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Plasma protein binding of amiodarone in a patient population: measurement by erythrocyte partitioning and a novel glass-binding method

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1 Amiodarone is an effective antiarrhythmic drug whose therapeutic usefulness is limited by variable pharmacokinetics and considerable toxicity. Total plasma concentrations are not reliably related to therapeutic effect, but if plasma protein binding varies between patients, then free drug concentrations may provide a better measure of drug effectiveness.

2 The plasma protein binding of amiodarone was measured by erythrocyte partitioning, and found to be the same in six healthy subjects and eight patients being treated for cardiac arrhythmias (mean = 99.98%; range 99.97–99.99%). The free fraction of amiodarone was independent of the total drug concentration ($r = -0.41$, $P > 0.50$) and albumin level ($r = -0.31$, $P > 0.50$).

3 These data show no advantage in monitoring free concentrations of amiodarone. On the other hand, the patients in this study did not receive very high doses of amiodarone, and were free from drug side effects and biochemical abnormalities. Possibly a more heterogeneous group of patients would show variability in amiodarone binding. This should be examined, especially for patients with variations in α_1-acid glycoprotein, a major ligand for basic drugs and a likely major binding protein for amiodarone.

Keywords amiodarone plasma protein binding therapeutic monitoring CT scans

Introduction

Amiodarone is an effective class III antiarrhythmic agent used for both supraventricular and ventricular arrhythmias unresponsive to other agents (Sloskey, 1983). Adverse effects are common and can be severe, which limits the clinical usefulness of the drug. There is no simple relationship between dose and either therapeutic effect or toxicity (Heger *et al.*, 1983; Latini *et al.*, 1984), no doubt at least partly because of the drug's inconvenient pharmacokinetic characteristics. The oral bioavailability is erratic, it accumulates extensively in body tissues, and is only slowly eliminated from the body with a terminal half-life of weeks or months after long-term therapy (Sloskey, 1983; Latini *et al.*, 1984). In addition, it is metabolised to desethylamiodarone, which may also be pharmacologically active, and is also highly tissue bound and slowly eliminated (Holt *et al.*, 1983). As would be expected from these pharmacokinetic properties, amiodarone shows a marked delay in onset and offset of pharmacological effect, which further

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confounds attempts at rational dosage adjustment (Latini *et al.*, 1984).

In order to reduce this pharmacokinetic variability, studies have been made of the blood drug concentration-response relationship. Total plasma drug concentrations of between 0.5 and 2.5 $\mu g\ ml^{-1}$ are effective in most patients, but others may require as little as 0.1 $\mu g\ ml^{-1}$ or as much as 11.9 $\mu g\ ml^{-1}$ (Latini *et al.*, 1984). We have previously found a lack of correlation between plasma drug concentrations of amiodarone or desethylamiodarone and antiarrhythmic effect, measured by prolongation of QTc interval (Markos *et al.*, 1985). Kannan *et al.* (1987) also found that serum amiodarone concentrations were not correlated with the drug's efficacy or toxicity. In reviewing the literature, Mason (1987) concluded that since the upper limit of the therapeutic range was not well established, measurement of the plasma concentration of amiodarone would be useful only to ensure adequate dosage and compliance, and to monitor withdrawal. Greenberg *et al.* (1987) found that monitoring the plasma concentrations of amiodarone and desethylamiodarone was of limited clinical value in assessing electrophysiological effects and predicting toxicity. On the other hand, another large study found that toxicity, but not apparent therapeutic failure (sudden death or recurrence of arrhythmias) was correlated with the serum amiodarone concentration (Falik *et al.*, 1987). These authors found that a serum amiodarone concentration of 2.5 $\mu g\ ml^{-1}$ or more had a 76% predictive value for identifying patients with an adverse drug effect. However, therapeutic failure was not attributable to low serum concentrations of amiodarone, as uncontrolled patients had a mean concentration of 2.4 $\mu g\ ml^{-1}$.

Amiodarone has been found to bind extensively to plasma proteins *in vitro*, apparently to albumin and lipoproteins (Lalloz *et al.*, 1984; Neyroz & Bonati, 1985). It is the free, or unbound, concentration of drug in plasma which is pharmacologically active, and variations in the fraction of drug bound can result in different total concentrations of drug being pharmacologically equivalent (Tozer, 1984). For drugs whose plasma protein binding varies significantly between patients, the free drug concentration can provide a more accurate measure of therapeutic effectiveness. Such drugs include phenytoin (Peterson *et al.*, 1985), carbamazepine (Levy & Schmidt, 1985) and disopyramide (Lima *et al.*, 1985). This phenomenon also occurs with the extremely highly bound (usually > 99.9%) hormone thyroxine, where binding abnormalities can result in very high serum thyroxine values in euthyroid subjects who have normal free thyroxine levels (Croxson *et al.*, 1985). Recently, Greenberg *et al.* (1987) have suggested that therapeutic monitoring of amiodarone may be improved by using free drug concentrations.

The objectives of this investigation were (1) to measure the plasma protein binding of amiodarone in patients undergoing treatment with the drug in order to assess the variability in binding, and (2) to use this to investigate the relationship between the free plasma concentration and tissue uptake, using hepatic amiodarone concentrations estimated by computerised tomography (CT) scanning (Markos *et al.*, 1985). The protein binding of amiodarone could not be satisfactorily measured using the classical methods of equilibrium dialysis and ultrafiltration because of extensive losses of drug to the apparatus. Protein binding was measured by erythrocyte partitioning (Ho Ngoc-Ta Trung *et al.*, 1984) and verified using a novel glass-binding method.

Methods

Chemicals

Amiodarone hydrochloride and [^{14}C]-amiodarone (50 $mCi\ mmol^{-1}$) were gifts from Labaz, Brussels, Belgium. The radiochemical purity of [^{14}C]-amiodarone was > 99% following a high performance liquid chromatographic (h.p.l.c.) clean-up procedure using the conditions described previously for plasma assays (Markos *et al.*, 1985). Initial attempts to purify amiodarone by thin layer chromatography (t.l.c.) on silica gel led to decomposition of the drug on the t.l.c. plate. Desethylamiodarone oxalate was supplied by Reckitt and Coleman, Pharmaceutical Division (NSW, Australia). All other chemicals were analytical grade, and water was glass distilled.

Liquid scintillation counting

Aliquots (100–400 μl) of plasma, plasma water and buffer were assayed for radioactivity by liquid scintillation (LS) spectrometry after mixing with 5 ml liquid scintillation fluid (Biofluor, New England Nuclear). The disintegrations per minute ($d\ min^{-1}$) were calculated after correction for background and quenching effects. Whole blood and erythrocyte suspensions were decolourised before counting by the following procedure. Aliquots (100 μl) were added to 0.5 ml of a 1:2 v/v mixture of Protosol (New England Nuclear) and ethanol in a glass LS counting vial which was loosely capped and heated in a shaking

water bath (60° C, 1 h). After cooling, the sample was bleached by dropwise addition of 0.5 ml 30% hydrogen peroxide, and re-incubated (60° C, 30 min). Then 15 ml Biofluor was added, followed by 0.5 ml of 0.5 M HCl, and the vial shaken and stored overnight in the dark (to extinguish chemiluminescence) before counting.

Equilibrium dialysis

Aliquots (0.8 ml) of fresh plasma were placed in 1 ml Teflon dialysis cells (Spectrum Medical Industries, Los Angeles, CA, USA) and spiked with radiolabelled amiodarone (2 μg; 324,000 d min^{-1} in 10 μl ethanol), and the chamber flushed with carbogen before stoppering. Then 0.8 ml 0.067 M phosphate buffer (pH 7.4) was placed in the other half of the dialysis cell, and the cells rotated (15 rev min^{-1}) at 37° C for up to 24 h. The dialysis membranes used were Spectropor 2 (MW cut-off 12000–14000) or Spectropor 6 (MW cut-off 25000). The unbound fraction was calculated from d min^{-1} buffer/d min^{-1} plasma. After removal of aliquots of buffer for counting, several drops of 5 M HCl were added to the residue as a check for leakage of protein from the other side of the dialysis membrane. Loss of amiodarone to the apparatus was determined using buffer in both half cells, with radiolabelled amiodarone added to one side.

Ultrafiltration

Fresh plasma (1.0 ml) spiked with radiolabelled amiodarone (2.5 μg; 405,000 d min^{-1} in 10 μl ethanol) was placed in the upper chamber of an Amicon MPS-1 micropartition system (Danvers, MA, USA) fitted with a YMT ultrafiltration membrane, and 0.1 ml removed for counting. The device was then centrifuged (1100 *g*, 25 min) at 37° C to produce approximately 0.2 ml ultrafiltrate, and 0.1 ml counted. The unbound fraction was calculated from d min^{-1} ultrafiltrate/d min^{-1} plasma. The EMIT free level ultrafiltration system (Syva, Palo Alto, CA, USA) was also tried. Loss of [^{14}C]-amiodarone to the apparatus was determined using buffer in place of plasma.

Erythrocyte partitioning

In this method it is assumed that the unbound concentration of drug in plasma is in equilibrium with that in erythrocytes, and the unbound fraction in plasma is estimated by comparing the partitioning of drug into red cells from plasma and from buffer (Garrett & Hunt, 1974). This has been found to be reproducible and precise when compared with the classical methods of equilibrium dialysis and ultrafiltration (Ho Ngoc-Ta Trung *et al.*, 1984).

Radiolabelled amiodarone (4 μg; 648,000 d min^{-1} in 10 μl methanol) was added to 5 ml fresh heparinised blood which was then incubated (37° C, 10 min) with gentle orbital shaking. The haematocrit (H) was measured, and duplicate 100 μl aliquots taken for counting whole blood d min^{-1} (WB). After centrifugation (2500 *g*, 2 min) duplicate 100 μl aliquots of plasma were counted (P). Erythrocytes from the same blood sample were obtained by centrifugation (1200 *g*, 10 min), and gently washed four times with two volumes of isotonic phosphate buffer (0.067 M, pH 7.4) before being resuspended in sufficient buffer to give the same haematocrit as the original blood sample. As for whole blood, radiolabelled amiodarone was added to 5 ml erythrocyte suspension which was incubated and duplicate 100 μl aliquots of the suspension taken for counting (S). The haematocrit was checked and, after centrifugation, duplicate 300 μl samples of the supernatant buffer counted (B).

The ratio of drug concentrations in erythrocytes and plasma (E/P) was calculated from the following equation (Ho Ngoc-Ta Trung *et al.*, 1984):

$$\frac{E}{P} = \frac{(WB/P) - (1-H)}{H} \quad (1)$$

Similarly, the erythrocyte/buffer concentration ratio is (assuming linearity at concentrations up to therapeutic levels):

$$\frac{E}{B} = \frac{(S/B) - (1-H)}{H} \quad (2)$$

The unbound fraction of drug (fu) in plasma can then be calculated.

$$fu = \frac{E/P}{E/B} \quad (3)$$

The bound fraction is, of course, $1-fu$.

A standard curve of erythrocyte amiodarone concentration (E) as a function of drug concentration in buffer (B) was constructed for a range of total amiodarone concentration in erythrocyte suspension from 0.4–31.5 μg ml^{-1} (achieved by including an appropriate amount of unlabelled amiodarone with the addition of [^{14}C]-amiodarone). The erythrocyte concentration was calculated from equation (2).

Other experiments examined the time course of equilibration, and the influence of haematocrit on the distribution, for whole blood and erythrocyte suspensions. The precision of the method

was estimated by repetitive determinations of E/P and E/B using blood from the same donor.

Glass-binding studies

From the failure of the equilibrium dialysis and ultrafiltration methods of measuring protein binding (see results) it was apparent that amiodarone binds extensively to the surface of many materials, especially plastics. Further experiments showed that amiodarone binds significantly to glass, and almost quantitatively when the glass has been silanised (Siliclad, Clay Adams, Parsippany, NJ, USA). This observation suggested a novel method of measuring protein binding, since it was hypothesised that only the free drug in solution would be available for binding to glass surfaces. The fraction of unbound drug in plasma could then be calculated by comparison of the binding of amiodarone to glass from plasma and from buffer. This method assumes that other constituents in plasma do not interfere with the binding of amiodarone to glass.

To each of a series of 1 ml glass tubes, treated with Siliclad, was added 0.9 ml isotonic phosphate buffer (0.067 M, pH 7.4) containing radiolabelled amiodarone (from 0.3 to 14.2 ng; 50–2300 d min^{-1}). The tubes were stoppered and incubated in an orbital shaking water bath (37° C, 2 h). The solutions from each tube were discarded, and the tubes rinsed twice with 0.6 ml buffer and dried by centrifuging upside down on filter paper (2500 *g*, 5 min). The glass-bound amiodarone was removed by two washes with 0.5 ml methanol (with 60 s vortex mixing), and was counted with 5 ml Biofluor. The free (unbound) concentration in buffer (B_f) was calculated by subtracting the amount bound to the glass tube (G) from the total amount placed in each tube. A standard curve was then plotted relating B_f and G, with slope x:

$$B_f = \frac{G}{x} \quad (4)$$

The same procedure was used to measure glass-binding of amiodarone from fresh plasma samples, except a larger amount of [^{14}C]-amiodarone (0.5 μg; 81,000 d min^{-1}) was added. For equal drug binding to glass, it was assumed that the free concentrations in plasma (P_f) and buffer (B_f) were equal, thus:

$$P_f = B_f = \frac{G}{x} \quad (5)$$

and the fraction unbound in plasma (*fu*) could be calculated knowing the total drug concentration in plasma (P):

$$fu = \frac{P_f}{P} \quad (6)$$

The completeness of the buffer rinse was checked by adding 5 M HCl to the second rinse; no precipitation was found. In other experiments, the time course of equilibration was determined for plasma and buffer, and the influence on binding of the total amiodarone added (from 0.2 to 10.0 μg 0.9 ml^{-1}). The precision of the method was estimated by replicate determinations using the same plasma sample.

Patients

Eight patients (seven males, one female) who were receiving amiodarone for cardiac arrhythmias participated in the study after giving written informed consent. Coronary artery disease was the underlying abnormality in five of the patients. The arrhythmia was ventricular in five cases, supraventricular in three, and both in one case. The mean age of the patients was 60 years (range 38–77); seven patients were currently taking 200 mg amiodarone daily, and the remaining patient took 400 mg. The mean duration of therapy was 20 months (range 1.5 to 52 months), the mean cumulative dose was 136 g (range 11 to 330 g), and the mean duration of stable therapy at the time of the study was 13 months (range 0.1 to 35 months). Each patient was taking at least one other drug, and the major drugs used were: digoxin, β-adrenoceptor blockers, captopril, enalapril, glibenclamide, allopurinol, diuretics, nitrates and haloperidol. Details are available from the authors. Haematological, thyroid and liver function tests were performed on all patients. There were no abnormalities, except for a small elevation of hepatic serum enzyme levels in two patients. Liver computerised tomographic (CT) scans were carried out to estimate the hepatic accumulation of amiodarone and desethylamiodarone (Markos *et al.*, 1985).

Plasma drug concentrations

Venous blood was collected immediately before the usual morning dose of amiodarone to estimate the trough concentrations of drug and metabolite. The blood was collected in heparinised tubes and a portion centrifuged within 1 h and the plasma stored at −20° C until assayed by h.p.l.c. (Markos *et al.*, 1985). The remaining blood was kept at 4° C and used within 1 h to determine the free fraction of amiodarone by the erythrocyte partitioning method. The free amiodarone concentration in each blood sample was calculated from the total plasma drug concentration and the unbound fraction.

Results

Binding to normal plasma

Classical methods Attempts to measure the protein binding of amiodarone by equilibrium dialysis and ultrafiltration were not successful, as other workers have found (Andreason *et al.*, 1981; Neyroz & Bonati, 1985). Even after dialysis for 24 h, the radioactivity on the buffer side of the cell was no greater than the background level, while there was a 30% loss of counts from the plasma. In a control experiment, buffer spiked with [^{14}C]-amiodarone (500 ng ml^{-1}) was dialysed against blank buffer. After 24 h, the original concentration had fallen to 15 ng ml^{-1} while 10 ng ml^{-1} was found in the blank buffer, leaving 98% of the added radioactivity unaccounted for. Rinsing the dialysis membrane recovered 43% of radiolabelled drug. Both Spectropor 2 and 6 membranes gave similar results. There was some movement of fluid from buffer to plasma during the long periods of dialysis. This was not corrected for, as it did not affect the overall result.

Ultrafiltration of spiked plasma samples gave filtrates of background activity. When buffer solutions spiked with [^{14}C]-amiodarone (2500 ng ml^{-1}) were used, the recovery of drug in the ultrafiltrate was 5% for the EMIT system, and zero for the Amicon device. Rinsing the ultrafiltration membranes with methanol yielded 25–33% of the added drug. A buffer solution of [^{14}C]-amiodarone (50 ng ml^{-1}) left to stand in the plastic reservoir (styrene-acrylonitrile) at 37° C for 30 min lost 46% of drug, indicating significant adsorption to the walls of the chamber.

Erythrocyte partitioning Preliminary experiments showed that 5 min was sufficient for complete equilibration of amiodarone between buffer or plasma and erythrocytes, and that the concentrations remained constant for 60 min. The concentration of amiodarone in erythrocytes (E) was linearly related to that in the buffer (B) up to a total of erythrocyte suspension concentration of 31.5 μg ml^{-1} (Figure 1). Similar linear relationships have been reported for valproic acid (Shirkey *et al.*, 1985); imipramine, amitriptyline, lignocaine and propranolol (Ho Ngoc-Ta Trung *et al.*, 1984); phenytoin (Kurata & Wilkinson, 1974); and quinidine (Hughes *et al.*, 1975; Ho Ngoc-Ta Trung *et al.*, 1984). The ratios of amiodarone concentrations in erythrocyte/buffer (E/B) and erythrocyte/plasma (E/P) were reproducible when replicate determinations were made on the same blood sample, as was the calculated unbound fraction, *fu* (Table 1). In this blood sample *fu* was 2.4×10^{-4}, with a coefficient of variation (C.V.) of 9.3% ($n = 5$).

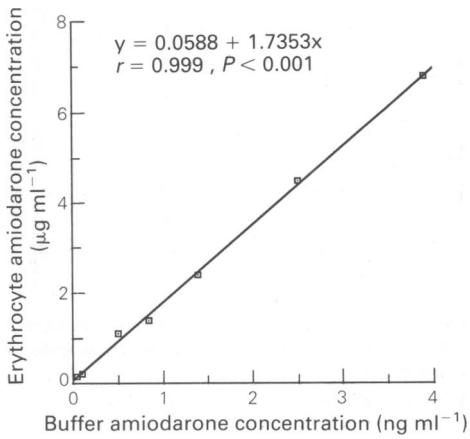

Figure 1 Distribution of amiodarone between buffer and erythrocytes.

The influence of haematocrit (H) on the distribution of amiodarone (total concentration 0.8 μg ml^{-1}) into red cells from buffer and plasma

Table 1 Reproducibility of amiodarone erythrocyte distribution and the estimated plasma protein binding, using blood from one individual

Sample number	*H*	*E/B*	*E/P*	*fu* ($\times 10^{-4}$)	*% free*
1	0.41	1818	0.404	2.22	0.022
2	0.41	1705	0.381	2.23	0.022
3	0.41	1435	0.352	2.45	0.024
4	0.41	1523	0.341	2.24	0.022
5	0.41	1650	0.450	2.73	0.027
Mean		1626	0.386	2.37	0.024
± s.d.		±151	±0.044	±0.22	±0.002
C.V. (%)		9.3	11.3	9.3	9.3

H = haematocrit; E/B = erythrocyte/buffer concentration ratio; E/P = erythrocyte/plasma concentration ratio; *fu* = fraction of amiodarone unbound in plasma calculated from E/P ÷ E/B.

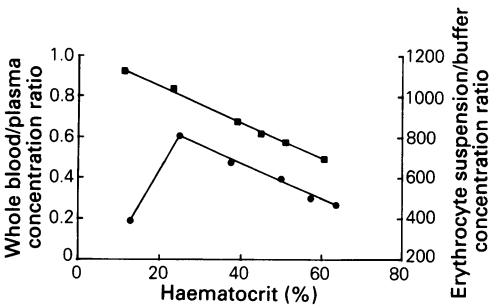

Figure 2 Effect of haematocrit on the distribution of amiodarone: ■ whole blood/plasma; ● erythrocyte suspension/buffer.
A typical experiment is shown.

was studied in blood from three subjects, with identical results. According to equation (1), if E/P is constant, then H should influence the whole blood/plasma ratio (WB/P) in a linear fashion. Similarly, by equation (2), if E/B remains constant then the erythrocyte suspension/buffer ratio (S/B) should vary linearly with H. Figure 2 shows that WB/P varied linearly with H, but that S/B was only linear at haematocrits above 0.25. Presumably in erythrocyte suspensions of lower haematocrits the cells become saturated with amiodarone, whereas this does not happen with whole blood because of the small fraction of unbound drug which is available for uptake by the cells. Even for buffer, however, the relationship is linear over the normal clinical range of haematocrits, and equations (1) and (2) are valid.

Blood was taken from six healthy subjects and E/P and E/S measured after adding a therapeutic concentration (0.8 μg ml^{-1}) of amiodarone to whole blood or erythrocyte suspensions. The mean unbound fraction of amiodarone by this method was 2.3×10^{-4} (s.d. = 0.5×10^{-4}, n = 6), with range 1.8×10^{-4} to 3.1×10^{-4} (Table 2).

Table 2 Amiodarone erythrocyte distribution and plasma protein binding by the erythrocyte partitioning method in healthy subjects

Subject	*E/B*	*E/P*	*fu* ($\times 10^{-4}$)	*% free*
1	1437	0.295	2.05	0.020
2	1915	0.440	2.30	0.023
3	2254	0.431	1.91	0.019
4	2366	0.599	2.53	0.025
5	2226	0.407	1.83	0.018
6	1577	0.488	3.09	0.031
Mean	1962	0.443	2.29	0.023
± s.d.	±385	±0.099	±0.47	±0.005

E/B = erythrocyte/buffer concentration ratio; E/P = erythrocyte/plasma concentration ratio; *fu* = fraction of amiodarone unbound in plasma calculated from E/P ÷ E/B.

Glass-binding method Preliminary experiments showed that 1 h was sufficient time for complete equilibration of radiolabelled amiodarone between buffer or plasma and silanised glass surfaces. The binding of [^{14}C]-amiodarone to glass varied linearly with buffer concentration, with no evidence of saturability with the addition of up to 14.2 ng drug to 0.9 ml buffer (Figure 3). The slope (x) was 2.084, indicating that about 70% of the amiodarone added was bound to the glass. The uptake of [^{14}C]-amiodarone to glass from plasma was also linear with additions of up to 10 μg per 0.9 ml plasma. When 500 ng amiodarone was added to plasma, the mean amount of glass-bound drug (G) was 1.76 ng (s.d. = 0.31, n= 5).

Using these values for x and G in equation (5) enables calculation of the free concentration of amiodarone in plasma:

$$P_f = \frac{G}{x} = \frac{1.76}{2.084} = 0.84 \text{ ng/0.9 ml plasma}$$

Hence, from equation (6) the free fraction of amiodarone in plasma:

$$fu = \frac{P_f}{P} = \frac{0.84 \text{ ng}}{500 \text{ ng}} = 0.00168$$

Thus the mean unbound fraction of amiodarone by this method was 16.8×10^{-4} (s.d. = 3.0×10^{-4}, n = 5). The C.V. of this method was 18% (n = 5).

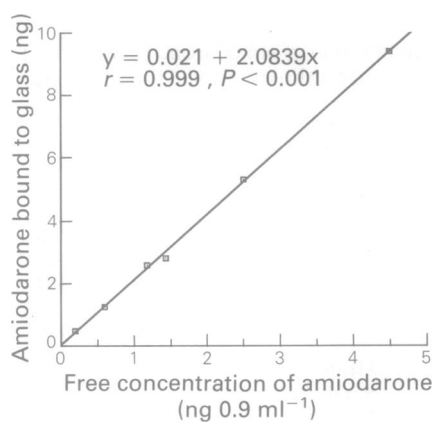

Figure 3 Relationship between the amount of amiodarone bound to siliconised glass tubes and the concentration of drug in buffer solution.

Clinical study

All patients had their arrhythmia well-controlled, and none suffered serious adverse effects from amiodarone. In six patients who had been stabilised on 200 mg amiodarone day^{-1} for at least 2 months, the mean total plasma concentration of drug was 0.98 $\mu g\ ml^{-1}$ (s.d. = 0.61), and the mean desethylamiodarone concentration was 0.67 $\mu g\ ml^{-1}$ (s.d. = 0.25).

The plasma protein binding of amiodarone was measured in each patient by the erythrocyte partitioning method (Table 3). The mean unbound fraction (fu) in patient plasma was 2.3×10^{-4} (s.d. = 0.6×10^{-4}), identical with that found in plasma from healthy subjects. The range of fu in patients (1.5×10^{-4} to 3.1×10^{-4}) was also the same as that found in healthy subjects (1.9×10^{-4} to 3.1×10^{-4}).

The total concentration of amiodarone in patient plasma correlated moderately well with the free concentration ($r = 0.84$, $P < 0.01$), but not with the free fraction ($r = -0.41$, $P > 0.50$) (Table 3). There was no correlation between the unbound fraction and albumin level measured by the dye-binding method ($r = -0.31$, $P > 0.50$). There was a close correlation between total amiodarone concentration and desethylamiodarone concentration ($r = 0.96$, $P < 0.001$). There was no significant correlation between the total amiodarone concentration and either current maintenance dosage or total cumulative dose.

The mean liver CT density was 64 (s.d. = 11), at the top of the normal range (50–65 units) (Table 2). An elevated CT density was present in four patients, but three were only marginally raised and these patients had liver/spleen CT density ratios in the normal range (0.9–1.3). The one patient who had a markedly elevated liver CT density (84, with liver/spleen CT ratio of 1.4) had also taken the largest cumulative dose of amiodarone.

There was a moderately good correlation between total cumulative dose of amiodarone and liver CT density ($r = 0.79$, $P < 0.02$) but not spleen CT density ($r = 0.13$). However, no significant correlation was found between liver (or spleen) CT density and daily dosage or average monthly dosage of amiodarone. Liver CT density did not appear to be related to plasma concentrations of amiodarone ($r = 0.18$), desethylamiodarone ($r = 0.13$), or (since CT density reflects the deposition of both drug and metabolite) the sum of the two ($r = 0.17$). No significant correlation was found when free concentrations of amiodarone were compared with CT densities ($r = 0.08$).

Discussion

Binding to normal plasma

Equilibrium dialysis can be used to measure protein binding even when some drug is lost to the membrane or chamber, but with amiodarone the loss was so extensive that the remaining drug was hard to measure, and equilibration appeared to be very slow, introducing problems with volume changes and microbial growth. We conclude that equilibrium dialysis is not a suitable method for measuring the protein binding of amiodarone. Others (Andreason *et al.*, 1981; Neyroz & Bonati, 1985) reached the same conclusion, but Lalloz *et al.* (1984) used equilibrium dialysis to determine the binding of [^{125}I]-amiodarone to serum proteins. Several flaws in this study's methodology deserve attention. The recovery of drug varied from 13.6 to 61.9% and no data were given on equilibration times, suggesting that equilibrium conditions may not have been reached. There was no reported check for protein leakage across the dialysis membrane (which could act as a carrier), and no data were given on the radiochemical purity of the [^{125}I]-amiodarone used. We found that unpurified [^{14}C]-amiodarone contained radioactive impurities which readily crossed the dialysis membrane leading to a gross underestimation of the extent of protein binding. Loss of drug to the ultrafiltration apparatus generally invalidates the use of this method to study protein binding. Andreason *et al.* (1981) were also unsuccessful with a variation of the technique in which plasma was filtered under pressure through a cellophane membrane. Amiodarone is extremely hydrophobic in both the neutral and charged forms (Chatelain & Laruel, 1985), and this is probably why so little crosses dialysis membranes, even when the nominal MW cutoff is for much larger molecules.

The erythrocyte partitioning method showed that the fraction of amiodarone bound to plasma proteins at the therapeutic level is 0.99977. The method had good reproducibility with a coefficient of variation of 9.3%. Similar methods have reported coefficients of variation of 2.3 to 10.0% for five basic drugs (Ho Ngoc-Ta Trung *et al.*, 1984) and 15% for proquazone (Roos & Hinderling, 1981).

The extremely large E/B value of 1962 indicates substantial binding of amiodarone to red cell structures, such as the cell membrane or haemoglobin. Other drugs have been reported to bind haemoglobin and carbonic anhydrase (Roos & Hinderling, 1981; Hinderling, 1984). The E/P value of 0.44 was slightly higher than the previously reported figures of 0.31 (Heger *et al.*,

Table 3 Amiodarone dosage, plasma concentrations, protein binding and CT densities in a group of patients

Patient	*Amiodarone dosage*			*Total trough plasma concentrations ($\mu g\ ml^{-1}$)*		*Amiodarone % free‡*	*Free concentration amiodarone ($ng\ ml^{-1}$)*	*CT density*	
	Total duration (months)	*Total dose (g)*	*Duration of current dose† (months)*	*Amiodarone*	*Desethylamiodarone*			*Liver*	*Spleen*
1	52*	330	35	0.58	0.38	0.021	0.12	84	58
2	13	80	12	0.39	0.60	0.027	0.10	61	42
3	4	25	3	1.60	0.78	0.015	0.24	45	49
4	1.5	11	0.1	0.88	0.59	0.028	0.25	66	70
5	30	220	1	3.00	1.74	0.016	0.47	67	50
6	27	190	21	1.88	1.11	0.031	0.58	64	56
7	6	55	2	0.82	0.57	0.019	0.16	59	45
8	30	180	29	0.62	0.59	0.027	0.16	66	65
Mean	20	136	13	1.22	0.79	0.023	0.26	64	54
s.d.	17	112	14	0.88	0.44	0.006	0.17	11	10

†Currently 200 mg day^{-1}, except patient no. 4 (400 mg day^{-1}).
*Includes a 5 months break in therapy.
‡By the erythrocyte partitioning method.

1984) and 0.33 (Escoubet *et al.*, 1986). This discrepancy could be due to their different method of measuring erythrocyte concentrations of amiodarone (haemolysis, extraction and h.p.l.c.). It should also be noted that Heger *et al.* (1984) had very variable results, with E/P ranging from 0 to 2.84! Although these authors measured red cell concentrations of amiodarone in an attempt to improve therapeutic monitoring of the drug, they did not carry out the additional experiments with erythrocyte suspensions in buffer to estimate the unbound concentration of drug in plasma.

Our finding of 99.977% protein binding of amiodarone is considerably higher than figures reported by others. Lalloz *et al.* (1984) found about 96%, but their methods seem unreliable, as discussed above. Neyroz & Bonati (1985) gave a similar figure of 96.3% using an ultrafiltration technique. However, they did not report a coefficient of variation for the method, and they selected only the deepest fraction of the sediment as binding protein. Non-albumin proteins such as lipoproteins, which are probably responsible for much of the binding of amiodarone to plasma proteins (Lalloz *et al.*, 1984), do not always sediment on centrifugation and this could explain the lower plasma protein binding reported by Neyroz & Bonati (1985). Furthermore, they used unlabelled amiodarone which would have been difficult to measure at unbound concentrations.

The glass-binding method was developed both to check the figure found for the protein binding of amiodarone, and as a potentially general method for measuring the protein binding of other very hydrophobic drugs where membrane separation techniques are unsatisfactory. The unbound fraction of amiodarone determined by glass binding (16.8×10^{-4}) was much greater than that found by erythrocyte partitioning (2.3×10^{-4}). However, both values support the conclusion that the free fraction of amiodarone in plasma is much lower than has previously been reported. The glass-binding method was less reproducible (C.V. = 18%) than the erythrocyte partitioning method (C.V. = 9.3%). The critical measurement in the glass-binding method was the amount of drug bound to the glass from plasma, since this was a very small fraction of the total drug in the tube (1.76 ng from 500 ng). Any residual protein on the glass would lead to overestimation of the free concentration of amiodarone in plasma (P_f) and consequently underestimation of the fraction bound. The glass was silanised to facilitate the washing process, and also to enhance the binding of amiodarone to the tube wall. Nevertheless, unremoved protein may have introduced a systematic error which could explain the discrepancy between the glass binding and erythrocyte partitioning methods. Because of this, and the greater reproducibility of the latter method, the best estimate of the fraction of amiodarone bound to plasma proteins is 0.99977 (s.d. = 0.00005).

Clinical study

The fraction of a particular drug bound to plasma proteins may be fairly constant amongst healthy subjects, yet vary significantly in a patient population because of other drug therapy and different physiological and pathological states. This happens with phenytoin, where one in five patients can show abnormal protein binding (Peterson *et al.*, 1982). Therefore, the plasma protein binding of amiodarone was measured in a group of patients who were taking a variety of other drugs. The binding was not different from that found in blood from healthy subjects, but only eight patients were studied and the constancy of fractional binding of amiodarone should be confirmed in a much larger number of patients.

Liver CT density was used as a measure of tissue deposition of amiodarone, and was found to correlate with total dose, probably because of the extremely slow elimination of amiodarone. Spleen CT density did not correlate well with amiodarone dosage. Although the concentration of amiodarone and desethylamiodarone is less in the spleen than in the liver (Adams *et al.*, 1985), this is probably sufficient to confound the use of liver/spleen CT density ratios to estimate hepatic drug uptake. Previously, we have found that plasma concentrations of desethylamiodarone, but not amiodarone, correlated moderately well ($r = 0.65$) with liver CT density (Markos *et al.*, 1985). In the present study, no significant correlations were found between liver CT density and plasma amiodarone (total or free) or desethylamiodarone, although two patients had been taking their current dose of amiodarone for less than 2 months and were unlikely to be at steady state. These findings should be verified in a much larger group of patients taking a greater range of doses, but they are consistent with other reports of a lack of correlation between blood concentrations of amiodarone and therapeutic and toxic effects (Heger *et al.*, 1983; Latini *et al.*, 1984). Although Heger *et al.* (1984) concluded that red cell concentrations of amiodarone were related to the occurrence of adverse effects, they found greatly variable E/P ratios (0 to 2.84) whereas ours varied much less (from 0.233 to 0.603 amongst the patients studied). It is difficult to interpret an E/P value of zero (indicating no uptake of amiodarone by erythrocytes) except as

an analytical error, possibly due to variability in the extraction of drug from haemolysate. Thus there does not seem to be convincing evidence at present for the value of plasma or red cell monitoring of amiodarone.

The main goal of the present study was to measure the individual variability in the binding of amiodarone to plasma proteins in order to see whether the unbound concentration could be more useful than the total drug concentration for therapeutic monitoring. The binding was found to be very extensive at 99.977%, and fairly constant with only a two-fold variation in the small free fraction amongst both healthy subjects and patients being treated for arrhythmias. There was no difference in binding between control subjects and patients, indicating that the binding was unaffected by the presence of the other drugs taken (see patient details in **Methods**). Not unexpectedly, the fraction bound did not vary with albumin levels, since other proteins, especially α_1-acid glycoprotein, are more important ligands for hydrophobic basic drugs (Routledge, 1986). This protein was not measured in the present study, but its levels are known to vary widely with disease and future investigations should examine the effect of changing levels of this critical protein on amiodarone binding. The unbound fraction of amiodarone was also unrelated to total plasma amiodarone concentration, indicating that the binding is not concentration-dependent over the normal therapeutic range.

This study examined the protein binding of amiodarone in only eight patients, due to the small number of suitable patients available in Hobart. In order to better assess the value of monitoring free concentrations of amiodarone, its protein binding should be measured in a much larger group of patients, especially across a wider range of patient ages and doses of amiodarone, and with a greater variety of attendant medical conditions and concomitant drug therapy.

An incidental outcome of this study was the development of a novel method of measuring plasma protein binding of a drug by using its property of binding strongly to glass. This method may be useful for other drugs which stick to surfaces, and whose protein binding cannot be satisfactorily measured by ultrafiltration or equilibrium dialysis. However, we found the erythrocyte partitioning method to be more reproducible and should perhaps be tried first.

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