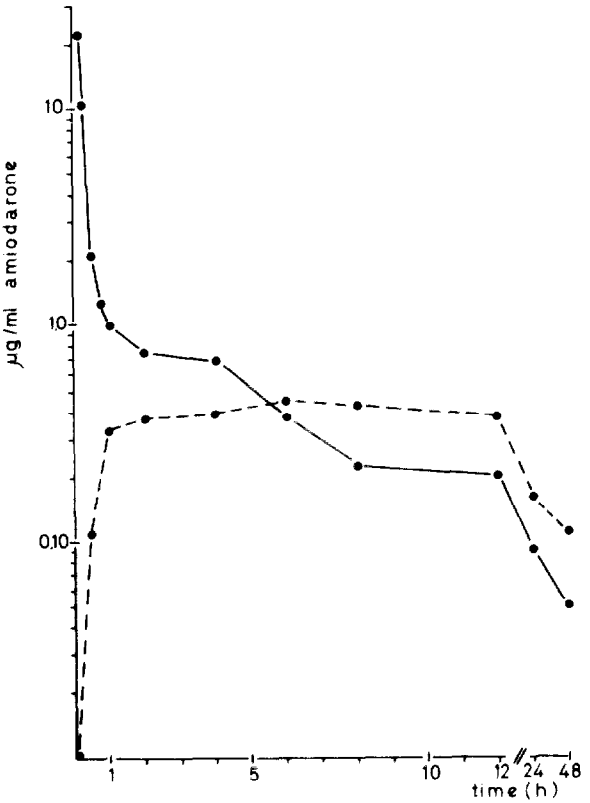


Fig. 4. Plasma concentration—time curves of amiodarone from a healthy volunteer after a single oral (•— — —•) and intravenous (•———•) dose of 400 mg of amiodarone.

Neither amiodarone nor desethylamiodarone were detected in urine samples of several patients on long-term amiodarone treatment. Very high levels were found in lung and liver tissue of a post-mortem case, with concentrations of 112 μg/g and 307 μg/g in the liver and 178 μg/g and 541 μg/g in the lung for amiodarone and desethylamiodarone, respectively. In other tissues investigated such as kidney, aortic arch, esophagus and heart, concentrations of amiodarone ranging from 8 to 16 μg/g and of desethylamiodarone ranging from 13 to 53 μg/g were observed.

DISCUSSION

Reversed-phase chromatography was used by several investigators for the separation of drugs like imipramine, maprotiline, chloroquine and lidocaine and their respective demethylated or deethylated metabolites [20—23]. In this type of liquid chromatography the dealkylated metabolites will usually be eluted before the parent drugs, which is advantageous with respect to the detection limit of these metabolites.

As desethylamiodarone, the major metabolite of amiodarone, is more polar and always present in considerable concentrations in plasma and tissues of patients on long-term amiodarone treatment, it is obvious that for the simultaneous assay of these compounds reversed-phase liquid chromatography should be chosen as the separation method. After investigation of several reversed-phase systems, a Radial-PAK C_{18} column in combination with a radial compression separation system (RCSS, Waters Assoc.) and a mobile phase consisting of methanol—0.7% ammonia 25% (v/v) was found most suitable for the separation of amiodarone, desethylamiodarone and the internal standard. The Radial-PAK C_{18} column was preferred to the conventional Bondapak C_{18} steel column because of its superior economy and convenience, enhanced reproducibility and reliability, high efficiency at higher flow-rates for faster analysis, lower back-pressure, and complete elimination of column voiding and channelling. For the normal routine analysis of steady-state levels of amiodarone and desethylamiodarone the 10-μm cartridge was preferred to the 5-μm one, because it has sufficient efficiency for complete separation of both compounds, it operates at a much lower back-pressure (25 versus 70 bars), it is far less prone to contamination with biological material from the sample and it has a substantially lower price. The 5-μm C_{18} cartridge was used in cases where higher efficiency (about 9000 versus 5000 plates for the 10-μm C_{18} column) and detection of low levels ($<$ 100 ng/ml of plasma) are required, for instance with metabolic and single-dose pharmacokinetic studies.

In order to determine the optimum chromatographic conditions, the influence of the ammonia concentration in the mobile phase on the retention behaviour and the capacity factors of amiodarone, its metabolites, desethylamiodarone, L6355, L3937, and the internal standard was investigated. The effect of the increase of the ammonia content in the mobile phase from 0.05% to 1% on the capacity factors (k') of amiodarone and its metabolites is shown in Fig. 5. It can be seen that the k' values decrease sharply from 0.05% (v/v) to about 0.35% (v/v) of ammonia 25% and then decrease gradually with further increase in the amount of ammonia. At an ammonia content of 0.05% all compounds were separated; however, under these conditions, retention times were long (L3937, 8.1 min; L6355, 9.9 min; amiodarone 11.4 min; desethylamiodarone 12.9 min; and internal standard, 20 min) and the peaks were unacceptably broad. Increase of the ammonia content to 0.1% resulted in a reduction of the retention times of about 26% for all compounds; however, the peaks were still rather broad and no separation was observed between amiodarone and desethylamiodarone with retention times of 8.6 min and 9.2 min, respectively. A further rise in the amount of ammonia to 0.35% (v/v) resulted again in a substantial decline of the retention times by another

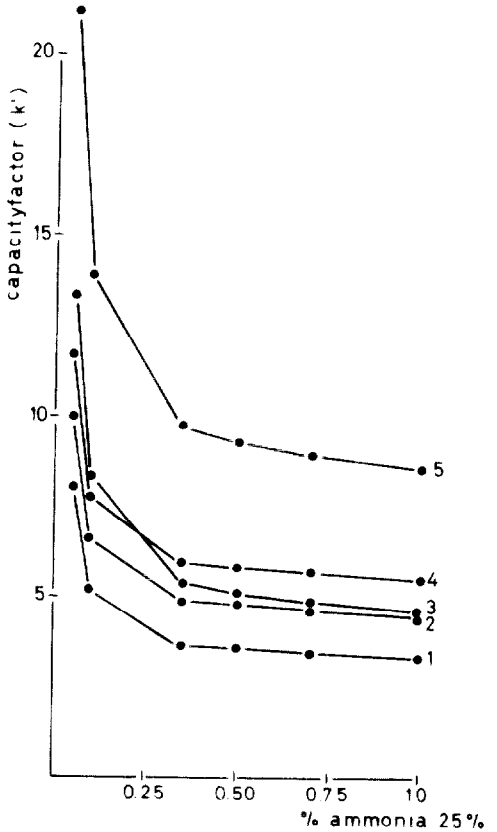


Fig. 5. Plot of capacity factors (k') of amiodarone (4), desethylamiodarone (3), two potential metabolites, L6355 (2) and L3937 (1), and the internal standard (5) against ammonia 25% content of the mobile phase. Unretained compound: methanol. Flow-rate: 2.0 ml/min. Chromatographic conditions as described in the text.

29% for all substances. At this concentration, however, the elution order of amiodarone and the desethyl metabolite was reversed with retention times of 6.5 min and 6.0 min, respectively, and the compounds showed a partial separation with L6355 (t_R = 5.5 min) and a complete separation with L3937 (t_R = 4.3 min) and the internal standard (t_R = 10.0 min). Using a mobile phase with an ammonia content of 0.70% (v/v) a further decrease in retention times of all compounds by ca. 12% was observed and sharp symmetrical peaks were obtained. At this ammonia concentration desethylamiodarone and amiodarone were completely separated (k'_{DA} = 4.88, k'_{A} = 5.71, R = 1.02 and α = 1.17); however, no separation could be obtained between desethylamiodarone and L6355 (k' = 4.65). A further increase of the ammonia content to 1.0% (v/v) resulted only in a small decrease of ca. 2% of the retention times of the compounds investigated and did not lead to a markedly better separation between amiodarone and its desethyl metabolite (R = 1.11) or between the latter compound and L6355 (Fig. 5). The effect of the ammonia increase in the mobile phase on the chromatographic behaviour of the drugs involved may be explained by a suppression of the dissociation of these compounds by the in-

creasing ammonia concentration, resulting in a separation on the reversed-phase column based upon the respective lipophilic character of the drugs using almost pure methanol as eluant. In addition, the increase of the ammonia content to 1% also led to a pH of the mobile phase of 11.0, which may cause gradual deterioration of the column efficiency and reduction of the column life. Consequently, methanol with 0.70% ammonia 25% (v/v) was chosen as the final mobile phase for the simultaneous assay of amiodarone and desethylamiodarone. Using this condition, however, it should be noted that the potential metabolite L6355 may interfere with the assay of desethylamiodarone. This possible risk of interference, however, is insignificant in clinical practice, since in plasma and tissue samples only amiodarone and desethylamiodarone have been detected so far and, furthermore, in urine specimens neither amiodarone nor any of its potential metabolites have been found [13, 14]. If the presence of L6355 is suspected, samples can be analysed using a mobile phase with 0.35% ammonia.

For the detection of amiodarone three different wavelengths, 242, 254 and 280 nm, have been described [13–15, 19]. Using these different wavelengths it was shown that the highest assay sensitivity was at 242 nm, the λ_{max} of amiodarone in the mobile phase, and therefore this wavelength was selected for the detection of amiodarone and its potential metabolites.

Several procedures have been reported for the extraction of amiodarone from plasma or serum [13–15, 19]. The procedure of Flanagan et al. [13] involved acidification of the sample to pH 6.0 with phosphate buffer, followed by a single extraction with diisopropyl ether and direct injection of the supernatant on to the HPLC column. Cervelli et al. [15] and Lesko et al. [19] used a double solvent extraction with diethyl ether and hexane, respectively, after acidification of the plasma to pH 3.8 with an acetate buffer. After evaporation of the organic layer and reconstitution in mobile phase, the solutions were injected into the liquid chromatograph. Instead of extraction of amiodarone from plasma with an organic solvent, Andreasen et al. [14] used a simple deproteinization of plasma with ethanol, then centrifugation, followed by direct injection of the supernatant on to the HPLC column. Recoveries reported for these various procedures varied from about 80% for the extractions at pH 3.8 with diethyl ether and hexane to about 99% for the extraction at pH 6 with diisopropyl ether and for the deproteinization with ethanol. Using the same procedures for the extraction of desethylamiodarone, recoveries of about 8% at pH 3.8 and of 82% at pH 6.0 were observed, suggesting that for quantitative recovery of both drugs a pH of at least 6 or even higher should be used. This observation was supported by preliminary experiments of Flanagan et al. [13], indicating pH 7.4 as optimal for the extraction of desethylamiodarone. For the above-mentioned reason and to prevent losses due to extraction, a simple deproteinization with acetonitrile at the pH of plasma (7.4) was chosen as the method for the simultaneous assay of amiodarone and its desethyl metabolite in plasma. Acetonitrile was selected as protein precipitant because it is the most effective of the organic solvents used for deproteinization and it provides virtually complete removal of plasma proteins [24]. Using this protein precipitating method a quantitative recovery was observed for both amiodarone and desethylamiodarone, as shown in

Table III, and also for the potential metabolites L6355 and L3937. In addition an adequate precision (Table II), sufficient sensitivity and identical mean calibration curves in plasma (r = 0.9999) were obtained for both drugs. Besides, preliminary experiments have shown that the described method may easily be applied to 200 μl of plasma sample, using 400 μl of acetonitrile and 50 μl of injection volume, with the same sensitivity, accuracy and precision as obtained by the use of 1.0-ml plasma samples. Endogenous substances or a variety of drugs concomitantly used in amiodarone therapy have not been found to interfere with the analysis of amiodarone and its metabolites, indicating the versatility of the method.

Using the deproteinization method for urine specimens, even after injection of 10 μl of the supernatant a very broad solvent peak in the chromatogram was observed, caused by endogenous UV-absorbing substances from urine and limiting the detection of amiodarone and desethylamiodarone in urine to about 2 μg/ml.

To obtain cleaner urine extracts and a better sensitivity, an extraction at pH 7 with a mixture of diisopropyl ether—acetonitrile (95:5) using disposable Clin-Elut columns appears to be the most suitable. Using this procedure an acceptable solvent peak was shown in the chromatogram (Fig. 2), recoveries for amiodarone and its metabolites were adequate (Table III) and urine levels of 0.1 μg/ml for both drugs could easily be detected. In addition, by reconstitution of the residue with 750 μl of solvent instead of 3 ml a sensitivity of about 0.03 μg/ml could be obtained.

In contrast to urine, the direct plasma deproteinization method was applicable with appropriate sensitivity and selectivity to the analysis of amiodarone and its metabolites in various tissues (Figs. 2 and 3). The mean recoveries of both compounds of ca. 94% in the various tissues investigated (Table III) and the lack of interference from endogenous substances (Fig. 2) showed that the homogenization with 50% ethanol and the subsequent deproteinization with acetonitrile is an efficient and effective procedure, which has proved its feasibility for post-mortem tissue analysis. The data on amiodarone plasma concentrations after single and repeated oral administration are in good agreement with the levels observed by Andreasen et al. [14] and Flanagan et al. [13]. No comparable data are available on the concentration of desethylamiodarone in plasma after repeated oral administration. Our data indicate that in the steady-state, plasma levels of both compounds were in the same range, whereas in tissues concentrations of desethylamiodarone were approximately 2.5 times higher than the amiodarone concentration. So far, in urine, no measurable concentration of amiodarone and desethylamiodarone was found after single or repeated oral administration, which agrees with data reported previously [13, 14].

Furthermore, no detectable amounts were present ($\leqslant$ 0.05 μg/ml or μg/g) of the two potential metabolites L3937 and L6355 (Fig. 1) in urine and tissues of patients on amiodarone treatment.

Finally, the simultaneous assay of amiodarone and desethylamiodarone has been used for over a year and has shown to be simple, reliable and accurate. The Radial-PAK C_{18} column may be continuously used for about

three months with 40 injections daily, without any apparent loss in column efficiency.

The method could be a valuable tool for the further elucidation of amiodarone disposition and pharmacokinetics. In addition, it may be suitable for routine monitoring of plasma levels of amiodarone as well as its major metabolite desethylamiodarone, which is of great importance for establishing the relation between these levels and clinical effects or possible side-effects during chronic amiodarone therapy.

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