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PROTEIN BINDING OF GP53,633: A BASIC NON-STEROIDAL ANTI-INFLAMMATORY DRUG

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Abstract—The protein binding of GP53,633 [2-tert.butyl-4(5)-phenyl-5(4)-(3-pyridyl)-imidazole], a basic non-steroidal anti-inflammatory drug (NSAID), has been investigated. Although GP53,633 is a base (pK_a 6.4) rather than an acid, the binding in plasma or serum was totally accounted for by binding to albumin. Scatchard analysis of the binding to albumin suggested the presence of one high affinity site and a number of low affinity sites. GP53,633 and its major metabolite, CGP8716, displaced site-I fluorescent probes (DNSA and warfarin) but not the site-II probe dansylsarcosine. Binding studies by equilibrium dialysis showed that GP53,633 and its metabolite displaced site-I drugs but not site-II drugs, and ^{14}C-GP53,633 was itself displaced by site-I but not site-II drugs. As with other site-I drugs, the binding of GP53,633 was enhanced by addition of oleic acid at molar ratios of up to 2:1 with albumin. Albumin binding of GP53,633 was markedly increased by raising the pH from 6.0 to 8.5 suggesting that only the unionised drug can bind at site-I. The data are consistent with the major part of the binding energy at site-I being due to hydrophobic interactions and also suggest that there is a cationic centre on the protein at or near site-I which precludes the binding of positively charged drugs.

Most non-steroidal anti-inflammatory drugs (NSAID) are acidic compounds which are highly protein bound in plasma. They bind mainly to one of two relatively selective binding sites on human serum albumin [1–5]. By contrast, most basic drugs bind primarily to alpha$_1$-acid glycoprotein or to various lipoproteins in plasma [6–15]. However, some basic drugs, particularly those with low pK_as such as diazepam, bind primarily to albumin [16, 17].

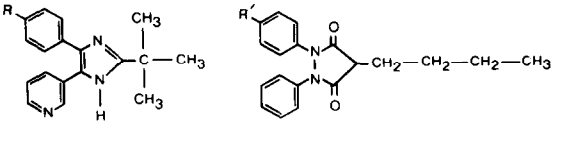
Albumin functions as a binding protein for a large variety of drugs, organic ligands and some endogenous substances. The binding sites on albumin are relatively non-specific but there is now good evidence for the existence of binding sites on albumin with considerable structural selectivity for both endogenous and exogenous compounds. A number of these sites have been localised on the albumin molecule [1–5, 18, 19]. Two distinct binding sites, I and II, for anionic drugs on human serum albumin have previously been demonstrated using fluorescent probe techniques. The specific fluorescent probes are 5-dimethylaminonaphthalene-1-sulfonamide (DNSA) and warfarin for site-I and dansylsarcosine for site-II [3, 4].

GP53,633 is a new NSAID shown to possess analgesic and anti-inflammatory activity in animals and, like other NSAID, it is an inhibitor of prostaglandin synthesis in various test systems. GP53,633 is a phenylbutazone analogue (Fig. 1) but is a base rather than an acid. Both phenylbutazone and GP53,633 are hydroxylated at the same position on the phenyl ring to produce oxyphenbutazone and CGP8716 respectively. CGP8716 is the major known metabolite.

This paper reports studies on the binding of GP53,633 to human plasma and albumin. The effects of the major metabolite CGP8716, other drugs, fatty acids and alteration in pH on the protein binding of GP53,633 are also presented.

MATERIALS AND METHODS

Human serum albumin (HSA), Essentially Fatty Acid Free (lot number 19C-7050), Fraction V (lot number 47C-04421), DNSA and dansylsarcosine were purchased from Sigma Chemical Co. (St. Louis, MO). HSA solutions were prepared in 0.1 M NaH_2PO_4 buffer. The mol. wt of albumin was taken as 66,500 [20]. Binding studies were also carried out using a normal control serum purchased from Ortho Diagnostics Inc., (lot number 5S-220), Blood Bank citrated plasma and heparinised plasma obtained from 12 healthy male subjects aged 19–32 yr. Plasma or serum fatty acid concentrations were measured by the method of Duncombe [21]. Albumin concentrations were measured by the bromocresol green



GP53,633 : R = H
CGP8716 : R = OH
(major metabolite)

Phenylbutazone : R' = H
Oxyphenbutazone : R' = OH

Fig. 1. Chemical structures of GP53,633 [2-tert.butyl-4(5)-phenyl-5(4)-(3-pyridyl)-imidazole], phenylbutazone and their major metabolites

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method and total protein concentrations by the Biuret reagent.

GP53,633 and CGP8716 were donated by Ciba-Geigy Ltd., who also supplied ^{14}C-labelled GP53,633 (sp. act. 13.7 μCi/mg). Thin layer chromatographic analysis of the labelled compound showed only one radioactive component corresponding to GP53,633. Labelled warfarin (^{14}C, 51 μCi/μmole) was purchased from the Radiochemical Centre (Amersham, U.K.). [^{14}C]-Salicylic acid (416 μCi/mg) was purchased from New England Nuclear (Boston, MA). ^{14}C-labelled ibuprofen (22.8 μCi/mg) and flurbiprofen (22.3 μCi/mg) were gifts from the Boots Co. Ltd. (Nottingham, U.K.). [^{14}C]-Indomethacin (35.7 μCi/mg) was donated by Merck, Sharpe & Dohme Research Lab. (Rahway, NJ). The radiochemical purity of these chemicals as stated by the manufacturers was >99%. The radiochemical purity of free (unbound) drug was measured by thin layer chromatography of the drug on the buffer side after equilibrium dialysis and in all cases was better than 98%.

All experiments were performed using 0.1 M sodium phosphate buffer pH 7.4 containing 0.9% (w/v) NaCl, unless otherwise stated. When necessary, drugs were dissolved initially in a small volume of 0.1 M NaOH or 0.1 M HCl. The final pH of the stock solutions of drugs was adjusted to pH 7.2–7.6. The displacement of fluorescent probes, i.e. DNSA, warfarin and dansylsarcosine, by drugs was measured as previously described [3]. Fluorescence measurements were made at room temperature (22°) using a Perkin–Elmer model 3000 spectrofluorometer.

Binding was measured by equilibrium dialysis at 37° against 0.1 M isotonic phosphate buffer adjusted to pH 7.4 unless otherwise stated. Dialysis was performed using a Dianorm apparatus (Diachema A.G., Zürich, Switzerland) with 1 ml capacity cells and Spectrapor 2 membrane tubing (Spectrum Medical Industries, Los Angeles, CA). Each measurement was performed with at least two and sometimes more replications.

The dialysis time used was 3 hr as longer equilibration periods did not alter the degree of binding. After dialysis, 0.5 ml aliquots from both sides of the dialysis cells were mixed with 5 ml of PCS (a complete Phase Combining System, the Radiochemical Centre, Amersham) for liquid scintillation counting of radioactive aqueous samples. Radioactivity was determined using a Searle liquid scintillation counter (Model 6892).

Displacement of GP53,633 by various drugs was studied using drug concentrations reported to occur at therapeutic doses. The L-tryptophan concentration used was similar to that reported in untreated rheumatoid arthritis patients [22]. In order to compare the relative displacement effects of other drugs, studies were also done with 100 μM HSA and various drugs added to equimolar concentrations (100 μM). Unless otherwise stated, the concentration of GP53,633 used in binding and displacement experiments was 14.5 μM (4 mg/l).

Binding data for GP53,633 was analysed by a curve-fitting procedure using the non-linear least-squares programme MLAB [23], with r as the dependent variable and $[D]$ as the independent variable. Two independent sets of binding sites were assumed and data were fitted to the expression:

$$r = \frac{N_1K_1[D]}{1 + K_1[D]} + \frac{N_2K_2[D]}{1 + K_2[D]}$$

where: r = concentration ratio of bound drug to albumin; N_1 = number of high affinity sites; K_1 = association constant for the high affinity sites; N_2 = number of low affinity sites; K_2 = association constant for the low affinity sites; $[D]$ = unbound (free) drug concentration.

Results are presented as means ± standard deviation (S.D) unless otherwise stated. When appropriate, statistical analysis was performed using Student's t test for unpaired samples. The limit of significance was taken as $P < 0.05$.

RESULTS

Binding of GP53,633 in albumin solutions, serum and plasma

The extent of binding of GP53,633 was examined with two HSA preparations and various samples of serum and plasma (Table 1). The drug was 96–98.4% bound in serum and plasma. The degree of binding was similar with Fraction V HSA suggesting that albumin is the major binding protein for the drug in plasma. The degree of binding was lower with Fatty Acid Free HSA than with Fraction V HSA (1.6 moles fatty acid/mole HSA) indicating that, as with warfarin, fatty acids may enhance the binding of this drug to albumin.

Table 1. GP53,633 binding to human serum albumin (HSA) and plasma from various sources

Sample	GP53,633 free fraction (×100)	Albumin (g/l.)	Total protein (g/l.)	Fatty acids (μM)
4% Essentially Fatty Acid Free HSA (Sigma)	7.17 ± 0.21	40	40	45
4% Fraction V HSA (Sigma)	3.23 ± 0.13	40	40	880
Pooled Blood Bank plasma	4.07 ± 0.20	38	64	380
Ortho control serum	1.62 ± 0.08	37	61	570
Plasma samples from 12 healthy subjects	2.50 ± 0.37 (range 2.00–3.28)	42.7 ± 2.5	69.8 ± 4.6	340 ± 120 (range 150–590)

Table 2. Binding characteristics of GP53,633 to Essentially Fatty Acid Free and Fraction V HSA

HSA	N_1	$K_1 \times 10^{-3}$ (M^{-1})	N_2	$K_2 \times 10^{-3}$ (M^{-1})
Essentially Fatty Acid Free (Sigma)	0.25 ± 0.09	69 ± 37	7.52 ± 0.41	2.2 ± 0.3
Fraction V (Sigma)	0.87 ± 0.13	52 ± 12	3.92 ± 0.08	2.6 ± 0.3

Results are given as means ± standard error.

Effects of heparin, storage conditions and GP53,633 concentration

GP53,633 binding was similar in serum and heparinised plasma and was not altered by storage at −20° for up to 2 weeks. The binding was constant over the GP53,633 concentration range of 1–12 mg/l. This corresponds to the range of plasma concentrations reported after recommended doses of the drug.

Scatchard analysis of GP53,633 binding to HSA

Scatchard plots for the binding of GP53,633 to Essentially Fatty Acid Free HSA and Fraction V HSA were used to derive the number of binding sites and the association constants (Table 2). Fraction V HSA contained a relatively high concentration of free fatty acids (the concentration ratio of free fatty acid to HSA was 1.6). In both cases, the non-linearity of the plots indicated that the binding sites were heterogeneous. With Fraction V albumin the data indicates one high affinity binding site and at least four further binding sites with lower affinity for GP53,633.

With Fatty Acid Free albumin the high affinity site was virtually not distinguishable but the number of low affinity sites was increased. In both cases, the dependency values for the parameters were high, illustrating the difficulty in obtaining a unique fit to binding data when there are a number of heterogeneous sites. However, assuming one high affinity site and a dissociation constant of 19 μM for Fraction V HSA, it was calculated that with 14.5 μM GP53,633 and 600 μM HSA the free fraction should be 0.0317. This is in agreement with the measured value of 0.0323 (Table 1) giving confidence to the values derived by the curve-fitting procedure at least in relation to the high affinity binding site.

Effects of other drugs on GP53,633 binding

A number of other drugs were tested for effects on the binding of GP53,633 (Table 3). When Essentially Fatty Acid Free albumin (100 μM) was used and drugs were added at equimolar concentrations with albumin, marked displacement was observed with warfarin, diflunisal, phenylbutazone and sulphinpyrazone. Clofibric acid and salicylic acid produced small, but significant displacements. An interesting finding was that GP53,633 binding was enhanced by ibuprofen, flurbiprofen, indomethacin, L-tryptophan and naproxen. Most of these latter compounds have previously been characterised as binding to site-II on albumin [1, 2, 4].

To further define the effects of other drugs on GP53,633 binding, the same drugs were added at therapeutic concentrations to control serum (Table 3). The results were qualitatively similar with both displacement and enhanced binding being observed. Salicylic acid at a concentration of 2172 μM (300 mg/l), the top of the usual therapeutic range, is present at a molar ratio of 3.8:1 with albumin, and under these conditions caused marked displacement of GP53,633. Other compounds such as warfarin and sulphinpyrazone did not displace at therapeutically realistic plasma concentrations. CGP8716, the major metabolite of GP53,633, markedly displaced the parent drug suggesting that their binding characteristics are similar.

The effects of increasing concentrations of the site-I drugs phenylbutazone, warfarin and sulphinpyrazone, and the site-II drugs flurbiprofen and ibuprofen on the binding of GP53,633 in whole serum is shown in Fig. 2. Progressive displacement of GP53,633 occurred as the concentrations of site-I drugs were increased to approximately equimolar

Table 3. Effects of various drugs on the binding of GP53,633 to Essentially Fatty Acid Free HSA and to Ortho control serum

Drug	GP53,633 free fraction as per cent of control	
	Albumin*	Serum†
Warfarin	133	105.1 (15.1)
CGP8716	131.1	103.4 (17)
Diflunisal	128.5	157.3 (199.8)
Phenylbutazone	122.5	253.4 (486.4)
Sulphinpyrazone	118.5	100.6 (61.8)
Clofibric acid	111.6	151.2 (599)
Salicylic acid	104.7	336 (2172)
Ibuprofen	67.3	73.5 (252.3)
Flurbiprofen	85.6	83.9 (53.5)
Indomethacin	88.9	102.4 (8.4)
L-Tryptophan	93.5	79.7 (73.5)
Naproxen	94.3	76.8 (325.7)

* The concentrations used were—Essentially Fatty Acid Free HSA 100 μM, GP53,633 14.5 μM and the other drugs were added to a final concentration of 100 μM. The GP53,633 free fraction in the absence of other drugs was 0.273.

† Ortho control serum was used (albumin concentration 570 μM). The GP53,633 concentration was 14.5 μM and the other drugs were added at the concentrations (μM) shown in parentheses. The GP53,633 free fraction in the absence of other drugs was 0.016.

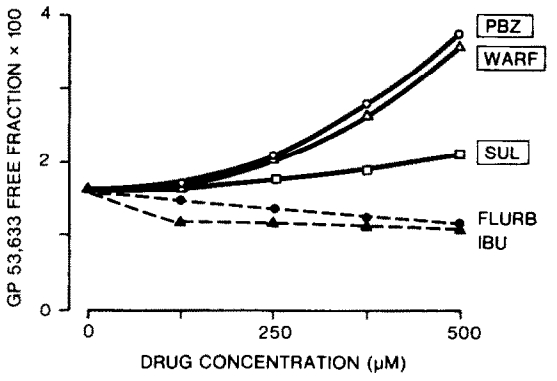

Fig. 2. Effects of site-I and site-II drugs on the binding of GP53,633 in Ortho control serum as measured by equilibrium dialysis. The drugs used were phenylbutazone (PBZ), warfarin (WARF), sulphinpyrazone (SUL), flurbiprofen (FLURB) and ibuprofen (IBU).

concentrations with albumin. Displacement by sulphinpyrazone was less marked than with the other site-I drugs but was statistically significant at higher concentrations (375 and 500 μM). Addition of site-II drugs, caused a statistically significant increase in binding of GP53,633 rather than a displacement. The free fraction of GP53,633 decreased from 0.016 to 0.011 with flurbiprofen ($P < 0.005$) and to 0.01 with ibuprofen ($P < 0.005$).

Displacement of warfarin and ibuprofen by GP53,633 and its metabolite

Both GP53,633 and CGP8716 added to serum at concentrations up to 500 μM caused a progressive increase in warfarin free fraction from 0.02 to 0.041 (GP53,633) and to 0.037 (CGP8716). Over the same concentration range neither compound had any effect on ibuprofen binding.

Fluorescent probe studies of GP53,633 binding

Figure 3 shows the effects of GP53,633 and its major metabolite, CGP8716, on the fluoresence of DNSA and warfarin (site-I probes) and dansylsarcosine (site-II probe). Effects of the site-I drug phenylbutazone and the site-II drug ibuprofen [1, 2, 4] are shown for comparison. Both GP53,633 and CGP8716 caused a marked displacement of warfarin and DNSA with the effect being maximal at approximately a 1:1 concentration ratio of drug to albumin. By contrast neither GP53,633 nor its metabolite caused any change in dansylsarcosine (site-II) fluorescence. As expected phenylbutazone displaced the site-I probes but not dansylsarcosine whereas ibuprofen displaced only dansylsarcosine.

Effect of fatty acids on binding of GP53,633

Oleic acid caused a progressive enhancement of GP53,633 binding when added to Fatty Acid Free HSA at concentrations which were less than equimolar with albumin (Fig. 4a). The free fraction of GP53,633 decreased from 0.273 without added oleic acid to 0.123 at 100 μM oleic acid (equimolar with albumin). Above equimolar concentrations oleic acid caused a progressive displacement of GP53,633 which reached a maximum at 4:1 molar ratio of oleic acid to albumin.

In plasma from 12 healthy adult male subjects, there was a good linear correlation between GP53,633 free fraction and the concentration of fatty acids ($r = -0.876$, $P < 0.001$). In this relatively homogeneous group 77% of the variability in GP53,633 binding was accounted for by the variability in fatty acid concentration (Fig. 4b). Neither plasma albumin concentration nor total protein concentration showed a significant correlation with GP53,633 free fraction in this group of subjects.

Effects of pH on GP53,633 binding

GP53,633 has two basic functions, one on the imidazole ring and the other on the pyridyl ring. Phenylbutazone has a single acidic function on the pyridazoline ring (Fig. 1). The ultraviolet spectrum of GP53,633 showed marked changes with pH in the 200–250 nm region. The absorbance at 200 nm and

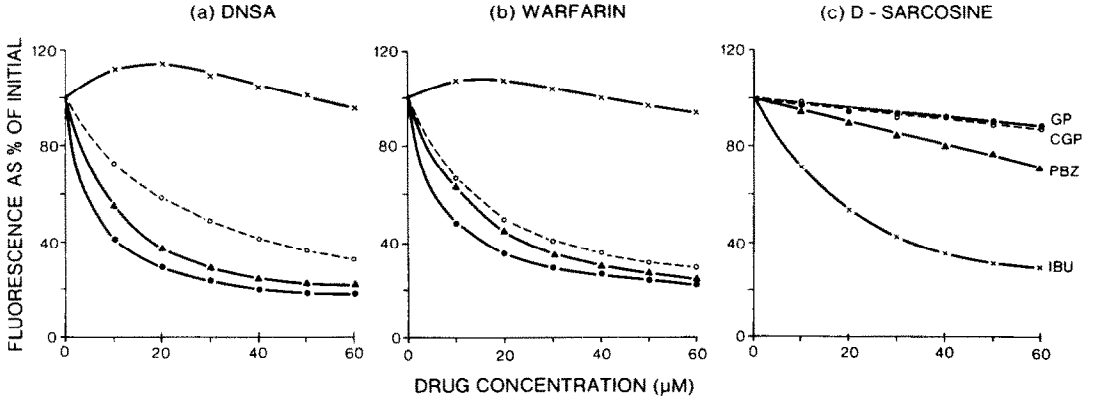

Fig. 3. Effects of GP53,633 (GP; ●), CGP8716 (CGP; ○), phenylbutazone (PBZ; ▲) and ibuprofen (IBU; ×) on the fluorescence of probes selective for site-I or site-II. Essentially Fatty Acid Free HSA was used at a concentration of 20 μM in 0.1 M sodium phosphate buffer with 0.9% NaCl, pH 7.4. DNSA, warfarin and dansylsarcosine concentrations were 2 μM. Fluorescence was measured at 475 nm (DNSA and dansylsarcosine) or 375 nm (warfarin) with excitation at 350 nm (DNSA and dansylsarcosine) or 315 nm (warfarin). Fluorescence is expressed as a percentage of that before addition of displacing drugs.

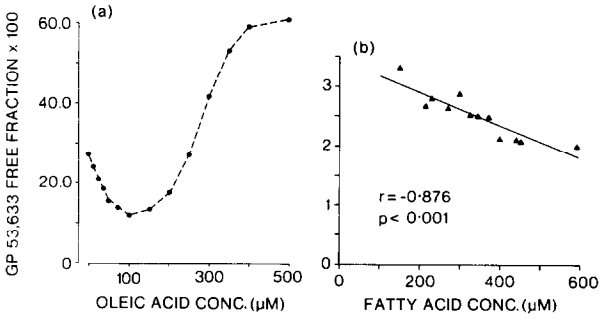

Fig. 4. Effects of fatty acids on the protein binding of GP53,633. (a) Effect of added oleic acid on GP53,633 binding to Essentially Fatty Acid Free HSA. (b) Correlation between fatty acid concentration and GP53,633 free fraction in normal plasma from 12 healthy subjects.

the albumin binding of GP53,633 change as a function of pH. The change in UV absorbance with pH indicates the ionisation of a group (probably the imidazole moiety) with a pK_a of approximately 6.4. Over the pH range 8.5 to 6.0 there was a 6-fold increase in GP53,633 free fraction (Table 4). The mid-point of the pH vs free fraction curve was about pH 6.5, very close to that observed for the UV absorbance titration.

DISCUSSION

GP53,633 differs from most other NSAID in that it is a base rather than an acid. Despite this, it shares with other NSAID the property of being highly protein bound. The free fraction of GP53,633 in plasma of 12 healthy subjects ranged from 0.02 to 0.033 with a mean of 0.025.

Most basic drugs bind primarily to alpha$_1$-acid glycoprotein or to lipoprotein fractions rather than to albumin. The data in Table 1 show that GP53,633 is largely, if not completely, bound to albumin. The free fraction of GP53,633 remained constant over the range of plasma concentrations reported after recommended human doses showing that binding is not saturable in this range of concentration.

Scatchard analysis of the binding of GP53,633 to Fraction V HSA indicated the presence of one high affinity binding site and a number of further sites with lower affinity for GP53,633. However, the high dependency values for the parameters indicated that other combinations of parameters might also give a good fit to the experimental data. At low concentrations of drug and with albumin present in large molar excess, binding is largely restricted to the high affinity site(s) on albumin. Therefore, one high affinity site was assumed for Fraction V HSA and the GP53,633 free fraction was calculated for 600 μM HSA and a GP53,633 concentration of 14.5 μM, assuming an association constant of $52 \times 10^3\,M^{-1}$ (from Table 2). The calculated free fraction was in close agreement with the measured value, suggesting that the parameters derived from the Scatchard analysis are valid.

The studies on displacement of fluorescent probes selective for sites I and II on HSA indicate clearly that both GP53,633 and its major metabolite bind to site-I. In equilibrium dialysis studies GP53,633 and its metabolite both displaced warfarin (site-I) but not ibuprofen (site-II) and GP53,633 was displaced by phenylbutazone, warfarin or sulphinpyrazone (site-I), but not by flurbiprofen or ibuprofen (site-II). A number of compounds which bind to site-II on albumin actually enhanced GP53,633 binding, indicating an allosteric interaction between site-II drugs and the binding of GP53,633. A similar interaction between binding sites I and II has been reported previously [2, 4].

Displacement of GP53,633 by other drugs was tested initially with displacing drugs added at equimolar concentrations with albumin (Table 3). Under these conditions displacement occurred primarily with drugs which bind to site-I on HSA. When displacement interactions were studied in whole serum with therapeutic drug concentrations, a somewhat different picture was seen. Salicylic acid which is present at high concentrations increased GP53,633 free fraction 2.4-fold, whereas it was a weak displacer at lower concentrations. On the other hand, other compounds (e.g. warfarin, sulphinpyrazone) which displaced at higher concentrations had little effect when present at therapeutic concentrations. These results emphasise the importance of using therapeutically realistic concentrations of drugs in binding experiments.

The GP53,633 free fraction was 2.2-fold higher with Fatty Acid Free albumin than with Fraction V HSA (Table 1), suggesting a 2.2-fold decrease in affinity at the primary site in the absence of fatty acids. This was consistent with the loss of the high affinity site on the Scatchard plot when Essentially Fatty Acid Free HSA was used.

It has been shown for other drugs such as warfarin, which bind to site-I on HSA, that removal of fatty acids causes a 2–3-fold decrease in binding affinity at this site [24–27]. The stoichiometry of this effect for GP53,633 was similar to that reported for other site-I drugs with maximum binding enhancement being observed at 1–2 mole of oleic acid per mole of albumin. Higher concentrations of oleic acid caused a progressive displacement of GP53,633. The free fatty acid concentration appears to be an important determinant of GP53,633 binding *in vivo* as there was a high negative correlation between these parameters in normal plasma from healthy subjects (Fig. 4).

Table 4. Association constants for unionised GP53,633 at various pHs

pH	GP53,633 free fraction (×100)	Association constant for unionised drug ($\times 10^{-3}$)* (M^{-1})	Association constant for total drug ($\times 10^{-3}$)* (M^{-1})
6.0	58.1	31.2	7.5
6.8	39.3	24.8	16.5
7.4	23.8	39.5	35.1
7.8	16.5	59.5	56.5
8.5	9.6	107.5	107.5

* The calculations were made from the GP53,633 free fractions assuming one high affinity binding site and a pK_a of 6.5 for the initial ionisation of the drug.

Unlike other compounds which bind to site-I on albumin, GP53,633 is a base rather than an acid. Binding of acidic drugs to HSA has been assumed to involve interaction of an anionic centre on the drug with a positively charged group on the protein surface with, however, the bulk of the binding energy being supplied by hydrophobic interactions [4, 28–30]. The effect of pH on the binding of GP53,633 suggests that the presence of a cationic centre on the drug precludes binding at the high affinity site. Binding was greatly reduced as the pH was decreased from 8.5 to 6.0 and the results suggested the involvement of a group with a pK_a of 6.5. Changes with pH in the UV spectrum of GP53,633 also indicated the ionisation of a group, probably on the imidazole ring, with a pK_a of 6.4. If one high affinity site is assumed, it is possible to calculate from the free fraction an association constant for *unionised* GP53,633 at each pH (Table 4).

The calculated association constants based on total drug (i.e. assuming ionisation has no effect on binding) are shown for comparison. Even allowing for the effect of ionisation, there is still an increase in association constant (for unionised drug) as the pH increases from 6.8 to 8.5. This is consistent with the reported increase in affinity of site-I drugs, such as warfarin, as the pH is increased over the range 6 to 9. This increase in binding affinity has been ascribed to a change in the conformation of albumin (the neutral-base transition) which occurs over this pH range [31–33].

The results presented in this communication show that the basic NSAID, GP53,633, binds to albumin at drug binding site-I. Binding at this site is usually associated with acidic drugs but the data show that basic compounds can bind with high affinity so long as they are in the unionised form. This suggests that non-polar (hydrophobic) interactions contribute the major part of the binding energy for drugs at this site. The marked effect of pH on the binding of GP53,633 is consistent with the existence of a cationic group on the protein surface close to or at drug binding site-I.

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