

Red Blood Cell Partitioning, Protein Binding and Lipophilicity of Six Phenothiazines

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Abstract—Hexane-water partition coefficients, pK_a values, protein binding and red blood cell partitioning were studied with six phenothiazine drugs. Red cell partitioning was independent of drug concentration, and there was no correlation between partitioning and physicochemical characteristics. Red cell partitioning could be used indirectly to estimate protein binding, but two potential pitfalls of general importance were found. Failure to consider drug binding of glassware and haematocrit changes were shown to induce incorrect estimates of both red cell partitioning and protein binding, as well as hexane-water partition coefficients.

In spite of the complexity of the interaction of drugs and biological control systems, many drug responses correlate with simple physicochemical properties of the drug molecules concerned, such as pK_a and lipophilicity (Albert 1962, 1979; Hansch et al 1987). This is especially true with the older centrally-acting drugs, such as anaesthetics, sedatives and tranquillizers (Green 1967; Murthy & Zograf 1970; Rojratanakiat & Hansch 1990). This applies to both parent drugs and drug metabolites. In regard to phenothiazine drugs, it has been shown that a wide variety of drug responses, including anticholinergic, antidopamine, antihistamine and antiadrenergic properties are correlated with lipophilicity (Gaudette & Brodie 1959; McMahan 1964; McMahan & Easton 1964; Kohl et al 1964; Krieglstein et al 1972; Gabay & Huang 1974; Whelpton 1989; Curry et al 1989).

Among the metabolites of phenothiazine drugs, hydroxy-chlorpromazine has been shown to be the only exception to the rule that metabolism reduces activity by increasing polarity (Curry 1984, 1986). In many systems this compound shows activity higher than would be predicted from its lipophilicity, although still less than that of the parent drug. In the most recent publication in this field, a study of anticholinesterase activity among six metabolites of chlorpromazine, including 7-hydroxychlorpromazine, plus the parent drug, the K_i values were inversely and perfectly correlated with $\log P$ values; a seventh very weak and highly polar metabolite was displaced by one in the rank order correlation (Whelpton 1989).

One crucial, and often neglected, area of drug disposition is red cell penetration and binding (red cell partitioning). Drugs can enter red cells, or adhere to the surfaces of red cells, or do both, with distribution governed by both biological and physical properties, and reach distribution ratios between red cells and plasma water which can markedly affect interpretation of plasma and whole blood concentrations. Since no adequate reports of red cell localization of phenothiazine drugs exist in the literature, we have conducted a study of the distribution of six phenothiazines between red cells, plasma, and buffer solutions. No correla-

tion with lipophilicity was found. In conducting this work, we discovered several potential artefacts, of possible general importance, in some of the standard red cell localization calculations.

Materials and Methods

Materials

The following reference samples of drugs were used: acepromazine maleate (Fort Dodge Laboratories, Fort Dodge, IO, USA), promazine hydrochloride (Wyeth Laboratories, Philadelphia, PA, USA), trifluoperazine dihydrochloride (Smith Kline and French Laboratories, Philadelphia, PA, USA), mesoridazine and thioridazine hydrochlorides (Sandoz Pharmaceuticals, E. Hanover, NJ, USA), and fluphenazine dihydrochloride (The Squibb Institute for Medical Research, Brunswick, NJ, USA). Reagents, all of analytical grade, were acetonitrile, hexane, ammonium acetate, sodium acetate, disodium phosphate, sodium hydrogen phosphate, sodium hydroxide, hydrochloric acid and toluene, all from the Fisher Scientific Company (Pittsburgh, PA, USA). All glassware was silanized with hexamethyldisilazane from SCM Specialty Chemicals (Gainesville, FL, USA).

Apparatus

The HPLC system consisted of a Waters Solvent Delivery System Model 6000, a WISP automatic Injector Model 710 A, a Data Module Model M730 (Waters Associates, Millford, MA, USA), a Fisher Recordal 5000 series recorder (Fisher Scientific Company, Pittsburgh, PA, USA), and an ESA Model 5100A Coulochem Electrochemical Detector (ESA Inc., Bedford, MA, USA, coulometric mode). Also used was a Zorbax CN HPLC column, 13 cm long, (MacMod Analytical Inc., Chadds Ford, PA, USA).

Analysis of phenothiazines in biological fluids

To a standard, or to a sample of 0.5–2 mL biological fluid (plasma or whole blood) 0.1 mL 1 M NaOH was added to make the pH alkaline. This alkaline sample was then extracted with 5 mL hexane for 1 h. After centrifugation, the hexane phase was removed and evaporated to dryness at 25°C under a constant stream of nitrogen. If any emulsion

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persisted after centrifugation, gentle stirring with a glass rod followed by further centrifugation solved the problem. The residue was redissolved in an appropriate volume of mobile phase, usually 250 μL , and an aliquot of this reconstituted residue was injected into the chromatographic system.

The mobile phase consisted of either 90:10 acetonitrile:0.2 M ammonium acetate pH 6.9, or 75:25 acetonitrile:0.1 M acetate buffer pH 4.75. The only exception for these conditions was with mesoridazine where the mobile phase consisted of 75:25 acetonitrile:0.1 M phosphate buffer pH 6. The flow rate was 1.2 mL min^{-1} .

The oxidation potential was set at 0.7 V for the analytical cell and 0.75 V for the guard cell. Table 1 shows sample calibration data for the six compounds in aqueous solutions.

Table 1. Typical equations describing calibration graphs for each phenothiazine assayed by the HPLC system.

Drug	Slope	SES ^a	Intercept	SEI ^b	R ^c
Acepromazine	0.7200	0.0300	0.133	0.046	0.933
Fluphenazine	0.0070	0.0005	0.686	0.680	0.992
Thioridazine	0.0036	0.0002	0.408	0.135	0.989
Trifluoperazine	0.0002	0.0001	0.017	0.005	0.993
Mesoridazine	0.1800	0.0090	0.033	0.047	0.994
Promazine	0.0020	0.0001	0.070	0.120	0.984

^a Standard error of the slope. ^b Standard error of the intercept. ^c Correlation coefficient.

Note: the y-axis in these calibrations was peak height ratio, which was affected by concentrations of both drug and internal standard in any particular application, and by the detector sensitivity to each compound.

Determination of the partition coefficients between hexane and phosphate buffer pH 7.4

Solutions of the different phenothiazines were prepared by adding 0.1 mL of a 100 $\mu\text{g mL}^{-1}$ aqueous stock solution to 10 mL phosphate buffer pH 7.4. The concentration of each solution was measured by HPLC before and after extraction with various volumes of hexane ranging from 0.1 to 10 mL (0.1 mL for thioridazine, trifluoperazine and promazine, 1 mL for acepromazine and fluphenazine and 10 mL for mesoridazine). Samples (0.05–0.5 mL) of the hexane were taken and dried under a constant stream of nitrogen and reconstituted in mobile phase. The concentration of the phenothiazine in the buffer and the mobile phase were determined by HPLC from standard calibration curves in buffer and mobile phase, respectively.

The partition coefficient (D) was calculated in two ways:

$$D = [C_h]/[C_{bu}] \quad (1)$$

where $[C_h]$ is the concentration in hexane and $[C_{bu}]$ is the concentration in buffer solution at equilibrium, and

$$D = (([C_b]_b - [C_b]_a)/([C_b]_a)) \times (V_a/V_o) \quad (2)$$

where $[C_b]_b$ is the buffer concentration before extraction, $[C_b]_a$ is the buffer concentration after extraction, V_a is the volume of the aqueous phase, and V_o is the volume of the organic phase. pK_a values considered were those published in reference literature (Martindale 1989).

Red blood cell/plasma distribution coefficient determination

From fresh blood obtained from a blood bank, packed red

blood cells were obtained by centrifugation. They were washed three times with isotonic saline and finally resuspended in isotonic phosphate buffer pH 7.4. To these samples, or to whole blood, was added different amounts of the phenothiazine of interest so that the final concentration was 300, 500 or 1000 ng mL^{-1} (with the exception of mesoridazine—see Table 3). The haematocrit was measured after equilibration of the samples for 60 min at 37°C. After centrifugation for 20 min at 3000 rev min^{-1} , the haematocrit of the red blood cell phase was measured to determine how much supernatant was left after centrifugation. Additionally, an aliquot of the red blood cell phase was taken and diluted with equal amounts of water in order to lyse the cells so that it became possible to measure their drug content. Both the supernatant and the red blood cell phase were analysed.

Appropriate calibration curves in both supernatant and red blood cells were constructed by adding to blank supernatant and blank red blood cell suspensions different amounts of the corresponding phenothiazine and the internal standard so that the final concentrations would be between 1000 and 25 ng mL^{-1} . Since phenothiazine drugs are notorious for binding to glassware (Curry 1968), red blood cell partitioning was evaluated in 3 different ways:

$$D = [C_{rbc}]/[C_{pw}] \quad (3)$$

$$D = [A_{tot} - V_b(1 - H)]/HV_b.C_{pw} \quad (4)$$

$$D = [C_{rbc} - C_{pw}(1 - H)]/H'.C_{pw} \quad (5)$$

where D is the red blood cell to supernatant partition coefficient, C_{rbc} is the concentration of the drug in the red blood cells, C_{pw} is the concentration of the drug in the plasma or supernatant, A_{tot} is the total amount of the drug added to the sample, and V_b is the volume of the sample. Note that the volume of supernatant can be calculated as $(1 - H) \cdot V_b$ with H being the haematocrit of the sample before adding the drug solution; H' is the haematocrit of the red blood cell phase after separation of the two phases. Haematocrit values were measured using micro-haematocrit capillary tubes (Fisher Scientific Company, Pittsburgh, PA, USA). The three results calculated from the three different methods were compared to determine significant glass binding and the extent to which it affected the results.

Extent of protein binding calculated from the red blood cell partitioning studies

The extent of protein binding was calculated from the

Table 2. Hexane/phosphate buffer (pH 7.4) partition coefficients for six phenothiazines.

Drug	Mean ^a (s.d.)	Partition coefficient ^b
Acepromazine	9.1 (1.1)	8.8 (1.2)
Thioridazine	152.0 (18.0)	113.0 (9.5)
Trifluoperazine	193.0 (32.0)	197.0 (26.0)
Fluphenazine	14.5 (1.7)	12.8 (4.6)
Mesoridazine	0.0 (—)	0.01 (0.001)
Promazine	405.0 (80.0)	85.4 (8.7)

^a From measurement of the concentrations in the two phases (eqn 1). ^b From the formula in the text (eqn 2). Data are means of five determinations.

difference between the red blood cells and between phosphate buffer and red blood cells. The fraction of drug bound to proteins was calculated from:

$$f = 1 - K_d/D \quad (6)$$

where f is the fraction of drug bound to proteins, K_d is the red blood cell partition coefficient between plasma and the red blood cell phase, and D is the red blood cell partition coefficient between the phosphate buffer and the red blood cells. The extent of protein binding was determined at a concentration of 1000 ng mL^{-1} in plasma.

Results and Discussion

Partition coefficients between hexane and phosphate buffer pH 7.4

The apparent partition coefficients between hexane and phosphate buffer pH 7.4 calculated in two ways are shown in Table 2. As expected mesoridazine, being a polar metabolite of thioridazine, had the lowest partitioning in hexane. Indeed, the concentration in the hexane was so low that the use of equation 1 was precluded. The most lipophilic compound was trifluoperazine (using eqn 2) or promazine (using eqn 1). It is notable that the greatest variability in the results was observed with these two relatively lipophilic phenothiazines. This could have been due to the fact that the more lipophilic the phenothiazine, the greater is the glass binding, thus introducing more difficulty and more variability into the assessment techniques. However, it was with these compounds that the 0.1 mL hexane sample was

Table 3. Distribution of six phenothiazines between red blood cells (r.b.c.) and pH 7.4 buffer.

Drug	Concn (ng mL ⁻¹)	Partition ratio between red cells and buffer (mean (s.e.m.))		
		Partition coefficient ^a	Corrected for presence of plasma water in r.b.c. ^b	By calculation ^c
Acepromazine	300	5.57 (0.32)	6.08 (0.32)	9.72 (0.57)
	500	3.83 (0.26)	4.48 (0.30)	5.95 (0.37)
	1000	4.39 (0.25)	5.11 (0.19)	6.44 (0.18)
	WB (1000)	1.26 (0.17)	1.31 (0.20)	— (—)
Fluphenazine	300	3.87 (0.41)	4.55 (0.52)	4.08 (0.49)
	500	3.47 (0.19)	3.99 (0.24)	7.43 (0.34)
	1000	3.17 (1.02)	4.61 (0.81)	9.45 (1.32)
	WB (1000)	1.39 (0.003)	3.83 (0.81)	1.44 (0.005)
Thioridazine	300	4.66 (0.49)	5.61 (0.64)	8.9 (1.44)
	500	5.63 (0.51)	6.83 (0.65)	12.12 (2.80)
	1000	5.77 (0.21)	6.3 (0.23)	11.79 (0.53)
	WB (1000)	0.38 (0.03)	0.28 (0.03)	0.47 (0.11)
Trifluoperazine	300	5.53 (—)	6.82 (—)	7.74 (—)
	500	4.66 (0.41)	5.39 (0.50)	6.24 (0.28)
	1000	7.48 (0.71)	9.78 (0.34)	11.19 (1.53)
	WB (1000)	1.57 (0.06)	1.70 (0.08)	3.77 (0.18)
Promazine	300	5.66 (0.03)	6.38 (0.28)	8.26 (0.17)
	1000	5.95 (0.19)	6.52 (0.19)	7.21 (0.29)
	WB (1000)	1.26 (0.07)	1.41 (0.11)	1.29 (0.10)
Mesoridazine	5000	2.51 (0.10)	2.78 (0.11)	6.15 (0.54)
	10000	2.36 (0.09)	2.62 (0.14)	3.72 (0.16)
	WB (1000)	0.62 (0.03)	0.51 (0.04)	1.26 (0.16)

$n = 4-8$ except trifluoperazine at 300 ng mL^{-1} ($n = 2$). WB is whole blood. ^a Equation 3. ^b Equation 4. ^c Equation 5.

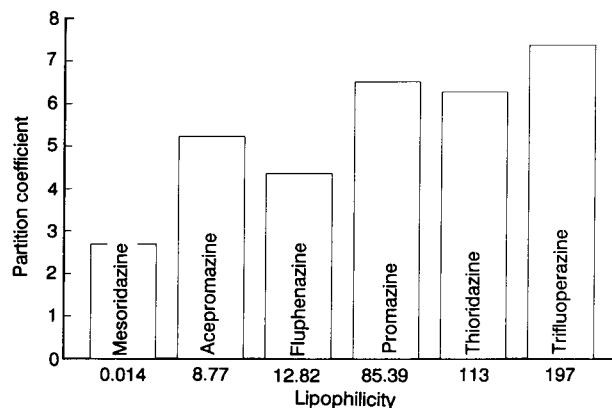


FIG. 1. Plot of the red cell partition coefficient against lipophilicity for the six phenothiazines of interest.

assayed. Scrupulous care was taken to prevent any variations in the volumes of these samples caused by evaporation. Instinctively, we believe that the equation 2 approach is more likely to be affected by binding of the drugs to glass surfaces, so place greater credence in the data using equation 1.

Red blood cell partitioning and estimates of plasma protein binding

The partition coefficients for the various phenothiazines between red blood cells and plasma or buffer are listed in Table 3. Fig. 1 shows the lack of an important relationship between lipophilicity as measured by the partition coefficient between hexane and phosphate buffer using equation 2 and the red blood cell partition coefficient ($r = 0.27$). Data from equation 2 were used for this diagram to avoid the need to use zero for the partition coefficient of mesoridazine. The use of either equation 1 or equation 3 in this context does not affect the conclusion.

It can be seen that the red blood cell partition coefficients for all of the phenothiazines studied (except for mesoridazine) lie within the same range (around 5). The fact that D values exceeded unity implies that these drugs associate with components of erythrocytes over and above that which would result from volume equilibration. It was also observed that these values were independent of concentration. The lack of relationship with lipophilicity is contrary to expectation because it was thought that the more lipophilic the phenothiazine is, the easier it should be for the drug to bind to or partition into red blood cells. The nondependency might be explained by the fact that at pH 7.4 all phenothiazines, being amines, will be largely in their ionized forms. The existence of the positive charge on the amine will impart a certain degree of polarity which will be the same for all members of this class of drugs; this could overrule lipophilicity in partitioning. Thus, because of the positive charge, the various drugs will most probably have the same physical characteristics and thus will all partition in the same way. Another reason for the nondependence of the red blood cell partitioning on lipophilicity might be due to the fact that the drug was not partitioning into the cell itself but binding to either the cell membrane or to some component inside the cell. In this case, lipophilicity would not be a major factor in contributing to the magnitude of the partition coefficient and other factors would play a much more important role.

Table 4. Extent of protein binding for the phenothiazines studied as determined from their red blood cell partitioning.

Drug	Bound (%)	Range ^a
Acepromazine	74.0	70.0-79.0
Fluphenazine	83.0	80.0-86.0
Thioridazine	95.5	94.9-96.6
Trifluoperazine	82.6	80.9-84.0
Promazine	78.3	76.0-80.6

^aThe range was calculated as follows: the lower range was: $1 - (K_d + s.e.) / (D - s.e.)$, the upper range was: $1 - (K_d - s.e.) / (D + s.e.)$.

Note: the concentration for fluphenazine was 333 ng mL⁻¹; mesoridazine is not included in this table because the concentration in whole blood was different from the concentration used in the red blood cell suspension in this example.

From a general, methodological, point of view, it is notable that for drugs that are very lipophilic and that exhibit a great degree of glass binding, the red blood cell partition coefficient cannot be calculated by just measuring the concentration in the buffer phase and calculating the concentration or amount in the red blood cells by difference. This will result in an overestimation of *D* as is seen and confirmed in our studies. This overestimation is due to the fact that the drug that is not found in the supernatant phase is often assumed to be bound to the red cells. However, for drugs that undergo glass binding, there is a three way partitioning between the glass, the red blood cells and the glass walls. From the results presented in Table 3, *D* was overestimated when it was calculated by difference (eqn 5) as compared with the value obtained by the actual measurement of both phases (eqns 3, 4). This overestimation was almost 100% in certain cases such as thioridazine where the partition coefficient was approximately 6 by actual measurement and approximately 12 by difference. On the other hand, *D* can be underestimated if it is assumed that the red blood cell phase is completely made of red blood cells and there was no plasma water present between cells (eqn 3). It was found that the red blood cell phase is not made of 100% red cells but that around 10-20% of the volume was plasma water. This resulted in an underestimation of the concentration in the red blood cell phase because the concentration measured was a combination of the concentration in the blood cells and the concentration in the residual plasma water. This underestimation is found only in the case where the drugs partition highly into or onto the red blood cells such as is seen with the phenothiazines; the actual concentration measured is smaller than the concentration would be if the red blood cell fraction was 100% red cells.

Extent of protein binding

It has been shown that the extent of protein binding of drugs can be estimated indirectly using red cell partitioning (Hinderling et al 1974), although, generally speaking, this technique is only applicable at relatively high, non-medicinal concentrations. The results of our calculations are summarized in Table 4. The results obtained agree with those previously reported in the literature for one compound, promazine, using totally different methods (Hu & Curry 1989). Hu & Curry determined the plasma protein binding of promazine by ultracentrifugation and found that approximately 25% of the drug was unbound. From our calcula-

tions, the free fraction of this drug was 22%. As for the other phenothiazines, no information is available at the concentration range studied. Since calculation pitfalls similar to those encountered with red cell partitioning can occur with protein binding measurements made this way, and since this approach is often only applicable at non-medicinal concentrations, direct assessment by means of equilibrium dialysis and/or ultrafiltration is recommended.

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