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The uptake of fentanyl by erythrocytes

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Fentanyl passed rapidly into and out of erythrocytes to equilibrate with plasma concentration, and a red cell/plasma partition coefficient of 1·01 ± 0·0083 s.e.m. was found in 15 normal subjects. Most of the binding of fentanyl by red cells was by haemoglobin. 10% was bound by the cell membrane. Partition was unaffected by haematocrit, pH, or the concentration of fentanyl up to 0·5 mg ml^{-1} of blood. Dilution of plasma proteins, and replacement of plasma by buffer showed that uptake of fentanyl by red cells is a linear function of the concentration of free drug in plasma. A partition coefficient for red cells/buffer of 4·91 ± 0·032 s.e.m. was found. This relation was confirmed where binding to plasma proteins was altered in uraemia or hyperlipoproteinaemia, or by competitive displacement of fentanyl by aspirin and phenylbutazone thereby changing the size of the free fraction of fentanyl in plasma. Quinidine, however, inhibited the binding of fentanyl to plasma proteins and red cells equally, to maintain a partition coefficient of unity.

The importance of plasma protein binding for the interpretation of plasma concentrations of drugs, and for pharmacokinetic data, is now appreciated. Such considerations also apply to the uptake of drugs by red cells. The relation of plasma concentration to blood concentration is significant in kinetic studies, particularly where drug clearance may be governed by restrictive binding to plasma proteins and erythrocytes. Factors which influence the extent of such binding may therefore have pharmacokinetic consequences.

The properties of uptake of the opioid fentanyl by erythrocytes was therefore examined in health, in uraemia and hyperlipoproteinaemia, and in the presence of drugs known to displace fentanyl from binding sites on plasma proteins (Bower 1981).

MATERIALS AND METHODS

Fentanyl

Tritiated fentanyl (8 Ci mM^{-1}) was obtained from I.R.E. Brussels. [^{3}H]Fentanyl (0·6 ng ml^{-1}) of blood was used routinely to reflect the clinical concentration range found in the post-distributive phase after injection of 100 μg of the drug (Bower et al 1976). The concentration was increased to 0·5 mg ml^{-1} to test the effect of drug concentration on uptake into red cells. The stability of [^{3}H]fentanyl was monitored throughout by specific anti-fentanyl antibody (Bower 1981).

Partition coefficient of fentanyl between red cells and plasma

Fresh heparinized blood was obtained from 15 healthy volunteers. The percent binding of fentanyl to whole plasma of these samples, and the effect of dilution of plasma on binding has been reported by Bower (1981). Concentrations of total plasma proteins, serum albumin, fasting cholesterol and triglyceride, haemoglobin and the haematocrit were all within normal limits.

50 μl of [^{3}H]fentanyl was added to 1 ml aliquots of freshly drawn whole blood in plastic Eppendorf tubes, mixed thoroughly and incubated at 37 °C in a waterbath for 10 min. The samples were then centrifuged (Jobling microcentrifuge) for 2 min at 9000 *g*. 50 μl of plasma was then removed, and the activity of [^{3}H]fentanyl counted in 14 ml of scintillation cocktail. A Searle Tricarb liquid scintillation counter was used, and an external standard method of quench correction applied.

The time required for fentanyl to reach equilibrium out of red cells was also established by removal of 0·5 ml plasma after incubation and centrifugation as above and replacement of 0·5 ml of control plasma from the same subject. The blood samples were then mixed thoroughly, and reincubated for 2 and 10 min to compare the time needed for equilibration from red cells to plasma.

The partition coefficient, λ, of [^{3}H]fentanyl between red cells and plasma was calculated from the formula

$$\lambda = \frac{\text{(Counts added/unit vol blood)—[Counts recovered in equal vol plasma} \times (1\text{–H})]}{\text{(Counts recovered in equal vol plasma} \times \text{H}}$$

where H is the haematocrit.

The effect of haematocrit

To study the effect of haematocrit on λ, whole blood

was diluted with the subject's own plasma. A range of haematocrit of 0·0459–0·459 was examined.

The effect of pH

The effect of pH on the value of λ was studied over the range 5·04–7·56 by addition of trace amounts of 1 M citric acid to whole blood. pH was then measured in separated plasma with a Radiometer 27 pH meter.

The effect of plasma protein concentration

The red cell fraction was separated and the buffy coat removed. The cells were washed four times by resuspension in 0·9% w/v NaCl and repeated separation to remove residual plasma protein. The red cells were then resuspended to a haematocrit of 0·5 in the subject's own plasma, which had been diluted up to tenfold with 0·1 M sodium phosphate buffer, pH 7·4, or replaced entirely by buffer. The value of λ between red cells and different concentrations of plasma, and between red cells and buffer, was estimated as before.

λ by equilibrium dialysis

To confirm the values of λ found by the separation method, the partition coefficient for red cells/plasma and red cells/buffer was also estimated by equilibrium dialysis using the system described by Bower (1981). 0·5 ml of washed red cells were dialysed against 0·5 ml of plasma or buffer of known [^{3}H]fentanyl concentration for 16 h at 37 °C. λ was then calculated as follows:

$$\lambda = \frac{\text{(Counts/unit vol plasma/buffer before dialysis)—(Counts/unit vol after dialysis)}}{\text{(Counts/unit vol plasma/buffer after dialysis)}}$$

The effect of drugs

Aspirin (2 mg ml^{-1} whole blood), phenylbutazone (1 mg ml^{-1}) and quinidine (2 mg ml^{-1}) were added to whole blood to test their effect on the value of λ. Similar concentrations of these drugs have been shown to give a 10% fall of percent binding of fentanyl to whole plasma (Bower 1981).

The value of λ in hyperlipoproteinaemia and uraemia

Fresh heparinized blood was taken from 4 patients with hyperlipoproteinaemia and 9 uraemic patients. The value of λ was found by the separation method. The percent binding of fentanyl to plasma proteins in these samples has been reported (Bower 1982).

Preparation of red cell membranes and haemoglobin solution

To distinguish an affinity of fentanyl for haemoglobin from that for the red cell membrane, washed red cells were lysed by addition of distilled water to restore the original haemoglobin concentration of the whole blood. The lysed blood was then ultracentrifuged (Beckman L2) for 15 min at 150 000 *g*. The haemoglobin solution was removed and the sediment of erythrocyte ghosts was resuspended in distilled water, mixed thoroughly and washed four times by repetition of the centrifugation and resuspension procedure. After the final spin, the ghosts were resuspended in a volume of 0·1 M sodium phosphate buffer, pH 7·4, equal to half the original volume of the blood sample so that their concentration was equal to, or slightly greater than, their concentration in whole blood.

The protein concentration of the ghost suspension was estimated according to Lowry et al (1951) and the total lipoprotein calculated assuming 63% w/w of protein (Saunders 1974).

The suspension of ghosts and the haemoglobin solution were each dialysed for 16 h at 37 °C against 0·1 M sodium phosphate buffer, pH 7·4, of known fentanyl concentration, in the equilibrium dialysis cells. The value of λ was calculated from the fentanyl concentration in buffer before and after dialysis, as above. Percent binding (B%) was calculated from the value of λ.

$$B\% = \frac{\lambda - 1}{\lambda} \times 100$$

Statistical analysis

Students *t*-test was used to test for significant difference between results. Correlation of results with values such as the haematocrit, percent of free drug in plasma, etc, was examined by linear regression analysis. All estimates were in quadruplicate, and the separation method was used, unless otherwise stated.

RESULTS

The equilibration of fentanyl between plasma and red cells, in both directions, was complete within 2 min. No significant difference was found in the counts recovered in separated plasma (and therefore the value of λ) with prolonged incubation times up to 1 h, so 10 min was therefore adopted. As the counts recovered were also not affected by the duration of centrifugation from 1–30 min, or by allowing the plasma to separate by standing for 4 h at 21 °C, the 2 min spin was used routinely.

Partition of fentanyl between plasma and red cells in 15 normal subjects gave the result $\lambda = 1{\cdot}01 \pm 0{\cdot}0083$ s.e.m. ($n = 60$). There was no significant correlation of λ with haematocrit, and therefore with haemoglobin concentration, or with percent of drug free in plasma, over the narrow range of values of λ found in

normal subjects. The count rate recovered per unit volume of plasma was not affected by the proportion of red cells to plasma in the sample, and therefore λ remained unity over a range of haematocrit of 0·0459–0·459. Similarly the recovered activity, and therefore λ, were independent of pH over the range 5·04–7·56. λ was not affected by fentanyl concentrations up to 0·5 mg ml^{-1} in whole blood.

Table 1. The effect of plasma protein concentration on the partition of fentanyl between red cells and plasma or buffer.

Plasma dil.	λ	F%	Tr	Tp	Fp	Tr/Fp
—	1·02	21	0·505	0·495	0·104	4·85
×2	2·11	40	0·679	0·322	0·129	5·26
×5	3·18	64	0·761	0·239	0·153	4·97
×10	4·33	84	0·812	0·188	0·159	5·11
Plasma replaced by buffer						
	4·91	100	0·831	0·169	0·169	4·91
						5·02 ± 0·036 Mean ± s.e.m. (n = 20)

Correlation of Tr with Fp r = 0·987 P < 0·001
Correlation of λ with F% r = 0·998 P < 0·001

* Tr is the mean fraction of total blood fentanyl which is in red cells.
* Tp is the mean fraction of total blood fentanyl which is in plasma.
F% is the mean % of plasma fentanyl in the free state (n = 3) (Bower 1981).
Fp is the mean fraction of total blood fentanyl which is free in plasma, calculated from Tp and F%.
* Calculated from λ.

With increasing dilution of plasma proteins, the concentration of fentanyl in the red cells rose as a linear function of the free concentration in plasma (r = 0·981, $P < 0·001$, Table 1). The value of λ for total drug in red cells/free drug in plasma was 5·02 ± 0·036 s.e.m. (n = 20) (Table 1). The values of λ red cells/plasma and λ red cells/buffer were not significantly different when obtained by the separation method or by equilibrium dialysis.

Complete recovery of [^{3}H] fentanyl added to 1 ml plasma and incubated for 10 min in an Eppendorf tube, or added to buffer and incubated overnight in the equilibrium dialysis blocks served as control and showed no adsorption of drug to the apparatus.

In the presence of the acidic drugs aspirin and phenylbutazone, which displaced fentanyl from binding sites on plasma proteins (Bower 1981), the value of λ for fentanyl in red cells/plasma (Table 2) was compatible with the relation of λ to the free concentration of drug in plasma found by dilution of plasma proteins (Table 1). Addition of the base quinidine had no effect on λ for whole blood, but decreased its value between red cells and buffer (Table 2). The two acidic drugs did not affect uptake of fentanyl from buffer, since λ red cells/buffer was not significantly different from normal in the presence of these drugs (Table 2).

The value of λ in patients with hyperlipoproteinaemia and uraemia also showed significant correlation ($P < 0·001$) with the percent of free drug in plasma (Table 3). There was no significant correlation of λ with haematocrit or haemoglobin concentration in these patients.

Table 2. The effect of aspirin, phenylbutazone and quinidine on partition of fentanyl between red cells and plasma, and red cells and buffer.

Drug(s)	†† F% (mean ± s.e.m.)	λ red cells/ plasma (mean ± s.e.m.)	λ red cells/ buffer (mean ± s.e.m.)
[^{3}H]Fentanyl 0·6 ng ml^{-1}	20·84 ± 0·16	1·02 ± 0·023	4·79 ± 0·034
+ aspirin 2 mg ml^{-1}	30·55 ± 0·28	1·42 ± 0·016**	4·94 ± 0·042†
+ phenylbutazone 1 mg ml^{-1}	28·88 ± 0·48	1·35 ± 0·021*	4·54 ± 0·028†
+ quinidine 2 mg ml^{-1}	30·02 ± 0·43	1·00 ± 0·024†	3·30 ± 0·024**

†† F% is the percent of plasma fentanyl which is in the free state, (n = 3) (Bower 1981).
† The difference is not significant; * the difference is significant at the 1% level; ** the difference is significant at the 0·1% level with respect to the corresponding value for fentanyl alone.

Table 3. The partition of fentanyl between red cells and plasma in hyperlipoproteinaemia and uraemia.

	Haemoglobin g%	Haematocrit	F%* (mean±s.e.m)	λ (mean±s.e.m)
Hyperlipoproteinaemic patients				
L2	15·1	0·439	15·60 ± 0·40	0·95 ± 0·021
L3	15·3	0·445	14·67 ± 0·26	0·92 ± 0·030
L5	14·1	0·398	16·40 ± 0·32	0·98 ± 0·019
Uraemic patients				
R7	5·9	0·184	19·97 ± 0·29	0·98 ± 0·025
R8	6·7	0·203	18·33 ± 0·19	0·95 ± 0·014
R9	6·5	0·193	27·33 ± 0·88	1·16 ± 0·018
R10	5·3	0·170	21·34 ± 0·32	1·01 ± 0·026
R11	5·6	0·198	22·30 ± 0·57	0·99 ± 0·023
R12	6·2	0·186	28·07 ± 0·33	1·24 ± 0·017
R13	6·6	0·200	26·80 ± 0·42	1·18 ± 0·025
R14	6·4	0·191	24·62 ± 0·53	0·99 ± 0·032
R15	5·7	0·188	28·62 ± 0·34	1·11 ± 0·028

* F% is the percent of plasma fentanyl which is in the free state (n = 3) (Bower 1982).
Correlation of λ with F% r = 0·868 $P < 0·001$.
Correlation of λ with haemoglobin or haematocrit is not significant.

Binding of fentanyl to red cell membranes was 11·73 ± 0·29% s.e.m. and 8·21 ± 0·34% s.e.m. in two normal subjects in whom the protein contents of the membrane preparation were respectively 4·32 and 3·37 mg ml^{-1} and the concentration of lipoprotein 6·86 and 5·35 mg ml^{-1}; the pH was 7·4. Fentanyl was 56·68 ± 0·78% s.e.m. bound to a solution of 15 g% haemoglobin (Table 4). The value of percent binding to haemoglobin fell linearly with $\log_{10}$ dilution factor of the haemoglobin solution (r = 0·993, $P < 0·001$).

Table 4. The binding of fentanyl (mean ± s.e.m.) to a solution of 15 g% haemoglobin in distilled water, and to diluted preparations of this solution, by equilibrium dialysis.

Dilution of Hb solution	Log_{10} dilution factor	B%
—(×1)	0·00000	56·68 ± 0·78
×2	0·30103	34·20 ± 1·15
×4	0·60205	19·11 ± 1·32
×8	0·90309	5·02 ± 1·18
		pH 7·4

Correlation of B% with log_{10} dilution factor r = 0·993 $P < 0·001$

DISCUSSION

The use of the method of plasma separation to study partition of fentanyl between red cells and plasma or buffer seems free from error introduced by the nature or duration of separation since the results obtained agree with those of equilibrium dialysis, and recovery of added [^{3}H]fentanyl in control experiments is complete. This simple method therefore gives a rapid and reliable estimate of the partition coefficient λ.

The rapid passage of fentanyl into and out of red cells is compatible with its highly lipophilic nature. The rate of entry of a drug into red cells is known to be correlated with its lipid-water partition coefficient (Schanker et al 1961).

Since λ is unity, and independent of haematocrit, normal blood appears to function as a homogeneous medium for the carriage of fentanyl. Changes in the pH of the blood, which affect the fraction of fentanyl bound to plasma proteins (Bower 1981), do not affect the value of λ. It seems, therefore, that changes of pH, which modify the intra- and extracellular environment of the blood, affect binding of the drug in or on the red cell in a manner similar to the way they affect binding of the drug to plasma protein. Therefore, where changes of the bound-to-free ratio of drug within the red cells and in the plasma are similar, and because the free concentrations in the red cells and plasma are equal, the ratio of total drug (λ red cells/plasma) will remain unity.

There is no evidence of saturation of red cell uptake of fentanyl, since the value of λ remains unity up to a drug concentration of 0·5 mg ml^{-1}. Individual variation in 15 healthy adults was low (λ = 1·01 ± 0·0083 s.e.m., n = 60).

When the plasma protein concentration is diluted, the value of λ rises as more fentanyl is carried within the red cells (Table 1) and it reaches a value of 4·9 when the plasma is replaced by buffer at pH 7·4. Dilution of plasma protein shows that total drug concentration in the red cells is a linear function of the free concentration in plasma. A consistent concentration ratio of 5·02 ± 0·036 s.e.m. is maintained (Table 1). This ratio is applicable at all concentrations of fentanyl up to 0·5 mg ml^{-1}, as red cell uptake was shown to be independent of fentanyl concentration within this range. Fentanyl is therefore bound in or on the red cells, and is not just in solution in the aqueous phase of the cells.

In normal plasma, fentanyl is 79·16 ± 0·16% s.e.m. bound to plasma proteins (Bower 1981). If the concentration of free drug in plasma and red cells is assumed to be equal, then the total-to-free (5:1) and bound-to-free (4:1) ratios within the red cells are similar to those of plasma in normal blood, supporting the hypothesis that binding to red cells resembles binding to plasma proteins, and is similarly influenced by factors such as changes in pH. A bound-to-free ratio of 4 will be maintained within the red cells at all concentrations of fentanyl up to 0·5 mg ml^{-1} blood at pH 7·4. The Scatchard plot of red cell binding of fentanyl will therefore be parallel to the abscissa, with an intercept on the ordinate of 4. This value is the product of the association constant K, and q, the number of binding sites. A Scatchard plot parallel to the abscissa, with an intercept of qK = 4 was also found for binding of fentanyl to whole plasma (Bower 1981).

Approximately equal binding to plasma proteins and to red cells, with λ of unity, has also been found for pentazocine (Ehrnebo et al 1974) and for imipramine (Bickel 1975). A linear relation between total ligand concentration in red cells and free concentration in plasma has been shown for phenytoin by Borondy et al (1973) and Kurata & Wilkinson (1974). These authors suggest that partition of drug between plasma and red cells gives a rapid screening procedure to detect altered binding of drug to plasma proteins, e.g. in renal and liver disease, and in displacement by other drugs. Such screening is valuable since phenytoin toxicity is more highly correlated with its free concentration in plasma than its total plasma concentration (Booker & Darcy 1973). The results are satisfactory for phenytoin, where red cell concentration appears to be solely a function of the free concentration in plasma. However, Fremstad (1977) has shown that partition of quinidine in anuric rats is governed by plasma protein binding, and also by altered uptake by the red cells.

For fentanyl, addition of the acidic drugs aspirin and phenylbutazone in concentrations shown to inhibit binding to plasma proteins by about 10%

(Bower 1981) gave a significant ($P < 0.01$) increase in the value of λ (Table 2), which agrees with the relation of λ to the percent of free drug in plasma expressed in Table 1. The increase in λ is therefore entirely due to inhibition of fentanyl binding to plasma proteins, and not to an altered uptake of the opoid by the red cells themselves. This is confirmed by the lack of effect of the anti-inflammatory drugs on the uptake of fentanyl by red cells where plasma is replaced entirely by buffer (Table 2). The highly anionic nature of the drugs at physiological pH will depress their entry into red cells within the time of incubation.

Addition of the base quinidine in a concentration shown to give about 10% inhibition of plasma protein binding of fentanyl (Bower 1981), did not affect the value of λ for red cells/plasma (Table 2). Quinidine enters red cells rapidly, with a value of $\lambda = 0.82$ red cells/plasma and binds to haemoglobin (Hughes et al 1975). The inhibitory effect of quinidine on the binding of fentanyl to plasma proteins therefore seems to be associated with a similar degree of inhibition of binding to the red cells to maintain a uniform distribution of ligand between the two phases. This is confirmed by the inhibitory effect of quinidine on uptake of fentanyl from buffer by the red cell fraction (Table 2).

The values of λ found in patients with chronic renal failure and hyperlipoproteinaemia also showed significant correlation of the value of λ with the percent of free drug in plasma (Table 3). There was no evidence of impaired red cell uptake of fentanyl in these conditions.

The lipoproteins of the red cell membrane might be expected to bind fentanyl in view of the attraction of the drug plasma lipoprotein fractions (Bower 1981). The ghost suspension bound approximately 10% of fentanyl at pH 7·4. The washing procedure may alter the membrane from the in vivo state, to cause reduced binding, but the major contribution to erythrocyte binding was made by haemoglobin (Table 4). Percent binding to haemoglobin showed a linear relation of log dilution factor of the haemoglobin solution. This relation was also found for binding of fentanyl to whole plasma (Bower 1981). Binding to the haemoglobin solution appeared lower than to corresponding dilutions of plasma since 15% haemoglobin in distilled water represents the haemoglobin concentration in whole blood, and is therefore approximately one half the concentration in the red cell fraction. Also the red cell membrane contributes 10% of total erythrocyte binding.

Other basic, lipophilic drugs have been shown to bind to the red cell membrane or contents. Bickel (1975) showed that imipramine and chlorpromazine bind to the membrane. Weak binding of chlorpromazine to the red cell contents was also observed, but the extent was negligible for imipramine. Certain acidic drugs also bind to red cells. For phenytoin, λ red cells/buffer = 4·83 (Kurata & Wilkinson 1974), but partition between red cells and plasma fell to $\lambda = 0.22$ to reflect a greater affinity of phenytoin for binding to plasma proteins than to red cells.

For fentanyl, partition between red cells and plasma is unity, and 80% of fentanyl in plasma is bound to plasma proteins (Bower 1981). Therefore in normal blood 50% of fentanyl is in the red cells, 40% is bound to plasma proteins and 10% is free in plasma water. Only the 10% of drug which is free in plasma is immediately available for equilibration throughout the body to the receptor sites and for elimination. The pharmacokinetics of fentanyl will therefore depend on whether binding to plasma proteins and red cells acts as restrictive binding, or whether distribution of the free fraction from the plasma compartment is followed by very rapid dissociation of binding to restore equilibrium with the free concentration in plasma water. This would effectively release the entire blood pool of drug for equilibration into the tissues, to sites of clinical action and elimination.

The magnitude of the changes in λ red cells/plasma found in hyperlipoproteinaemia and uraemia, and in the presence of other drugs, would not be expected to affect the clinical response to fentanyl in these conditions.

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