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INTERACTION OF 3-CARBOXY-4-METHYL-5-PROPYL-2-FURANPROPANOIC ACID, AN INHIBITOR OF PLASMA PROTEIN BINDING IN URAEMIA, WITH HUMAN ALBUMIN

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Abstract—The furan dicarboxylic acid 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (5-propyl FPA) accumulates in uraemic plasma and is a potent inhibitor of the binding of other anionic ligands to albumin. The interaction of 5-propyl FPA with human albumin has been investigated by equilibrium dialysis at 37° and pH 7.4. Analysis of the binding data on the basis of a two-site model gave binding parameters of $n_1 = 0.6$ and $K_1 = 4.8 \times 10^6\ M^{-1}$ for the primary binding site. 5-Propyl FPA binding was observed to decrease as the pH was raised from 6.4 to 8.3 which emphasizes the need for pH control of whole plasma or serum. Temperature, however, had little effect on binding as assessed by equilibrium dialysis at 10°, 25° and 37°. The high affinity of 5-propyl FPA for albumin explains its retention in uraemic plasma, its potency as a binding inhibitor and points to active tubular secretion as the mechanism by which it is normally excreted by the kidney.

3-Carboxy-4-methyl-5-propyl-2-furanpropanoic acid (5-propyl FPA; Fig. 1) was first detected in normal human urine in 1979 [1] and was later isolated and identified in human blood [2]. This furan dicarboxylic acid has subsequently been found to accumulate in uraemic plasma [3–6] reaching concentrations in the range of 60 to 370 μM [5] and it is therefore quantitatively as important as other uraemic metabolites such as indoxyl-3-sulphate and hippuric acid. Typical concentrations of these two metabolites in uraemic plasma are 45–202 μM for indoxyl-3-sulphate and 18–883 μM for hippurate [7]. Recent evidence suggests that 5-propyl FPA is a breakdown product of longer chain furanoid acids (F-acids) which are found in both the diet [8] and are also produced from unsaturated fatty acids [9]. The function of 5-propyl FPA and the reason for its increase in concentration in patients with renal failure are unknown, although accumulation in uraemia is caused, at least in part, by diminished excretion by the failing kidney.

The plasma protein binding of anionic albumin-bound drugs, dyes and endogenous ligands is reduced in uraemia [10]. The concomitant increase in fraction unbound and hence the likelihood of toxicity is one of the reasons why the dose of drugs with low therapeutic indices such as phenytoin and warfarin may need to be reduced in patients with renal impairment. The most widely accepted explanation for the drug binding defect of uraemic plasma is that retained endogenous solutes act as inhibitors of binding [7, 11]. 5-Propyl FPA inhibits the binding to whole human plasma and isolated albumin of a wide range of ligands including warfarin, diazepam, salicylic acid, phenytoin and *o*-methyl red [12–14]. Furthermore, 5-propyl FPA is the most potent uraemic binding inhibitor yet studied in experiments with human albumin and *o*-methyl red as the test ligand [11, 12]. At an inhibitor to albumin ratio of one, 5-propyl FPA was over 10 times more potent at displacing *o*-methyl red than indoxyl-3-sulphate and over five times more potent than hippuric acid [11, 12].

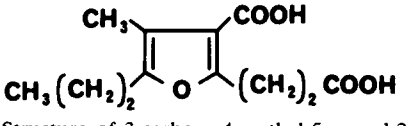

Fig. 1. Structure of 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid.

The inhibitory potency of 5-propyl FPA indicated that it should have a high affinity for human albumin so this has been measured directly by equilibrium dialysis at 37° and pH 7.4 under similar conditions to those in our earlier investigations of uraemic binding inhibitors. The binding of *o*-methyl red, a typical marker for the uraemic-induced reduction in binding [15, 16], to the same batch of albumin was also determined for comparison. The effect of pH and temperature on 5-propyl FPA binding was also investigated, the latter to provide some information about the thermodynamics of the 5-propyl FPA–albumin interaction. A preliminary account of some of this work has appeared [17].

MATERIALS AND METHODS

Chemicals. Crystalline human albumin (fraction V, M_r 66,500, lot 66F-9333 (for Scatchard plots) and lot 76F-9353 (all other experiments), crystalline bovine albumin (M_r 66,200; several lot numbers)

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and *o*-methyl red (sodium salt), were obtained from the Sigma Chemical Co. (Poole, U.K.). 3-Carboxy-4-methyl-5-propyl-2-furanpropanoic acid was a generous gift from Prof. G. Spiteller (University of Bayreuth, F.R.G.) and had been synthesized as described [18]. The purity of 5-propyl FPA was 97% by HPLC. Trifluoroacetic acid (Spectrosol) was obtained from BDH Chemicals Ltd (Poole, U.K.). All other HPLC solvents were "HPLC grade" and were obtained from FSA Laboratory Supplies (Loughborough, U.K.). Water used for HPLC was double distilled and deionized. All other reagents were of "Analar" grade and were obtained from BDH Chemicals Ltd.

Equilibrium dialysis. All solutions for dialysis were prepared in isotonic sodium phosphate–chloride buffer, pH 7.4 (5.75 g Na_2HPO_4, 1.5 g $NaH_2PO_4 \cdot 2H_2O$ and 2.075 g NaCl per litre). The pH was adjusted to pH 6.4 and 8.3 where necessary with 10 M NaOH or 10 M HCl. The aqueous solubility of 5-propyl FPA is limited so a stock 5-propyl FPA solution (1 mM) was prepared in acetonitrile : water (4 : 6 by volume) and stored at 4° until required. In order to avoid the presence of an organic solvent such as ethanol in the dialysis experiments, aliquots of stock 5-propyl FPA solution (1 mM) were freeze-dried and dissolved in a solution of buffered human albumin (1%) in a shaking water bath (37° for 1 hr) to give the desired concentration of 5-propyl FPA. The concentration of each solution was determined by HPLC (see below) to account for variable losses during the freeze-drying process. All glasswear used for 5-propyl FPA was silanized with dichlorodimethylsilane (5% in toluene).

Binding experiments were carried out with a "Dianorm" apparatus (Diachema AG, Switzerland) in waterbaths calibrated to 37°, 25° and 10° (±0.3°) as appropriate. Semipermeable cellulose membranes (visking tubing, size 9, molecular weight cut-off 12,000; Medicell International Ltd, London, U.K.) were soaked for 12 hr in distilled water, immersed in 30% (v/v) ethanol–water for 30 min, thoroughly rinsed with distilled water and transferred to buffer of relevant pH before use. Half-cells of 1 mL capacity were used and triplicate or quadruplicate aliquots (0.9 mL) of the test solution (albumin plus ligand) were dialysed against equal volumes of buffer at a rotation speed of about 9 rpm. The concentration of albumin was 150 μM (1%) as there was shown to be no osmotic volume shift from buffer to albumin compartment at this concentration by the method of Giacomini *et al.* [19]. Albumin concentration was determined from a bovine serum albumin standard curve by the biuret method [20]. Dialysis of albumin alone (150 μM) was performed to confirm the absence of albumin transfer across the membrane. The limit of detection of the biuret method was less than 1 μM.

o-Methyl red and 5-propyl FPA were dialysed for 3 and 5 hr, respectively, during which time the unbound ligand was shown to have reached equilibrium in control experiments under identical conditions. The mean recovery of *o*-methyl red (six concentrations over the range 20 to 300 μM) from the dialysis system in the absence of albumin was 93.1 ± 2.2%. The recovery of 5-propyl FPA (1 mM) was 92.1 ± 5.7% (N = 3). Unbound *o*-methyl red was assayed spectrophotometrically at 525 nm after the addition of an equal volume of 0.1 M hydrochloric acid [15]. Unbound 5-propyl FPA was measured directly by HPLC (see below) while the 5-propyl FPA concentration of the protein compartment was also determined by extraction of albumin-bound 5-propyl FPA by a modification of the methods of Mabuchi and Nakahashi [21] and Takeda *et al.* [6] as follows. An aliquot (0.2 mL) of human albumin (20% w/v) was added to the sample (0.8 mL) in a screw-topped plastic microcentrifuge tube (1.5 mL; Sarstedt Ltd, Leicester, U.K.) to produce a more concentrated (5.8% w/v) albumin solution which gelled on boiling. Samples were boiled for 5 min to denature the albumin and release bound 5-propyl FPA, triturated with a spatula, centrifuged at 11,500 *g* for 30 min ("Microcentaur" microcentrifuge, MSE Scientific Instruments, Crawley, U.K.) and the supernatant assayed for 5-propyl FPA by HPLC. After correction for both sample dilution and the 5-propyl FPA content of the human albumin preparation (0.5 ± 0.5 μM in the 5.8% w/v solution; N = 14), the deproteinization recoveries for 5-propyl FPA (four concentrations over the range 10 to 500 μM) were in excess of 94%.

Measurement of 5-propyl FPA by HPLC. The method was based on those published by Mabuchi and Nakahashi [21] and Takeda *et al.* [6]. A Pharmacia LKB HPLC system (Pharmacia LKB, Bromma, Sweden) was used along with a Nucleosil 5-C_{18} guard column (5 μM, 5 × 0.46 cm i.d.) and main column (5-C_{18}, 25 × 0.46 cm i.d.) both obtained from Technicol (Stockport, U.K.). The mobile phase was acetonitrile : water : trifluoroacetic acid (40 : 60 : 0.05 by vol., pH 2.4) and the elution was isocratic at a flow rate of 1.0 mL/min at room temperature. The samples (10 or 50 μL) were injected onto the column via a Rheodyne 7125 valve and the 5-propyl FPA in the column effluent was monitored by UV absorbance at 265 nm (model 2150 variable wavelength UV detector, Pharmacia LKB). Quantitative calculations were based on peak height measurements.

Standard curves for 5-propyl FPA (1–1000 μM) dissolved in acetonitrile : water (4 : 6 v/v) were linear and passed through the origin. The retention time was 12.8 min and the minimum detectable concentration of 5-propyl FPA was 200 nM. Although both the within-day and between-day coefficients of variation in peak height were less than 6% for both high (100 μM) and low (10 μM) 5-propyl FPA concentrations, three or four standards, chosen to encompass the range of experimental concentrations, were assayed each day and a standard curve constructed from which the 5-propyl FPA concentrations were calculated. (The mean correlation coefficient of these standard curves was 0.999 ± 0.002, N = 67.) HPLC standards and all other samples were stored in silanized vials at −20° prior to assay.

Analysis of binding data. The concentration of bound ligand, D_b, was calculated from the total concentration of ligand (D_t) in the protein compartment at the end of dialysis and the concentration of unbound ligand (D_u) in the buffer

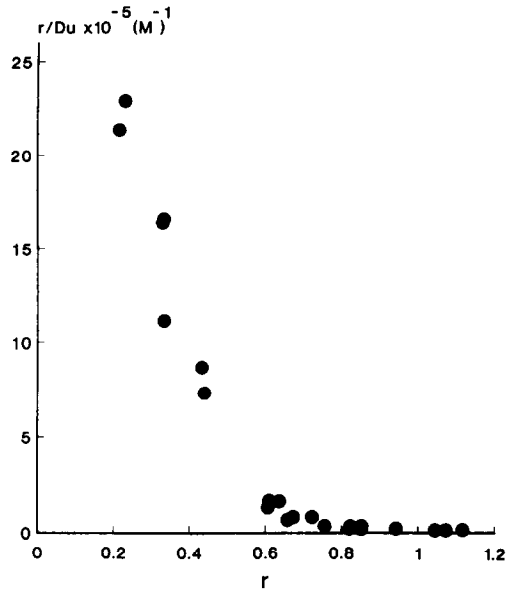

Fig. 2. Scatchard plot for the binding of 5-propyl FPA (initial concentration 30–300 μM) to human albumin (150 μM) at 37° and pH 7.4. Each point represents data from a single dialysis cell. See Table 1 for binding constants.

compartment at this time, as $D_b = D_t - D_u$. The percentage of ligand bound was calculated from the equation % bound = $D_b/D_t \times 100$. The molar ratio of bound drug to protein, r, was then determined for Scatchard-type analysis as follows: $r = D_b/$[albumin]. The values of D_u and r were then subjected to least squares non-linear regression with the program BMDPAR run on a mainframe computer at the University of Leeds. The data were analysed in terms of a two-site model to provide estimates of the apparent association constants for the high and low affinity sites (K_1 and K_2) and the number of high and low affinity binding sites (n_1 and n_2).

Results are expressed as the mean ± SD and statistical comparison was made by the non-paired Student's t-test.

RESULTS

Scatchard analysis

The Scatchard plot obtained for the binding of a range of concentrations of 5-propyl FPA (30–300 μM) to human albumin (150 μM) under physiological conditions (pH 7.4 and 37°) is presented in Fig. 2. These data and similar data obtained for *o*-methyl red (25–1000 μM) under the same conditions (Scatchard plot not shown) fitted a two-site model over the concentration range studied and the estimated binding parameters are given in Table 1. It can be seen that 5-propyl FPA bound primarily to a single high affinity site, its affinity (K_1) being an order of magnitude greater than that of *o*-methyl red for its primary site (Fig. 2 and Table 1). 5-Propyl FPA had rather low affinity however for a number of secondary binding sites, in contrast to *o*-methyl red which had an affinity for its secondary sites (K_2) over five times greater than that of 5-propyl FPA (Table 1). This explains why 5-propyl FPA is more highly bound than *o*-methyl red at low ligand concentrations (where binding will take place mainly to the primary sites in the system), and less highly bound than *o*-methyl red at higher concentrations where the primary sites are becoming saturated and binding takes place increasingly to secondary sites (Fig. 3). Hence at a total ligand concentration of 75 μM, 97% and 88% of 5-propyl FPA and *o*-methyl red respectively, were bound. At a ligand concentration of 200 μM however, 70% of 5-propyl FPA was bound compared to 79% of methyl red (Fig. 3).

Table 1. Binding parameters for the interaction of 5-propyl FPA and *o*-methyl red with human albumin (150 μM) at 37° (pH 7.4)

Binding parameter	5-Propyl FPA (30–300 μM)	*o*-Methyl red (25–1000 μM)
K_1 (M^{-1})	4.8×10^6	2.2×10^5
n_1	0.6	0.7
K_2 (M^{-1})	0.6×10^3	3.3×10^3
n_2	10.3	5.4

The mean square errors for the computer fits for 5-propyl FPA and *o*-methyl red were 5.1×10^{-4} and 2.2×10^{-3}, respectively.

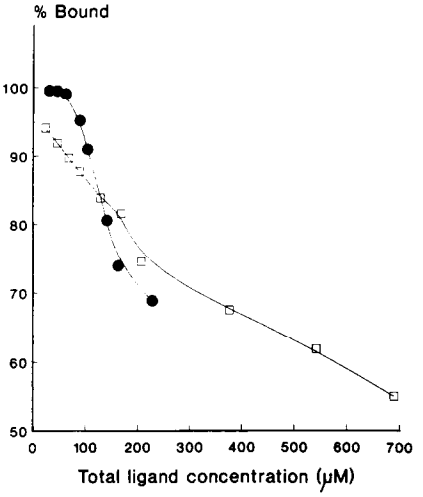

Fig. 3. Variation in extent of binding of 5-propyl FPA (●; initial concentration 30–300 μM) and *o*-methyl red (□; initial concentration 25–1000 μM) to human albumin (150 μM) at 37° and pH 7.4. Each point is the mean of duplicate (*o*-methyl red) or triplicate (5-propyl FPA) dialysis cells. All SD values were less than 1.3%.

Effect of pH *and temperature on 5-propyl FPA binding*

The 5-propyl FPA concentrations used to assess the effect of pH and temperature on binding were chosen to give values of r (0.5–0.7) likely to be found

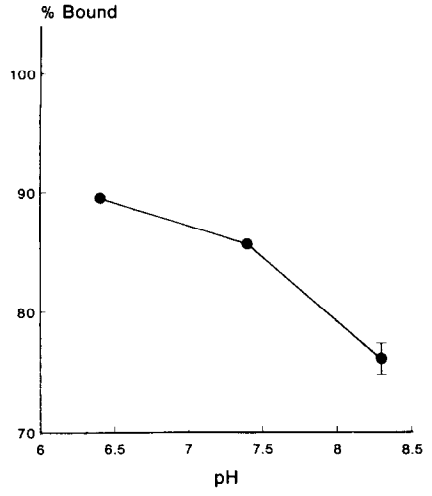

Fig. 4. Effect of pH on the binding of 5-propyl FPA (133 μM) to human albumin (150 μM) at 37°. Each point is the mean of three or four dialysis cells and the SD is shown where it exceeded 1%.

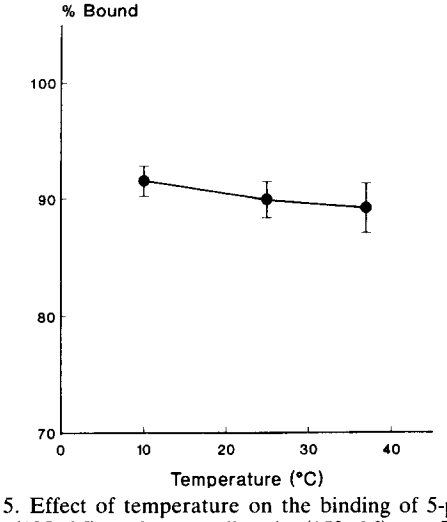

Fig. 5. Effect of temperature on the binding of 5-propyl FPA (105 μM) to human albumin (150 μM) at pH 7.4. Each point is the mean of four dialysis cells ± SD.

in uraemic patients. Figure 4 illustrates the decrease in binding to albumin (150 μM) of 5-propyl FPA (133 μM) with increased pH, from 89.5% at pH 6.4 to 76.1% at pH 8.3 ($P < 0.001$). The unbound concentration of 5-propyl FPA doubled over this pH range.

Temperature had little effect on the binding of 5-propyl FPA (105 μM; Fig. 5) and binding decreased by only 2.3% as the temperature was raised from 10° to 37°. A similar reduction in binding of 2.5% was observed for 123 μM 5-propyl FPA (data not shown). Control experiments showed that the unbound concentration of 5-propyl FPA had reached equilibrium at all temperatures during the 5 hr dialysis. Furthermore, temperature had little effect on the pH of the sodium phosphate–chloride buffer used, its pH was 7.4 at 25° and changed to 7.49 and 7.3 at 10° and 37°, respectively. This pH change was calculated to cause a 1.5% increase in % bound (a 1 μM reduction in the unbound concentration) as the temperature increased from 10° to 37° and therefore would not influence the observed lack of effect of temperature on binding.

A thorough investigation of the thermodynamics of the interaction would require full binding isotherms to be obtained for at least two other temperatures besides 37°, the temperature at which the data for the Scatchard plot were obtained (Fig. 2). The restricted supply of 5-propyl FPA precluded this approach and so the results from Fig. 5 were used to calculate approximate binding constants from the equation $K_1 = (D_b)/(D_u \times ([\text{albumin}] - D_b))$ and a van't Hoff plot ($\ln K_1$ vs $1/T$) constructed. The slope of the plot was marginally short of being statistically different from zero ($r = 0.556$, $t = 2.12$; the value of t required for statistical significance at $P < 0.05$ was 2.23). Bearing in mind this limitation of the data the thermodynamic constants calculated by standard methods [22] were: −9 kJ/mol for the change in standard enthalpy ($\Delta H°$), + 69 J/mol/°K for the change in entropy ($\Delta S°$) and −29 kJ/mol for the standard free energy change ($\Delta G°$). These values are similar to those for warfarin [23] for example but in marked contrast to those for pirprofen [24]. Ligands such as 5-propyl FPA and warfarin, however, pose an intrinsic problem in relation to a thermodynamic analysis by equilibrium dialysis because their binding changes relatively little with temperature and there is a limit to how much this can be varied in this context.

DISCUSSION

Analysis of the binding data (Fig. 2) suggested that 5-propyl FPA bound primarily to one site on human albumin with an association constant (K_1) of the order of $10^6\ M^{-1}$ (Table 1). The binding constants which we obtained by equilibrium dialysis at 37° (Table 1) and an albumin concentration of 150 μM were similar to those ($K_1 = 2.5 \times 10^6\ M^{-1}$, $n_1 = 0.8$; $K_2 = 2.7 \times 10^3\ M^{-1}$, $n_2 = 3.0$) reported by Mabuchi and Nakahashi [14] who used ultrafiltration of albumin (105 μM) at room temperature. This is further confirmation that temperature has little influence on binding (see Fig. 5). The binding of *o*-methyl red was also analysed for comparison because it has frequently been used as a model ligand with which to study ligand–albumin interactions, in the context of both fundamental mechanisms and as a marker for the drug binding defect of uraemic plasma or serum [11, 15, 16]. 5-Propyl FPA bound to albumin with an affinity greater not only than that of *o*-methyl red but also many other drugs and exogenous ligands whose K_1 values are listed in reviews [10, 25]. This explains the inhibitory potency of 5-propyl FPA towards *o*-methyl red and other albumin-bound ligands [11]. Only endogenous substances such as the long-chain fatty acids and bilirubin have notably higher primary association constants [10, 26]. The high affinity of 5-propyl FPA for albumin (Table 1; Fig. 2) and the high

concentrations (approaching 400 μM) found in uraemic plasma [4–6] indicate that this ligand is likely to be responsible for a substantial proportion of the uraemic binding defect which is not accounted for by indoxyl-3-sulphate and hippuric acid [7].

The effect of pH (Fig. 4) emphasizes the need for adequate pH control, particularly when whole plasma or serum is used since pH increases on storage because of the loss of dissolved carbon dioxide. The pH dependence of 5-propyl FPA-albumin binding parallels the neutral to base (N–B) transition of albumin, a hydrogen ion induced conformational change in the albumin molecule over the pH range 6 to 9 [27]. At pH 6, albumin is almost completely in the N-conformation whereas at pH 9 the B-conformation predominates. The results in Fig. 4 suggest that 5-propyl FPA like suramin [28] has a greater affinity for the N-form than for the B-conformation, which is in direct contrast to ligands such as warfarin [29], diazepam [30] and L-tryptophan [31], which have an increased affinity for human albumin in the B-form.

At pH values below 6, the basic amino acids arginine, lysine and histidine are combined with a hydrogen ion to form quaternary nitrogen ions which confer a net positive charge on the albumin molecule. However, as the pH increases above the isoelectric point (about pH 5) of human albumin, the acidic amino acids such as glutamic acid lose a hydrogen ion and form carboxylate ions which exceed the number of quaternary nitrogen ions at pH values above 6 and so impart a net negative charge to the protein [29]. According to the Tanford equation describing the relationship between pH and the anionic charge on serum albumin [32], the number of anionic charges per mole of albumin increases from 13 at pH 6.4 to 29 at pH 8.3. The decrease in binding of 5-propyl FPA (with two ionized carboxylic acid groups) is therefore likely to be due to greater electrostatic repulsion at higher pH. This effect is expected to be more pronounced for 5-propyl FPA (two anionic charges) than it is for the weakly acidic warfarin molecule (with no strongly ionizing groups) and could therefore explain the divergent effect of pH on these two ligands. The change in 5-propyl FPA binding as a function of pH may also be attributed to a change in the state of ionization of the dicarboxylic acid if one or both of its pK_a values are greater than 6 and the compound is not fully ionized over the pH range of these investigations.

Evidence from the use of fluorescent probes [33] and competitive binding studies [13] suggests that warfarin and other site I drugs were more readily displaced by 5-propyl FPA than site II drugs such as L-tryptophan. The experimental data presented in this paper concur with a number of studies conducted into the binding interaction at the warfarin site (site I) which suggest that although hydrophobic forces are important for ligand binding, forces of an electrostatic nature also exist [34, 35]. Indeed, Li *et al.* [34] postulate the existence of two cationic groups (lysine and arginine) in the warfarin binding site so it is possible that these could interact with the two anionic carboxylic acid groups of 5-propyl FPA, thereby stabilizing any hydrophobic and hydrogen bonds such that these forces together could account for the high affinity of 5-propyl FPA for albumin (Table 1). It is therefore possible that site I represents the high affinity site of 5-propyl FPA although this requires experimental verification.

5-Propyl FPA is a final excretion product in human urine [9] and no conjugates with either glucuronic acid or glycine have been detected [36]. The kidney must therefore possess an efficient active tubular secretion mechanism for 5-propyl FPA, in view of its high affinity for albumin, and we have preliminary evidence that it is taken up into rat kidney slices *in vitro* to a similar extent to *p*-aminohippuric acid, a classical marker for the renal anion transport system [37]. This means that 5-propyl FPA is likely to inhibit the active renal excretion of a range of other anionic substances and may itself be a useful prototype for highly protein-bound endogenous ligands which undergo active tubular secretion. Its strong affinity for albumin could explain the slow decline in the elevated levels of 5-propyl FPA and hence the gradual reduction in the binding defect after successful transplantation in patients with renal failure [38]. The highly protein-bound nature of 5-propyl FPA also accounts for the ineffectiveness of haemodialysis and related techniques for its removal [4, 21]. The consequent accumulation of 5-propyl FPA and inhibition of anion transport at various sites may therefore be responsible for at least part of the toxicity observed in patients with renal failure.

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