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Reversible binding of the novel anti-tumour agent 5,6-dimethylxanthenone-4-acetic acid to plasma proteins and its distribution into blood cells in various species

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Abstract

The plasma protein binding and distribution in blood cells of the novel anti-tumour agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA) has been investigated in-vitro using filtration and an HPLC method to measure DMXAA. DMXAA (500 μM) was extensively bound in plasma from all species with an unbound fraction (f_u) of 4.61 ± 1.10 (mouse), 2.59 ± 0.32 (rat), 2.02 ± 0.48 (rabbit) and 2.07 ± 0.23 % (human). The binding was concentration dependent with DMXAA concentrations $\geqslant 1000$ μM markedly increasing the f_u in the plasma from all species. The estimated number of binding sites in plasma were 2.4 ± 0.2 (mouse), 1.7 ± 0.2 (rat), 0.8 ± 0.1 (rabbit) and 2.1 ± 0.2 (human). The major binding protein in human plasma was albumin, with negligible binding to γ-globulin and α_1-acid glycoprotein. There was a significant linear relationship between the bound:free DMXAA concentration ratio (C_b/C_u) and albumin concentration in human serum albumin solution ($r = 0.955$; $P < 0.05$) and in healthy human plasma ($r = 0.998$; $P < 0.05$), but not in plasma from cancer patients ($n = 5$), nor across species. In cancer patients ($n = 5$) DMXAA had a significantly higher ($P < 0.05$) f_u (4.60 ± 0.42 %) compared with healthy human plasma (2.07 ± 0.23 %). In human plasma, the f_u of DMXAA (500 μM) was significantly reduced by 500 μM diazepam ($P < 0.05$), but not by warfarin, phenylbutazone, salicylic acid, ibuprofen or clofibric acid at that concentration. DMXAA significantly reduced the binding of dansylsarcosine (a Site-II binder) to HSA, but significantly increased the binding of dansylamide (a Site-I binder). Within species, the blood:plasma concentration ratio (C_{BL}/C_P) of DMXAA was relatively constant (mouse, 0.581 ± 0.005; rat, 0.667 ± 0.025; rabbit, 0.637 ± 0.019; human, 0.673 ± 0.103) over the range 50–1000 μM, but increased significantly at DMXAA concentrations > 1000 μM in all species except the rabbit. These results indicate that significant alterations in DMXAA plasma binding and distribution into blood cells occur with increasing concentrations of DMXAA in all species, and also that significant interspecies differences exist. It would be more appropriate to compare plasma unbound concentrations when assessing DMXAA exposure in cancer patients or when extrapolating across species.

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Introduction

The unbound drug in plasma is considered to be responsible for the pharmacological activity of a drug, and binding to plasma proteins is considered to be a critical factor affecting the intensity and duration of drug action (du Souich et al 1993). The protein binding of drugs may be modified by conditions such as cancer, or co-administration of other highly protein-bound drugs, possibly leading to

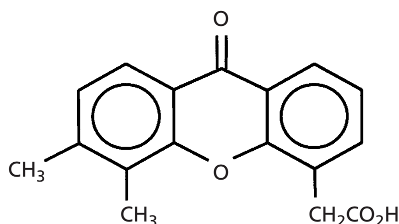

Figure 1 The chemical structure of 5,6-dimethylxanthenone-4-acetic acid (DMXAA).

changes in a drug's pharmacokinetics, and thus pharmacodynamics (e.g. anti-tumour activity or toxicity) (Fichtl et al 1991; Olson & Christ 1996). The novel anti-tumour agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA) (Figure 1) exhibited high plasma protein binding which was concentration-dependent and with significant variation between species (McKeage et al 1991; Webster et al 1995; Kestell et al 1999). It has been suggested that the concentration-dependent protein binding may contribute in part to the observed non-linear pharmacokinetics of DMXAA, although other factors relating to its metabolism and biological modifying effects may be more important (Kestell et al 1999). These include its metabolism to form an acyl glucuronide with subsequent hydrolysis to reform parent drug, and also its complicated vascular and immune modulatory effects (Zwi et al 1994; Baguley et al 1997). The latter is thought to be responsible for rapid vascular collapse in the tumour leading to necrosis (Zwi et al 1994), and the induction of cytokines such as tumour necrosis factor-α (TNF-α), serotonin and nitric oxide (Thomsen et al 1990; Philpott et al 1995; Baguley et al 1997). The DMXAA concentrations required to elicit these effects have been investigated in-vitro. The onset of TNF-α secretion in mouse macrophages occurs at approximately 30 μM DMXAA with optimal secretion at 300 μM (Perera et al 1994). Similar concentrations have been reported to stimulate and sustain the production of nitrite from activated mouse macrophages (Thomsen et al 1990). In mouse splenocytes, 100 μM DMXAA is necessary to induce mRNA of TNF-α, whereas 300–2000 μM is required to produce similar effects in human HL-60 cells (Ching et al 1994). Similar concentrations were also used to investigate the elimination and metabolism of DMXAA using the rat isolated perfused liver model (Webster et al 1995). In the latter study, DMXAA perfusate concentrations were affected by uptake into the red blood cells, implying that the disposition of DMXAA in the blood is complex. In-vivo mouse studies have indicated that DMXAA can exert potent anti-tumour activity only at doses that are close to the maximum tolerated doses (30 mg kg^{-1}), which results in plasma concentrations of 100–550 μM (Kestell et al 1999). The maximum DMXAA concentrations achieved in the plasma of patients in a Phase-I clinical trial ranged from 1000–2000 μM (Jameson et al 2000). Thus, a greater knowledge of DMXAA's protein binding and its distribution to blood cells may be important in the comparison of anti-tumour/toxicity effects across species, and to achieve optimal results in patients.

The aims of this study were to characterize the in-vitro binding of DMXAA to plasma proteins and distribution in red blood cells in various species and to investigate and compare the protein binding of DMXAA in plasma from healthy donors with that from cancer patients. In addition, with the popularity of multiple drugs in cancer treatment, the effect of some extensively protein-bound drugs on the protein binding of DMXAA in human plasma was investigated.

Materials and Methods

Chemicals

DMXAA sodium salt and 2,5-dimethylxanthenone-4-acetic acid (the internal standard; IS) were synthesized in the Auckland Cancer Society Research Centre (Rewcastle et al 1989). DMXAA was protected from light exposure to avoid degradation (Rewcastle et al 1990). Warfarin, diazepam, digitoxin, ibuprofen, phenylbutazone, cyproheptadine, bromcresol green, Brij 35, bicinchoninic acid (BCA), citric acid, essentially fatty acid-free human serum albumin (HSA) prepared from fraction V, human γ-globulin, and human α_1-acid glycoprotein (AAG) were purchased from Sigma-Aldrich Chemicals Co. (Auckland, New Zealand). The Centrisart micropartition device with 20000 molecular weight cut-off was from Sartorious AG (Goettingen, Germany). All other reagents were analytical or HPLC grade as appropriate.

Blood and plasma

Male Wistar Kyoto rats (200–250 g, n = 20), male C57B1/6 mice (25–32 g, n = 50) and male white New Zealand rabbits (3–3.4 kg, n = 3) were anaesthetized with halothane, and blood was collected into heparinized glass tubes (Venoject, Terumo Co., Tokyo, Japan) by cardiac puncture for rats and rabbits, and via the ocular sinus for mice. Heparinized blood was obtained from healthy human subjects (n = 6) with no known intake of drugs over the previous 4 weeks, and from

cancer patients (n = 5) before the DMXAA infusion during a Phase I trial. Ethical approval was obtained from the Northern New Zealand Research Ethics Committee and all subjects gave written informed consent. Plasma was separated by centrifugation at 1000 *g* for 15 min at 4°C. Plasma from a single healthy donor and pooled plasma from each animal species were used to characterize concentration dependence in the binding of DMXAA. Total protein and albumin concentrations in the plasma were determined using the BCA method (Smith et al 1985) and the bromcresol green dye binding method, respectively (Doumas & Biggs 1972).

HPLC assay for DMXAA

DMXAA concentrations in plasma, albumin solution and blood were determined by HPLC with fluorescence detection (345 and 409 nm for excitation and emission wavelengths, respectively). Plasma, albumin solution or whole blood (100 μL) was mixed with 50 μL methanol containing 20 μM IS, followed by 0.4 mL ice-cold acetonitrile–methanol (3:1, v/v). After centrifugation at 2500 *g* for 15 min to remove precipitated proteins, the supernatant was removed and evaporated to dryness under nitrogen. The residue was dissolved in 200 μL mobile phase, and 50 μL was injected into the HPLC. A Luna C18 guard column and a 5-μm Spherex C18 analytical column (150 × 4.6 mm; both from Phenomenex, NZ Ltd, Auckland) was used with a mobile phase of acetonitrile–10 mM ammonium acetate buffer (24:76, v/v, pH 5.8). For filtrates, a 100-μL sample was mixed with 50 μL of 0.1 M phosphate buffer (pH 7.4) containing 10 μM IS, and 50 μL was injected into the HPLC system. Calibration curves (0.5–40 μM) were constructed from the peak area ratio of DMXAA:IS versus known DMXAA concentrations in plasma, albumin solution, whole blood or phosphate buffer. Linear least-squares regression analysis was used to determine the slope, intercept and coefficient of determination. The validation of the HPLC method indicated acceptable accuracy (85–115% of true values) and precision (intra- and inter-assay coefficients of variation $< 15\%$). Samples with DMXAA concentrations > 40 μM were diluted with 10 mM ammonium acetate buffer (pH 5.0) to ensure that the concentrations were within the assay range.

Determination of the plasma protein binding of DMXAA

The adsorption of DMXAA to the Centrisart filtration device was investigated with DMXAA concentrations (0.5–500 μM) in phosphate buffer. DMXAA concentrations in the pre- and post-centrifuged phosphate buffer were similar, with the ratio ranging from 0.99 ± 0.02 to 1.01 ± 0.03 (n = 4), indicating that there was no nonspecific binding of DMXAA to the Centrisart devices. Filtration studies with normal human plasma containing 500 μM DMXAA indicated that centrifugation at 2000 *g* for 30 min at 37°C (Beckman J-6M centrifuge) was appropriate for the determination of the unbound fraction.

The protein binding of DMXAA was determined in plasma from mice, rats, rabbits, healthy subjects, cancer patients and various human protein solutions. Plasma and HSA solutions (20–60 g L^{-1}) containing 25–2000 μM DMXAA were incubated for 30 min at 37°C. A 100-μL sample was taken to determine the total DMXAA concentration by HPLC. A further 400-μL sample was transferred to the filtration device, centrifuged at 2000 *g* for 30 min at 37°C. Samples were capped to minimize changes in pH during filtration. The DMXAA concentration in the filtrate was determined by HPLC. The unbound fraction (f_u) of DMXAA was calculated by the ratio of the DMXAA concentration in the filtrate to that in the plasma before filtration. Similarly, individual solutions of HSA (40 g L^{-1}), human γ-globulin (10 g L^{-1}), and AAG (1 g L^{-1}) in 0.1 M phosphate buffer (pH 7.4) were incubated with 500 μM DMXAA for 30 min at 37°C, and the protein binding determined by filtration.

Effects of various drugs on the protein binding of DMXAA

The effects of various drugs on the protein binding of DMXAA were investigated in normal drug-free human plasma from a single healthy donor. Salicylic acid, warfarin (both dissolved in water), phenylbutazone, diazepam, ibuprofen, digitoxin and cyproheptadine (dissolved in ethanol) were investigated at concentrations ranging from 50–5000 μM. DMXAA (500 μM) was added to plasma, followed by the test drug, and incubated for 30 min at 37°C with gentle shaking. The DMXAA unbound fraction was then determined by filtration. Preliminary experiments demonstrated that the addition of a second sample of water or ethanol at a final concentration of 1% (v/v) had no significant effect on the plasma protein binding of DMXAA.

Effects of DMXAA on the protein binding of specific probes

Specific probes for Site I and Site II, dansylamide and dansylsarcosine (Sudlow et al 1976), were used to determine the binding of DMXAA to specific sites on

HSA. The fluorescence of solutions containing 2 μM probe and 20 μM HSA (1.23 g L^{-1}) was measured before and after the addition of DMXAA at 20, 40 and 60 μM using a fluorescence spectrophotometer at 370 and 475 nm (excitation and emission wavelength respectively) (Hitachi Co., Chiyoda-ku, Tokyo). Results are expressed as a percentage of initial fluorescence.

Distribution of DMXAA in blood

The blood:plasma concentration ratio of DMXAA (C_{BL}/C_P) was determined in incubations of fresh heparinized whole blood containing DMXAA (50–2000 μM) with slow shaking for 30 min at 37°C. A sample (100 μL) was taken to determine the total blood DMXAA concentration, and a further sample (400 μL) was centrifuged (1000 *g* for 15 min) at 37°C to obtain plasma. DMXAA concentrations were determined in whole blood and in plasma as described above. The haematocrit for each blood was also measured, and used to determine the fraction of DMXAA distributed to the red blood cells in each species.

Data analysis

Data were expressed as mean ± s.d. Models were applied to the protein binding data using both weighted least squares (Prism 3.0 program package, Graphpad Software Co., CA) and extended least squares (MKMODEL package, Elsevier, Cambridge, UK) (Holford 1985). Single- and two-binding-site models with or without non-specific binding, and a modified two-binding-site model (Semmes & Shen 1990) were used to describe the protein binding of DMXAA, according to equations 1–5:

$$C_b = N \times C_u/(K_d + C_u) \quad (1)$$

$$C_b = N \times C_u/(K_d + C_u) + NS \times C_u \quad (2)$$

$$C_b = N_1 \times C_u/(K_{d1} + C_u) + N_2 \times C_u/(K_{d2} + C_u) \quad (3)$$

$$C_b = N_1 \times C_u/(K_{d1} + C_u) + N_2 \times C_u/(K_{d2} + C_u) + NS \times C_u \quad (4)$$

$$C_b = N_1 \times C_u/(K_{d1} + C_u) + (N_2/K_{d2}) \times C_u \quad (5)$$

where C_b is the concentration of bound DMXAA, obtained from subtracting unbound (C_u) from total (C_t) DMXAA concentration; K_d is the apparent dissociation constant for the binding site; N is the concentration of binding sites on plasma protein; NS is the constant accounting for the nonspecific and non-saturable binding; and subscripts 1 and 2 represent the first and the second type of sites. The choice of model for weighted least-squares analysis was confirmed by comparing and reviewing the relative residuals and the standard error of the parameter estimates from the non-linear regression analysis. Akaike's information criterion (AIC) was also used as the criterion for comparing the models (Yamaoka et al 1978). The equation with minimum AIC is regarded as the best representation of the model. Schwarz criterion was used for model determination by extended least squares (Holford 1985).

The fraction of DMXAA in blood that is distributed to red blood cells (f_{BC}) is calculated by equation 6:

$$f_{BC} = 1 - (1 - H) \times C_P/C_{BL} \quad (6)$$

where H is the haematocrit and C_P and C_{BL} are the concentration of DMXAA in plasma and whole blood, respectively. Student's *t*-test was used to test for differences at a significance level of 5% ($P < 0.05$). Linear correlation analysis was performed to test for statistically significant correlation between two parameters at the significance level of 5% ($P < 0.05$).

Results

Species differences in the plasma protein binding of DMXAA

DMXAA (500 μM) was extensively bound in plasma from all species, but showed a 2.3-fold variation in the unbound fraction (f_u) across species (Figure 2A). Plasma albumin concentrations also varied considerably between species, but there was no significant relationship between C_b/C_u or f_u and plasma albumin concentration across species (r = 0.858 and 0.805, respectively, $P >$ 0.05) (Figure 2B). The binding of DMXAA was concentration dependent with concentrations $\geqslant$ 1000 μM markedly increasing the f_u of DMXAA in the plasma from all species investigated (Figure 3A). The one-binding-site model with non-specific binding was the best fit for the binding of DMXAA to plasma in all species (Figure 3B). The binding parameters from this model are presented in Table 1.

Binding of DMXAA to human plasma proteins

Albumin was the predominant plasma binding protein for DMXAA; whereas the binding by human γ-globulin was considerably less and negligible with AAG. There was a significant correlation between C_b/C_u of DMXAA and albumin concentration in HSA solution (r = 0.955, $P < 0.05$). The f_u of DMXAA at 500 μM in HSA solution was inversely proportional to the albumin concentration (r = −0.963, $P < 0.05$). The unbound DMXAA fraction in fresh plasma from healthy subjects was significantly less than that observed in cancer patients (Table 2). Plasma albumin concentrations varied considerably between healthy subjects and cancer patients. In healthy

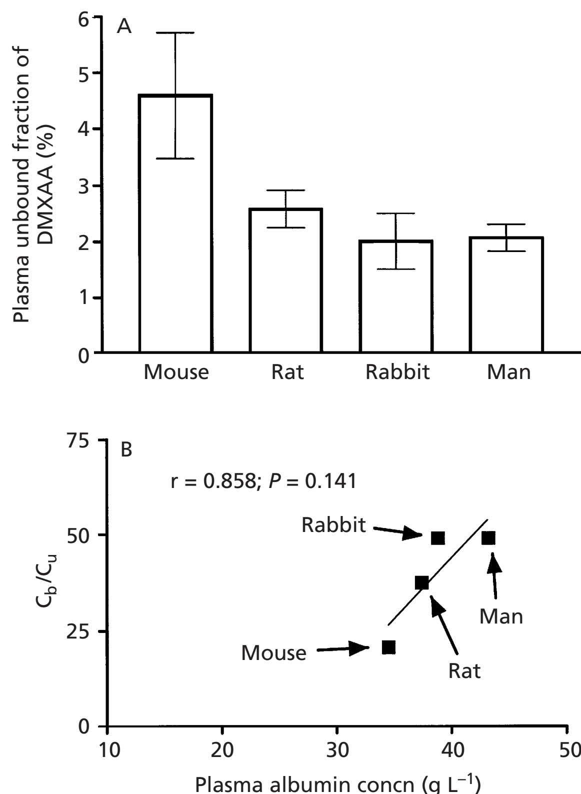

Figure 2 A. The unbound fraction of DMXAA (500 μM) in fresh plasma from mouse, rat, rabbit and man (n = 6). B. The relationship between binding ratio (C_b/C_u) of DMXAA and albumin concentration in plasma across species.

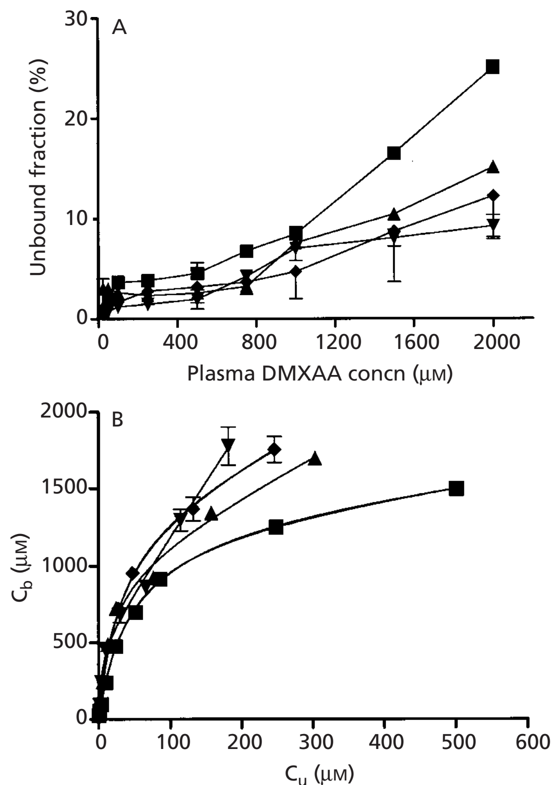

Figure 3 A. The relationship between the unbound DMXAA fraction (f_u) and total plasma concentration in mouse (■), rat (▲), rabbit (▼) and man (◆). B. Plots of the plasma bound concentration of DMXAA as a function of unbound concentration in mouse (■), rat (▲), rabbit (▼) and man (◆); the curves represent the best fit for a one binding-site model with non-specific binding. Each point represents mean ± s.d. of at least three determinations.

human plasma, there was a significant correlation between C_b/C_u or f_u for DMXAA and plasma albumin concentration (r = 0.998, $P < 0.05$). However, there was no significant relationship between C_b/C_u or f_u with plasma albumin concentration in cancer patients (r = 0.804 and 0.817, respectively, $P > 0.05$).

A significant increase in DMXAA f_u in healthy human plasma was observed with diazepam at 500 μM ($P < 0.05$), but not with warfarin, phenylbutazone, salicylic acid, ibuprofen or clofibric acid (Figure 4A). Diazepam, clofibric acid, salicylic acid and ibuprofen significantly increased the unbound DMXAA fraction when present at 5000 μM. At therapeutic concentrations, salicylic acid (2000 μM), but not clofibric acid, significantly increased DMXAA f_u in plasma ($P < 0.05$). Digitoxin, which is bound at Site III (Sjoholm et al 1980), did not alter the protein binding of DMXAA. There was no significant change in DMXAA f_u in plasma with the addition of warfarin, phenylbutazone or cyproheptadine at 500 or 5000 μM.

DMXAA reduced the fluorescence of the Site-II binding agent dansylsarcosine by 42%, when present at a ratio of 3 : 1 of drug-to-HSA concentration (Figure 4B). In contrast, a 49% increase in the fluorescence of the Site-I binding agent dansylamide was observed, suggesting increased binding of the probe in the presence of DMXAA.

Distribution of DMXAA in blood

The blood : plasma DMXAA concentration ratio (C_{BL}/C_P) was similar between species and also relatively constant over the blood concentration range 50–500 μM. At concentrations > 1000 μM, the C_{BL}/C_P increased significantly in all species except the rabbit (Figure 5A). There was a significant relationship between the C_{BL}/C_P and plasma f_u in the mouse, rat and man (r = 0.965, 0.913 and 0.925, respectively, $P < 0.05$) but not in the rabbit (r = 0.111, $P > 0.05$). Species differences in the fraction of DMXAA distributed to blood cells (f_{BC})

Table 1 Estimated binding parameters for DMXAA in plasma from mouse, rat, rabbit and man, and human serum albumin solution (HSA, 40 g L^{-1}).

Parameters	Mouse	Rat	Rabbit	Human	HSA
N (μM)	1273 (64.6)	999.7 (132.7)	495.2 (52.4)	1350 (110.7)	1425 (93.8)
K_d (μM)	42.7 (4.2)	16.8 (4.8)	3.9 (1.3)	30.0 (4.1)	53.1 (4.9)
NS[a]	0.655 (0.121)	2.503 (0.481)	7.066 (0.391)	2.211 (0.380)	2.288 (0.258)
Albumin concn (μM)	535 ± 14	580 ± 8	601 ± 19	644 ± 15	620
No. of SBS[b]	2.4 (0.2)	1.7 (0.2)	0.8 (0.1)	2.1 (0.2)	2.3 (0.2)

Values are mean ± s.d. The values in parentheses are the standard error of the nonlinear estimation. [a]NS = non-specific non-saturable binding. [b]No. of SBS = number of specific binding sites, assuming that albumin is the only binding molecule.

Table 2 Unbound DMXAA fraction (%) in plasma from healthy subjects and cancer patients.

No.	Sex/age (yr)	f_u (%)	Albumin concn (g L^{-1})	Total protein concn (g L^{-1})	n
Fresh plasma from healthy subjects					
1	M/38	1.55 ± 0.09	47.87 ± 3.22	75.35 ± 5.89	3
2	M/35	1.65 ± 0.73	47.20 ± 3.51	74.38 ± 2.46	3
3	M/45	2.18 ± 0.18	41.56 ± 0.98	73.21 ± 3.26	3
4	M/25	2.17 ± 0.18	41.73 ± 5.12	72.98 ± 7.75	3
5	F/37	2.97 ± 0.22	37.44 ± 1.80	67.45 ± 3.13	3
6	F/32	1.87 ± 0.38	44.56 ± 7.73	73.21 ± 4.73	3
Mean ± s.d.		2.07 ± 0.23	43.39 ± 6.65	72.76 ± 3.94	3
(range)		(1.55–2.97)	(37.44–47.87)	(67.45–75.35)	
Cancer-patient plasma					
1	F/48	5.32 ± 0.72	28.53 ± 3.78	54.35 ± 4.48	3
2	M/57	3.94 ± 0.15	40.01 ± 1.45	67.38 ± 2.21	3
3	F/51	7.29 ± 0.06	30.16 ± 2.23	56.21 ± 3.04	3
4	F/40	2.77 ± 0.58	38.85 ± 6.54	64.98 ± 5.76	3
5	F/37	3.69 ± 0.61	37.58 ± 4.69	62.45 ± 3.88	3
Mean ± s.d.		4.60 ± 0.42*	35.02 ± 9.15*	61.07 ± 5.29*	3
(range)		(2.77–7.29)	(28.53–40.01)	(54.35–67.38)	

Data were expressed as mean ± s.d., n = 3. *$P < 0.05$ compared with fresh healthy human plasma.

were observed with the smallest f_{BC} in mice and the largest in rats, with man and rabbits intermediate at 50 μM DMXAA. Increasing whole blood concentrations to 2000 μM resulted in 8-, 2- and 3-fold increases in the f_{BC} in the mouse, rat and man respectively. However, the f_{BC} remained relatively constant (15.0–20.8 %) in the rabbit with increasing DMXAA concentrations (Figure 5B). A significant correlation between f_{BC} of DMXAA and f_u in plasma was also observed in the mouse, rat and man (r = 0.958, 0.871 and 0.882, respectively, $P < 0.05$), but not in the rabbit (r = 0.218, $P > 0.05$). In addition, f_{BC} was significantly correlated with total blood concentration in the mouse, rat and man (r = 0.881, 0.854 and 0.928, respectively, $P < 0.05$), but not in the rabbit (r = 0.284, $P > 0.05$). The % distribution of total DMXAA in the various blood compartments with increasing DMXAA concentration in the mouse, rat, rabbit and man is shown in Figure 6.

Discussion

Our results indicate that DMXAA is highly bound in animal and human plasma with a small f_u (< 5%), which is in agreement with a previous report (Kestell et al 1999). Interspecies differences were apparent with the mouse exhibiting a 2.3-fold greater f_u compared with healthy subjects. These differences may impact on the extrapolation from animal studies to man, as the mouse was the laboratory animal used for the pre-clinical development of DMXAA. The differences in free fraction must be taken into account when comparing plasma

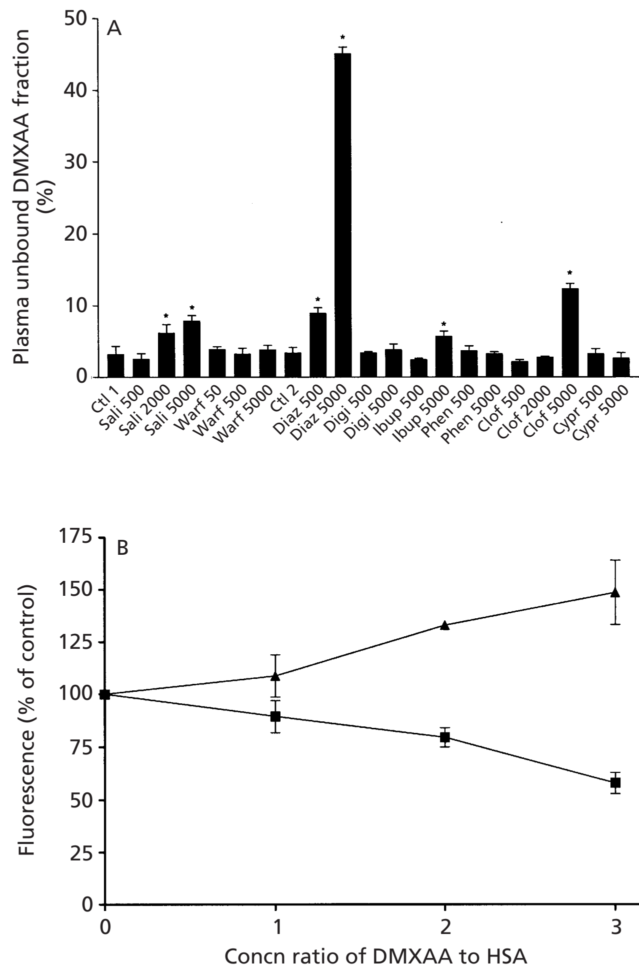

Figure 4 The effects of various drugs on the protein binding of DMXAA at 500 μM in plasma from healthy subjects. Salicylic acid (Sali) and warfarin (Warf) were compared with control 1 (Ctl 1, 1% water), and diazepam (Diaz), digitoxin (Digi), ibuprofen (Ibup), phenylbutazone (Phen), clofibric acid (Clof) and cyproheptadine (Cypr) were compared with control 2 (Ctl 2, 2% ethanol). The concentration unit for ligands was μM. $*P < 0.05$. B. DMXAA-induced alterations in the fluorescence of fluorescent probes dansylsarcosine (■) and dansylamide (▲) in human serum albumin solution. Results represent the mean ± s.d. of three determinations.

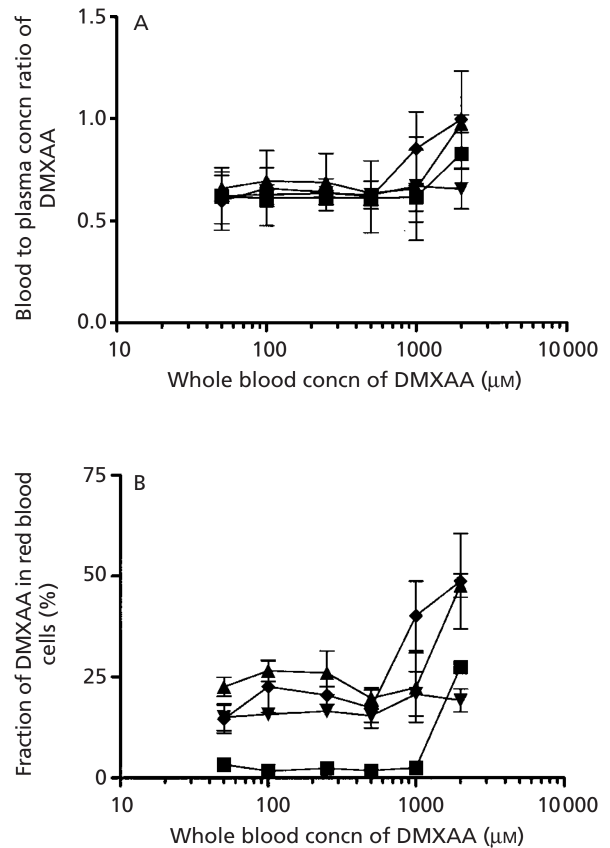

Figure 5 A. The blood-to-plasma concentration ratio (C_{BL}/C_P) of DMXAA over the concentration range of 50–2000 μM in mouse (■), rat (▲), rabbit (▼) and man (◆). B. The fraction of DMXAA in the blood (f_{BC}) that distributed to the red blood cells over the concentration range 50–2000 μM in fresh blood from mouse (■), rat (▲), rabbit (▼), and man (◆). Each point represents mean ± s.d. of at least three determinations.

concentration–time curves for therapeutic or maximum tolerated doses in mice and man. However the difference between mouse and man may not be so marked in cancer patients, as the plasma f_u may be significantly increased in the latter. Several factors may be responsible for this, including the reduction in albumin concentration, the presence of other drugs, metabolites, and endogenous ligands such as free fatty acids, and possibly post-translational modification of albumin binding sites in cancer patients. In our small study population, the mean serum albumin concentration in cancer patients was significant lower than those of healthy subjects. The lack of correlation between DMXAA C_b/C_u or f_u with albumin concentration ($P > 0.05$) in cancer patients suggests that other factors are involved in the reduced DMXAA binding, and that albumin concentration may not be useful as an index of f_u in these patients. However, the latter must be interpreted with caution, as the number of patients was small and the HSA concentrations were not well distributed within the range.

Our results indicate that albumin is the predominant binding protein for DMXAA in human plasma. The binding primarily involves Site II, as DMXAA displaced dansylsarcosine from HSA, but may influence the binding of ligands at Site I, as it increased the binding of dansylamide. Presumably by binding to Site II, DMXAA may cause a conformational change in albumin which may alter the affinity for a ligand at Site I.

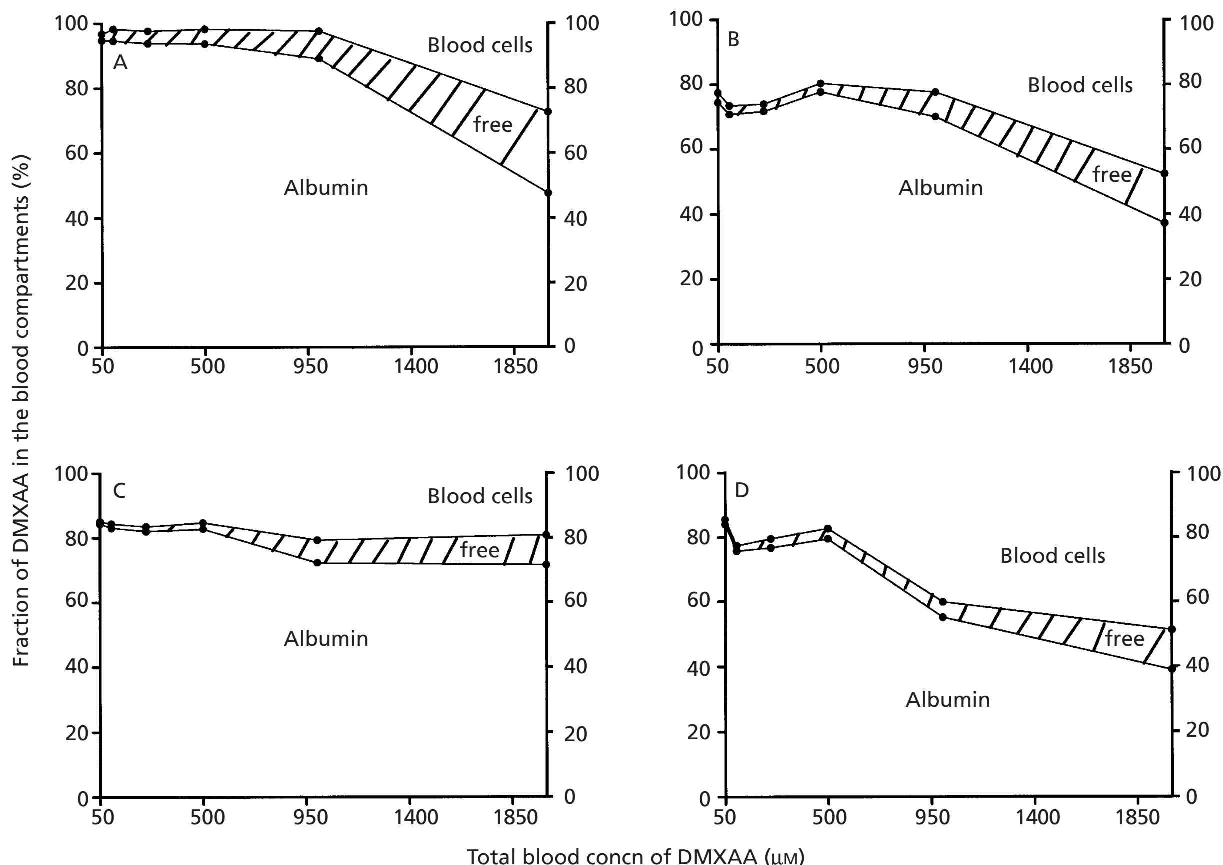

Figure 6 The distribution of DMXAA in the blood compartments (albumin and blood cells) in mouse (A), rat (B), rabbit (C) and man (D).

Further evidence for Site-II binding by DMXAA is suggested by the reduction in plasma binding induced by diazepam, clofibric acid and ibuprofen, but not by other Site-I and Site-III ligands such as warfarin and phenylbutazone or digitoxin. However, salicylate (a reported Site-I ligand) also reduced DMXAA plasma binding, perhaps due to an indirect effect on Site II. The clinical implications of these displacement interactions would be expected to be limited, as DMXAA undergoes restrictive elimination by the liver (data from the Phase-I trial showed that the DMXAA blood clearance in cancer patients ranges from 2.1–6.7 mL min^{-1} kg^{-1} (Jameson et al 2000), which is much smaller than human hepatic blood flow (20 mL min^{-1} kg^{-1}, Boxenbaum 1980)). An increase in the plasma f_u of DMXAA in cancer patients will, in theory, facilitate the distribution of DMXAA out of the plasma compartment and therefore increase the apparent volume of distribution, and also increase the plasma clearance (based on total concentrations). However, the DMXAA free concentration will remain relatively unchanged, in the absence of any alteration in intrinsic clearance. Thus, the pharmacodynamic effect (such as anti-cancer activity) of DMXAA would be expected to be unaltered by plasma binding changes.

The fraction of DMXAA within red blood cells was greater than the fraction unbound in plasma in all species except the mouse, indicating binding components, binding compartments or active uptake into the red blood cells. However, the linear relationship between the f_{BC} and the f_u suggests that the former is more likely, and that DMXAA is taken up by the blood cells by passive diffusion. Thus, with increasing DMXAA concentration, the saturation of the plasma binding results in a predictable rise in red blood cell uptake of DMXAA in all species (except the rabbit). It appears from the analysis of the plasma binding curves that the rabbit, although it had a single high-affinity binding site, had a large capacity for non-specific non-saturable binding of DMXAA. Thus, greater DMXAA concentrations do not lead to major alterations in redistribution of DMXAA into blood cells in the rabbit. In contrast, the

mouse, with the lowest nonspecific binding, resulted in an 8-fold increase in the distribution into blood cells at 2 mM DMXAA. This increased distribution of DMXAA will have an impact on the pharmacokinetics in-vivo as the total plasma concentration will rise to a lesser extent with increasing dose than would be expected from plasma in isolation (assuming that the hepatic intrinsic clearance does not change). The problems presented by variable plasma binding between species and decreased plasma binding with increasing concentration during pharmacokinetic studies can be circumvented by using the unbound plasma drug concentration, which is independent of plasma protein binding and dependent only on intrinsic clearance.

In conclusion, DMXAA exhibited extensive concentration-dependent plasma protein binding in all species, but with considerable interspecies differences in the unbound fraction and the fraction distributed to blood cells. Albumin was the major protein involved with DMXAA binding, primarily at Site II, but with influences on Site-I binding. The unbound fraction was significantly greater in plasma from cancer patients compared with that from healthy subjects, but albumin concentration cannot be used to predict the unbound fraction in patients. It is suggested that unbound plasma concentration should be used for interspecies comparison and dose comparison of pharmacokinetics and pharmacodynamics of DMXAA.

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