Protein kinase A phosphorylation assays were performed in a reaction mixture (80 μL) containing Histone II_A (60 μg/mL), $MgCl_2$ (5 mM), [γ^{32}P]ATP (10 μM, 2000–4000 cpm/pmol), Tris/HCl buffer (pH 7.0), protein kinase A (1 μg), and inhibitor at different concentrations.

For each kinase, when histones were used as substrates, reactions were run at 30 °C for 7 min and stopped by trichloroacetic acid (12% w/v) in presence of bovine serum albumin (0.9 mg) as a carrier. After centrifugation (10 min at 3000 rpm) supernatant containing [γ^{32}P]ATP and unprecipitable inhibitors were discarded, and the pellet was dissolved in 1 M NaOH and precipitated a second time by trichloroacetic acid. Radioactivity incorporated into histones was counted by scintillation spectrometry with Aqualyte reagent. All experiments were carried out in triplicate.

When peptides (GS 1-12 or kemptide) were used as substrates, the procedure differed as follows: after the reactions were stopped with trichloroacetic acid, [γ^{32}P]ATP was removed as described by Glass et al.[19] by spotting an aliquot (50 μL) of each sample onto phosphocellulose paper which was washed three times in H_3PO_4 (75 mM). Squares were dried and the radioactivity was quantified by scintillation spectrometry.

Registry No. 1, 129786-26-1; **2**, 129786-27-2; **3**, 129786-28-3; **4**, 129786-29-4; **5**, 129786-30-7; **6**, 129786-31-8; **7**, 129786-32-9; **8**, 129786-33-0; **9**, 129786-34-1; **10**, 84468-24-6; 11, 129786-35-2; **12**, 129786-36-3; **13**, 84468-17-7; **14**, 129786-37-4; **15**, 129786-38-5; **16**, 129786-39-6; **17**, 129786-40-9; PKC, 89800-68-0; PKA, 9026-43-1; H-β-Ala-OH, 107-95-9; BOC-β-Ala-OH, 3303-84-2; BOC-β-Ala-OMe, 42116-55-2; $BrCH_2CH_2COOMe$, 3395-91-3; $Br(CH_2)_4COOMe$, 5454-83-1; $H_2NCH_2CH_2NH_2$, 107-15-3; piperazine, 110-85-0; succinic anhydride, 108-30-5; 5-isoquinolinesulfonyl chloride, 84468-15-5; dansyl chloride, 605-65-2.

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(Acyloxy)alkyl Carbamate Prodrugs of Norfloxacin

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As a new prodrug approach to norfloxacin (NFLX) we prepared the acetoxyalkyl carbamates of the type NFLX–CO–OCHR–OAc by the reaction of sodium or mercuric acetate on NFLX α-chloroalkyl carbamates. These prodrugs did not have the bitter taste of NFLX. In vitro, the acetoxyethyl carbamate exhibited activity only against *Staphylococcus* spp. and was inactive against Gram-negative organisms. However, in the presence of serum and intestinal homogenate, esterase-catalyzed hydrolysis of the ester bond in these modified carbamates led to a cascade reaction resulting in the rapid regeneration of NFLX. At high oral doses of the prodrug, the acetaldehyde produced as a side product in the breakdown of the promoiety caused a slight decrease in alcohol metabolism in a mouse model. The bioavailability of NFLX from the acetoxyethyl carbamate was lower compared to an equivalent dose of NFLX when given as an oral suspension in rhesus monkeys, presumably because of the lower aqueous solubility of the prodrug.

Norfloxacin (**1a**) is a synthetic second generation quinolone antibacterial agent which exhibits remarkable activity against a broad spectrum of Gram-positive and Gram-negative bacteria.[1] Since only 35–40% of a typical oral dose of the drug is absorbed in human subjects, attempts were made to improve the therapeutic efficacy of the drug by several different methods of prodrug modification.[2] The desirability of masking the somewhat disagreeable taste of norfloxacin was an added impetus. Both the carboxylic acid group[3a,4] and the secondary nitrogen of the piperazino substituent[3a,4-6] have been used as loci for latentiation. The carboxylic acid esters were ineffective, either because they were too unstable chemically or because they failed to liberate norfloxacin after absorption. Reduction of the carboxylic acid to the corresponding formyl group was reported[4] to result in improved absorption in mice, leading to increased serum levels of norfloxacin after oxidation in vivo. The N-blocked norfloxacin prodrugs,[5-7] viz., *N*-[(5-methyl-2-oxo-1,3-dioxol-4-yl)methyl] and *N*-oxoalkyl derivatives, release the parent drug in vivo by oxidative dealkylation.

In general, for ionizable molecules the rate of transport through biomembranes appears to be proportional to the concentration of undissociated molecules in solution and the lipid solubility. So, a prodrug strategy that involves latentiation of the amino function which will change the zwitterionic nature of norfloxacin is expected to produce a more readily absorbable form. Analogues which carry *N*-alkyl substitution such as pefloxacin (**1b**) have enhanced oral absorbability.[3] A prodrug strategy involving carbamylation of the piperazino nitrogen would have the additional advantage that most carbamates do not ionize under normal physiological pH. However, success with carbamate ester latentiation requires that it must be hydrolyzed to a carbamic acid and an alcohol after penetration through the biological barrier. This is especially true of carbamates of secondary amines, the rate of hydrolysis of which are 10^5 to 10^9 times slower than that of

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Table I. Solubility of Norfloxacin and Prodrugs

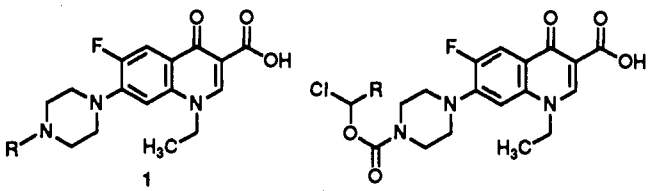
medium	solubility, mg/mL, at 25 °C		
	norfloxacin	3a	3b
water	0.19		0.06
pH 5 citrate buffer (0.1 M)	8	0.03	0.005
pH 7.4 phosphate buffer (0.1 M)	0.2	0.06	0.05
pH 8.5 bicarbonate buffer (0.05 M)	0.21	0.15	0.41

the corresponding primary amines. A carbamate ester specific enzyme does not seem to exist in mammals. Though cholinesterases hydrolyze carbamates and become reversibly inhibited in the process, the rates are too slow for practical uses. Not surprisingly, the ethyl and formylbenzyl carbamates of norfloxacin were found not to undergo hydrolysis in mouse blood.[3a]

In this paper we report the synthesis and evaluation of bioreversible (acyloxy)alkyl carbamates of norfloxacin[8] with an enzymatically hydrolyzable ester function on the promoiety. Esterase-catalyzed hydrolysis of these modified carbamates triggers the regeneration of the parent amine through the intermediate formation of an unstable carbamic acid.[9] The side products arising from the promoiety are a carboxylic acid, an aldehyde, and carbon dioxide.

Chemistry

The (acyloxy)alkyl carbamates **3a** and **3b** were prepared by a two-step process. Reaction of norfloxacin (**1a**) with



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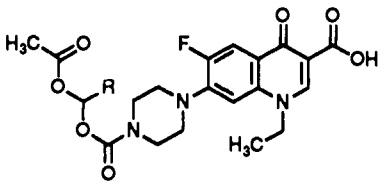

3

a, R = H; **b**, R = Me

chloromethyl[10] and α-chloroethyl chloroformate[11] in the presence of 1,8-bis(dimethylamino)naphthalene gave the corresponding chloroalkyl chloroformates **2a** and **2b**. Triethylamine was unsuitable as a base for this reaction because of the decomposition of the chloroformates in its presence. The chloromethyl carbamate **2a** was converted to the acetoxymethyl carbamate **3a** by reaction with sodium acetate in dimethylformamide or by silver acetate in acetic acid. The α-chloroethyl carbamate **2b** gave unreacted starting material or norfloxacin under similar conditions, the latter, presumably, arising from eliminative decomposition. Attempts to convert **2b** to the corresponding iodo compound, using sodium iodide, also resulted in similar decomposition. However, **2b** could be converted cleanly to the acetoxyethyl carbamate **3b** by reaction with mercuric acetate in acetic acid. The mercuric salts of a variety of carboxylic acid were found to be effective for this type of acyloxylation.[9a]

Table II. Hydrolysis (Half-Lives) of Norfloxacin Prodrugs at 37 °C

prodrug	medium	$t_{1/2}$, min
3a	rat serum	1.5
	dog serum	6.0
3b	rat serum	1.5
	dog serum	6.0
	human serum	116.0
	rat intestinal homogenate	2.6
	pH 7.4 phosphate buffer	7670.0

Table III. Comparison of the Antibacterial Activity of Norfloxacin and Prodrug **3b**

microorganism	MB no.[a]	MIC, μg/mL	
		3b	norfloxacin
S. aureus GmR Meth^R	4310	2.0	1.0
S. aureus	2868	1.0	1.0
S. aureus	2865	2.0	0.25
Strep. faecalis	2864	>128.0	8.0
E. coli TEM 2+	4351	>128.0	0.03
E. coli TEM 2+	4351	>128.0	0.03
E. coli TEM 2+ DC2	4352	>128.0	0.5
E. coli DC2	4353	>128.0	0.5
E. coli	2891	128.0	0.06
Sal. typhimurium	3860	>128.0	0.5
Ent. cloacae 99+	2646	>128.0	1.0
Ent. cloacae P99–	2647	>128.0	0.25
Ent. aerogenes	2828	>128.0	0.5
K. pneumoniae K1+	4354	>128.0	8.0
K. pneumoniae	4005	32.0	0.06
Prot. vulgaris	2829	>128.0	0.06
Prot. morganii SmR	2833	8.0	0.06
Prot. mirabilis GmR	2830	>128.0	0.06
Ps. aeruginosa RPL11+	3350	>128.0	1.0
Ps. aeruginoa	2835	>128.0	1.0
Ps. aeruginoa	4279	>128.0	1.0
Ser. marcescens	2840	>128.0	4.0

[a] Bacteria from Merck culture collection.

Biological Results

The masking of the secondary nitrogen of the piperazino group in norfloxacin as an (acyloxy)alkyl carbamate resulted in the disappearance of its bitter taste. However, this led to decreased solubility in water and buffers at pH 5–8 (see Table I) and increased solubility in organic solvents.

To be effective prodrugs, these modified carbamates must revert rapidly and quantitatively to norfloxacin in animal tissues. The hydrolysis half-lives of **3a** and **3b** were measured in rat, dog, and human serum and pH 7.4 phosphate buffer (see Table II). In pH 7.4 phosphate buffer **3b** exhibited good hydrolytic stability with $t_{1/2}$ of 128 h at 37 °C. The esterase-catalyzed hydrolysis in plasma was complete with the typical decrease in rate going from rat to dog to human plasma. Hydrolysis in rat intestinal homogenate also was very rapid, showing the potential for reconversion in the intestinal epithelium during absorption.

A comparison of the in vitro antibacterial activities of **3b** and norfloxacin presented in Table III showed that the prodrug had good intrinsic activity only against *Staphylococcus* spp. and was, in general, not active against Gram-negative organisms. These data indicated that the prodrug was not hydrolyzed or only slowly hydrolyzed, resulting in little or no active parent compound in this in

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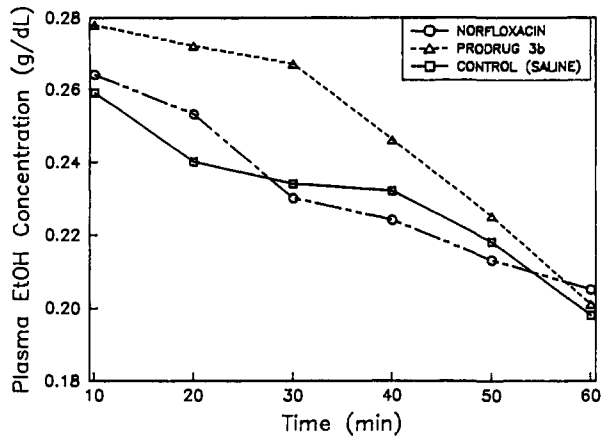

Figure 1. Comparison of the DLE of norfloxacin and prodrug **3b**.

Table IV. Oral Bioavilability of Norfloxacin in Rhesus Monkeys[a]

	norfloxacin,[b] μg/mL	prodrug 3b,[c] μg/mL
serum concn at 3 h	0.76 ± 0.29	0.36 ± 0.05
urine concn 0–5 h	175.86 ± 154.16	25.03 ± 8.85
urine concn 5–24 h	33.78 ± 25.56	21.97 ± 15.47
% dose recovered in urine	16.30 ± 4.29	7.23 ± 1.36

[a] Dose of 25 mg/kg of norfloxacin or equivalent. [b] $n = 5$. [c] $n = 3$.

vitro system, in accordance with the stability data above. A free basic amino group appears to be present on the C-7 substituent on the majority of second generation quinolone antibacterial agents[12] which have a broad spectrum.

A disulfiram-like effect (DLE) has been associated with certain antibiotics, especially β-lactam antibiotics with the *N*-methyltetrazolyl side chain, when taken in conjunction with alcohol.[13] The symptoms of DLE are believed to result from accumulation of acetaldehyde caused by an inhibition of acetaldehyde dehydrogenase. Since acetaldehyde is one of the side products in the breakdown of the prodrug **3b** to norfloxacin, a study to compare the ability of the prodrug and norfloxacin to induce any DLE was conducted using an animal model developed by Fromtling and Gadebusch.[14] The model is based on changes in plasma ethanol concentration over time as determined by an enzyme assay, in mice pretreated with the antibiotic and injected with ethanol. Norfloxacin or the prodrug did not produce DLE over the 60 min assay period when orally administered at 100 mg/kg compared to saline controls. But, a statistically significant ($p = 0.039$) increase in plasma ethanol levels compared to those of control animals was observed during the initial 40 min following oral administration of **3b** at 100 mg/kg (Figure 1). At 25 and 50 mg/kg there was no observable difference.

The bioavailability of norfloxacin from the prodrug **3b** was compared with that of the parent in a cross-over design in male rhesus monkeys. The drugs were administered as suspensions in 5% Tween 80 in 5% ethanol at a dose of 25 mg of norfloxacin per kilogram body weight or equivalent, via gastric tube to the monkeys that had been fasted overnight. The data (Table IV) indicate that **3b** was not absorbed and/or hydrolyzed very efficiently in this species, resulting in lower serum concentrations, lower urinary concentrations in the 0–24-h samples, and lower urinary recovery of norfloxacin in 24 h than those obtained following a comparable dose of the parent drug. The data also show the wide variability in absorption of norfloxacin and the prodrug in the rhesus monkeys. The lower bioavailability is believed to be due to poor absorption as a result of the decreased solubility of the prodrug compared to that of norfloxacin.

Conclusion

(Acyloxy)alkylcarbamylation of the secondary piperazino nitrogen of norfloxacin gave bioreversible prodrugs which underwent rapid hydrolysis catalyzed by rat and dog serum esterases to regenerate the parent drug in vitro. The hydrolysis was slower in human serum. The chemical stability of these prodrugs, which depends on the hydrolytic stability of the ester group, is very high at physiological pH. The prodrug modification resulted in the repression of the bitter taste of norfloxacin. However, the acetoxymethyl and acetoxyethyl carbamates that we synthesized were less water soluble than the parent drug. The decreased bioavailability of the prodrug **3b** compared to norfloxacin in rhesus monkeys is presumed to be the result of poor absorption because of the low dissolution rate. It might be possible to improve the bioavailability by increasing the intrinsic solubility of the prodrug. This might be achieved by the introduction of solubilizing groups on the promoiety or by the proper choice of acyl groups to yield prodrugs with lower melting points.

Experimental Section

Chemistry. The following spectrometers were used: IR, Nicolet 5DX; ^{1}H NMR, Varian T-60; MS, Riber R_{10}-10 interfaced with a PDP-8A computer. Chemical shifts are reported in parts per million relative to tetramethylsilane as internal reference. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Reagents and solvents were purchased from common commercial suppliers and were used as received. Yields were not optimized.

1-Ethyl-6-fluoro-1,4-dihydro-4-oxo-7-[4′-[(chloromethoxy)carbonyl]-1′-piperazinyl]quinoline-3-carboxylic Acid (2a). Norfloxacin (3.2 g, 10 mmol) was suspended in chloroform (175 mL), and Proton Sponge (2.15 g, 10 mmol) was added to it. The suspension was cooled in an ice bath and chloromethyl chloroformate[10] (1.3 g, 10 mmol) was added. A clear solution was formed in 15 min. After stirring at room temperature for 16 h, the reaction mixture was washed with water and brine and evaporated to furnish a solid (3.3 g). It was crystallized from chloroform to give **2a** (2.45 g, 62%); mp 207–208 °C; ^{1}H NMR (DMSO-d_6) δ 1.45 (t, 3 H), 3.2–3.8 (m, 8 H), 4.61 (q, 2 H), 5.93 (s, 2 H), 7.21 (d, 1 H, $J = 8$ Hz), 7.90 (d, 1 H, $J = 14$ Hz), and 8.93 (s, 1 H). Anal. ($C_{18}H_{19}N_3ClFO_5$) C, H, N.

1-Ethyl-6-fluoro-1,4-dihydro-4-oxo-7-[4′-[(acetoxymethoxy)carbonyl]-1′-piperazinyl]quinolinecarboxylic Acid (3a). Norfloxacin chloromethyl carbamate (**2a**) (5.6 g, 14.1 mmol) was dissolved in dimethylformamide (50 mL) and heated with anhydrous sodium acetate (3.5 g, 42.6 mmol) for 20 h at 100–105 °C. The DMF was evaporated off in vacuum, and the residue was triturated with water (100 mL) and filtered. Crystallization of the resulting solid from chloroform–hexane gave pure **3a** (3.7 g, 60%): mp 248–250 °C; ^{1}H NMR ($CDCl_3$) δ 1.6 (t, 3 H), 2.15 (s, 3 H), 3.2–3.9 (m, 8 H), 4.38 (q, 2 H), 5.82 (s, 2 H), 6.86 (d, 1 H, $J = 7$ Hz), 7.99 (d, 1 H, $J = 13$ Hz) and 8.6 (s, 1 H); IR (KBr) ν 1762, 1635 cm^{-1}; MS *m/s* 435 (M^+), 391, 346, 320, 277, 233, 219.

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Anal. ($C_{20}H_{22}N_3FO_7 \cdot H_2O$) C, H, N.

1-Ethyl-6-fluoro-1,4-dihydro-4-oxo-7-[4'-[(1''-chloroethoxy)carbonyl]-1'-piperizinyl]quinoline-3-carboxylic Acid (2b). This compound was prepared in 65% yield from norfloxacin and α-chloroethyl chloroformate[11] as described for **2a**: mp 220 °C dec; ^1H NMR (CF_3COOH) δ 1.76 (d, 3 H), 1.83 (t, 3 H), 3.6–4.2 (m, 8 H), 4.85 (q, 2 H), 6.63 (q, 1 H), 7.36 (d, 1 H, J = 6 Hz), 8.25 (d, 1 H, J = 14 Hz), and 9.23 (s, 1 H). Anal. ($C_{19}H_{21}N_3ClFO_5$) C, H, N.

1-Ethyl-6-fluoro-1,4-dihydro-4-oxo-7-[4'-[(1''-acetoxyethoxy)carbonyl]-1'-piperzinyl]quinoline-3-carboxylic Acid (3b). The chloroethyl carbamate **2b** (3.0 g, 7.3 mmol) was suspended in acetic acid (150 mL) and stirred with mercuric acetate (3.2 g, 10 mmol) at room temperature for 72 h. The acetic acid was evaporated off and the residue dissolved in chloroform was washed with dilute sodium chloride solution. Removal of solvent gave a white solid (3.2 g) which was crystallized from chloroform–ethyl acetate to furnish pure **3b** (2.1 g, 64%): mp 215–217 °C; ^1H NMR ($CDCl_3$) δ 1.5 (d, 3 H), 1.55 (t, 3 H), 2.06 (s, 3 H), 3.2–3.9 (m, 8 H), 4.33 (q, 2 H), 6.66–7.0 (m, 2 H), 7.9 (d, 1 H, J = 12 Hz), and 8.6 (s, 1 H); MS m/e 449 (M^+); IR (KBr) ν 1735, 1715, 1627 cm^{-1}. Anal. ($C_{21}H_{24}N_3FO_7$) C, H, N, F.

Analytical Methods. A high-pressure liquid chromatographic assay was used to measure the physical properties and hydrolysis rates of the prodrugs. The chromatographic analysis was performed on a component system consisting of a Waters Associates Model 6000A solvent delivery system, a Kratos Spectraflo 773 variable-wavelength detector, and a Waters data module. A 20-μL injection of each sample and standard of similar concentration was made into the HPLC equipped with a 10-cm Spheri-5 RP-18 column (Brownlee Labs) and a similar 3-cm guard column at ambient temperature. The mobile phase was acetonitrile–tetrahydrofuran–water containing phosphoric acid (7 mL/L) and triethylamine (7 mL/L) in the ratio of 25:10:65 at a flow rate of 2.5 mL/min. The detector was set at 278 nm. The concentration was calculated from the areas of the sample and standard peaks and the known concentration of the standard by using an external standard procedure.

Microbiological Assay for Norfloxacin. The antibacterial activity in biological samples measured by a microbiological assay was used for quantitation of norfloxacin. The total bioactivity was determined in a standard disk diffusion assay followed by computer-assisted image analysis. *Klebsiella pneumoniae* MB 480 was used as the assay organism for serum samples (senstivity: 0.2 μg/mL) and *Bacillus subtilis* MB 964 for urine samples (sensitivity: 2.0 μg/mL).

Solubility. A suspension of the compound in the solvent was sonicated for 15 min and then kept gently rotating for 3 h at room temperature. The suspensions was filtered through a 0.2-μm filter. After appropriate dilution, 20 μL of the solution was injected into the HPLC.

Serum Hydrolysis. One milliliter of serum maintained at 37 °C was added to approximately 0.1 mg of prodrug dissolved in a small drop of dimethyl sulfoxide. The serum was placed in a temperature-controlled water bath at 37 °C. The disappearance of the prodrug was followed by direct injection of aliquots withdrawn at various intervals into an HPLC. Buffer hydrolysis was conducted similarly with pH 7.4 Sorensen's buffer instead of serum.

Antibacterial Activity in Vitro. The prodrug **3b** was compared with norfloxacin for activity against a panel of 21 aerobic bacteria. The prodrug was prepared at a concentration of 1.04 mg/mL in 0.62 M phosphoric acid. Subsequent serial dilutions were made in 0.5 M phosphoric acid and sterile water. Concentrations above 80 μg were suspensions. The solution of norfloxacin was prepared in 0.05 M phosphoric acid and diluted with sterile water. The drug dilutions were added to molten trypticase soy agar (TSA-BBL) to give final drug concentration of 128 μg or lower. When diluted with agar, **3b** was soluble at all concentrations except 128 μg/mL. The test cultures were first subcultures of TSA slants stored at −70 °C, incubated overnight in trypticase soy broth. The drug/agar plates were inoculated with cultures to result in a final inoculum of ca. 10^6 cfu/spot. Incubation was at 35 °C for 20 h. The minimum inhibitory concentration (MIC) was recorded as the lowest concentration showing no growth or less than 5 cfu/spot.

Measurement of Disulfiram-like Effect. Female CD-1 mice weighing approximately 20 g (from Charles River Laboratories) were orally administered 100 mg/kg of norfloxacin or prodrug on three occasions during a 24-h period (20 mice per group). Control animals received three subcutaneous injections of sterile 0.85% saline. Three hours after the third administration of the test compound, 1 mL of 7.0% ethanol was given by intraperitoneal injection. Blood samples (0.3 mL) were collected by heart puncture at 10, 20, 30, 40, 50, and 60 min following administration of ethanol and were mixed with 0.1 mL of 0.1 M sodium oxalate to prevent clotting. Samples from two to three mice were pooled at each time point and were centrifuged at 1500g for 5 min. The plasma (100 μL) was diluted with 4.9 mL of 0.9% saline and 100 μL of this solution was added to 2.6 mL of an enzyme reagent (Ethyl alcohol Stat-Pack, Calbiochem-Behring, San Diego). The mixture was incubated at 30 °C for 12 min, and the absorbance of the reaction mixture was measured at 340 nm. Ethanol concentration was calculated from the change in absorbance relative to absorbance of a saline blank. The plasma ethanol concentration (area under the curve for the 1-h test period) from control and test compound treated groups were compared and statistically analyzed by using the t-distribution test for unpaired data. Confidence limits of 95% ($p < 0.05$) were applied for significance. A compound which induced statistically significant increase in plasma ethanol concentration over the 1-h assay period was interpreted as causing DLE.