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Identification and measurement of desethylamiodarone in blood plasma specimens from amiodarone-treated patients

R. J. FLANAGAN, G. C. A. STOREY, D. W. HOLT*, AND P. B. FARMER†

Poisons Unit, Guy's Hospital, St Thomas' Street, London, SE1 9RT, †MRC Toxicology Unit, Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey SM5 4EF, UK.

Desethylamiodarone has been identified as the principal lipophilic metabolite of amiodarone present in plasma specimens from amiodarone-treated patients. This structure has been confirmed by probe-injection mass spectrometry of column effluent fractions and comparison with the authentic compound using both chromatographic and mass spectrometric techniques. Although there is no information available as to the pharmacological activity of desethylamiodarone in man, the plasma concentrations of this metabolite attained during chronic amiodarone therapy are similar to those of the parent compound (0·1–4 mg litre^{-1}).

No detailed information on the metabolism in either man or laboratory animals of amiodarone (1; Fig. 1), an orally-effective antiarrhythmic agent, is available. However, the occurrence of a presumed metabolite of this drug was observed during the development of a high-performance liquid chromatographic (h.p.l.c.) method for the measurement of amiodarone in blood plasma or serum (Flanagan et al 1980).

Probe-injection mass spectrometry of column effluent fractions obtained on analysis of extracts of pooled plasma from patients treated with amiodarone has now been performed. Desethylamiodarone (2; Fig. 1) has been identified as the principal lipophilic metabolite present, and this identification has been confirmed by comparison with the authentic compound using both chromatographic and mass spectrometric techniques.

MATERIALS AND METHODS

Materials and reagents

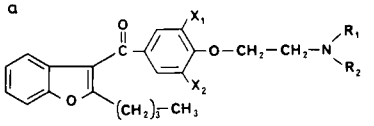
Amiodarone (1), desethylamiodarone (2) and the additional amiodarone analogues studied (Fig. 1) were obtained from Labaz (Brussels, Belgium). Perchloric acid (60%) (AR) was obtained from BDH (Poole, U.K.), and methanol, hexane and methyl t-butyl ether (all h.p.l.c. grade) from Rathburn (Walkerburn, U.K.).

* Correspondence: Poisons Unit, New Cross Hospital, Avonley Road, London SE14 5ER, U.K.

High performance liquid chromatography

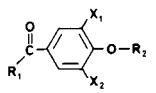
The solvent delivery system was a constant-flow reciprocating pump (Applied Chromatography Systems, Model 750/03) and sample injection was performed using a Rheodyne Model 7125 syringe-

a



Analogue No	R_1	R_2	X_1	X_2
1	C_2H_5	C_2H_5	I	I
2	C_2H_5	H	I	I
3	H	H	I	I
4	C_2H_5	C_2H_5	I	H
5	C_2H_5	H	I	H
6	H	H	I	H
7	C_2H_5	C_2H_5	H	H
8	C_2H_5	H	H	H
9	H	H	H	H

b



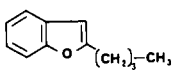
Analogue No	R_1	R_2	X_1	X_2
10		H	I	I
11	··	H	H	I
12	··	H	H	H
13	··	$(CH_2)_2$–N$(C_2H_5)_2$	I	I
14	OH	$(CH_2)_2$–N$(C_2H_5)_2$	I	I
15	OH	··	H	I

FIG. 1. (a) (b) Structural formulae of amiodarone, desethylamiodarone and the other amiodarone analogues studied.

loading valve fitted with a 2·0 ml sample loop. Stainless-steel tubing (0·25 mm i.d.) was used to connect the outlet port of the valve to the column, a stainless-steel tube, 250 × 8 mm (i.d.) packed with either Spherisorb S5W Silica or Spherisorb S50DS1 (both from Hichrom, Woodley, U.K.) and used at ambient temperature (normally 22 °C). The column effluent was monitored at 240 nm (Applied Chromatography Systems, Model 750/11). The eluent was methanol containing perchloric acid (0·04% (v/v), 3·7 mmol litre^{-1} (silica column) or 0·02% (v/v), 1·85 mmol litre^{-1} (ODS column)), and the flow-rate was 4·0 ml min^{-1}, maintained by a pressure of approximately 80 bar.

The chromatography on these systems of amiodarone and the amiodarone analogues studied (Fig. 1) is shown elsewhere (Flanagan et al 1982a).

Sample preparation—mass spectrometry

Pooled plasma specimens from amiodarone-treated patients were analysed by gas-liquid chromatography to ensure the absence of interference from diazepam and nordiazepam using the method of Rutherford (1977). Subsequently, portions (10 ml) of the pooled plasma specimens were extracted with methyl t-butyl ether (2 × 20 ml) for 15 min on a rotary mixer. The combined extracts from each pooled specimen were evaporated to dryness under a stream of air, and the residues were redissolved in methanol (1 ml) before h.p.l.c. analysis.

Column effluent fractions corresponding to the chromatographic peaks of interest (cf. Fig. 2a) were collected manually from the detector outlet into 10 ml glass conical test-tubes. Each effluent fraction was treated to remove perchloric acid by the addition of 2 ml glass-distilled water, evaporation of the organic component of the eluent (methanol) under a stream of compressed air, and extraction of the remaining contents of the tube with 2 × 5 ml portions of hexane. The combined hexane extracts were subsequently evaporated to dryness and reconstituted in 10 μl methanol before deposition onto the direct insertion probe of the mass spectrometer.

Mass spectrometry

Samples were admitted via the direct insertion probe into a VG Micromass 70–70F double focussing mass spectrometer. For electron impact (EI) operation the ionizing voltage was 70 eV and trap current 200 μA. For chemical ionization (CI) isobutane was used as reagent gas, the ionizing voltage was 50 eV and the emission current 500 μA. The source temperatures were 190–200 °C for EI and 140–160 °C for CI. An accelerating voltage of 3 kV and a scan speed of 1 s decade^{-1} were used.

RESULTS AND DISCUSSION

Before the development of h.p.l.c. methods for the measurement of plasma or serum amiodarone concentrations (Flanagan et al 1980; Andreasen et al 1981; Lesko et al 1981; Riva et al 1982), the only information available concerning the pharmacokinetics of amiodarone was work performed using ^{131}I-labelled drug (Broekhuysen et al 1969). Similarly, the metabolism of this compound was not known save for suggestions that the excretion of iodide anion was increased during amiodarone therapy (Andreasen et al 1981) and that amiodarone accounted for only 15–30% of the extractable, organic-bound, non-thyroxine iodine in plasma specimens from amiodarone-treated patients (Levis et al 1980).

The only additional information available concerning the metabolism of amiodarone has been the observation of a presumed metabolite during the development of an assay for the parent compound in blood plasma or serum (Flanagan et al 1980). The 'metabolite' was present in extracts of specimens obtained from patients treated chronically with amiodarone, and increased in concentration with time in samples from a patient soon after the commencement of therapy. The uv absorption spectrum of the 'metabolite' was found to be similar to that of amiodarone itself by measuring the relative

Table 1. Capacity factors (k') for amiodarone, desethylamiodarone and the other analogues studied. (For chromatographic conditions, see legend to Fig. 2).

Analogue No. (cf. Fig. 1)	Silica Column(1) k'	ODS Column(2) k'
10	0·2	0·7
11	0·2	0·5
12	0·2	0·4
3	2·1	1·4
2 (Desethylamiodarone)	2·5	2·2
6	2·7	1·4
9	2·7	1·1
13	2·9	3·5
8	3·0	1·8
5	3·2	2·0
14	3·5	1·9
1 (Amiodarone)	4·1	5·4
15	4·2	1·7
4	4·9	4·9
7	5·2	4·4

(1) Retention time of amiodarone = 10·2 min, retention time of non-retained peak = 2·0 min
(2) Retention time of amiodarone = 11·6 min, retention time of non-retained peak = 1·8 min

peak heights of the two compounds obtained on analysis of extracts of the same specimen at different wavelengths. However, the extraction efficiency of the 'metabolite' was found to be greater at pH 7·4 than under the acidic (pH 4·5) conditions optimal for the extraction of amiodarone, although it was difficult to interpret this latter finding in view of the apparently anomalous behaviour of the tertiary amine amiodarone.

High performance liquid chromatography

(i) *H.p.l.c. of amiodarone and analogues 2–15 (Fig. 1)*

To investigate further the nature of the 'metabolite' observed in our original work (Flanagan et al 1980), we have studied the chromatography of amiodarone and a range of analogues (Fig. 1) using both silica and octadecylsilyl-bonded silica columns with methanol containing perchloric acid as eluent (Table 1).

The characteristics of these non-aqueous ionic eluent systems have been discussed in detail (Flanagan et al 1982a) and include (i) in general, basic compounds only are retained under conditions where they are appreciably ionized, and (ii) for a range of structurally similar compounds, those substances of apparently greater polarity are often eluted before the remaining compounds. It is clear from the data presented in Table 1 that those analogues in which the amine side-chain of amiodarone has been lost (Fig. 1b, Analogues 10–12) are for practical purposes not retained on the silica system. The separation of amiodarone and the remaining analogues (Table 1) is clearly dominated by two factors, the degree of alkylation of the ethylamine moiety and the degree of iodination. On the silica column the elution order for a series of amines is 1°<2°<3°, e.g. di-desethylamiodarone (3)<desethylamiodarone (2) <amiodarone (1), whereas progressive loss of iodine normally produces increases in retention (e.g. amiodarone (1) <deiodoamiodarone (4) <di-deiodoamiodarone (7). On the ODS-bonded column, however, although the elution order for a series of amines remains 1°<2°<3°, the progressive loss of iodine results in decreased retention and thus, for example, the elution order for amiodarone and its de-iodinated analogues is reversed when compared with the elution order of these compounds on the silica column (Table 1).

(ii) *H.p.l.c. of sample extracts*

Chromatographic analysis of sample extracts was performed both to provide information concerning the chromatography of the presumed amiodarone metabolite present in sample extracts and to prepare column effluent fractions for subsequent probe-injection mass spectrometry. Extracts of pooled plasma from amiodarone-treated patients were prepared as discussed in the methodology section and analysed on both the silica and ODS-bonded silica systems, although only effluent fractions from the silica system were collected for mass spectrometry. The chromatograms obtained are illustrated in Fig. 2.

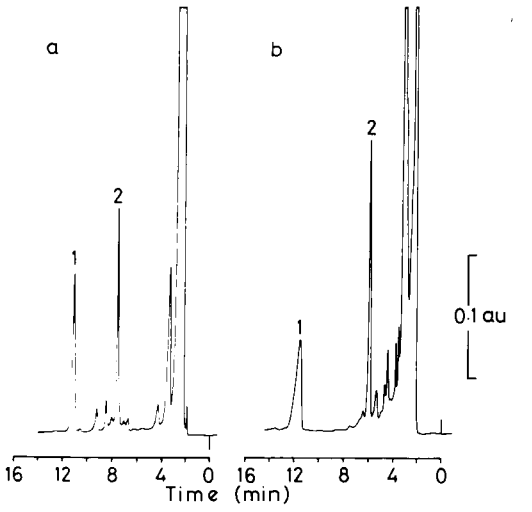


FIG. 2. Chromatogram obtained on analysis of a methyl t-butyl ether extract of pooled plasma (10 ml) from amiodarone-treated patients on a 250 × 8 mm (i.d.) column. Injection: 1·0 ml of extract re-constituted in methanol. Eluent: Methanol containing perchloric acid. Flow rate: 4·0 ml min^{-1}. Detection: uv, 240 nm. Peaks: 1 = amiodarone (Fraction 'A' for mass spectrometry from the silica column), 2 = desethylamiodarone (Fraction 'B' for mass spectrometry).
(a) Column packed with Spherisorb S5W Silica. Eluent perchloric acid concentration 0·04% (v/v) (3·7 mmol litre^{-1}) (The amiodarone and desethylamiodarone concentrations present in a portion of this same pooled plasma specimen were 0·8 and 0·6 mg litre^{-1}, respectively).
(b) Column packed with Spherisorb S50DS1. Eluent perchloric acid concentration 0·02% (v/v) (1·75 mmol litre^{-1}).

Only two major peaks with retention times of 5 min or greater were obtained on both chromatographic systems, and each peak was well-resolved from other components of the extract showing uv absorption at 240 nm except for a small shoulder eluting at the leading edge of Peak 2 (Fig. 2a). The retention time of Peak 1 (Fig. 2) corresponded to that of authentic amiodarone (1, Fig. 1a) on both systems, while it was found that the remaining major peak had retention times corresponding to those of authentic desethylamiodarone (2, Fig. 1a) (retention time relative to amiodarone 0·68 and 0·49 on the silica and ODS-bonded silica systems, respectively).

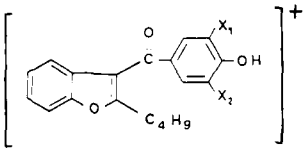
Since *N*-dealkylation is a common mode of foreign compound metabolism (Mandel 1971), and desethylamiodarone is well-resolved from the remaining analogues studied, the chromatographic data thus support the assignment of Peak 2 (Fig. 2) as desethylamiodarone.

Only 3 of the amiodarone analogues studied (Nos. 10–12, Fig. 1b) had retention times <4 min (Table 1), and it is not possible to draw conclusions about the presence or otherwise of these poorly-retained compounds on the sample-extract chromatograms (Fig. 2). However, some of the peaks represented on these chromatograms (Fig. 2) with retention times >4 min may represent additional lipophilic amiodarone metabolites, although no further fractions were collected for mass spectrometric analysis in view of the difficulties encountered in obtaining adequate sensitivity for amiodarone and the amiodarone metabolite studied when using probe-injection mass spectrometry of column effluent fractions (vide infra). In particular, the compound eluting at retention times of 0·82 and 0·47 relative to amiodarone on the silica and ODS systems, respectively (Fig. 2), may correspond to deiododesethylamiodarone (5, Fig. 1a). The presence of de-iodinated amiodarone metabolites might be expected from studies suggesting the excretion of free iodine during amiodarone therapy (Andreasen et al 1981). However, it must be remembered that these peaks may not represent further metabolites, although care was taken to ensure the absence of potential interference from diazepam and nordiazepam in the specimens analysed (Flanagan et al 1980).

Probe-injection mass spectrometry

(i) *Mass spectra of amiodarone and analogues 2–9 (Fig. 1a)*

In the EI mass spectra of these compounds the base peak was the ion $(CH_2{=}NR_1R_2)^+$ with the exception of analogue 3 where it was $(CH_2CH_2NR_1R_2)^+$ (Table 2). Molecular ions for the compounds with R_1 or $R_2{=}C_2H_5$ were weak or absent. High mass fragments were also not abundant although the ion



was observed for amiodarone and analogues 2, 3, 4, 8 and 9.

In the CI mode, using isobutane as the reagent gas, intense MH^+ ions were seen (Table 2). Abundant ions were observed following the cleavage of the bonds α- or β- to the nitrogen atom in the $R_1R_2NCH_2CH_2$ side chain. Thus for example the ion $[(MH)-(CH_2{=}CHNR_1R_2)]^+$ was present in all the spectra. $(CH_2CH_2NR_1R_2)^+$ and $(CH_2{=}NR_1R_2)^+$ were also abundant, the former of these ions being the base peak for amiodarone and desethylamiodarone and the latter ion being the base peak for analogues 4, 5, 7 and 8. The situation with analogues 3, 6 and 9 was difficult to interpret as the $(CH_2CH_2NR_1R_2)^+$ and $(CH_2{=}NR_1R_2)^+$ ions (*m/z* 44 and 30, respectively) were obscured by the CI reagent gas background. For this reason the ion intensities shown in Table 2 represent relative values only for ions of *m/z* values greater than 50.

(ii) *Mass spectra of analogues 14 and 15 (Fig. 1b)*

The EI mass spectra of analogues 14 and 15 are dominated by the formation of the $(CH_2{=}NR_1R_2)^+$ ion (*m/z* 86, 100%) (Table 2). All other ions of mass greater than this were of relative intensity less than 2%. MH^+ ions were observed in the CI spectra (14, 15·4%. 15, 15·6%) but again side chain fragmentation to *m/z* 86 (100%) and *m/z* 100 (14, 71·2%. 15, 85·0%) was very abundant.

(iii) *Mass spectra of analogues 10–13 (Fig. 1b)*

For analogues 10–12, which do not possess the $CH_2CH_2NR_1R_2$ side chain, the molecular ions and high mass fragments in the EI spectra were of much greater intensity than those observed for amiodarone and analogues 2–9, 14 and 15 (Table 3). A prominent fragmentation was to ion A (Table 3). The CI spectra showed considerably less fragmentation and in all cases MH^+ was the base peak. The only other fragment of significance was ion A (Table 3).

Analogue 13 (amiodarone *N*-oxide) appeared to undergo a ready thermal breakdown to analogue 10 (Fig. 1b). No molecular ion was seen in EI or CI and the mass spectra obtained were similar to those for analogue 10.

(iv) *Mass spectrometry of h.p.l.c. effluent fractions*

H.p.l.c. effluent fractions were analysed by CI mass spectrometry as it was found that the EI spectra gave little information concerning the molecular species present.

The isobutane CI spectrum of the material in h.p.l.c. fraction 'A' (Peak 1 Fig. 2a), showed *m/z* 646, 547, 100 and 86 and was very similar to the spectrum of amiodarone (Table 2). In view of the spectral similarity and the identity of retention time

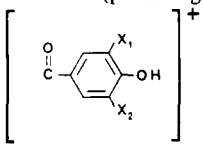
Table 2. Significant ions in the EI and CI (isobutane) mass spectra of amiodarone and some of the analogues studied.

	EI			CI (isobutane)			
Analogue No. (cf. Fig. 1)	M^+	$(M-CH_3)^+$	Base peak	MH^+	$[(MH)-(CH_2=CHNR_1R_2)]^+$	$(CH_2CH_2NR_1R_2)^+$	$(CH_2=NR_1R_2)^+$
1	645 (1·6)[a]	630 (1·7)	86 (100)	646 (8·6)	547 (12·6)	100 (100)	86 (88)
2	617 (0)	602 (0)	58 (100)	618 (68.1)	547 (18·5)	72 (100)	58 (65·8)
3	589 (2·7)	574 (0)	44 (100)	590 (13·6)	547 (5·6)	—(–)[b]	—(–)[b]
4	519 (0)	504 (0·4)	86 (100)	520 (11·6)	421 (4·2)	100 (98·5)	86 (100)
5	491 (0)	476 (0)	58 (100)	492 (68·7)	421 (8·1)	72 (31·7)	58 (100)
6	463 (61·8)	448 (0)	30 (100)	464 (100)	421 (7·9)	—(–)[b]	—(–)[b]
7	393 (0)	378 (0·5)	86 (100)	394 (48·4)	295 (5·7)	100 (75·8)	86 (100)
8	365 (2·4)	350 (0)	58 (100)	366 (40·7)	295 (4·8)	72 (20·2)	58 (100)
9	337 (88·1)	322 (0)	30 (100)	338 (100)	295 (6·4)	—(–)[b]	—(–)[b]
14	489 (0)	474 (1·2)	86 (100)	490 (15·4)	391 (6·7)	100 (71·2)	86 (100)
15	363 (0)	348 (0·8)	86 (100)	364 (15·6)	265 (2·9)	100 (85·0)	86 (100)

a. *m/z* value (percentage relative intensity).
b. Relative intensities of ions in the CI spectra are only quoted for ions of *m/z* values greater than 50.

Table 3. Significant ions in the EI and CI (isobutane) mass spectra of amiodarone analogues 10–12 (Fig. 1b).

	EI			CI (isobutane)	
Analogue No. (cf. Fig. 1b)	M^+	$(M-CHO)^+$	Ion A[b]	MH^+	Ion A[b]
10	546 (100)[a]	517 (68·3)	373 (44·9)	547 (100)	373 (18·6)
11	420 (100)	391 (93)	247 (79·1)	421 (100)	247 (12·8)
12	294 (57·1)	265 (83·3)	121 (100)	295 (100)	121 (34·9)

a. *m/z* value (percentage relative intensity).
b. 

of the peak corresponding to fraction 'A' with that of amiodarone, the compound in this fraction may be identified as unchanged amiodarone (1, Fig. 1).

Fraction 'B' (Peak 2 Fig. 2a) gave a CI mass spectrum with an MH^+ ion at *m/z* 618 (45·6%). Fragmentation was again to *m/z* 547 (20·6%) indicating (Table 2) that the amiodarone structure had been modified only in the diethylaminoethoxy side chain. This was confirmed by the absence of *m/z* 100 and the presence of a new fragment ion at *m/z* 72 (100%). It is apparent therefore that the material in fraction 'B' is the monodesethyl analogue of amiodarone (2, Fig. 1). Additionally, the retention time of authentic desethylamiodarone was the same as that of the peak corresponding to fraction 'B', which confirms the assignment of the latter's structure as the de-ethylated drug.

Considerable difficulty was experienced in the early stages of this work in obtaining adequate sensitivity and freedom from interference when performing probe-injection mass spectrometry of column effluent fractions. It was found initially that removal of perchloric acid from the column effluent before deposition onto the mass spectrometer probe gave rise to enhanced sensitivity. Secondly, reconstitution of the methyl t-butyl ether plasma extract in methanol before analysis by h.p.l.c., while taking up all of the amiodarone and desethylamiodarone present, was found to leave a considerable methanol-insoluble residue. Mass spectrometric analysis of column effluent fractions performed using these methanol-reconstituted extracts were found to give rise to less interference on the spectra of the compounds of interest than obtained on previous occasions when using extracts reconstituted in methyl t-butyl ether.

Desethylamiodarone concentrations attained during chronic amiodarone therapy

Although the desethylamiodarone plasma concentrations attained within 2 days of the commencement of oral amiodarone therapy are usually much lower than those of the parent compound, the plasma desethylamiodarone concentrations often approach those of amiodarone itself after only 5 days (Flanagan et al 1982b). Thereafter, the plasma concentra-

tions of the two compounds remain approximately equivalent (w/w), and this is clearly shown by the data summarized in Table 4. Furthermore, preliminary data on the single-dose pharmacokinetics of amiodarone (Holt & Storey 1982) and observations in patients withdrawn from chronic amiodarone therapy (Wilkinson et al 1982) suggest that both amiodarone and desethylamiodarone have a long terminal half-life of elimination (ca 30–50 days).

Table 4. Daily dose of amiodarone and mean (with s.d.) plasma concentration of amiodarone and desethylamiodarone in patients receiving the drug for one month or longer.

Amiodarone dose (mg day^{-1})	Plasma concentration (mg litre^{-1})		
	Amiodarone	Desethylamiodarone	n
200	0·95 (0·40)	0·99 (0·31)	50
400	2·15 (0·82)	2·00 (0·54)	33
600	3·37 (1·57)	2·69 (1·26)	16

Since desethylamiodarone is present in such relatively large amounts and may possess pharmacological activity, it is obviously important to consider the presence of this compound when evaluating assay procedures for the measurement of amiodarone in biological fluids. Thus, not only should desethylamiodarone be considered as a potential source of interference, but also the simultaneous measurement of both compounds may prove useful. To this end, we have modified our original chromatographic system to eliminate potential interference from nordiazepam in the measurement of desethylamiodarone (Flanagan et al 1982b).

Conclusions

Desethylamiodarone has been identified as the principal lipophilic metabolite of amiodarone present in blood plasma specimens from amiodarone-treated patients, and the concentrations of this metabolite attained during chronic amiodarone therapy are similar to those of the parent compound. Thus, the presence of desethylamiodarone should be considered not only in relation to the pharmacological effects of amiodarone therapy but also when evaluating methods for the measurement of amiodarone in biological fluids.

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