High-performance Liquid Chromatographic Isolation and Fast Atom Bombardment Mass Spectrometric Identification of Di-*N*-desethylamiodarone, a New Metabolite of Amiodarone in the Dog

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Amiodarone is an antiarrhythmic drug which has received considerable attention in recent years. It has been suggested that the unusual pharmacodynamic characteristics of this drug may be due in part to the influence of active metabolites. Using fast atom bombardment (FAB) mass spectrometry we have identified a new metabolite of amiodarone, the di-*N*-desethyl analog (DDEA). This metabolite was present in the blood of dogs treated with the parent drug, and showed a greater affinity for myocardium than did the parent drug. The unique features of FAB mass spectrometry over electron impact mass spectrometry was an essential element in facilitating the identification of this new metabolite. Whether or not this metabolite has pharmacologic activity or is responsible for some of the side effects occurring during amiodarone administration is not known.

INTRODUCTION

Amiodarone (2-butyl-3-(4-diethylaminoethoxy-3,5-diiodobenzoyl)benzofuran hydrochloride) (Fig. 1) was first developed as an agent for the treatment of angina pectoris. However, now it is used primarily as an antiarrhythmic drug.[1]

The metabolic fate of amiodarone has not been totally elucidated; mono-*N*-desethylamiodarone (MDEA) is the only metabolite which has been positively identified in plasma from patients taking amiodarone (Fig. 1).[2]

Since amiodarone is a tertiary amine, one can anticipate N-dealkylation to be a significant route of metabolism. It has been shown that tertiary amines usually undergo a two-step N-dealkylation giving rise first to a secondary and subsequently a primary amine.[3,4] Using model compounds, McMahon[5] has shown that the N-demethylation of tertiary amines is faster than that of their secondary analogs.

While the mono-*N*-desethyl metabolite (MDEA) of amiodarone has been identified, the di-*N*-desethyl metabolite (DDEA) has not been reported previously. We have detected this metabolite in plasma and myocardium from dogs and herein describe the methods for its isolation and identification.

Flanagan *et al.*[2] have used low-resolution electron impact (EI) mass spectrometry and chemical ionization to characterize amiodarone and some of its possible metabolites in samples of pooled patient plasma. We have chosen to use fast atomic bombardment (FAB) mass spectrometry, a technique recently developed to study the structures of involatile and thermally or chemically labile compounds.[6–8]

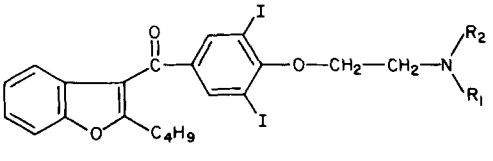
This method was found to be more suitable for the analysis of these compounds in biological samples for the following reasons: (1) high relative intensity of the pseudomolecular ion, (2) structurally significant fragmentation pattern at the higher masses, (3) less background interferences in the spectra and (4) mass spectra obtained without chemical derivatization.

After separation and isolation of these compounds by high performance liquid chromatography (HPLC), we have analysed, by FAB mass spectrometry, samples of plasma and myocardium from chronically treated dogs, and plasma and whole blood from patients being treated with oral amiodarone.

EXPERIMENTAL

Chemicals

Amiodarone hydrochloride, mono-*N*-desethylamiodarone (MDEA) and di-*N*-desethylamiodarone



Compound	R_1	R_2
Amiodarone	C_2H_5	C_2H_5
MDEA	C_2H_5	H
DDEA	H	H

Figure 1. Structural formulas of amiodarone, mono-*N*-desethylamiodarone (MDEA) and di-*N*-desethylamiodarone (DDEA).

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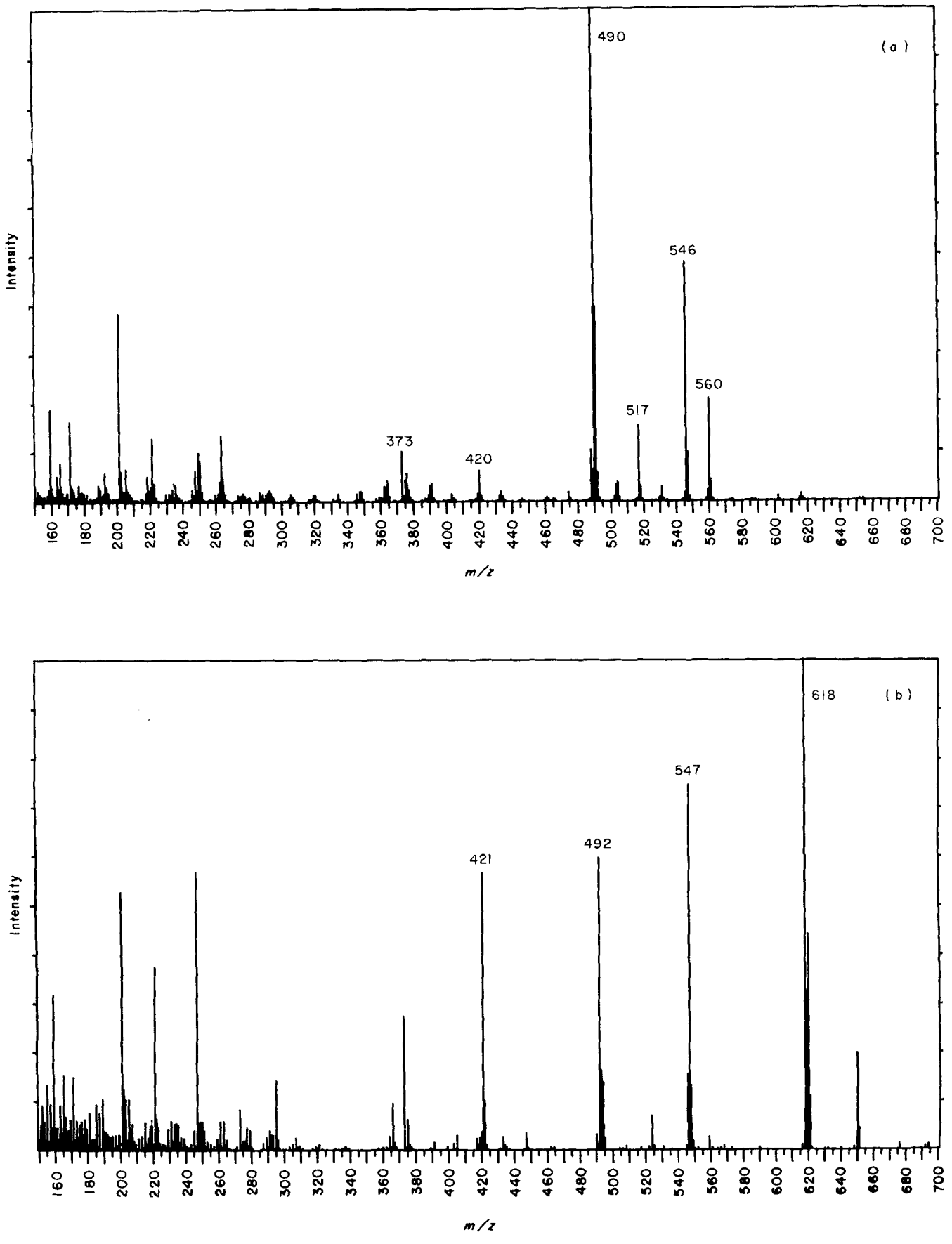


Figure 2. Electron impact (a) and positive ion FAB mass spectra (b) of mono-*N*-desethylamiodarone. The pure compound was isolated by HPLC before mass spectrometric analysis.

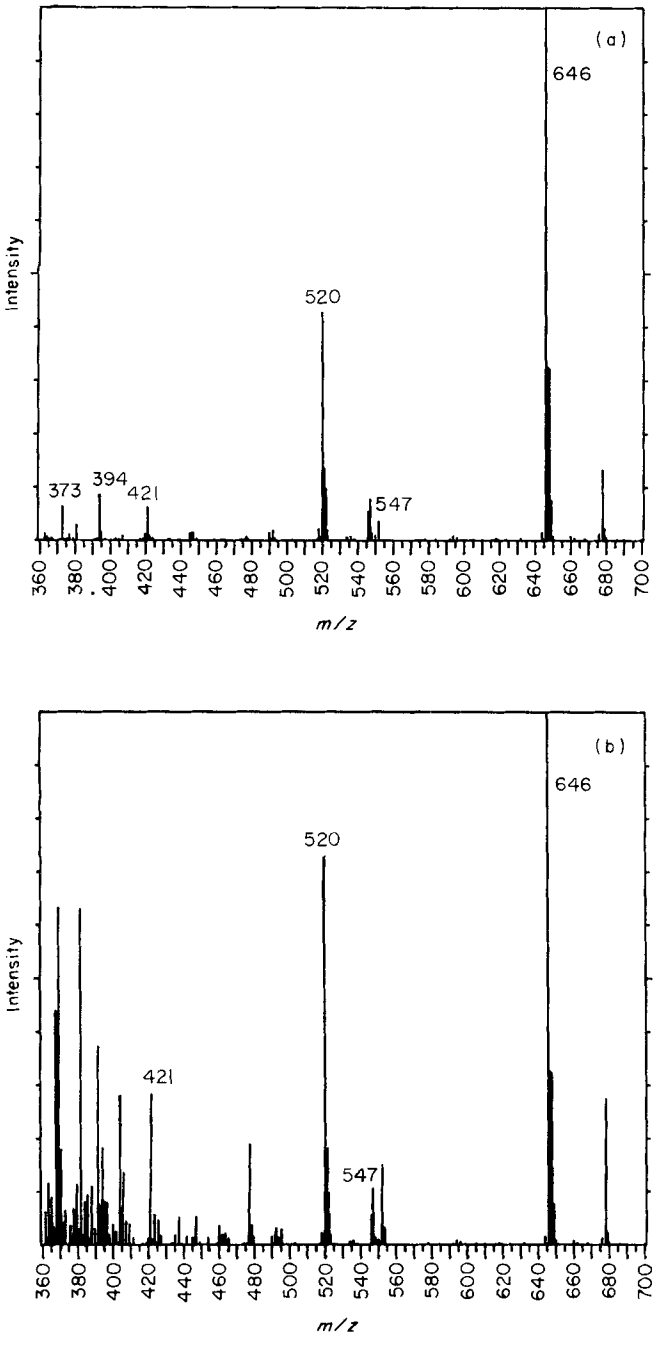


Figure 3. Positive ion FAB mass spectra of amiodarone external standard (a) and isolated from plasma of patients chronically treated with amiodarone (b).

(DDEA) oxalate were provided by SANOFI (Bruxelles, Belgium).

Spectrophotometric grade glycerol and oxalic acid (reagent grade) were obtained from Aldrich Chemical Co. (Wisconsin, USA).

Dogs

Four mongrel dogs were treated chronically for 5 weeks orally with amiodarone 400 mg twice daily. Experimental conditions and sampling protocols have been reported elsewhere.[9]

Patients

Four patients with ventricular tachycardia resistant to other drugs, were chronically treated with amiodarone hydrochloride (400–600 mg/day orally) for at least 1 month. Morning blood samples were obtained.

Isolation and purification of compounds

Amiodarone and its metabolites are extracted from plasma, whole blood or myocardium into an organic solvent and then separated and quantitated by HPLC. In brief, to aliquots (0.5 ml) of plasma or blood is added 0.5 μg of internal standard, and 2 ml of phosphate buffer (pH 5.4). This is extracted twice with 5 ml of hexane. The aqueous phase is discarded and the pooled organic phases evaporated to dryness at 35 °C under nitrogen. The residue is then redissolved in 0.1 ml of methanol and an aliquot (20–50 μl) injected onto the chromatographic column. Myocardial tissue samples are homogenized in 2 ml of methanol. Internal standard is added. The methanol is then evaporated and the residue redissolved in 2 ml of phosphate buffer and extracted as described above. The chromatographic column used is an Alltech Lichrosorb RP-8 (25 cm × 3.2 mm i.d.). The mobile phase is a mixture of 80% acetonitrate and 20% acetate buffer (pH 5.4). The flow rate is 2–3 ml min^{-1}. Compounds are detected by UV absorbance at a wavelength of 240 nm.

Amiodarone, MDEA and DDEA were isolated from the biological samples by fractional collection of the HPLC effluent, appropriate fractions were identified by monitoring UV absorbance at 240 nm.

The fractions were dried at 60 °C under a stream of nitrogen and then redissolved in 1 ml of 0.1 M phosphate buffer, pH 7.4. Five ml of benzene were added and shaken for 30 min. The tubes were centrifuged to separate the benzene and buffer. The benzene was removed to another tube and evaporated to dryness under nitrogen. The benzene extracts then were redissolved in 20 μl of methanol prior to mass spectrometric analysis. Both the biological samples and pure standards were processed via this isolation and purification scheme.

Mass spectrometry

Low-resolution EI mass spectrometry was performed on a Kratos MS-9. FAB mass spectrometry was performed on a Kratos MS-50S mass spectrometer equipped with a 23 kg magnet. A standard Kratos 8 keV Xe FAB gun and ion chamber were used to obtain a neutral xenon primary FAB beam.

Analytical conditions were: scan rate 30 and 100 s/decade, dynamic resolution M/ΔM 3000.

The samples were dissolved onto a glycerol/oxalic acid matrix (5% oxalic acid in glycerol) and deposited on a copper target surface ('tip'). The mass spectrometer was interfaced with a LOGOS II data system, permitting real-time assignment of masses.[10] Calibration of the mass spectrometer was achieved using Ultramark 1621 (PCR, Gainesville, Florida).

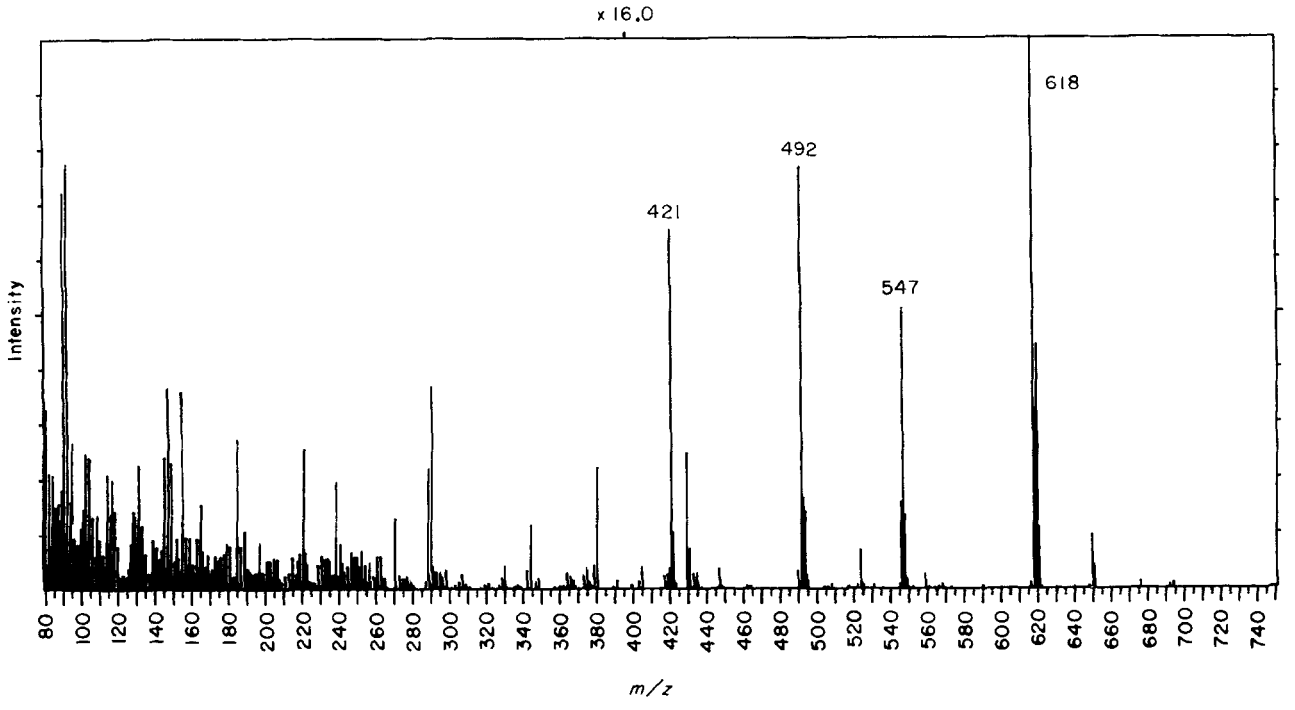


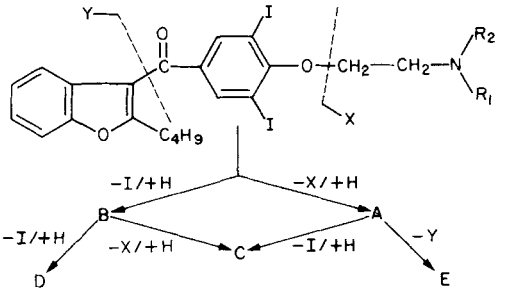
Figure 4. Positive ion FAB mass spectra of mono-*N*-desethylamiodarone isolated from plasma of patients chronically treated with amiodarone.

RESULTS

Analysis of standards

Electron impact spectra of amiodarone, MDEA and DDEA were found to have similar characteristics to those reported by Flanagan *et al* (1982).[2] The molecular ions are quite weak and the high mass fragments also are not abundant. This is shown for MDEA in Fig. 2(a). Only one characteristic and relatively abundant fragment, due to the loss of one iodine from the molecular ion, was detected at the high masses (*m/z* 518, 490, 462 for amiodarone, MDEA and DDEA respectively).

The positive ion FAB mass spectra of amiodarone, MDEA and DDEA show a rather abundant pseudomolecular ion and some interesting high mass fragments (Figs 2(b), 3, 4 and 5). Two fragmentation patterns are evident (Scheme 1): one due to the sequential loss of the two iodines, giving rise to specific ions for each of the three substances, and the other due to the loss of the amine group with the aliphatic chain. These last fragments are non-specific. Two other non-specific fragments are observed at *m/z* 373 and 421.



	AMIO	MDEA	DDEA
$[MH]^+$	646 (17.5)	618 (25.1)	590 (36.3)
A	547 (1.7)	547 (21.1)	547 (43.7)
B	520 (8.3)	492 (16.7)	464 (14.2)
C	421 (1.7)	421 (17.2)	421 (15.2)
D	394 (2.0)	366 (6.1)	338 (1.1)
E	373 (1.3)	373 (5.0)	373 (18.4)

Scheme 1. Fragmentation patterns of amiodarone and its metabolites due to positive ion FAB mass spectrometry. The masses of the significant ions are reported along with their relative abundance in parentheses.

Analysis of biological samples

Amiodarone and MDEA could be detected by HPLC both in plasma and myocardium of the treated dogs. A well-defined peak with a retention time corresponding to DDEA was evident in chromatograms of myocardial extracts (Fig. 6).

The measured concentrations of the three compounds in plasma and myocardium are reported in Table 1. Myocardial concentrations of the three compounds are

Table 1. Amiodarone, MDEA and DDEA concentrations in plasma and myocardium of dogs chronically treated with the drug

	Concentration ($\mu g\ ml^{-1}$)					
	Amiodarone		MDEA		DDEA	
Dog	Plasma	Myocard.	Plasma	Myocard.	Plasma	Myocard.
1	0.41	30.88	0.21	47.91	0.18	34.41
2	0.80	20.14	0.13	11.96	0.06	5.70
3	1.27	22.46	0.13	10.59	0.09	7.20

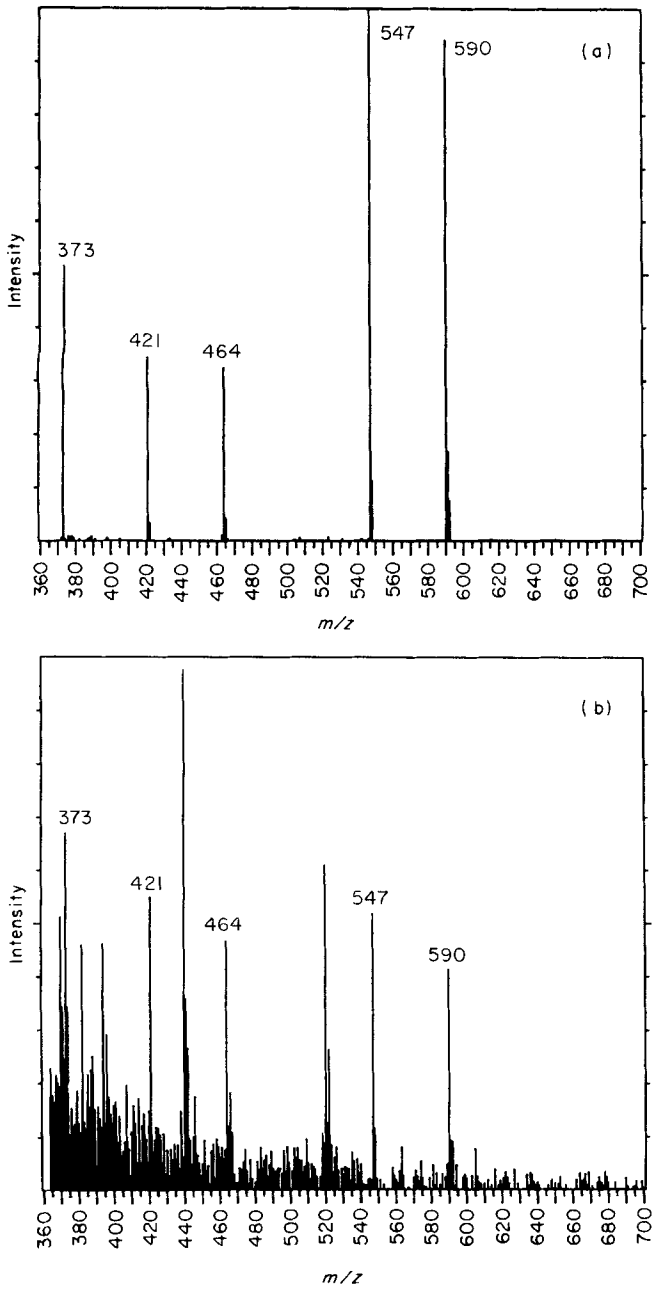


Figure 5. Positive ion FAB mass spectra of di-*N*-desethylamiodarone external standard (a) and isolated from myocardium of dogs chronically treated with amiodarone (b).

much higher than the plasma concentrations; moreover the two metabolites accumulate more in the myocardium. The myocardial/plasma concentration ratios ranged from 18 to 75 for amiodarone, from 81 to 228 for MDEA and from 80 to 191 for DDEA.

While amiodarone and MDEA were both present in patient plasma and whole blood, DDEA was not present in measurable quantities. A small peak eluting with a retention time corresponding to that of DDEA was evident in only a few chromatograms (Fig. 6). Plasma concentrations of amiodarone and MDEA in patients ranged respectively from 0.81 to 1.97 $\mu g\ ml^{-1}$ and from 0.45 to 0.62 $\mu g\ ml^{-1}$. Similar, but slightly higher levels were found in whole blood.

Due to the higher concentrations present in myocardium, the analyses by FAB mass spectrometry have been limited to these samples. Amiodarone, MDEA and DDEA were clearly identified in the samples examined; the spectrum of DDEA extracted and purified by HPLC from dog myocardium is shown in Fig. 5(b). Two unidentified ions at m/z 440 and 520 are present.

The identification of the di-N-desethylated metabolite of amiodarone was based on the peaks corresponding to the pseudomolecular ion and to the sequential loss of the two atoms of iodine, as already described (Scheme 1).

Even though there is a complete correspondence of fragments m/z between the standards and the samples, the relative intensities of the peaks are different.

DISCUSSION

Using FAB mass spectrometry combined with HPLC, it was possible to identify in the dog a new metabolite of amiodarone, its di-N-desethylated derivative, DDEA.

This finding was not unexpected, since the metabolic pathway involved in N-dealkylation reduces tertiary amines to secondary amines and finally to a primary amine. This has been described for many drugs.[11]

It has also been shown that some tertiary amines are dealkylated at a faster rate than their secondary analogs.[5] Based on these data, it was hypothesized that, during chronic treatment with a tertiary amine,[3] the secondary amine metabolite should accumulate more than the primary one and eventually also more than the parent compound. This seems to be the case for amiodarone in man, where MDEA plasma concentrations during chronic therapy are sometimes higher than those of amiodarone.[2] However, the presence of DDEA in human plasma has not been reported. MDEA concentrations in plasma of dogs during chronic oral amiodarone treatment are relatively lower than in man; the ratio between plasma concentrations of MDEA and amiodarone in dogs ranges between 0.12 and 0.60.[9] The primary amine metabolite of amiodarone, DDEA, is also present in dog plasma at concentrations higher than in man. Its high uptake into myocardium made the isolation and mass spectrometric identification of the compound, from this tissue, easier and more reliable.

FAB mass spectrometry has proven to be a very useful technique for the identification of amiodarone and its metabolites in biological samples. Some interesting characteristics of the FAB mass spectra reported should be emphasized. All the main fragmentations at high masses seem to involve a hydrogen rearrangement process, similar to the one reported for the chemical ionization mass spectra of these compounds for the ion m/z 547 $[MH-(CH_2{=}CH{-}NR_1R_2)]^+$.[2] Of specific and considerable interest in our case are the fragments 126 mass units apart, corresponding to the loss of an iodine atom with hydrogen rearrangement. The same effect has been noticed for other iodinated compounds (Burlingame, unpublished data). The relative abundance of the fragments seems to increase with increasing polarity and decreasing basicity of these compounds, that is going from the tertiary down to the primary amine. The relative abundances of the molecular ion and of the high mass fragments are not strictly constant, even within the same sample. This is due to factors that are currently under

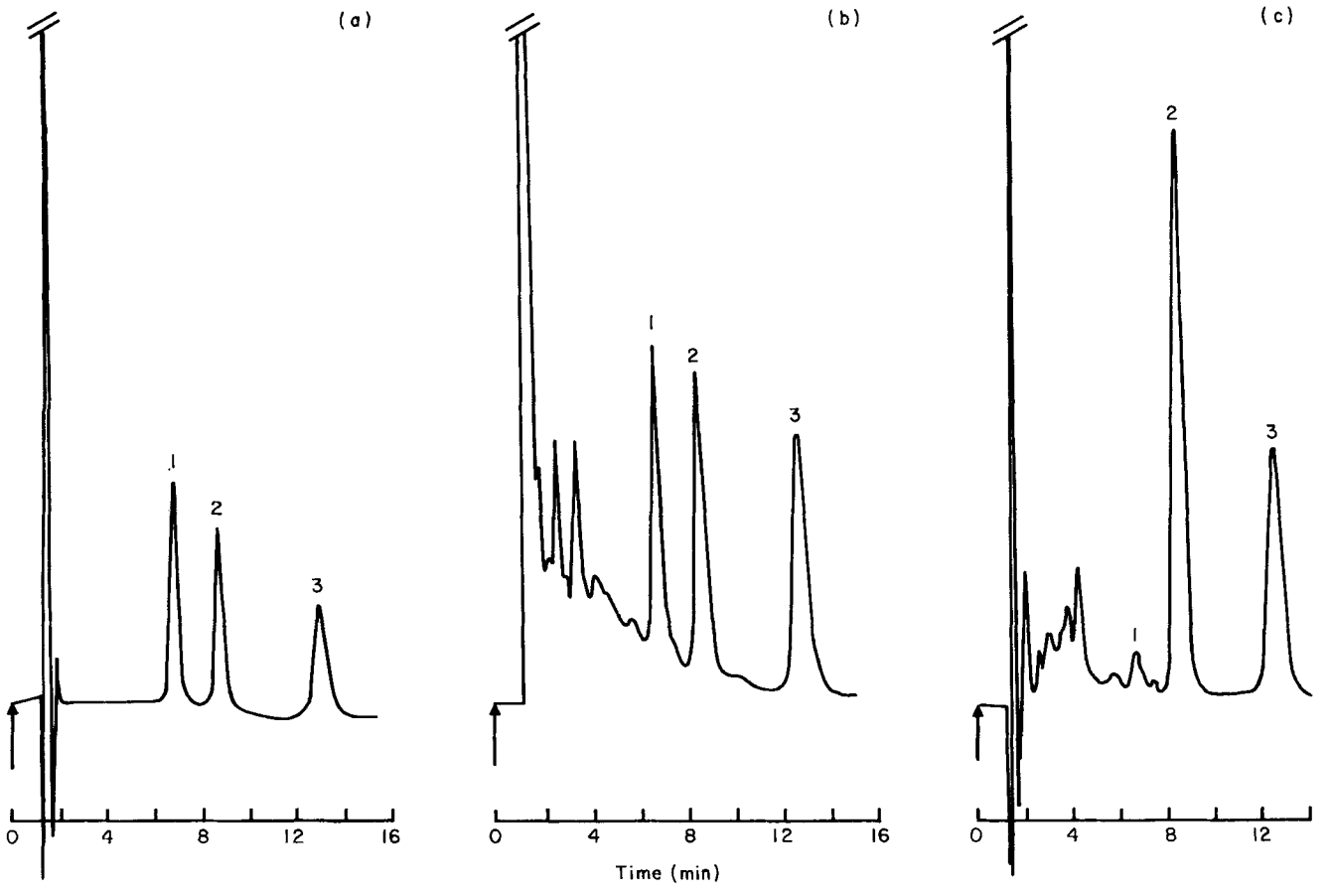


Figure 6. Reversed-phase HPLC chromatograms of a standard (a), a dog myocardium extract (b) and a patient plasma extract (c). The peaks correspond to di-N-desethylamiodarone (1), mono-*N*-desethylamiodarone (2) and amiodarone (3).

investigation. Possible factors include the flow density of the primary ion beam, and the relative amount of the compound in the sample analysed.

In conclusion, we have identified a new metabolite of amiodarone using fast atomic bombardment mass spectrometry. The unique features of FAB mass spectrometry over electron impact mass spectrometry was an essential element in facilitating the identification of this new metabolite. The two N-desethylated metabolites of amiodarone might be active in analogy to other antiarrhythmic drugs.[12] Whether they have pharmacologic activity or are responsible for some of the side effects occurring during amiodarone administration it is not known. Their accumulation in tissues, to levels higher than those of the parent drug may produce some effects even in the absence of high plasma levels.

Acknowledgement

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