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Ionization constants, octanol partition coefficients and cholinesterase inhibitor constants for chlorpromazine and its metabolites

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Abstract—The relationship between the lipophilic character of chlorpromazine and seven of its metabolites and their ability to inhibit horse serum cholinesterase (K_i) has been investigated. Log(1/K_i) values were correlated with log P octanol partition coefficients ($r = 0.88$, $P < 0.01$, $n = 8$). The inhibitor values ranged from 2.7×10^{-6} M for chlorpromazine to 48.6×10^{-6} M for monodesmethylchlorpromazine sulphoxide. Ionization constants were determined by limiting solubility, spectrophotometry and pH-partition characteristics. Demethylated metabolites were more basic than the tertiary amines and the sulphoxides were slightly less basic than the corresponding sulphides.

It has long been known that chlorpromazine (CPZ) is a competitive inhibitor of cholinesterase. Courvoisier et al (1953) and other workers, e.g. Usdin et al (1961) and Michalek (1973) have determined K_i values for other phenothiazines. In their review paper, Hansch & Dunn (1972) correlated the anticholinesterase activities of eight bis-(quaternary ammonium) compounds with their calculated octanol partition coefficients. Purcell et al (1966) demonstrated a linear relationship between log I_{50} and log (partition coefficient) for ten N-alkyl-substituted amide anti-cholinesterases.

As metabolism generally leads to less lipophilic products, it seemed reasonable to suppose that chlorpromazine metabolites would have reduced anticholinesterase activities and that these may correlate with their octanol partition coefficients. Ionization constants were determined, as values for the desmethyl compounds and 7-hydroxychlorpromazine were not known. Murthy & Zografi (1970) found the pKa of chlorpromazine sulphoxide (CPZSO) to be 9.0 whereas Beckett et al (1974) obtained a value of 9.3. Accurate ionization constants are needed to calculate the partition coefficients of the non-ionized species.

Materials and methods

Materials. CPZ hydrochloride was a gift from May and Baker Ltd, Dagenham, Essex, UK. Chlorpromazine metabolites were provided by Dr A. Manian, National Institute of Mental Health, Rockville, Maryland, USA. Horse serum cholinesterase (Type IV-S, 23.6 units mg^{-1}), butyrylthiocholine, 5,5-dithiobis-(2-nitrobenzoic acid) and 1-octanol were purchased from Sigma Chemical Company, Poole, Dorset, UK. Other reagents were analytical grade.

Ionization constants. A Vibret Model 46A pH meter with a glass micro-electrode (E.I.L. 1140) was used. The meter was standardized with pH 7 buffer and 0.05 M disodium tetraborate solution (pH 9.23 at 20°C). The ionization constants of CPZ, monodesmethylchlorpromazine (nor_1CPZ) and didesmethylchlorpromazine (nor_2CPZ) were determined using the limiting solubility method (Albert & Serjeant 1962; Green 1967). The ionization constants of the sulphoxide metabolites were determined spectrophotometrically (Albert & Serjeant 1962).

pH-Partitioning between aqueous buffers and n-heptane was used to obtain the pKa values for 7-hydroxychlorpromazine (7-OHCPZ). Using the data of Whelpton (1978) the volumes of the phases were chosen so that approximately 50% of the solute would be distributed in the organic layer. The absorbance (257 nm) of 7-OHCPZ in heptane (ca. 10 $\mu g\ mL^{-1}$) before and after partitioning was used to determine the apparent partition coefficient (APC) as a function of pH. The pKa values were obtained using an iterative curve fitting procedure (Whelpton 1989).

Partition coefficients. Octanol/water partition coefficients were determined as described by Leo et al (1971). The pH of the buffer and the volume ratio octanol:buffer were chosen so that approximately 25–75% of the total solute was extracted from the aqueous layer into the organic. To test for ion-pair extraction the APC values for CPZ and nor_1CPZ were obtained at pH values between 4.0 and 7.4. The partitioning of CPZ was determined at pH 5.0 in the presence of 0.1 M chloride and 0.1 M bromide. The partition coefficients of the non-ionized forms (true partition coefficient, TPC) were calculated as described previously (Curry & Whelpton 1983).

Inhibition of cholinesterase. Cholinesterase activity was determined by the colorimetric method of Ellman et al (1961). The reaction was monitored at 412 nm in a Pye Unicam SP 800 equipped with a four-place, thermostatted cell holder and autochanger. Reagents were prepared in 0.1 M phosphate buffer, pH 7.7. Stock solutions of butyrylthiocholine (500 mM) and horse serum cholinesterase (1 mg mL^{-1}) were stored frozen until required. The colorimetric reagent (0.25 mM) was prepared immediately before use. Ellman's reagent (3 mL), prewarmed to 37°C, was pipetted into 10 mm polystyrene cuvettes and enzyme solution (50 $\mu g\ mL^{-1}$, 0.1 mL) and inhibitor solution (0, 50, 100, 200 μL) added. The volume was made up to 3.3 mL with buffer, the contents stirred and the cells placed in a thermostatted holder in the SP 800 for 5 min before substrate (0.1 mL) was added. The contents of the cells were immediately mixed and the reaction monitored for 5 to 10 min after the first min. The experiment was conducted at three substrate concentrations.

The substrate concentration was determined by hydrolysing a dilution (approximately 2.5 mM) of the stock to completion with excess enzyme. The initial concentration was calculated using an extinction coefficient of 13 600 $M^{-1}\ cm^{-1}$.

Results and discussion

In order to determine the partition coefficient of the non-ionized species (TPC) of a weak electrolyte one must either: 1) obtain an experimental value at a pH such that the solute is essentially non-ionized or 2) calculate the TPC value from a knowledge of APC, the pH at which the APC was determined and the pKa of the ionizing species.

For very lipophilic compounds, such as CPZ, the TPC may be too large to allow accurate determination experimentally and the second approach must be used. The TPC for a base is given by:

$$TPC = APC\ (1 + 10^{(pKa - pH)})$$

where pH is the value at which the APC was determined.

Because not all the pKa values were known, it was necessary to determine them (Table 1). The ultra-violet spectra of CPZ, nor_1CPZ and nor_2CPZ showed little or no change in absorbance as a function of pH, so their ionization constants were obtained

Table 1. Ionization constants (±scatter), log(octanol partition coefficient) and cholinesterase inhibition by chlorpromazine and its metabolites.

Compound	pKa	Log P	$K_i \times 10^6$
CPZ	9·3±0·1	5·30	2·7
Nor_1CPZ	10·21±0·07	4·94	2·9
Nor_2CPZ	9·9±0·2	4·47	7·7
7-OHCPZ	9·16±0·05[a]	4·63	5·7
	10·00±0·05[a]		
CPZNO	4·7[b]	2·73	13·6
CPZSO	8·96±0·04	2·32	16·0
Nor_1CPZSO	9·72±0·03	1·82	48·6
Nor_2CPZSO	9·50±0·09	1·41	14·6

[a] ±s.d., [b] From Beckett et al (1974).

by determining their aqueous solubilities as a function of pH. The pKa for CPZ, 9·3±0·1, was in agreement with the value of 9·3 obtained by Green (1967) and Murthy & Zografi (1970) using the same technique. The desmethyl compounds were more basic. The increase in pKa to 10·2 on demethylation of the parent compound is similar to that observed for desipramine which has a pKa of 10·2 compared to 9·5 for imipramine (Green 1967).

Although not large, the spectral shift given by the sulphoxides, was sufficient to allow it to be used for pKa determination. Sulphoxidation reduced the pKa values of the corresponding sulphides by about 0·4 units. The value for CPZSO of 8·96±0·04 is in good agreement with Murthy & Zografi's (1970) value of 9·0 obtained by potentiometric titration. Again the demethylated compounds were more basic. The pKa of the secondary amine was about 0·7 units higher than CPZSO whereas the value for primary amine, nor_2CPZSO, was only 0·5 units greater. These differences are in keeping with those observed with the sulphides.

The spectral shift obtained with 7-OHCPZ was smaller than that for the sulphoxides, and although this has been used for pKa determination (Whelpton 1978), it is not clear which group is responsible for the shift. Both the basic and acid moieties may contribute. Consequently, an alternative approach was sought. There was insufficient material for solubility studies so pH-partitioning was employed. The method proposed by Butler (1953) required experimental determination of the partition coefficient at a pH such that it approximates to the TPC. This is not possible with an amphoteric compound such as 7-OHCPZ where the pKa values are similar. However, if several APC values are obtained for a range of pH values then the ionization constants and TPC can be obtained iteratively (Whelpton 1989). The experimental points and the fitted curve are shown in Fig. 1. A pKa of 9·2 for the basic group is similar to that for CPZ, while a value of 10·0 is not unexpected for a phenol.

Because of the large range of values that are often studied it is usual to quote log(partition coefficients). Values for log P (octanol/water) for CPZ determined by Murthy & Zografi (1970) and Leo et al (1971) were 5·4 and 5·35, respectively. This study obtained a log P of 5·30. A partition coefficient of 200 000 cannot be determined experimentally and has to be calculated from an APC value determined at a pH value much lower than the pKa of the solute. The calculation assumes ideal behaviour and that only the neutral molecule extracts into the organic phase. Very lipophilic compounds are prone to ion-pair extraction and, if the extrapolation from APC to TPC is large, a very small degree of ion-pairing can have a marked effect on the TPC obtained. This is illustrated by the data of Table 2. At pH 4 ion-pair extraction of CPZ leads to a calculated log P of 6·4 but at values above 5 the contribution due to ion-pairing is negligible. That ion-pairing was responsible was confirmed by adding 0·1 M chloride or bromide when the APC increased from 11·5 to 48·8 and 70·2, respectively. Ion-pairing of nor_1CPZ was a problem at

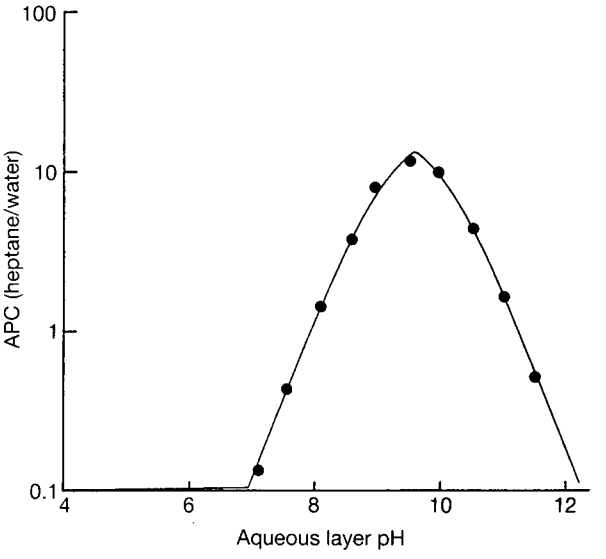

FIG. 1. Apparent partition coefficients of 7-OHCPZ as a function of pH. The solid line represents the least squares fit which gave $pKa_1 = 9·16$, $pKa_2 = 10·00$ and TPC (heptane/water) = 17·8.

Table 2. Effect of pH on determination of log P.

Compound	pH	APC	Log P
CPZ	4·0	8·42	6·23
	5·0	11·5	5·4
	6·0	63·9	5·1
	7·0	793	5·2
Nor_1CPZ	5·0	6·6	6·02
	6·0	11·2	5·25
	7·0	54·8	4·94
	7·4	138	4·94

higher pH values than those observed with CPZ, as would be expected with this more basic and, hence, more ionized compound.

Not surprisingly, the sulphoxides were considerably less lipophilic than their corresponding sulphides, log P being 3–3·1 units lower. Reduction in log P on demethylation was in the order of 0·4–0·5 units per methyl group.

The Michaelis constant for the enzyme-substrate complex was $5·3 \times 10^{-4}$ M at pH 7·7 and 37°C. Using horse serum cholinesterase and acetylthiocholine as substrate, Michalek (1973) obtained a value of $K_m = 2·9 \times 10^{-4}$ M. Szasz (1968) obtained a value of $K_m = 1·1 \times 10^{-4}$ M with butyrylthiocholine as substrate at pH 7·2 and 25°C.

The inhibition of cholinesterase by CPZ and its metabolites was competitive; the lines of the Dixon plot intersecting at a point $1/V_{max}$ above the abscissa. The inhibitor constant values ranged from $2·7 \times 10^{-6}$ M, for CPZ to $48·6 \times 10^{-6}$ M for nor_1CPZSO (Table 1). The K_i values for the first 7 compounds were in the same rank (reverse) order as their log P values, however the didesmethyl sulphoxide was more active than nor_1CPZSO and CPZSO.

Linear regression of log $(1/K_i)$ versus log P showed that 78% of the variation in log $(1/K_i)$ is accounted for by the change in log P. Although the slope of the regression line was only 0·25 it was significant ($P < 0·01$). The dependence of K_i on partition coefficient is similar to that found by Purcell et al (1966) who found that over 75% of the change in K_i correlated with P. The correlation found by Hansch & Dunn (1972) was much higher ($r = 0·972$) and the slope was close to 1. However, their compounds were from an homologous series so the change in

anti-cholinesterase activity may have been due to changes in chain length rather than partition coefficient.

The correlation for CPZ metabolites may indicate that their ability to inhibit cholinesterase is due in part to hydrophobic bonding to the enzyme. The K_i values are relatively high which suggests that the bonding is not very specific.

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Incorporation of dyes into low density lipoprotein in the presence of non-ionic surfactants

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Abstract—The interaction of low density lipoprotein (LDL) with two model dyes in the presence or absence of non-ionic surfactants has been studied. The water soluble dye, thymol blue, only weakly interacts with LDL to cause slight increases in particle size and the interaction can be reversed on column chromatography and dialysis. The oil-soluble dye sudan III reacts strongly with LDL to produce marked changes in particle size the complex formed being stable to dialysis and gel exclusion chromatography. These interactions are not affected by the presence of surfactants. The results indicate that even small quantities of lipophilic materials will easily partition into the particle, with or without the aid of surfactants and can have a marked effect on the measured size of the particle.

Several approaches have been made to establish methods for the delivery of cytotoxic agents specifically to neoplastic cells. Such targeting should reduce toxicity due to unwanted effects on normal healthy cells. To achieve this aim drugs have been linked to a variety of carrier molecules (Garnett & Baldwin 1986; Halbert et al 1987) or encapsulated in colloidal carriers (Rogerson et al 1988) to alter the distribution of the active species. One carrier system which has aroused interest is the low density lipoprotein (LDL) particle, a natural component of plasma (Brown & Goldstein 1986) which accumulates in certain tumour cells to a greater degree than normal cells, both in-vitro (Gal et al 1981) and in-vivo (Hynds et al 1984). LDL is the principal cholesterol transport lipoprotein in human plasma and is an almost spherical particle around 22nm in diameter with a molecular weight of approximately $2{\cdot}5 \times 10^6$ Daltons. Each LDL particle contains a non-polar core of cholesterol esters surrounded by a monolayer of phospholipid, unesterified cholesterol and apoprotein B, which is the receptor protein recognized by LDL receptors on cell surfaces (Deckelbaum et al 1977).

Various drugs, such as aclacinomycin (Rudling et al 1983), and chlorambucil (Firestone et al 1984), have been successfully incorporated into LDL using a technique involving freeze drying. The particle is first lyophilized in the presence of saccharides, then extracted with hexane to remove the cholesterol esters and the replacement core added in a volatile solvent which is evaporated before LDL reconstitution with an aqueous buffer.

We have previously shown that the interaction of LDL with surfactants causes the particle to swell and increase in size, a phenomenon that may be useful in assisting the incorporation of cytotoxic drugs into LDL (Tucker & Florence 1983). This work has demonstrated that the Brij (alkyl polyoxyethylene ether) surfactants produce the least alterations to lipoprotein properties whilst producing the greatest increase in size. They may

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