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The protein binding of timegadine determined by equilibrium dialysis

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1 The protein binding of timegadine to albumin, serum, plasma and plasma enriched with the acute phase reactants α_1-acid glycoprotein, α_1-anti-trypsin and C-reactive protein was determined by equilibrium dialysis.
2 The effects of other analgesic and anti-inflammatories (indomethacin, ketoprofen, paracetamol and sodium salicylate) and other basic drugs (disopyramide, lignocaine, propranolol and quinidine) on the binding of timegadine were also determined.
3 Timegadine binding was concentration-dependent up to 0.5 μg/ml, but independent above this level up to 10.0 μg/ml, the mean and standard error being 93.8 ± 0.5%.
4 Albumin accounted for only 32.4% of timegadine bound to plasma.
5 Plasma enrichment with the acute phase reactants led to significant increases in timegadine binding.
6 Simultaneous dialysis with other drugs caused significant decreases in timegadine binding.

Keywords protein binding acute phase reactants anti-inflammatory drugs

Introduction

Timegadine (*N*-cyclohexyl-*N''* -4-(2-methyl-quinolyl)-*N'*-2-thiazolyl guanidine) is a recently synthesised basic non-steroidal anti-inflammatory drug (NSAID) produced by Leo Pharmaceutical Products, Denmark. Timegadine acts by inhibiting both cyclo-oxygenase and lipoxygenase activity thus preventing the formation of prostaglandins (Ahnfelt-Ronne & Arrigoni-Martelli, 1980).

It is generally thought that only the free fraction of a drug is pharmacologically active and subject to elimination (Koch-Weser & Sellers, 1976; Brors *et al.*, 1983; Buss *et al.*, 1983). It therefore follows that the elimination half-life ($T_{½}$) could be greater for highly bound drugs.

Previous work with single doses of timegadine administered to healthy volunteers (George *et al.*, 1983) suggested a $T_{½}$ of 3.2 ± 0.3 h (mean ± s.e. mean), but multiple dose work in progress to study the steady state pharmacokinetics of timegadine suggest a $T_{½}$ of 6.6 ± 1.1 h (mean ± s.e. mean).

In view of these observations, and to further elucidate the pharmacokinetics of timegadine, it was decided to determine the plasma protein binding of timegadine.

We investigated the protein binding of timegadine to plasma (from a volunteer and normal drug free blood bank plasma), to serum, to plasma enriched with acute phase reactant proteins (APRP), and to albumin solutions of different concentrations. The APRP chosen were α_1-acid glycoprotein (AGP), α_1-anti-trypsin (AAT) and C-reactive protein (CRP) because in inflammatory disorders, for which timegadine would be prescribed, the concentration of these proteins is

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significantly elevated (Piafsky *et al.*, 1978; Waller *et al.*, 1982; Walker *et al.*, 1983). These increases could affect the binding of timegadine and so the rate of elimination together with the duration of action may be altered.

The effects of other commonly administered drugs (analgesics, anti-inflammatories and anti-arrhythmics) were also studied since these may also affect the binding kinetics when administered concomitantly with timegadine.

Methods

Plasma samples

Drug free blood bank plasma was collected, aliquoted into 30 ml glass universals and stored at −20°C prior to use. Plasma and serum was collected from a fasted healthy male volunteer by venepuncture and stored at −20°C prior to use. Total protein and albumin concentrations were determined on all samples by the cardiothoracic laboratory using a Gilford 203s automated analyser.

Purified proteins

The purified proteins: HSA, AGP, AAT, and CRP were all obtained from Sigma, Poole, UK.

Drugs

A selection of commonly administered basic, analgesic and anti-inflammatory drugs were used to discover whether or not displacement reactions occurred with timegadine. The drugs used were disopyramide, indomethacin, ketoprofen, lignocaine, paracetamol, propranolol, quinidine and sodium salicylate.

Timegadine and internal standard (SR 1410, a dimethyl analogue of timegadine) were gifts from Leo Pharmaceutical Products.

Equilibrium dialysis

Equilibrium dialysis was performed using a Dianorm equilibrium dialyser (MSE scientific instruments, Crawley, UK) in conjunction with Diachema membrane discs (molecular weight cut-off 5000) and Spectrapor membrane tubing (molecular weight cut-off 6000–8000). At a temperature of 37°C and a speed of 8 r/min, equilibrium was achieved between 2 and 3 h, and so a dialysis time of 3 h was used for all determinations. Previous single dose studies with timegadine (George *et al.*, 1983), and multiple dose work in progress, gave plasma concentrations between 0.05 and 2.32 μg/ml (mean 0.81 μg/ml), therefore 1.0 μg/ml was used for all binding work (except concentration effects).

The dialysis buffer was Sorensen's phosphate buffer pH 7.4 made by mixing 800 ml of Na_2HPO_4 (9.46 g/l) with 200 ml of KH_2PO_4 (9.07 g/l) and adjusting the pH using one or other of the salt solutions.

Timegadine protein binding was determined by dialysing 1.0 ml of plasma, serum, enriched plasma or albumin solution against 1.0 ml of drug solution(s) in buffer. The percentage of timegadine bound to plasma was calculated from:

$$\% \text{ bound} = (Ct - Cf)/Ct \times 100$$

where Ct is the concentration of timegadine in the plasma or protein solution and Cf is the timegadine concentration in the buffer after dialysis. All determinations were performed in triplicate for each variable monitored (the mean values presented in the tables). Statistical analysis was by the paired t-test.

Timegadine determination

Analysis was performed on a reverse phase column (Lichrosorb RP 18, 5 μm) 30 cm × 0.4 cm. Detection was at 300 nm, 0.02 AUFS. Eluent was 3% triethylamine in acetonitrile at a flow of 1.5 ml/min.

Into a 5.0 ml glass screw top tube, were placed 750 μl of albumin solution, plasma or enriched plasma, 100 μl of 4.0 μg/ml internal standard solution in methanol and 1.1 ml of 3% vol./vol. perchloric acid. This was vortexed for 30 s and centrifuged for 5 min at 3000 r/min. The supernatant was transferred to a clean stoppered centrifuge tube, made alkaline with 1.2 ml of 1.0 M sodium hydroxide and extracted with 3.0 ml of dichloromethane by spiromixing for 15 min. Following centrifugation as above, the organic phase was evaporated to dryness at 35°C under nitrogen. The residue was reconstituted in 50 μl of the eluent, and 40 μl were injected into the chromatograph.

For Sorensen's buffer, containing the free drug, the extraction was the same except that no acid deproteinisation step was required, and the volume of internal standard solution was 60 μl.

This system yielded retention times of 3.5 and 5.5 min for the drug and internal standard, respectively. Quantitation was based on a standard curve over the range 0–2.0 μg/ml. The assay was found to be linear over the range 0–10.0 μg/ml, recovery was 120.7% relative to the internal standard, and replicate analysis of 0.2 μg/ml and 0.5 μg/ml gave coefficients of variation of 3.9% and 6.3%, respectively.

Results

It was found that the overall recovery from the dialysis (as a percentage of the initial concentration) was less using the Diachema membranes (mean 68.7%) than with the Spectrapor membranes (mean 80.5%) for 1.0 μg/ml, and so the Diachema membranes were not used for further work. The 80.5% recovery for the Spectrapor membranes was constant over the range 0.1–10.0 μg/ml, the coefficient of variation being 8.5%.

Changing the ionic strength of the buffer by the addition of 0.6% sodium chloride made no significant difference to the binding of timegadine ($P > 0.05$).

Table 1 The effects of different anticoagulants on the protein binding of timegadine as compared to blood bank plasma. The % bound values are the means of three replicates.

Anticoagulant	*Sample*	*Timegadine (% bound)*	*% difference*
Blood bank	Plasma	95.4	0
Citrate	Plasma	95.3	0.1
EDTA	Plasma	95.4	0
Heparin	Plasma	95.7	0.3
Oxalate	Plasma	95.5	0.1
	Serum	95.6	0.2

The effect of timegadine concentration on protein binding

Timegadine protein binding was measured over the range 0.1–10.0 μg/ml (Figure 1). It can be seen that the binding is concentration-dependent up to 0.5 μg/ml, but independent above this level, 93.8 ± 0.5% (mean ± s.e. mean).

The effect of different anticoagulants on protein binding

The effect of different anticoagulants (citrate, EDTA, heparin and oxalate) was studied by filling different sample collection tubes with whole blood drawn from a volunteer by venepuncture. The results are summarised in Table 1 and show that anticoagulants had no significant effect on binding ($P > 0.05$).

The effect of albumin concentration

It can be seen from Figure 2 that albumin only accounted for 32.4 ± 0.4% (mean ± s.e. mean) of timegadine bound to total proteins in plasma. There was no correlation between the albumin concentration in plasma and the amount of timegadine bound to albumin ($r = 0.57$; $P > 0.05$). This suggests that albumin is not the sole, or main, protein that binds timegadine. Similarly the binding of timegadine did not correlate with the total protein concentration ($r = 0.56$; $P > 0.05$).

The effect of plasma enrichment

The enrichment of plasma by the addition of the purified APRP, AGP, AAT and CRP, resulted in significant increases in timegadine binding (Table 2 and Figure 3). It can be seen that CRP had the greatest influence on timegadine binding with the addition of 25 μg/ml causing a significant increase ($P < 0.01$). It was possible to correlate the increase in binding to the concentration of APRP added to plasma, AGP, $r = 0.88$; AAT, $r = 0.74$; and CRP, $r = 0.76$.

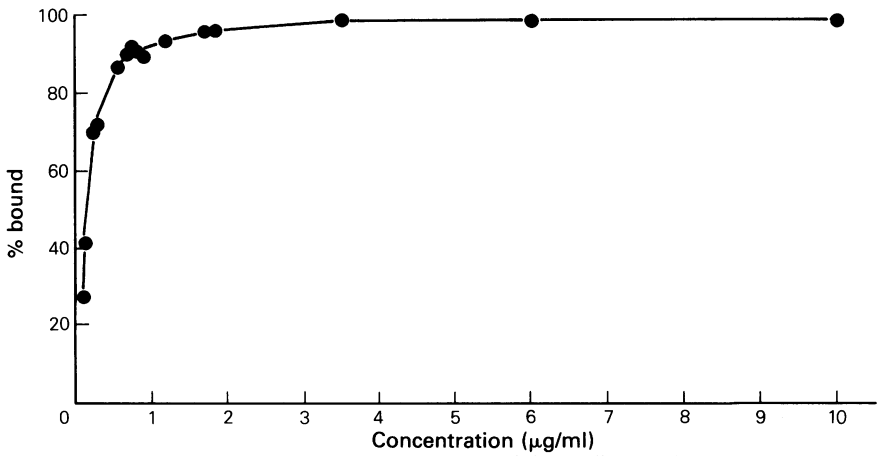

Figure 1 The effects of drug concentration on the binding of timegadine to plasma.

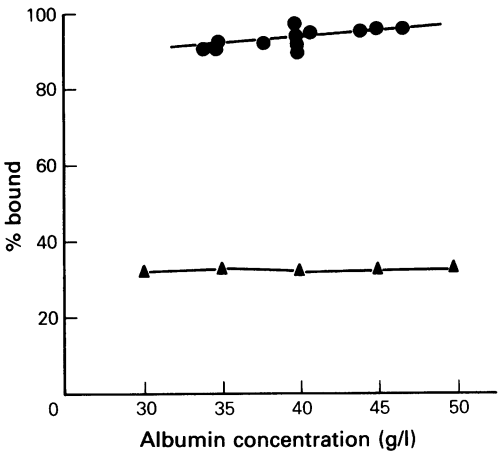

Figure 2 The effects of albumin concentration on the binding of timegadine to both plasma samples (●) and albumin solutions (▲).

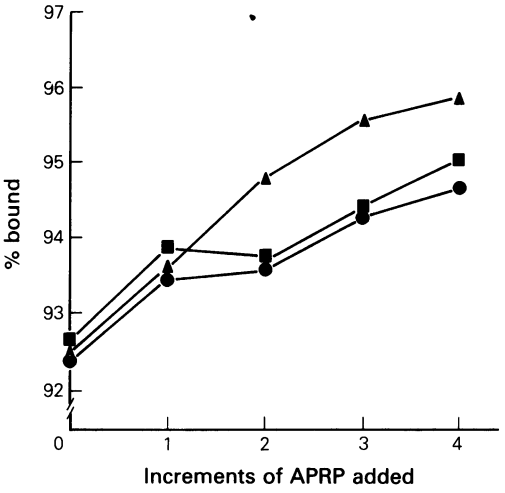

Figure 3 The effects of APRP on timegadine binding to plasma (see Table 2 for concentrations used). AGP (●), AAT (■), CRP (▲).

The effect of other drugs

The effects of various drugs on timegadine plasma protein binding are summarised in Table 3. The values given for the extent of protein binding of the drugs were taken from Wade (1977) and Taylor & Finn (1981).

All the drugs studied caused a significant decrease of timegadine binding with quinidine having the greatest effect—a 35% decrease ($P < 0.001$), at therapeutic concentrations. Propranolol at a concentration of 0.5 μg/ml also caused a significant decrease, a two-fold increase in free fraction, in the binding of timegadine.

Discussion

The degree of protein binding of a drug greatly influences the distribution, elimination and therefore the duration of action of the drug since only the free fraction exerts a pharmacological effect and can be eliminated. Ekstrand *et al.* (1979) proposed that the metabolic clearance of salicylate is proportional to the free fraction. Wanwimolruk *et al.* (1982) stated that the degree of protein binding is a major determinant of drug clearance and of drug concentrations at steady state. In view of these points, and

Table 2 The effects of plasma enrichment on the binding of timegadine as compared to normal plasma.

Plasma protein	*Concentration added (mg/ml)*	*Mean % bound*	*Mean % free*	P
Plasma	0	92.5	7.5	
CRP	0.0125	93.6	6.4	> 0.050
	0.0250	94.8	5.2	< 0.010
	0.0500	95.6	4.4	< 0.010
	0.1000	95.9	4.1	< 0.010
Plasma	0	92.4	7.6	
AGP	0.2	93.5	6.5	< 0.010
	0.4	93.6	6.4	< 0.010
	0.6	94.3	5.7	< 0.010
	0.8	94.7	5.3	< 0.010
Plasma	0	92.6	7.4	
AAT	0.5	93.9	6.1	< 0.050
	1.0	93.8	6.2	< 0.025
	1.5	94.4	5.6	< 0.010
	2.0	95.1	4.9	< 0.010

Table 3 The effects of other drugs on the protein binding of timegadine as compared to timegadine alone. The % free timegadine figure is the mean value.

Drug (% bound)	*Concentration used (μg/ml)*	*% free timegadine*	P
Timegadine (92.2)	1.0	7.8	
Disopyramide (~ 70)	3.0	8.9	< 0.010
Indomethacin (> 90)	5.0	10.3	< 0.025
Ketoprofen (> 95)	10.0	9.9	< 0.050
Lignocaine (60–80)	3.0	8.3	< 0.010
Paracetamol (~ 30)	200.0	9.5	< 0.010
Propranolol (85–95)	0.5	14.6	< 0.001
Quinidine (80–90)	3.0	31.6	< 0.001
Salicylate (~ 85)	250.0	11.0	< 0.050

because the pharmacokinetics of timegadine are still under research, the protein binding of timegadine was determined, using equilibrium dialysis.

In common with other NSAID (Craig & Buchanan, 1980; Verbeeck *et al.*, 1983) timegadine was found to be highly bound to plasma proteins, 93.8 ± 0.5% (mean ± s.e. mean) at the concentrations achieved in healthy volunteers but binding was found to be concentration-dependent at concentrations below 0.5 μg/ml. Leo Pharmaceutical Products (data on file) found that at a concentration of 2 μg/ml timegadine was 99% bound. Figure 1 shows that at this concentration we found timegadine to be about 98% bound. An interesting and important point is that the binding is the same in serum, freshly taken plasma, and drug free blood bank plasma.

It is well documented that acidic NSAID are bound primarily to albumin (Ekstrand *et al.*, 1979; Helleberg, 1981; Verbeeck *et al.*, 1983), whereas basic drugs such as propranolol and lignocaine are bound mainly to AGP (Piafsky *et al.*, 1978; Edwards *et al.*, 1982; Barchowsky *et al.*, 1982). In view of this, it was not surprising to find that albumin only accounted for 32.4% of timegadine bound to plasma proteins, or that the binding of timegadine could not be correlated to albumin concentration ($r = 0.57$).

Pike *et al.* (1983) used sera deficient in lipoproteins, albumin and orosomucoid (AGP) to determine the quantitative role of proteins in drug binding. In our research, it was decided to enrich plasma with APRP known to be increased in the inflammatory conditions for which timegadine would be administered. The proteins were AGP, AAT and CRP, and the concentrations used were chosen so as to exceed the normal range, virtually doubling the levels normally found in adults. The levels for normal plasma were taken from the table of human blood plasma proteins produced by Hoechst and is based on the normal concentrations found in European adults:

AGP mean 0.9 mg/ml (range 0.6–1.4)
AAT mean 2.6 mg/ml (range 1.9–3.5)

In the case of CRP the table gave no mean value, and 5.0 μg/ml as the top of the range. Pepys (1981) stated that the median value in 468 healthy adults was 0.8 μg/ml but that serum CRP levels may reach 300 μg/ml following the onset of inflammation. It was for this reason that additions of up to 100 μg/ml were studied.

Plasma enrichment led to very significant increases in timegadine binding, especially with AGP, not unexpectedly since timegadine is a basic drug. However the results of enrichment with CRP are quite surprising since a significant increase in binding ($P < 0.01$) was observed following the addition of only 25.0 μg/ml.

The APRP are present in elevated concentrations for some time, about 10 days for CRP (Pepys, 1981) and 20 days for AGP (Edwards *et al.*, 1982) in inflammatory conditions and trauma. The increased binding to APRP could therefore have a marked effect on the action of timegadine and its clearance from the body, indicating a possible need for monitoring the levels of timegadine at the time of commencing therapy.

The displacement of timegadine from protein by other drugs, summarised in Table 3, can be explained in part by the binding characteristics of the drugs involved. As stated previously NSAID are primarily bound to albumin, and since timegadine is oniy 32.4% bound to albumin, then the displacement of timegadine by NSAID should be slight. In fact NSAID were found to cause a mean increase of 31% in the free fraction of timegadine.

The basic drugs are mainly bound to AGP, but quinidine, propranolol and to a slight extent disopyramide, are bound to albumin as well

(Pike *et al.*, 1983), and these drugs could therefore be expected to have a greater effect on the binding of timegadine. In the case of quinidine the free fraction was increased by 305%, i.e. timegadine binding was reduced from 92.2% to 68.4%.

McElnay & D'Arcy (1983) stated that the importance of displacement interactions has been over-estimated and over-stated. It is true that the clinical relevance of these drug interactions *in vitro* is difficult to interpret, however, the results presented in this study for the effects of APRP and the effects of other drugs suggest a need for the monitoring of plasma concentrations of timegadine at least during the commencement of therapy.

We would like to express our thanks to the staff of the cardiothoracic laboratory for measuring the total protein and albumin concentrations in our samples, and to Leo Pharmaceutical Products for supplying us with timegadine and internal standard.

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