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SENSITIVE METHOD FOR THE MEASUREMENT OF AMIODARONE AND DESETHYLAMIODARONE IN SERUM AND TISSUE AND ITS APPLICATION TO DISPOSITION STUDIES*

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SUMMARY

A high-performance liquid chromatographic (HPLC) method for the measurement of amiodarone (AM) and its metabolite(s) in serum and tissues was developed. The method uses a 5-μm silica column, methanol containing 0.02% perchloric acid at pH 4 as the mobile phase, and ultraviolet detection at 240 nm. The standard curves for AM and desethylamiodarone (DAM) were linear for serum (range 0.025–6.0 μg/ml) and tissues (range 0.1–0.5 μg for 10–25 mg wet weight). There was a significant decrease as a function of time in AM and DAM concentrations in patients' sera left at ambient temperature in the presence of light. This HPLC method was applied to studies on serum AM elimination kinetics in patients and on tissue uptake during chronic AM administration to rabbits. The elimination half-life (5.8 h) of AM after a 5 mg/kg intravenous dose to a patient was similar to that after acute oral doses. AM, being lipophilic, accumulated maximally in the fat tissue (56 μg/g wet weight), followed by lung and liver in rabbits injected with AM for six weeks. The latter two tissues also contained nearly equal quantities of DAM. The high concentrations of AM and DAM in the liver and lungs may be related to the hepatic toxicity and pulmonary fibrosis associated with chronic AM therapy. Two new metabolites were found in the lung and bile of AM-treated rabbits, but these have not yet been identified.

INTRODUCTION

Amiodarone (AM, Fig. 1) is a drug effective in controlling various forms of cardiac arrhythmias[1], which has recently been approved in the U.S.A. for oral use. It undergoes hepatic metabolism to produce a major metabolite, desethylamiodarone (DAM), which can approach the concentrations of the parent drug during chronic therapy[2,3]. AM is characterized by its long serum elimination half-life ranging from 30 to 120 days[2,4] consistent with its slow onset and decline of action[5].

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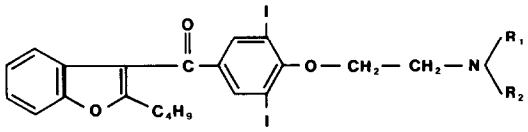


$R_1=R_2=C_2H_5$ AMIODARONE

$R_1=H$, $R_2=C_2H_5$ DESETHYLAMIODARONE

$R_1=R_2=H$ Di-N-desethylamiodarone

Fig. 1. Structure of amiodarone and its metabolites.

Several high-performance liquid chromatographic (HPLC) methods for the quantitation of AM in serum have been reported, and these have recently been summarized in a review[6]. While some of these methods can be used to quantitate AM and DAM simultaneously, they have not been used for the measurement of AM and its metabolite in tissue samples. The early work by Broekhuysen *et al.*[7] with radiolabeled AM had shown that the drug accumulates in several body tissues, particularly in fat and muscle. The major, serious side-effects of prolonged AM treatment have been reported to be pulmonary and hepatic toxicity[8,9]. Thus, it becomes important to study the metabolism of AM in these and other tissues during chronic AM therapy. Recently, an enzymatic method which requires a 16-h tissue digestion with collagenase has been used successfully to determine AM and DAM levels in autopsy tissue specimens[10].

In this communication, we describe a faster, non-enzymatic method for the determination of AM and DAM in several tissues. The method has been applied to a study of the tissue accumulation of AM in chronically AM-treated rabbits.

EXPERIMENTAL

Materials

Methanol and methyl *tert.*-butyl ether were obtained from Fisher Scientific (Tustin, CA, U.S.A.). Sodium hydroxide pellets (USP), perchloric acid, and sodium hydrogen phosphate were of reagent grade, also from Fisher Scientific. The reference compounds, amiodarone and desethylamiodarone, and the internal standard L8040, a brominated analogue, were kindly supplied by Sanofi (Brussels, Belgium). Fenethazine hydrochloride was obtained from Rhone-Poulenc (Paris, France).

Instrumentation and chromatographic conditions

A Waters (Millipore-Waters, Milford, MA, U.S.A.) liquid chromatograph, equipped with an M6000A pump, an M450 variable-wavelength detector set at 240 nm, and an Altex (Beckman Instruments, Palo Alto, CA, U.S.A.) 5-μm Ultrasphere Si column (25 cm $\times$ 4.6 mm I.D.), was used for analysis. The mobile phase consisted of methanol containing 0.02% perchloric acid and was adjusted to pH 4.0 with 0.1 *M* methanolic sodium hydroxide. The flow-rate was 0.9 ml/min. Peak areas were recorded by a Hewlett-Packard Model 3380A integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

Assay procedure

Serum. To 0.2 ml serum in a 12 × 75 mm polypropylene tube, 40 μl phosphate buffer (pH 4.5) and 0.5 μg fenethazine internal standard in 10 μl methanol were added. The serum was then extracted with 0.75 ml methyl *tert.*-butyl ether for 20 min by shaking in a mechanical shaker. After centrifugation at 10 000 *g* for 15 min, the supernatant layer was transferred to a 6 × 50 mm culture tube, dried under nitrogen and reconstituted in 25 μl methanol for HPLC analysis.

Standard curves were prepared for each batch of clinical specimens from duplicate analyses of normal human serum (Irvine Scientific, Irvine, CA, U.S.A.), containing varying concentrations of AM and DAM, ranging from 0.025 to 6 μg/ml.

Tissue. Tissue (20–25 mg wet weight) was minced and homogenized with 1 ml methanol, containing 0.5 μg L8040, in a hand homogenizer. The homogenizer was washed with an additional 1 ml methanol. The combined methanol suspensions were dried under nitrogen at 40°C and reconstituted in 0.2 ml control serum. This was followed by *tert.*-butyl ether extraction, as outlined above for serum. Fat tissue (10 mg wet weight) was homogenized in chloroform–methanol (2:1), containing 0.5 μg fenethazine as internal standard.

Animal experiments. Six male New Zealand White rabbits were injected intraperitoneally with AM (20 mg/kg body weight) for six weeks[11] while another group of six received saline. After collecting 2 ml blood from the ear vein, the rabbits were sacrificed under nembutal anesthesia (50 mg/kg). Liver, lung, heart, spleen, kidney, and brain were dissected, blotted dry, and weighed. Bile was collected in graduated tubes. Specimens of inguinal adipose tissue and thigh muscle were also collected. All tissues were kept frozen at −20°C until analyzed.

Intravenous kinetics. From one patient with supraventricular arrhythmias, injected intravenously with 5 mg/kg AM, blood samples were collected before and 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 h after the injection. The blood was centrifuged the same day and the serum was stored at −20°C until analyzed.

For studying the effects of storage on AM and DAM on serum concentrations, sera from two patients, who were on a maintenance dose of 400–600 mg per day for more than a year, were analyzed immediately after thawing of frozen specimens and after 12, 20, and 27 days at ambient temperature in the presence of light.

RESULTS

Varying amounts of AM and DAM were added to control human and rabbit sera and their concentrations were determined according to the extraction procedure for serum outlined in Experimental. Serum as well as tissue standard curves were prepared by plotting the peak-area ratio of AM (or DAM) to internal standard in the ordinate and to the amount of AM or DAM added to control serum in the abscissa. The standard curve for rabbit serum was linear from 0.025 to 6.0 μg/ml. The slope, intercept, and correlation coefficient were 1.127, 0.202, and 0.984, respectively, for AM and 1.087, 0.055, and 0.990, respectively, for DAM. The standard curves for normal human serum (not shown) were linear from 0.025 to 10 μg/ml. The slope, intercept, and regression for AM and DAM were 1.128, 0.039, 0.991 and 1.112, −0.012, 0.995, respectively. The lowest detection limit for both rabbit and human sera were 0.01 μg/ml for AM and DAM. The inter- and intra-assay coefficients of

TABLE I

EFFECT OF STORAGE ON AM AND DAM LEVELS IN HUMAN SERUM

Initial concentrations were measured in duplicate in freshly thawed frozen serum. Subsequent determinations were made on sera, stored in plastic vials at ambient temperature in the presence of light.

Patient	*Storage (days)*	*Concentration (μg/ml)*		*Recovery (%)*	
		AM	*DAM*	*AM*	*DAM*
G.S.	1	2.28	3.35	100	100
	12	2.19	1.77	98	53
	20	2.11	1.44	95	43
	27	1.90	1.40	83	42
P.F.	1	1.57	1.35	100	100
	12	1.30	1.16	82	86
	20	1.04	0.89	66	66
	27	0.78	0.97	50	71

variation were 2.1 ± 0.7 and 6.8 ± 1.1%, respectively, at an AM (DAM) concentration of 1 μg/ml.

Table I shows the data on stability of AM and DAM as a function of time in the serum of two patients (P.F. and G.S.), receiving 400–600 mg AM daily for over a year. The concentration at the initial measurement (day 1) from the freshly thawed frozen serum was taken as 100%. The sera were left at room temperature in the presence of light for 12, 20, and 27 days. A standard curve was also prepared for each day of serum analysis. The recovery of AM decreased with time with a significant decrease to 50% in P.F. and 83% in G.S. in 27 days. The DAM concentration also decreased significantly to 42 and 71%, respectively, in 27 days in the two patients.

The standard curves for AM and DAM in liver tissue were linear from 0.1 to 0.5 μg. This range was chosen to correspond to approximate levels expected in postmortem tissue specimens[4,12]. The intercept, slope, and correlation coefficient for AM were 1.282, −0.021, and 0.984 and those for DAM were 1.093, −0.032, and 0.986, respectively. In addition to serum and liver, standard cuirves were also obtained for heart, lung, kidney, muscle, spleen, and fat tissues. The standard curve parameters for all the above tissues both for AM and the metabolite are listed in Table II. As mentioned above for liver tissue, the curves were prepared by adding known amounts of AM and DAM to 20–25 mg wet tissue and using L8040 as internal standard during extraction. In the case of fat tissue, 10 mg tissue were used, and fenethazine served as the internal standard. Standard curve for DAM was not prepared for fat tissue, since this tissue contains negligible quantities of the metabolite[4]. As shown in Table II, the standard curve for all tissues studied were linear with a regression of >0.9 for AM and DAM.

Figs. 2 and 3 show representative chromatograms, obtained from extracts of lung and bile of a rabbit treated for six weeks with AM. In Fig. 2 one extra peak eluted before DAM was seen. In bile, there were two peaks in addition to DAM and AM (marked U). One of these unknown metabolites eluted before DAM (similar to that in the lung chromatogram) while the other had a retention time between those

TABLE II

STANDARD CURVE PARAMETERS FOR TISSUE ANALYSIS OF AMIODARONE AND DESETHYLAMIODARONE

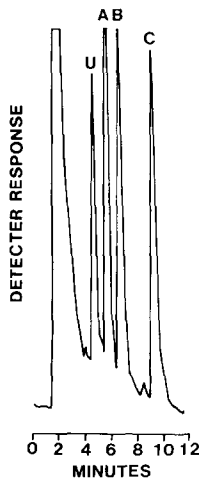
Data are derived from standard curves, prepared by adding varying amounts of AM and DAM to tissues from control rabbits. Two to four determinations were made for each concentration listed in the range column.

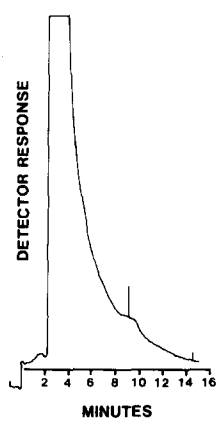
Tissue	*Amount*	*Range* (μg)*	*Amiodarone*			*Desethylamiodarone*		
			Slope	*Intercept*	*r*	*Slope*	*Intercept*	*r*
Serum	0.40 ml	0.05–6.0	1.128	0.202	0.984	1.087	0.055	0.990
Heart	25 mg	0.1 –0.5	0.930	−0.075	0.950	1.07	−0.067	0.935
Liver	25 mg	0.1 –0.5	1.282	−0.021	0.984	1.093	−0.032	0.986
Lung	25 mg	0.1 –0.5	1.466	−0.001	0.988	1.208	0.010	0.989
Kidney	25 mg	0.1 –0.5	1.481	−0.240	0.995	1.192	0.024	0.986
Muscle	25 mg	0.1 –0.5	1.351	0.004	0.991	1.015	0.021	0.990
Spleen	20 mg	0.2 –0.5	1.136	0.193	0.967	1.174	0.007	0.995
Fat	10 mg	0.2 –0.5	1.500	−0.037	0.982	–	–	–

* Same range for both amiodarone and desethylamiodarone.

of DAM and L8040 (Fig. 3). No attempts were made to characterize these peaks from lung and bile tissues.

Fig. 4 shows the intravenous kinetics of AM in a patient with supraventricular arrhythmia. After an intravenous dose of 5 mg/kg, serum concentrations were determined up to 24 h. The elimination kinetics of AM followed a biexponential function in this patient. The elimination half-life was 5.8 h. No metabolite was detected in serum after the intravenous dose.





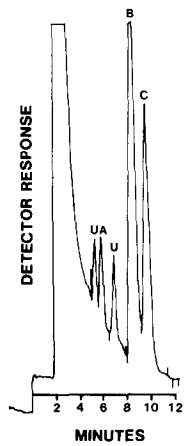


Fig. 2. Chromatogram of lung extract from a rabbit treated with amiodarone for six weeks. For procedure, see text. Peaks: A = desethylamiodarone; B = L8040; C = amiodarone; U = unknown metabolite(s).

Fig. 3. Chromatograms of bile extracts from a control rabbit (left) and from a rabbit treated with amiodarone for six weeks (right). For peak identification, see legend to Fig. 2.

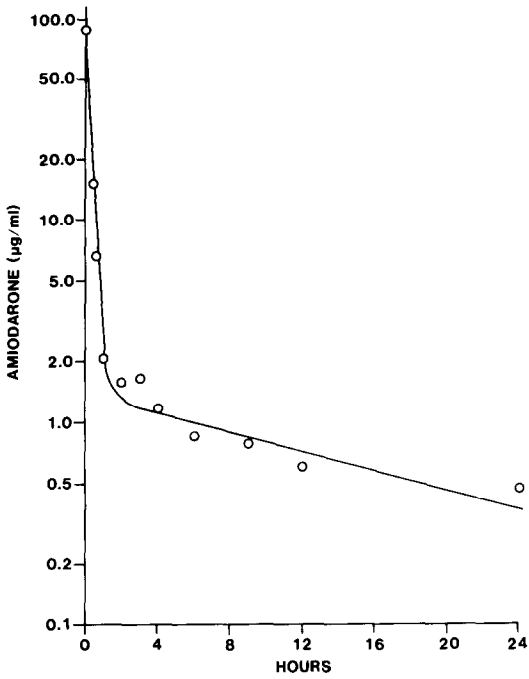


Fig. 4. Serum concentrations of amiodarone as a function of time in a patient injected intravenously with 5 mg/kg amiodarone.

Table III summarizes the tissue concentrations of AM and DAM in various organs of the rabbit, treated chronically with AM. The fat had the highest concentrations of AM and contained insignificant quantities of the metabolite. This was

TABLE III

AM AND DAM CONCENTRATIONS IN SERUM AND TISSUES AFTER CHRONIC AM ADMINISTRATION TO RABBITS

All values are mean ± S.E. from five to six rabbits except for bile (n = 3).

Tissue	*AM (μg/g)*	*DAM (μg/g)*	*AM/DAM ratio*
Fat	55.98 ± 8.88	Trace	–
Lung	23.03 ± 6.85	15.89 ± 4.41	1.51 ± 0.45
Liver	17.92 ± 7.43	18.56 ± 8.96	1.07 ± 0.58
Muscle	14.11 ± 5.28	2.67 ± 0.73	4.57 ± 0.70
Spleen	12.36 ± 4.20	10.49 ± 1.41	1.18 ± 0.27
Kidney	10.79 ± 1.69	6.81 ± 1.08	1.61 ± 0.33
Bile	7.05 ± 2.68 μg/ml	5.28 ± 1.17 μg/ml	1.24 ± 0.25
Ventricle	6.90 ± 1.00	3.12 ± 0.43	2.37 ± 0.50
Serum	1.42 ± 0.45 μg/ml	0.42 ± 0.20 μg/ml	3.95 ± 0.75
Brain	Trace*	Trace*	–

* AM and DAM in brain tissue were in insignificant concentrations, below the sensitivity of our HPLC method.

followed by lung, liver, and muscle (Table III). In the lung and liver, high concentrations of DAM were found so that the ratio of metabolite to parent drug was close to 1. The concentrations of AM (and metabolite) were the lowest in the heart, the site of action of the drug. Only traces of AM or DAM could be detected in the brain.

DISCUSSION

We have described an HPLC method for the measurement of AM and its chief metabolite in serum and in tissues and have demonstrated its application to clinical pharmacokinetics and tissue disposition in an animal model. The method uses a silica column and a mobile phase containing perchloric acid, first used by Flanagan *et al.*[13] for the separation of a variety of basic drugs. Solvent extraction of tissues instead of enzymatic digestion[10] reduced the time of analysis considerably, and only 10–20 mg of tissue were required for the assay. The linearity in the concentration range studied was satisfactory (Table III). No interfering peaks were seen in the chromatograms from any of the tissue, except fat, which had a large UV absorption extending from the solvent front to the metabolite peak. In this instance, fenethazine had to be used as an internal standard instead of L8040 to obtain better results. In the chromatograms of lung and bile an unidentified peak was observed with a retention time shorter than that of DAM. Another peak eluting between DAM and L8040 was also observed in the bile chromatogram (Fig. 3). Although these two peaks have not yet been identified, it is assumed that they correspond to di-N-desethylamiodarone and dideiodinated desethylamiodarone. These two metabolites have recently been characterized in the myocardium of chronically treated dogs[14] and in rabbit liver and intestine[15]. Staubli *et al.*[16] have presented evidence for the existence of a significant pool of iodine-containing metabolites other than DAM in the serum of chronically treated patients which had even longer elimination half-lives (98 ± 40 days) than AM or DAM.

Owing to an increasing number of reports documenting hepatic and pulmonary toxicity of AM[8,9,17,18], tissue distribution studies during chronic AM treatment have a special significance. The lung and liver in treated rabbits were found to have a high concentration of AM and its metabolite. The drug was not found in neural tissues, suggesting a blood brain barrier. The pattern of AM distribution in the rabbit seems to be generally similar to that in rats[19,20] and in post-mortem tissue specimens from man[4,21]. However, some aspects of AM metabolism in the rabbit are different from that in other species. Unlike human serum, where the metabolite/AM ratio is over 1 during chronic therapy, rabbit serum has a DAM/AM ratio of only 0.3. Although highly perfused tissues such as liver, lung, and kidney metabolize AM significantly, our failure to detect any uptake by the brain suggests that differential tissue binding (rather than perfusion rates) may be an important factor in species difference between the rabbit and other species.

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