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Oxazepam Esters. 2. Correlation of Hydrophobicity with Serum Binding, Brain Penetration, and Excretion

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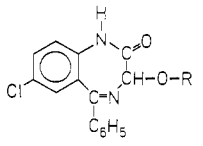
Pharmacokinetics of a series of prodrug-type oxazepam esters were studied in mice. The effect of hydrophobicity was investigated in relation to serum binding, brain penetration, tissue storage, and excretion. Binding to mouse serum and to human serum albumin was measured by equilibrium dialysis, and the changes in binding free energy were correlated with R_M values. Brain–blood partition of the esters did not change parallel with their serum binding. An indirect correlation exists between R_M of the esters and oxazepam brain accrual. Brain–blood concentration ratios of oxazepam prove that hydrolysis precedes brain penetration and hydrophobicity might primarily influence the hydrolysis rate. The amount of tissue storage and total excretion rates also correlate with hydrophobicity.

SAR studies on prodrugs require a pharmacokinetic aspect. Biotransformation is the factor studied most thoroughly in this field.[1] The role of hydrophobicity that is well known in QSAR studies, in general,[2] was briefly investigated for prodrugs.

The esters of oxazepam, a potent tranquillizer and anticonvulsant, were studied in the previous part of this work[3] to examine the role of hydrolysis in the brain accrual of oxazepam. A correlation was also demonstrated between the R_M values of the esters and the increased rate of oxazepam brain levels. This work attempts to explain the nature of this correlation and the role of hydrophobicity of oxazepam prodrugs in other pharmacokinetic processes, i.e., serum binding, tissue storage, and excretion as well.

Results and Discussion

Sixteen esters of oxazepam (7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-3*H*-1,4-benzodiazepin-2-one; see the structure where R = H), including two stereoisomers, were



studied. The acyl moiety contained an alkyl chain with varying length, successive branching in position 2 or 3, and/or an ω-phenyl group (Table I).

Serum Binding. Binding of oxazepam esters to mouse serum was investigated by equilibrium dialysis. The same ester concentration was used throughout, which was near the blood concentration in our in vivo experiments. Hydrolysis by serum esterases makes the measurement difficult; thus, the cells were equilibrated at 4 °C with continuous stirring. Diisopropyl fluorophosphate (DFP), a strong inhibitor of the esterases, was also included.[4] DFP was found not to influence the binding equilibrium of

Table I. Structure of Ester Substituents and Dose Data of the Esters

no.	R	dose, mg/kg[a]
1	H	5
2	$COCH_3$	10
3	$COCH_2CH_3$	10
4	$COCH(CH_3)_2$	5
5	$CO(CH_2)_2CH_3$	15
6	$CO(CH_2)_2COOCH_3$	5
7	$COCH_2CH(CH_3)_2$	
8	$COC(CH_3)_3$	10
9	$COCH(CH_2CH_3)_2$	10
10	$CO(CH_2)_2Ph$	5
11	$COCH(CH_3)CH_2Ph$	5
12	$CO(CH_2)_3Ph$	5
13	$COC(CH_3)_2CH_2Ph$	5
14[b]	$COCH(CH_3)CH_2Ph$	5
15[c]	$COCH(CH_3)CH_2Ph$	5
16	$CO(CH_2)_2COOH$	

[a] Expressed in mg-equiv of oxazepam. [b] Esterified with *d*-(+)-2-methyl-3-phenylpropionic acid. [c] Esterified with the *l*-(−) enantiomer of the acid.

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Table II. Binding of Oxazepam Esters to 22% Mouse Serum[a]

no.	PF_{exp} ± SE,[b] %	oxazepam content,[c] %	PF_{ester} ± SE[d]	RT ln [(100/PF_{ester}) −1], kJ
1	31.0 ± 0.3			1.84
2	28.5 ± 1.3	13.7	28.0 ± 1.6	2.17
4	14.7 ± 3.7	21.2	11.2 ± 1.7	4.77
5	14.9 ± 1.6	3.5	14.3 ± 1.6	4.12
6	29.9 ± 0.7	5.5	29.9 ± 0.7[e]	1.96
7	6.7 ± 0.3	0.3	6.6 ± 0.3	6.09
9	3.8 ± 0.8	1.7	3.4 ± 0.8	7.70
11	11.1 ± 1.2	14.6	8.0 ± 2.4	5.62
12	10.2 ± 2.4	24.5	4.3 ± 2.4	7.14
13	11.3 ± 2.0	8.2	9.5 ± 2.4	5.18

[a] Total concentration of the compounds = 10 μM. Data are averages of four to six determinations. [b] Percent free concentration, measured experimentally. [c] $[c_{ox}/(c_{ox} + c_{ester})]100$ in the serum samples. [d] Percent free ester concentration, corrected for the oxazepam content according to eq 7. [e] Not corrected for the other hydrolysis product, 20% oxazepam hemisuccinate.

Table III. Binding of Oxazepam Esters to 1% Human Serum albumin[a]

no.	PF_{HSA} ± SE, %	RT ln [(100/PF) − 1], kJ
1	10.5 ± 1.9	5.25
2	24.6 ± 3.7	2.74
4	7.8 ± 0.9	6.05
5	8.9 ± 0.6	5.70
6	17.2 ± 2.4	3.85
7	9.3 ± 0.8	5.58
9	6.0 ± 1.7	6.74
10	5.2 ± 3.1	7.11
11	5.1 ± 2.6	7.16
12	3.4 ± 0.9	8.20
13	2.2 ± 0.7	9.30

[a] Total concentration of the compounds = 10 μM. Data are averages of four to six determinations.

sterically hindered esters. After the equilibrium had been reached, oxazepam content of the dialysis cells was measured and the data (PF_{expl}) were corrected for the different binding of oxazepam (PF_{ester} in Table II).

The esters are strongly bound to serum proteins. Binding of the 1,4-benzodiazepines to serum albumin has been extensively investigated. A substantial part of the studies[5,6] stress the importance of hydrophobic binding; others[7,8] showed the role of polar interactions.

It was assumed that serum albumin is responsible for serum binding; therefore, binding to human serum albumin (HSA) was also examined at the same drug concentration (Table III).

As a rule, binding constants correlate with the hydrophobicity of the ligands[9] and binding free energies can be calculated from them according to eq 1, where c_b and c_f

$$\Delta F = -RT \ln \frac{c_b}{c_f c_p} \quad (1)$$

are the bound and free concentrations of the drug and c_p is the free protein concentration. As in our experiments, the total protein concentration (20 mg/mL for mouse serum; 10 mg/mL = 1.5×10^{-4} M for HSA) was in excess of the total ester concentration (10^{-5} M); c_p practically equals the total albumin concentration and, therefore, it is constant for the derivatives. Thus, the increments of the binding free energy ($\Delta\Delta F$) characterizing the binding of the acyl side chain will be proportional to:

$$RT \ln \frac{c_b}{c_f} = RT \ln \left(\frac{100}{PF} - 1 \right)$$

Correlation of this quantity with reverse-phase chromatographic R_M was demonstrated both for mouse serum (eq 2) and for HSA (eq 3). Oxazepam was excluded from the

$$RT \ln \left(\frac{100}{PF_{serum}} - 1 \right) = 3.29 + 5.20 R_M \quad (2)$$

$$n = 10;\ r = 0.846$$

$$RT \ln \left(\frac{100}{PF_{HSA}} - 1 \right) = 4.18 + 6.09 R_M \quad (3)$$

$$n = 10;\ r = 0.936$$

correlation for HSA. Its anomalously stronger binding can be accounted for by a stereospecific interaction. It is known that the enantiomers of oxazepam hemisuccinate bind stereospecifically to HSA.[10] Oxazepam, unlike its esters, racemizes rapidly in aqueous medium.[11] Binding of its preferred antipode shifts the racemic equilibrium, which results in increased binding, as was suggested by Sjödin et al.[7] The binding of oxazepam to mouse serum did not show this deviation. The interaction does not seem to be stereospecific, similar to the binding of oxazepam hemisuccinate to bovine serum albumin.[12]

It was previously demonstrated[13] that R_M values excellently correlate with the carbon number of the acyl moiety: $\Delta R_M = 0.166$ per methylene unit. $\Delta\Delta F$ values can be calculated from ΔR_M and from the regression coefficient of the correlation between RT ln [(100/PF) − 1] and R_M for the aliphatic esters ($n = 6$): $\Delta\Delta F$ is 1.0 kJ for HSA and 1.6 kJ for mouse serum per methylene unit. These values correspond to a weak hydrophobic interaction.[9]

Brain Penetration. It is known that penetration of a

Table IV. Ratio of the Brain and Blood Levels[a] of the Compounds Administered

time, min	ratio									
	1[b]	3	4	8	9	10	12	13	15	16[c]
1		2.9				0.5	0.2	0.5		0.1
2			1.1		2.1					
3	2.5	2.4		3.6		0.9	0.5	1.9	1.0	
5			1.7	6.9	5.1	1.5	0.9	2.3	5.6	
6	2.7	3.5								0.2
10	2.5		2.4	4.2	4.5	2.3	1.2	2.1	5.1	0.3
12		1.7								
20	2.8	1.6	1.2	6.2	4.2	3.7	1.5	1.4	6.5	
30	2.8			6.2	4.2					0.6
40			2.0		3.5	2.9	1.4		5.4	

[a] Data are averages of four mice. [b] Doses are in Table I. [c] Oxazepam succinate liberated after the administration of oxazepam succinate methyl ester.

Table V. Ratio of the Brain and Blood Levels[a] of Oxazepam Liberated

time, min	ratio								
	2	3	4	5	8	9	10	12	15
1	4.0	3.8		0.2			0.6	0.5	
2			0.7			0.7			
3	3.4	2.0		0.3	1.7		0.4	0.5	0.3
5	2.0		1.2		2.0	0.6	0.5	0.3	1.1
6		3.0		0.4					
10	2.4		1.8		2.2	1.0	0.5	0.4	1.6
12		2.4		0.8					
20		2.8	1.9	1.4	2.0	1.1	1.4	1.0	2.0
30	4.0				2.5	1.4			
40		2.8	2.3	2.0		1.7	2.2	0.9	2.1

[a] Data are averages of four mice.

Table VI. First-Order Excretion Rate Constants (K) and Tissue Storage Data (Ts)

no.	Ts, % of the dose	K, h^{-1}	log K
1	5[a]	4.33×10^{-2}	−1.36
2	0	5.78×10^{-2}	−1.24
3	19[b]	2.26×10^{-2}	−1.64
8	7[a]	3.47×10^{-2}	−1.46
10	26[a]	1.98×10^{-2}	−1.70
12	38[b]	1.90×10^{-2}	−1.72
14	34[b]	2.77×10^{-2}	−1.56

[a] Determined at 96 h as unexcreted. [b] Determined at 150 h as unexcreted.

drug into the tissues is proportional to its free concentration in the blood, other conditions being equal.[14,15] PF values of Table II regularly decrease with increasing hydrophobicity of the esters. It was examined how the increase in serum binding influences the blood–brain partition of the esters.

Brain–blood concentration ratios of the administered compounds were determined (Table IV). They do not show any systematic change with increasing hydrophobicity (3–15). That is, an opposite equilibrium factor, affinity of the esters for the lipoid brain tissue, roughly compensates the effect of serum binding. However, while the less hydrophobic compounds 1–8 instantly reach their steady-state ratio, partition of the ω-phenyl-substituted esters (10–15) is kinetically controlled and their ratios increase up to 10–20 min. The very polar oxazepam hemisuccinate (16) poorly penetrates into the brain.

Oxazepam brain accrual was previously demonstrated[3] to correlate with the hepatic microsomal hydrolysis rate of its esters. Brain penetration rate constants for oxazepam also correlated with R_M, as shown in eq 4. Brain–blood

$$\log k_1 = -0.7 - 3.3R_M + 2.5R_M^2 \quad (4)$$

$$n = 10$$

ratios of oxazepam further elucidated the role of hydrolysis (Table V). Longer-chain esters (4–12) result in a lower and increasing brain–blood ratio for oxazepam than does the administration of oxazepam (compare Table IV, first column). The concentration gradient points toward the brain, because hydrolysis precedes and controls the penetration of oxazepam. The high initial ratios of 2 and 3 show the significant contribution of brain hydrolysis that was demonstrated in vitro to have an opposite stereoselectivity to liver.[16] It results in an antiretarding effect for oxazepam acetate and oxazepam propionate; meanwhile, other esters retard oxazepam brain accrual.

It can be realized that the correlation between log k_1 and R_M (eq 4) cannot originate from hydrophobic effects in serum binding or brain penetration. Instead, it might reflect the correlation between the hepatic hydrolysis and R_M that was demonstrated in vitro.[17]

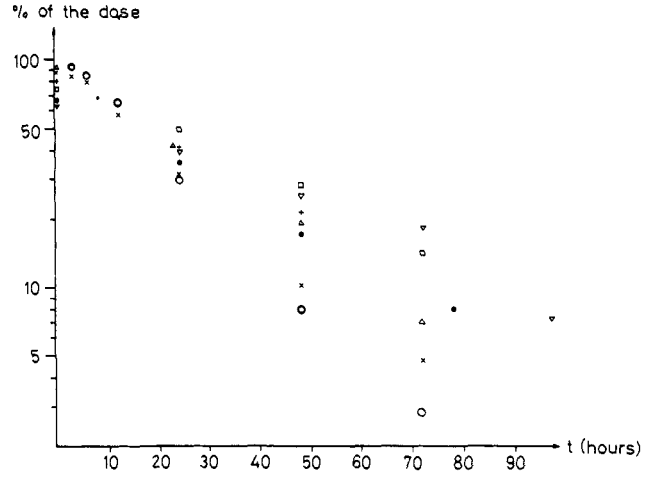

Figure 1. Total excretion data of oxazepam derivatives: 1 (×), 2 (○), 3 (+), 8 (△), 10 (□), 12 (▽), and 14 (●). Dose data are collected in Table I.

Excretion Rates. Biotransformation of oxazepam esters leads into one major route: hydrolysis to oxazepam, its conjugation to glucuronide, and excretion.[18] Therefore, we were interested to see if there are still differences in their excretion rates.

Excretion measurements were restricted to feces and urine, since the whole radioactivity resulting from the esters was excreted this way. Total excretion data were treated according to the "sigma-minus" method[19] and are collected in Figure 1. Tissue storage (Ts), defined as the fraction of the dose that remains unexcreted after 100–150 h, was subtracted from the points. First-order excretion rate constants (K) and tissue storage data (Table VI) correlated with R_M values of the esters (eq 5 and 6). Hy-

$$\log K = -1.44 - 0.34R_M \quad (5)$$

$$n = 7; r = 0.72$$

$$\mathrm{Ts} = 11.4 + 32.5R_M \quad (6)$$

$$n = 7; r = 0.88$$

drophobicity-dependent storage in lipoid tissue depots was demonstrated for long-chain steroid esters[20] and for 1,4-benzodiazepines as well.[21]

The excrements contained only trace amounts of the esters. Since lipophilic esters are not readily excreted,[22] their release from the depots is followed by transformation. However, hydrolysis is not rate determining, because the k_1 values are three orders of magnitude larger[3] than the K values.

Table VII. Preparative Data on Oxazepam Esters

no.	solv syst for col chrom[a]	mp,[b,c] °C	crystn solv[a]	yield,[a] %	sp act.,[c] mCi/mmol
1		201–202	EtOH	92	4.74
7	6:4	143–146	ether	45	4.77
10	1:1	192–193	*i*-PrOH	78	3.13
11	6:4	164–166	MeOH	53	2.33
12	1:1	168–169	*i*-PrOH	67	2.55
13	6:4	198–200	MeOH	73	2.80
14[d]	6:4	166–167	MeOH	78	2.27
15[e]	6:4	167–168	MeOH	81	2.24
16		152–153	EtOH-H_2O	74	

[a] Benzene–ether. [b] Data for 2–6, 8, and 9 can be found in ref 4. [b] C, H, and N analyses agreed with calculated values within ±0.2%. [c] Specific radioactivity of 2–6, 8, and 9 ranged from 1.5 to 4.8 mCi/mmol. [d] Esterified with *d*-2-methyl-3-phenylpropionic acid: $[\alpha]_D$ +19.4 (*c* 2, $CHCl_3$), $[\alpha]_D$ +17.8 (*c* 2, dioxane). [e] Esterified with *l*-2-methyl-3-phenylpropionic acid: $[\alpha]_D$ −19.0 (*c* 2, $CHCl_3$), $[\alpha]_D$ −18.1 (*c* 1.5, dioxane).

In conclusion, this study shows that hydrophobicity may differently influence single pharmacokinetic processes. QSAR studies can only demonstrate the result of diverse hydrophobic effects which is rarely enough to make conclusions about the transport-determining step, especially in the field of prodrugs, where biotransformation may precede or follow the penetration of the drug into the target organ. In the examination of brain penetration, the brain–blood concentration ratios are especially useful.[15,23]

Experimental Section

All melting points are uncorrected. Silica gel 60 (0.063–0.200 mm, Merck) was used for column chromatography. Acyl chlorides were prepared by means of thionyl chloride and purified by distillation. The 2-methyl-3-phenylpropionic acid was resolved by the method of Kipping and Hunter[24] by fractional crystallization of the quinine salt. Specific rotations for the enantiomers were $[\alpha]_D$ +17.9 (*c* 8, benzene) and $[\alpha]_D$ −17.1 (*c* 4, benzene). Their chlorides hydrolyzed in a solution of Na_2CO_3 yielded acids with the same specific rotations.

General Procedure for the Synthesis of Oxazepam Esters. A suspension of 20 mmol of oxazepam in 200 mL of dichloromethane was made alkaline with pyridine and a solution of 30–60 mmol of freshly distilled acyl chloride was added to it dropwise during 30–60 min. After stirring at room temperature for 3–5 h and after a subsequent 1–2 h of reflux when necessary (e.g., ω-phenyl-substituted esters), the reaction mixture was successively extracted with 2 N HCl, 10% $NaHCO_3$ solution, and water. After drying the mixture over $MgSO_4$, the solvent was removed and the residue was chromatographed on a silica gel column in a benzene–ether solvent mixture. The pure product was crystallized (see Table VII).

[2-^{14}C]Oxazepam and its acetic ester were synthesized as previously described.[25] Radioactive esters were prepared from 2 mmol of [2-^{14}C]oxazepam and they were identical with the inactive ones (melting point, thin-layer chromatographic R_f, and $[\alpha]_D$). Their radiochemical purity was 99.2–99.6%, checked by TLC in *n*-heptane–$CHCl_3$–AcOH–EtOH (5:5:1:0.3) and benzene–ether solvent mixtures (see in Table VII).

Pharmacokinetics. Male albino mice (18–22 g) were given 2-^{14}C-labeled oxazepam esters in the doses assigned in Table I in Me_2SO (35 μL of Me_2SO/20 g of body weight).

Tissue Levels. Total Metabolite Levels. Brain and blood samples were dried and weighed, and their radioactivity was measured using a carbon–tritium analyzer (Chinoin Ltd., Budapest).

Metabolite Levels. Brain and blood levels of the esters and oxazepam were determined according to a previous method.[16] Samples were homogenized in methanol, centrifuged, and chromatographed on thin-layer plates. The ratio of the radioactivities of the chromatographic spots corresponded to the ratio of the metabolite concentrations in the sample. Since no metabolites other than the ester and oxazepam contribute significantly to the levels within 0.5 h, knowledge of the sum and the ratio of the two concentrations permits the calculation of true ester and oxazepam levels.

Excretion Rate Measurements. Animals were kept separated in glass metabolic cages and fed ad libitum. Urine and feces were collected without separation. At certain intervals, excrements were removed and the cages carefully rinsed with distilled water. The feces were dried and ground, and the weighed samples were used for radioactivity measurement. Radioactivity of an aliquot of the washing combined with urine was also determined. Data points are averages of four to five animals.

Serum Binding Data. Mouse Serum. Equilibrium dialysis was used. Teflon cells (1-mL volume) separated with a membrane (Visking tubing, Medicell, U.K.) were rotated according to the method of Weder and Bickel,[26] where a revolving bubble facilitates the approach to equilibrium. Samples containing 22% mouse serum and 10^{-4} M DFP in 0.05 M phosphate buffer (pH 7.4) were equilibrated with the same buffer for 8–12 h at 4 °C. Radiolabeled substrates (10 μM) were added to both cells in 5 μL of Me_2SO.

Human Serum Albumin. HSA was used as purchased (Human, Hungary); its lipid content was less than 10 mg/g. HSA, 1%, in 0.05 M buffer was equilibrated with the buffer for 12–20 h at room temperature, applying the same concentration of the substrates. The ratio of radioactivities on the two sides corresponds to the ratio:

$$PF = \left(\frac{c_f}{c_f + c_b}\right) 100$$

After the equilibrium had been reached, radioactivity of the aliquots was measured in a scintillation spectrometer (Model 3003, Packard Instrument Co.). The equilibrated samples were extracted three times with ether and chromatographed on thin-layer plates. Recovery of the esters and oxazepam from the serum samples was practically the same (80–90%). Partial hydrolysis of the esters was taken into account. The experimental distribution is a weighed mean of the distribution of the compounds present (eq 7), where PF_{ester} is the true distribution of the ester

$$PF = \left(\frac{c_{ester}}{c_{ester} + c_{ox}}\right) PF_{ester} + \left(\frac{c_{ox}}{c_{ester} + c_{ox}}\right) PF_{ox} \qquad (7)$$

and PF_{ox} is that of oxazepam measured independently, and c_{ester} and c_{ox} are the analytical equilibrium concentrations in the solution. Data are averages of five to seven determinations. Reverse-phase thin-layer chromatographic R_M values were used as previously determined.[12]

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Effect of Structure on Phenothiazine Cation Radical Reactions in Aqueous Buffers

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The reactions of the cation radicals of 11 phenothiazine tranquilizers were examined in mildly acidic aqueous buffers. Of the 11, those having an aminopropyl side chain in the 10 position reacted to form 0.5 mol of sulfoxide and 0.5 mol of parent drug per mole of initial radical. Cation radicals with different side chains react to form additional products, which remain to be identified but probably result from hydroxylation of the phenothiazine ring. The decay kinetics of three of the cation radicals undergoing reactions with known stoichiometry, namely, chloropromazine, promazine, and triflupromazine, were studied in detail, and it was concluded that they all react via the same mechanism. The mechanism involves attack of the cation radical by a nucleophile, and radicals with electron-withdrawing groups in the 2 position react more quickly. Since the cation radicals with faster reaction rates with nucleophiles are more pharmacologically active, it is hypothesized that the cation radical–nucleophile interaction may be responsible for binding of phenothiazines to receptor proteins.

The mechanism of action of the phenothiazine class of major tranquilizers has remained a subject of intense study since the beginning of their use in the early 1950's.[1,2] Significant evidence has been presented that the drugs interact with dopamine receptors, and a good correlation exists between drug potency and the strength of this interaction.[2-4] In addition, several investigators have proposed that the cation radical formed by oxidation of a phenothiazine, such as chlorpromazine, is an important intermediate in the metabolism of the drug[1,5,6] and may be the active pharmacological entity.[1,5,7-9] The reasons for these hypotheses have been summarized previously and will not be repeated here.[10] The work presented herein is related to the question of radical involvement in metabolism and activity of phenothiazine major tranquilizers.

The influence of the 2-position substituent and the structure of the 10-position side chain on the antipsychotic activity of phenothiazines has been studied in detail, and it has been concluded that changes in drug structure at these positions cause variations in antipsychotic activity covering at least three orders of magnitude.[11,12] However, information about substituent effects on cation radical behavior is less prevalent due to the difficulty in studying the reactive radical cations. Given the likely involvement of the radical in the metabolism of the drugs and possibly in their activity, it is deemed useful to more fully investigate the behavior of the radicals as a function of structure.

The phenothiazine cation radicals have been studied by a variety of approaches by previous workers.[1,13-15] Due to rapid reactions of the radicals at physiological pH, all but a few of the previous studies have been carried out in strong mineral acids or in concentrated acetic acid. In one paper, an attempt was made to correlate cation radical stability in 1–9 N sulfuric acid solutions with clinical potency, but no correlation was found.[13] Until recently, the second-order kinetics of the radical decay in these solvent systems led workers to propose a disproportionation mechanism for the radical decay, leading to reduced phenothiazine and a sulfoxide.[1,5,13,14] Recent work in aqueous buffers in the pH range from 2 to 5 demonstrated that the cation radical of chlorpromazine (1) does not disproportionate but is attacked by a weak nucleophile.[15] The resulting adduct adduct is oxidized by a second molecule of the cation radical, and then decomposes to form reduced chlorpromazine and chlorpromazine sulfoxide as shown in Scheme where where $A^{+\cdot}$ represents the chlorpromazine cation radical, A represents chlorpromazine, AO its sulfoxide, and B^- a buffer anion. This mechanism is similar to that observed for thianthrene and 10-phenylphenothiazine cation radical reactions with pyridine in acetonitrile.[16,17]

In subsequent work, it was shown that the products and the rate of the reaction are highly dependent on the identity of the nucleophile, with some nucleophiles yielding hydroxylated products rather than sulfoxide.[10] Given this further knowledge about the importance of solution con-

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