J. Pharm. Pharmacol. 1981, 33: 507–514
Received February 24, 1981

0022–3573/81/080507–08 $02.50/0

Plasma protein binding of fentanyl

SUSANNE BOWER

Department of Anaesthesia, University of Newcastle upon Tyne, Queen Victoria Road, Newcastle upon Tyne, NE1 7RU, U.K.

Whole plasma of 15 normal volunteers bound 79·16% ± 0·16 s.e. (n = 45) of fentanyl, at a concentration of 0·6 ng ml^{-1}. Measurement was by equilibrium dialysis at 37 °C, pH 7·4. The largest contribution to binding appears to be due to serum albumin, since 45·52% ± 0·40 s.e. (n = 3) of fentanyl was bound to a solution of purified albumin at a concentration of 46 g litre^{-1} in buffer. Physiological concentrations of isolated very low density, low density and high density lipoprotein fractions in buffer bound 18·39% ± 0·65 s.e.; 39·14% ± 0·42 and 21·18% ± 0·51 (n = 15) respectively. A significant correlation was found between percent binding and serum albumin concentration (r = 0·745, P = 0·0022) and oestrogen and progestagen therapy (r = 0·766, P = 0·0014). There was no significant correlation with fasting serum cholesterol, triglyceride, age, sex or the concentration of total protein minus albumin. Binding to fibrinogen and α_1-acid glycoprotein did not occur. Binding increased with increasing pH, temperature and ionic strength of the buffer. The results were compatible with hydrophobic bond formation between fentanyl and proteins. Fentanyl concentration did not affect the percent bound to whole plasma or the protein fractions over a range of 0·6 ng–10 mg ml^{-1}. Dilution of plasma with buffer gave a linear relation of percent bound or free to log plasma dilution. The binding of fentanyl to pooled plasma of the normal subjects was not affected by a wide variety of anionic, cationic and uncharged drugs when these were tested at a concentration of 20 μg ml^{-1}. At higher concentrations, aspirin, phenylbutazone and quinidine caused inhibition of fentanyl binding. A linear relation was found between percent bound and concentration of the inhibitory ligand. For aspirin, r = 0·873, P <0·01; for phenylbutazone, r = 0·81, P <0·05; and for quinidine, r = 0·982, P <0·01. Aspirin and phenylbutazone inhibited binding of fentanyl to albumin, while quinidine caused inhibition of binding to lipoproteins of all three densities, but not to albumin. Changes in the concentrations of the common ions of plasma (except H^+), of free fatty acids and of creatinine did not affect fentanyl binding to whole plasma. 8 M urea reduced binding by 25% of the normal value.

Knowledge of the extent of plasma protein binding of drugs is essential in the interpretation of their plasma concentrations, and in pharmacokinetic studies. It is the free drug which is able to equilibrate with extracellular fluid and the tissues, and to initiate the pharmacological response. Therefore factors which influence the extent of plasma protein binding may have clinical significance. In particular, competition by other drugs or endogenous ligands may reduce the size of the bound fraction, and may enhance the pharmacological effect. Estimations of binding of the short-acting opioid fentanyl to plasma proteins have been limited to a non-clinical temperature (25 °C) (Schaer & Jenny 1970) and high plasma fentanyl concentrations (Hollt & Teschemacher 1975).

MATERIALS AND METHODS

Fentanyl

Crystalline fentanyl citrate (mol. wt 528·6) was obtained from Janssen Pharmaceutica, Beerse, Belgium. Tritium-labelled fentanyl was obtained from I.R.E., Brussels. The specific activity was 8 Ci mM.$^{-1}$ A concentration of 0·6 ng ml^{-1} [^{3}H] fentanyl in 0·1 M sodium phosphate buffer was used routinely for equilibrium dialysis and a higher concentration of 40 mg ml^{-1} was also used where competition by other ligands was studied. The concentrations were chosen to represent a typical plasma concentration in the post-distributive phase after i.v. injection of 100 μg of drug (Bower et al 1976), and a higher plasma concentration compatible with the high dosage regimes used in the technique of 'stress-free anaesthesia' (Wood 1978). Concentrations of fentanyl are quoted throughout as fentanyl citrate. 1·56 ng fentanyl citrate is equivalent to 1·0 ng active base.

Buffer

0·1 M sodium phosphate buffer was used. Thiomersal, 0·6 mM was added to inhibit bacterial action during equilibrium dialysis. The use of thiomersal did not affect binding. The buffer was also used to dilute plasma to examine the effect of plasma concentration on binding.

Drugs and endogenous ligands

Clinical preparations of the drugs tested were diluted to the required concentration in buffer. The three postulated metabolites of fentanyl, 4-anilino-1-

(2-phenethyl) piperidine, (Maruyama & Hosoya 1969), *N*-phenyl-*N*-(4-piperidinyl) propanamide, and 1-(2-*p*-hydroxyl-phenylethyl)-4-*N*-(*N*-proprionyl-anilino) piperidine (van Wijngaarden & Soudijn 1968) were also tested. These compounds were obtained from Janssen Pharmaceutica, Beerse, Belgium.

Oleic acid (99%), stearic acid (99%) and palmitic acid (99%), urea and creatinine sulphate were obtained from the Sigma London Chemical Company.

All ligands except fatty acids were prepared in buffer and [^{3}H] fentanyl (0·6 or 40 ng ml^{-1}) was added for equilibrium dialysis against plasma proteins. All concentrations quoted refer to the initial concentration of ligand in buffer unless otherwise stated. The concentrations of drugs were chosen to include or exceed likely clinical plasma concentrations. A concentration of 20 μg ml^{-1} was used to screen many of the ligands, but higher concentrations were used for those drugs which are usually given at higher dosages. The drugs which caused inhibition of fentanyl binding to whole plasma were also incubated with albumin and lipoprotein fractions in order to examine the site of the effect. Equimolar preparations of aspirin (acetylsalicylic acid) and sodium salicylate were compared to determine whether acetylation of albumin was the cause of inhibition by aspirin.

The concentrations quoted for endogenous compounds, except fatty acids, are the final concentrations of ligand added plus the original concentration in the pooled plasma.

Plasma

Plasma was obtained from the blood of 15 normal volunteers, collected into lithium-heparin tubes, and used for individual results in addition to data for pooled plasma. Three of the subjects were taking oral oestrogens and progestagens, the rest took no drugs. Total serum protein, albumin, urea and creatinine, fasting cholesterol and triglyceride concentrations were within normal limits in all cases. Total protein and lipoprotein electrophoresis showed normal patterns.

Serum albumin

Crystallized, lyophilized human serum albumin (Sigma, London) was prepared to a concentration of 46 g litre^{-1} in the buffer.

Isolation of lipoprotein fractions

The Very Low Density (VLDL), Low Density (LDL) and High Density (HDL) lipoprotein fractions were prepared from plasma by the flotation procedure in the preparative ultracentrifuge (MSE Superspeed 65) as described by Havel et al (1955). NaCl and KBr were used to adjust the density of the solution. The isolated lipoprotein fractions were washed to remove NaCl and KBr by fourfold concentration in a 2·5 ml Millipore Ultrafiltration cell (XX42 013 10, Millipore Corporation, U.K.). An ultrafiltrate was forced through a selective membrane at the base of the cell by application of nitrogen at 40 p.s.i. (276 KN m^{-2}). The Millipore PSED membrane was used. The original volume was restored with buffer and the process repeated three times. The lipoprotein fractions were then suspended in a volume of buffer equal to that of the original plasma sample to give physiological concentrations. The protein content of each fraction was estimated by the method of Lowry et al (1951). The concentrations of lipoproteins were calculated assuming that the apoprotein contributes 10% (w/w) of VLDL, 23% of LDL and 55% of HDL (Lewis 1976).

Removal of free fatty acid (FFA) from plasma

To test the effect of FFA on fentanyl binding, a portion of the pooled plasma was first defatted with charcoal (Norit SX-1) at pH 3, by the method of Chen (1967). 5 M HCl was used to lower the pH of the plasma to 3, and 5 M NaOH was added to restore the pH to 7·4 after removal of FFA. The excess NaCl so formed was removed by fourfold concentration of aliquots of plasma in a 2·5 ml Millipore ultrafiltration cell, (XX42 013 10, Millipore Corporation, U.K.). The application of nitrogen at 40 p.s.i. forced an ultrafiltrate of the plasma through a selective membrane (Millipore PSED membrane) at the base of the cell. The original volume was restored with buffer and the process repeated three times.

Preparation and assay of FFA in plasma

Oleic, stearic and palmitic acids were each added to distilled water to give a concentration of 25 mM. 5 M NaOH was added with continuous stirring and heating on a heated magnetic stirrer until a clear solution formed. The pH was restored to 7·4 with 5 M HCl. The preparations were then added to defatted plasma to give a final concentration of 4 mM. The final concentration of NaCl in the plasma after equilibrium dialysis was within the range of ionic strength found not to affect fentanyl binding. Direct addition of FFA to plasma for binding studies was also employed by Rudman et al (1971). The FFA content of the plasma before and after defatting was measured by the isotope dilution technique of Ho & Meng (1969).

TBEP

Trisbutoxyethylphosphate (TBEP) (Aldrich Chemical Company, Gillingham, Dorset) inhibits ligand binding to α_1-acid glycoproteins (Borga et al 1977). TBEP (100 μg ml^{-1}) was added to plasma, and mixed thoroughly, to test involvement of α_1-acid glycoproteins in the binding of fentanyl.

Measurement of protein binding

Perspex equilibrium dialysis blocks were assembled to divide a central chamber into two 1 ml compartments by an intervening Visking dialysis membrane (36/32). 0·5 ml of protein solution or plasma were introduced into one compartment with an E-Mil Lang-Levy pipette. 0·5 ml of buffer containing [^{3}H] fentanyl (and the additional ligands tested, where appropriate), were added to the other compartment.

To test the effect of fentanyl concentration on binding, unlabelled fentanyl was used up to a concentration of 10 mg ml^{-1} in buffer, with 0·6 ng ml^{-1} [^{3}H] fentanyl added as tracer.

The initial pH of the buffer was adjusted to give a final pH of 7·4 in each protein sample, or varied to test the effect of pH on binding over the range 6·4 to 7·8. pH control blocks, identical to the sample blocks but with an equal concentration of unlabelled fentanyl in place of tritiated ligand, were incubated simultaneously. The final pH of the plasma of the control blocks was measured with a Radiometer 27 pH meter.

The blocks were incubated routinely for 16 h at 37 °C in a waterbath. Temperatures between 4°–40 °C were also studied to examine the effect of temperature on binding.

After equilibration, 200 μl aliquots of buffer and plasma compartment samples were added to 13 ml of scintillation cocktail. The activity of the samples was counted in a Searle Tricarb liquid scintillation counter, and the external standard ratio method of quench correction was used. A toluene: Triton X-100 3:1 cocktail was used with 5 g litre^{-1} PPO puriss. (BDH Ltd., Poole, Dorset). Triton X-100 puriss was obtained from Koch-Light Ltd, Colnbrook, Bucks., and toluene, scintillation grade, from BDH.

The relevance of binding data given by this method to physiological ionic conditions was tested. An ultrafiltrate of pooled plasma was prepared in the Millipore apparatus and substituted for the buffer. The binding results obtained with buffer and ultrafiltrate were compared at the same final pH.

The percent binding was calculated by the formula

$$B\% = \frac{C_t - C_f}{C_t} \times 100 = \frac{C_b}{C_t} \times 100$$

where C_t is the concentration of total ligand given by the activity in the plasma compartment, C_f is the concentration of free ligand given by the activity in the buffer compartment, and C_b is the concentration of bound ligand. The assumption is made that the free ligand concentration is the same in both compartments.

Stability of [^{3}H] fentanyl

The integrity of the labelled fentanyl was monitored throughout by recognition of the intact fentanyl molecule by specific anti-fentanyl antibody. Over 95% of binding in excess antibody denoted unchanged fentanyl. Recognition of the products of hydrolysis was very low, in agreement with the results of Michiels et al (1977).

Statistical analysis

Unless otherwise stated, all results were obtained at 37 °C, pH 7·4, in triplicate and refer to whole pooled plasma. Forward stepwise multiple regression analysis (Midas package, Statistical Research Lab., University of Michigan) was used to examine the correlation of binding with the clinical variables measured. Analysis of variance was used to compare the effect of 28 drugs on fentanyl binding. Linear regression analysis was used to test the relation of concentration of aspirin, phenylbutazone and quinidine to percent of fentanyl bound. Student's *t*-test was used for comparison of other results. The values of variance were compared by the F ratio test to ensure validity of the *t*-test.

RESULTS

Interindividual variation of binding between 15 healthy adults was very low, 79·16% ± 0·16 s.e. (n = 45). Multiple regression analysis gave a significant correlation of oestrogen and progestagen therapy ($r = 0{\cdot}776$, $P = 0{\cdot}0014$) and serum albumin concentration ($r = 0{\cdot}745$, $P = 0{\cdot}0022$) with percent bound. There was no significant correlation of binding with fasting serum triglyceride, cholesterol, age, sex or total protein minus albumin.

Binding of fentanyl to whole plasma and albumin

increased with pH (Fig. 1), and therefore with the number of unionized molecules of drug. Purified albumin (46 g litre^{-1}) bound 45·52% ± 0·40 s.e. of fentanyl at pH 7·4. The three lipoprotein fractions bound fentanyl, VLDL 18·39% ± 0·65 s.e.; LDL

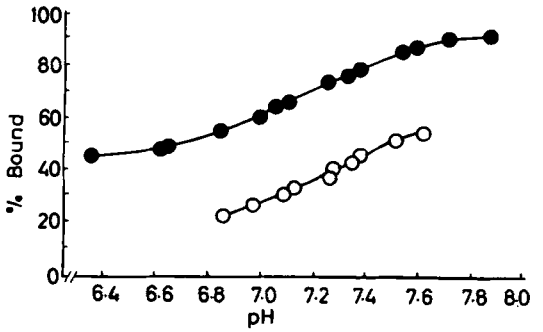

FIG. 1. The effect of pH on binding of [^{3}H]fentanyl to pooled whole plasma ● and human serum albumin, 46 g litre^{-1} ○.

Table 1. The percent binding (mean ± s.e.m.) of fentanyl to lipoprotein fractions of plasma.

Subject		VLDL	LDL	HDL
2		16·01±0·58	38·42±0·75	21·84±0·78
3		15·25±0·47	33·78±0·29	18·27±0·60
5		18·21±0·58	41·25±0·46	16·21±0·33
7		19·36±0·29	42·93±0·41	25·71±0·45
8		13·42±0·55	35·72±0·39	12·17±0·63
Mean		18·39±0·65	37·14±0·42	21·18±0·51
Pooled concentration (mg%)		124·1	398·6	416·3
*Normal values (range) (mg%)	M	159±116	369±81	275±79
	F	56±60	303±36	436±140

* From Hatch & Lees (1968).

37·14% ± 0·42; HDL 21·18% ± 0·51, and recovery of lipoproteins was compatible with physiological concentrations (Table 1). Significant binding to fibrinogen was excluded as there was no significant difference between percent binding to plasma and serum samples from the same individual when tested in two normal subjects. Similarly α_1-acid glycoproteins do not contribute to plasma protein binding of fentanyl as addition of TBEP (100 μg ml^{-1} plasma) had no effect on binding to whole plasma.

There was no change in percent binding of fentanyl to whole plasma, albumin, VLDL or LDL with increase of drug concentration up to 10 mg ml^{-1}. Binding increased with temperature between 4°–30 °C (Table 2). Most of the increase occurred between 25°–30 °C. The value was constant between 30°–40 °C.

Table 2. The effect of temperature on percent binding (mean ± s.e.m.) of fentanyl to whole plasma.

°C	n	Incubation time (h)	B%
4	3	78	60·12 ± 0·38
10	3	50	61·53 ± 0·46
15	3	29	63·47 ± 0·53
20	3	27	65·51 ± 0·58
25	3	24	66·63 ± 0·42
27	3	16	74·23 ± 0·56
30	3	16	79·24 ± 0·61
37	45	16	79·16 ± 0·16
40	3	16	79·08 ± 0·21

The percent binding of fentanyl to whole plasma was inversely proportional to plasma concentration at both constant ligand concentration and constant ligand to protein ratio (Fig. 2). There was a linear

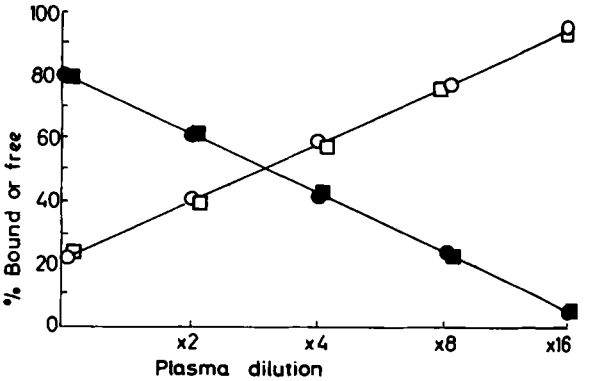

FIG. 2. The relation of percent bound and free fentanyl to plasma concentration at constant ligand concentration (bound ● free ○) and at constant ligand to protein ratio (bound ■ free □).

relation of total, bound and free fentanyl concentration to log plasma dilution at constant ligand concentration (Fig. 3). Total concentration fell slightly

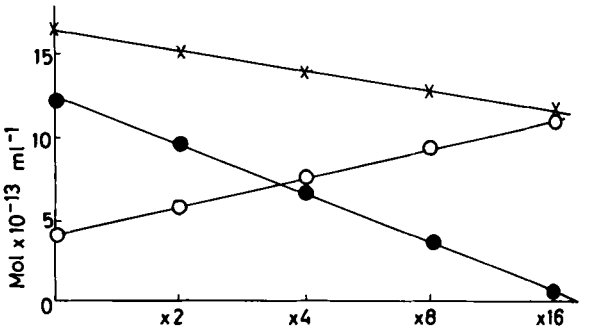

FIG. 3. The relation of total ×, bound ● and free ○ fentanyl concentration in the plasma compartment to plasma dilution at constant ligand concentration.

with plasma dilution as the concentrations refer to ligand in the plasma compartment only. Lower binding in the plasma compartment was associated with an increase in the free fraction in buffer, while total drug present in each block remained constant. At constant ligand to protein ratio only the concentration of free fentanyl showed a linear relation to log plasma dilution (Fig. 4).

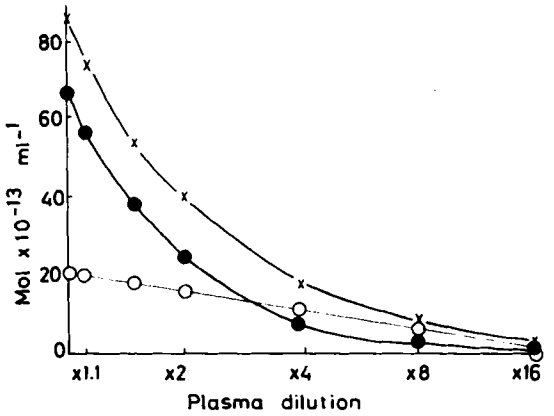

FIG. 4. The relation of total ×, bound ● and free ○ fentanyl concentration in the plasma compartment to plasma dilution at constant ligand to protein ratio.

Binding was not affected by the ionic strength of the buffer up to 0·14 M (Table 3), but was increased when 0·5 M buffer was used. There was no significant difference in binding results when an ultrafiltrate of whole pooled plasma was substituted for the buffer.

Table 3. The effect of the pH and molarity of phosphate buffer on percent binding (mean ± s.e.m.) of fentanyl to whole plasma.

Molarity	B% pH 6·6	pH 7·0	pH 7·4
0·05	†48·21±0·51	†68·22±0·91	†80·36±0·72
0·1	47·87±0·63	66·37±0·72	79·98±0·47
0·14	†48·38±0·29	†67·42±0·61	†79·80±0·86
0·5	*55·21±0·63	*73·34±0·54	*87·02±0·75

† The difference is not significant at the 5% level with respect to the results for 0·1 M buffer.
* The difference is significant ($P < 0·01$) with respect to the results for 0·1 M buffer.

The following drugs of varied chemistry did not inhibit binding of fentanyl (0·6 and 40 ng ml^{-1}) to whole plasma. The drugs were tested at a concentration of 20 μg ml^{-1} unless otherwise stated. Uncharged cyclic compounds: chloramphenicol, digoxin, hydrocortisone prednisolone. Anionic cyclic compounds: heparin (200 units ml^{-1}), penicillin G (300 μg ml^{-1}), phenytoin (80 μg ml^{-1}), sulphadiazine, thiopentone (80 μg ml^{-1}). Cationic drugs, acyclic compounds: pancuronium, suxamethonium, cyclic compounds: atropine, chlorpromazine, diazepam, droperidol, isoniazid, isoprenaline, lignocaine, morphine, naloxone, neostigmine, papaveretum, pethidine, physostigmine scopolamine, and the three possible metabolites of fentanyl. Analysis of variance showed no significant difference between the percent of fentanyl bound in the presence of these drugs, and where fentanyl alone was dialysed against the same pooled plasma.

Inhibition of fentanyl binding to whole plasma was caused by four drugs of higher therapeutic concentration, aspirin, sodium salicylate, phenylbutazone and quinidine. A significant correlation of percent fentanyl bound with concentration of these drugs was found. For aspirin $r = 0·873$, $P < 0·01$; for phenylbutazone $r = 0·810$, $P < 0·05$; and for quinidine $r = 0·982$, $P < 0·01$, (Table 4). The results obtained in the presence of sodium salicylate were not significantly different from those of an equimolar solution of aspirin.

Table 4. The effect of aspirin, phenylbutazone and quinidine on the percent binding (mean ± s.e.m.) of fentanyl to whole plasma at fentanyl concentrations of 0·6 and 40 ng ml^{-1}.

	Conc. (μg ml^{-1})	B% 0·6 ng ml^{-1}	40 ng ml^{-1}
Aspirin	20	79·26±0·18	79·02±0·43
	60	76·35±0·37	
	125	75·02±0·26	
	250	74·14±0·62	
	500	73·21±0·40	
	1000	71·19±0·53	
	2000 } 1·1 × 10^{-2} M	69·45±0·28	69·37±0·52
Na salicylate	1776 }	68·78±0·42†	69·14±0·43†
	Aspirin $r = 0·873$ $P < 0·01$		
Phenylbutazone	20	79·73±0·50	7·943±0·37
	60	76·83±0·35	
	125	75·02±0·41	
	250	72·87±0·68	
	500	72·53±0·52	
	1000	71·12±0·48	71·72±0·41
	Phenylbutazone $r = 0·810$ $P < 0·05$		
Quinidine	20	79·91±0·61	79·62±0·38
	60	79·42±0·24	
	125	78·91±0·36	
	250	76·28±0·33	
	500	73·47±0·48	
	1000	69·98±0·43	69·43±0·71
	Quinidine $r = 0·982$ $P < 0·001$		

† The difference is not significant at the 5% level.

The inhibitory action of phenylbutazone and aspirin was exerted on the binding of fentanyl to

albumin, while quinidine reduced fentanyl binding to all three lipoprotein fractions (Table 5).

Table 5. The effect of aspirin, phenylbutazone and quinidine on percent binding (mean ± s.e.m.) of fentanyl to albumin and lipoprotein fractions.

	B%			
	Fentanyl alone 0·6 ng ml^{-1}	Aspirin 2 mg ml^{-1}	Phenyl-butazone 1 mg ml^{-1}	Quinidine 0·5 mg ml^{-1}
Albumin 46 g litre^{-1}	45·52 ±0·40	41·27* ±0·29	38·64** ±0·58	45·63† ±0·23
VLDL	18·43 ±0·52	19·19† ±0·28	19·03† ±0·23	10·86** ±0·57
LDL	38·11 ±0·46	38·56† ±0·40	37·89† ±0·29	30·49** ±0·20
HDL	21·24 ±0·36	22·18† ±0·61	22·16† ±0·52	14·35** ±0·33

† The difference is not significant with respect to the binding of fentanyl alone.
* $P < 0·01$ ** $P < 0·001$ with respect to the binding of fentanyl alone.

Concentrations of sodium, potassium, calcium, chloride, bicarbonate and phosphate did not affect fentanyl binding when varied over a pathological range from 0·5 to 3 times the normal values in plasma.

In the concentration range usually found in renal failure, creatinine (up to 1200 μM) and urea (up to 1 M) had no significant effect on fentanyl binding. Percent bound was significantly lower than control in the presence of urea at concentrations of 2 M (76·81 ± 0·49 s.e. $P < 0·05$), 4 M (68·72 ± 0·56, $P < 0·01$) and 8 M (60·58 ± 0·41 $P < 0·01$).

Assay of FFA in the pooled plasma gave a concentration of 0·41 mM. After the defatting procedure no FFA was detectable but binding of fentanyl was unaffected, as there was no significant difference in percent bound before and after removal of FFA. Stearic, oleic and palmitic acids added singly to give a concentration of 4 mM in previously defatted plasma, had no effect on the binding of fentanyl.

DISCUSSION

Fentanyl is a highly lipophilic weak base of pK_a given variously as 7·8 (Hollt & Teschemacher 1975) or 8·43 (I.R.E. Brussels). The increase in binding with pH (Fig. 1) is therefore compatible with a contribution to the binding forces by hydrophobic bonds between unionized ligand and plasma protein. This is supported by the increased binding with increased ionic strength of the buffer to 0·5 M (Table 3) which is a feature of hydrophobic bonds (Kauzmann 1959). Cationic fentanyl is also bound, as percent bound at lower values of pH (Fig. 1) exceeds the percent unionized for both estimates of pK_a. The results are compatible with reduced affinity of the ionised drug.

The increase of binding to whole plasma with temperature also suggests involvement of hydrophobic bonds associated with an increase of the association constant with temperature (Kauzmann 1959). The particular increase between 25°–30 °C may be due to conformational changes in the proteins to give an increase in the number of binding sites. The value of binding at 25 °C agrees with that of Schaer & Jenny (1970). The effect of ionic strength, pH and temperature give no evidence of an electrostatic interaction between cationic fentanyl and protein carboxyl groups, or of hydrogen bond formation. The data therefore suggest that hydrophobic interaction may be an important factor in the binding forces.

Hydrophobic attraction of lipophilic drugs for plasma proteins is common, and binding of drug series has been shown to increase with length of hydrocarbon chain e.g. barbiturates (Goldbaum & Smith 1954) or with n-octanol–water partition coefficient e.g. penicillins (Bird & Marshall 1967).

Lipophilic bases commonly bind to lipoproteins in addition to albumin, e.g., chlorpromazine and imipramine (Bickel 1975), quinidine (Nilsen & Jacobsen 1975). Also correlation of binding with α_1-acid glycoprotein concentration has been shown for several drugs of this group, e.g. alprenolol and imipramine (Piafsky & Borga 1977) and lignocaine (Routledge et al 1980). Binding to the plasma protein fractions which show marked variation between individuals may therefore explain the scatter of percent binding of these drugs to whole plasma in normal subjects.

The highly consistent binding of fentanyl to plasma of 15 normal volunteers may suggest a predominant role for the more constant albumin fraction. The variable α_1-acid glycoprotein fraction does not appear to be involved in the binding of fentanyl. This view is supported by the correlation of percent binding with serum albumin concentration ($P = 0·0022$), and the lack of correlation with serum cholesterol and triglyceride. Binding is also correlated to oral oestrogen and progestagen therapy ($P = 0·0014$). Oestrogens are known to stimulate production of α_2 macroglobulins, transferrin, corticosteroid-binding globulin and thyroid-binding globulin (Lipsett 1971; Horne et al 1971). One or more of these fractions may contribute to the binding of fentanyl, though greater numbers of subjects should be investigated for confidence in a positive correlation.

Hollt & Teschemacher (1975) reported 69·5% binding of 10^{-7} M (52·8 ng ml^{-1}) fentanyl to whole plasma, and 65·8% to human serum albumin,

48 g litre^{-1}, at 37 °C and pH 7·4. The reason for the difference from the present results is not clear, and a similar equilibrium dialysis technique was used. These authors did not investigate binding to other protein fractions or the extent of individual variation.

The percent binding to whole plasma, albumin, VLDL and LDL was independent of total fentanyl concentration up to 10 mg ml^{-1}. This corresponds to a bound fentanyl concentration of approximately 10^{-4} M in each case. The relation of total plasma concentration of drug to the free, pharmacologically active fraction is therefore constant for all doses of fentanyl. The limit of solubility of fentanyl in buffer does not permit the study of molar concentrations of fentanyl bound to albumin in excess of the concentration of albumin ($6{\cdot}5 \times 10^{-4}$ M). No evidence of saturation occurs up to this concentration. However the concentration of fentanyl bound to VLDL and LDL greatly exceeds the concentration of lipoprotein complexes, and suggests the presence of multiple binding sites for fentanyl on each complex.

By the law of mass action, simple dilution of plasma with buffer gives reduced binding of ligand. Graphs of the linear relation of percent bound and free to log plasma dilution at constant ligand concentration, and constant ligand to protein ratio (Fig. 2) are superimposed since binding is independent of ligand concentration. Such independence clearly applies over the range of plasma concentrations tested. A comparison of the effect on fentanyl binding of plasma dilution with the hypoalbuminaemia of chronic renal failure has been made (in preparation).

The relevance of the results obtained to physiological ionic media was confirmed by the agreement of binding results where plasma ultrafiltrate was substituted for the buffer.

Since percent binding is not inhibited by competition of further molecules of fentanyl for the available binding sites, then competition by other drugs may be unlikely. Thus a wide range of drugs, and the three possible metabolites of fentanyl failed to inhibit fentanyl binding when tested at likely clinical concentrations.

At concentrations in excess of 60 μg ml^{-1}, phenytoin, thiopentone and penicillin G did not inhibit binding. At similar concentrations, inhibition was caused by aspirin, sodium salicylate, phenylbutazone and quinidine (Table 4). The linear relation between concentration of these drugs and percent binding of fentanyl is in accordance with the predicted relation given by the law of mass action to describe competitive inhibition.

Quinidine binds to lipoproteins and albumin (Nilsen & Jacobsen 1975) and α_1-acid glycoprotein (Fremstad et al 1976). Inhibition of fentanyl binding by quinidine acts at the site of binding to all three lipoproteins, but not to albumin (Table 5). The two alkaloids may therefore compete for identical or closely related binding sites on the lipoprotein complexes. Sites of quinidine binding have been found on LDL and HDL with association constants of the order of 10^4 (Nilsen 1976). Displacement of fentanyl by quinidine may reflect a lower affinity of fentanyl for lipoprotein. There may be distinct binding sites on albumin for fentanyl and quinidine as there is no evidence of competition (Table 5).

Aspirin, sodium salicylate and phenylbutazone are acidic drugs of high affinity for a common binding site on albumin (Anton 1960). Their inhibitory effect on fentanyl binding to whole plasma is exerted at the binding site on albumin (Table 5). A similar example of displacement of a base from albumin by an acidic drug is the inhibition of binding of imipramine and chlorpromazine by phenytoin (Borga et al 1969). However, coincidence of the acidic and basic drug binding site may not be exact, and inhibition may be due to overlap of sites or to an allosteric effect exerted on albumin by the acidic ligand.

The irreversible acetylation of human serum albumin by aspirin is known to cause decreased affinity of albumin for ligands such as flufenamic acid, and increased affinity of others, e.g. phenylbutazone (Chignell & Starkweather 1971). The similar inhibition of fentanyl binding to whole plasma by equimolar concentrations of aspirin and sodium salicylate (Table 4), suggests binding of the salicylate structure, and not acetylation of albumin, as the cause of inhibition.

The three-dimensional structure of protein is affected by high concentrations of urea (Boyer 1945). The binding of fentanyl to whole plasma fell by only 25% of the normal value in 8 M urea. This suggests that the native structure of plasma proteins is not critical for the binding of this drug. At the concentrations found in renal failure, urea and creatinine do not affect fentanyl binding.

Variation in the concentrations of the common ions of plasma (except H^+) has no effect on binding. The pharmacological effect of fentanyl is therefore not potentiated by inhibition of binding to plasma proteins in clinical conditions of electrolyte disturbance associated with these ions.

Although many drugs are not affected by changes in FFA concentration within the physiological range (Rudman et al 1971), inhibition of ligand binding to albumin by FFA within this range has been demon-

strated for thyroxine (Braverman et al 1969) and L-tryptophan (Lipsett et al 1973). Such ligands may be sensitive to the allosteric effect of FFA exerted on albumin (Soltys & Hsia 1977), rather than competition for a shared binding site since physiological concentrations of FFA are thought to be limited to the high affinity first class binding site for FFA on albumin, which is not shared by drugs (Goodman 1958). The binding of fentanyl to whole plasma is not affected even at FFA concentrations of 4 mM. This supports the hypothesis that the nature of the binding sites on albumin for fentanyl and the three competing anionic drugs (Table 5) are not identical, as these drugs are displaced by FFA at such concentrations (Rudman et al 1971).

The defatting procedure was effective in the removal of FFA from albumin. This process also removes many endogenous, and potentially competitive, ligands from normal plasma, and may therefore cause increased binding of the test ligand, e.g. chloramphenicol (Vodrazka et al 1978). The binding of fentanyl does not appear to be sensitive to the presence of such molecules as percent binding was unaffected by charcoal treatment of plasma.

In conclusion, fentanyl is free from the potential clinical consequences of a change in the free, active fraction of the drug in plasma, due to variation in binding between individuals, at different doses of fentanyl or in the presence of other drugs or endogenous ligands. Slight displacement is found only with a few drugs of higher dosage and is negligible at likely therapeutic plasma concentrations. In particular, none of the drugs in common use in anaesthesia interferes with binding when given singly. However care should always be exercised in polypharmacy, as drugs which cause little displacement when given alone may have a significant effect in combination (Anton 1973). The change in binding with pH is also slight over the clinical range of the pH of plasma.

Acknowledgement

I am grateful to Janssen Pharmaceutica for the gift of crystalline fentanyl citrate and the possible metabolites of fentanyl, and to Mr P. Sterling, Mr W. Hudson and Mr N. Burn for their technical assistance.

REFERENCES

Anton, A. H. (1960) J. Pharmacol. Exp. Ther. 129: 282–290

Anton, A. H. (1973) Ann. N.Y. Acad. Sci. 226: 273–292

Bickel, M. H. (1975) J. Pharm. Pharmacol. 27: 733–738

Bird, A. E., Marshall, A. C. (1967) Biochem. Pharmacol. 16: 2275–2290

Borga, O., Azarnoff, D. L., Forshell, G. P., Sjoqvist, F. (1969) Ibid. 18: 2135–2143

Borga, O., Piafsky, K. M., Nilsen, O. G. (1977) Clin. Pharmacol. Ther. 22: 539–544

Bower, S., Holland, D. E., Hull, C. J. (1976) B. J. Anaesth. 48: 1121

Boyer, P. D. (1945) J. Biol. Chem. 158: 715–716

Braverman, L. E., Arkey, R. A., Foster, A. E., Ingbar, S. H. (1969) J. Clin. Invest. 48: 878–884

Chen, R. F. (1967) J. Biol. Chem. 242: 173–181

Chignell, C. F., Starkweather, D. K. (1971) Mol. Pharmacol. 7: 229–237

Fremstad, D., Bergerud, K., Haffner, J. F. W., Lunde, P. K. M. (1976) Eur. J. Clin. Pharmacol. 10: 441–444

Goldbaum, L. R., Smith, P. K. (1954) J. Pharmacol. Exp. Ther. 111: 197–209

Goodman, D. S. (1958) J. Am. Chem. Soc. 80: 3892–3898

Hatch, F. T., Lees, R. S. (1968) Adv. Lipid Res. 6: 2–63

Havel, R. J., Eder, H. A., Bragdon, J. H. (1955) J. Clin. Invest. 34: 1345–1353

Ho, R. J., Meng, H. C. (1969) Anal. Biochem. 31: 426–436

Hollt, V., Teschemacher, H. (1975) Naunyn-Schmiedeberg's Arch. Pharmacol. 288: 163–177

Horne, C. H. W., Mallinson, A. C., Ferguson, J., Goudie, R. B. (1971) J. Clin. Pathol. 24: 464–466

Kauzmann, W. (1959) Adv. Prot. Chem. 14: 1–63

Lewis, B. (1976) in: Lewis, B. (ed.) The Hyperlipoproteinaemias, Ch. 3, pp 34–40. Blackwell, Oxford

Lipsett, M. B. (1971) Ann. Int. Med. 74: 251–263

Lipsett, D., Madras, B. K., Wurtman, R. J., Munro, H. M. (1973) Life Sci. Part 2: 12: 57–64

Lowry, C. H., Roseborough, N. J., Farr, A. L., Randall, R. J. (1951) J. Biol. Chem. 193: 265–275

Maruyama, Y., Hosoya, E. (1969) Keio J. Med. 18: 59–70

Michiels, M., Hendriks, R., Heykants, J. (1977) Eur. J. Clin. Pharmacol. 12: 153–158

Nilsen, O. G. (1976) Biochem. Pharmacol. 25: 1007–1012

Nilsen, O. G., Jacobsen, S. (1975) Biochem. Pharmacol. 24: 995–998

Piafsky, K. M., Borga, O. (1977) Clin. Pharmacol. Ther. 22: 545–549

Routledge, P. A., Barchowsky, A., Bjornsson, T. D., Kitchell, B. B. (1980) Clin. Pharmacol. Ther. 27: 347–351

Rudman, D., Bixler, T. J., Del Rio A. E. (1971) J. Pharmacol. Exp. Ther. 176: 261–272

Schaer, H., Jenny, E. (1970) Plasmakonzentration und Plasmaweissbindung von Droperidol und Fentanyl wahrend der Neuroleptanasthesie beim Menschen. In: Henschel, W. F. (ed.) Neue Klinische Aspekte der Neuroleptanalgesie. Schattaner-Verlag, Stuttgart

Soltys, B. J., Hsia, J. C. (1977) J. Biol. Chem. 252: 4043–4048

van Wijngaarden, I., Soudijn, W. (1968) Life Sci. Part 2, 7: 1239–1244

Vodrazka, Z., Jandova, D., Grafnetterova, J., Schuck, O., Kalousek, I., Tomasek, R., Lachmanova, J. (1978) Biochem. Pharmacol. 27: 1717–1720

Wood, C. (1978) Ed. 'Stress-Free Anaesthesia' Roy. Soc. Med. Int. Congr. Symp. Series No. 3, Ch. 1