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Quinazolines and 1,4-Benzodiazepines LXXXIX: Haptens Useful in Benzodiazepine Immunoassay Development

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Abstract □ The syntheses of some 1,4-benzodiazepines potentially useful as haptens are reported. These compounds are related to chlordiazepoxide, diazepam, nitrazepam, clonazepam, and some of their metabolites. The chemistry reported here is intended to support specific immunoassay development for these drugs.

Keyphrases □ Benzodiazepines—chemical synthesis, potentially useful haptens □ Tranquilizers—benzodiazepines, various, chemical synthesis, potentially useful haptens □ Haptens—benzodiazepines, chemical synthesis

Immunoassay (1, 2) is a powerful method for measuring drug levels in biological fluids. The extensive clinical use (3, 4) and continued development (4, 5) of benzodiazepines as a drug class make immunoassays for these compounds desirable. This paper reports some benzodiazepine hapten[1] syntheses. Many haptens reported here have been utilized (6–8) in the immunoassay development for clinically important benzodiazepines and their metabolites.

The synthetic compounds are presented in three groups. Where possible, the numerical sequence reflects the synthetic sequence. The end-products, XII–XXIV, in Group A are related to diazepam and metabolites of diazepam and chlordiazepoxide. Compounds XXXI–XXXVI in Group B are related to chlordiazepoxide. Group C contains compounds derived from nitrazepam, clonazepam, and their metabolites.

The synthetic methods are known either in the general art or in the special benzodiazepine chemistry described elsewhere (5, 9, 10). 5-Chloro-3-(4-hydroxyphenyl)-2,1-benzisoxazole (I) (11) and the corresponding 3-(4-aminophenyl) analog II (12–14) were the crucial starting materials for compounds in Groups A and B. All other compounds reported are new except for the following: IV (11, 15), VII (16), XXIV (6), XXV (17), XXXVIII (18), XXXVII (19), and XL (20). The preparation of VII, XXIV, and XL, however, is reported for the first time.

[1] Haptens are defined (1) as antigens that are coupled to larger molecules, usually proteins, to provoke an antibody response.

EXPERIMENTAL[2]

3-(4-Aminophenyl)-5-chloroanthranil (II) (12–14)—To a mixture of 100 g (0.662 mole) of *o*-nitrobenzaldehyde and 160 g (1.05 mole) of phosphorus oxychloride was added dropwise, with stirring, 100 g (1.08 moles) of aniline while the temperature was kept below 30°. After 3 hr at room temperature, the solution was heated at 75° for 18 hr and at 90° for 3 hr. (The reaction becomes exothermic when heated.)

The mixture was cooled, 200 ml of ethanol and 200 ml of concentrated hydrochloric acid were added, and the solution was heated to reflux for 3 hr with stirring. On cooling, the precipitate was collected and washed with acetone, resuspended in dilute ammonium hydroxide for 1 hr, and collected again. After crystallization from ethanol, 70 g (86%) of II was obtained. Recrystallization from ethanol gave orange rods, mp 208–211°.

4′-(5-Chloro-3-anthranilyl)-2,2,2-trifluoroacetanilide (III)—A mixture of 70 g (0.286 mole) of II, 75 g (0.357 mole) of trifluoroacetic anhydride, and 1 liter of tetrahydrofuran was heated to reflux for 30 min. After concentration to a small volume, ether (300 ml) was added. The solution was filtered to give 60 g of product and then was concentrated to give an additional 5 g. The filtrate was evaporated, and the residue was stirred with cold aqueous potassium carbonate solution and filtered.

The solid thus collected was dissolved in tetrahydrofuran and treated with charcoal. After filtration and concentration to a small volume, ether was added. A 10-g precipitate of III was collected to give a total yield of 75 g (77%). A sample recrystallized from tetrahydrofuran–hexane gave pale-yellow rods, mp 251–254°; IR (KBr): 3310 (NH) and 1705 (C=O) cm^{-1}.

Anal.—Calc. for $C_{15}H_8ClF_3N_2O_2$: C, 52.88; H, 2.37; N, 8.22. Found: C, 52.85; H, 2.32; N, 8.27.

2-Amino-5-chloro-4′-hydroxybenzophenone (IV) (11, 15)—To a solution of 83.7 g (0.34 mole) of 5-chloro-3-(4-hydroxyphenyl)-2,1-benzisoxazole (I) (11) in 1500 ml of acetic acid was added 45 g of iron filings. The mixture was stirred and heated on the steam bath for 20 min. Every 30 min, an additional 20 g of iron filings and 100 ml of water were added for 2.5 hr. After 30 min more, the reaction mixture was filtered while hot.

The collected precipitate was heated with acetic acid and filtered. The combined filtrates were diluted with ice water to precipitate 39.8 g (47%) of IV, mp 170–175°. Recrystallization from methanol–water gave yellow rods, mp 173–178°.

2-Amino -5- chloro-4′-(2,2,2-trifluoroacetamido)benzophenone

[2] Structural assignments are based on unambiguous spectral data and on related structures published elsewhere [R. I. Fryer, J. Blount, E. Reeder, E. J. Trybulski, and A. Walser, *J. Org. Chem.*, **43**, 4480 (1978)]. Only selected spectral data are presented here.

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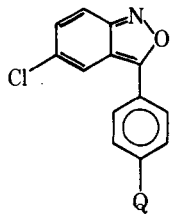
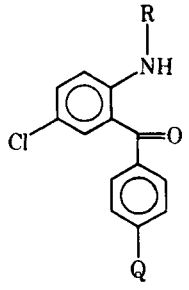
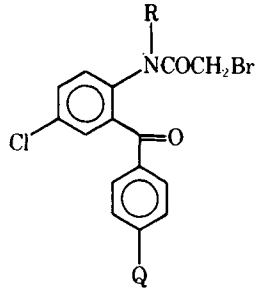
Structural Group A



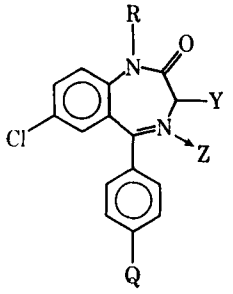
I: Q = OH
II: Q = NH_2
III: Q = $NHCOCF_3$



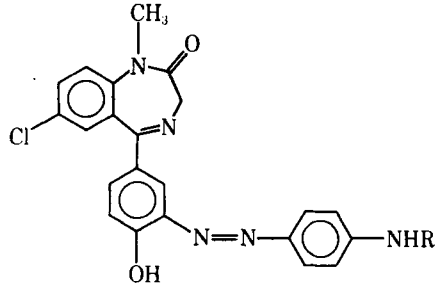
IV: Q = OH, R = H
V: Q = $NHCOCF_3$, R = H
VI: Q = $OCH_2CO_2CH_3$, R = H
VII: Q = OH, R = CH_3



VIII: Q = OH, R = H
IX: Q = $NHCOCF_3$, R = H
X: Q = $OCH_2CO_2CH_3$, R = H
XI: Q = OH, R = CH_3



XII: Q = OH, R = H, Y = H
XIII: Q = $NHCOCF_3$, R = H, Y = H
XIV: Q = $OCH_2CO_2CH_3$, R = H, Y = H
XV: Q = OH, R = CH_3, Y = H
XVI: Q = OCH_3, R = CH_3, Y = H
XVII: Q = OCH_2CONH_2, R = H, Y = H
XVIII: Q = $OCH_2CONHNH_2$, R = H, Y = H
XIX: Q = $NHCOCF_3$, R = H, Y = H, Z = O
XX: Q = NH_2, R = H, Y = H, Z = O
XXI: Q = $NHCOCF_3$, R = H, Y = $OCOCH_3$
XXII: Q = NH_2, R = H, Y = OH



XXIII: R = $COCH_3$
XXIV: R = H

synthetic sequences

I, IV, VI, X, XIV–XVII, and XVIII
II, III, V, IX, XIII, XIX, XX, XXI, and XXII
IV, VIII, XII, and XV–XVI
XV, VII, XI, and XV
XV, XXIII, and XXIV

(V)—Following the procedure described for IV, III afforded V in an 88% yield. A sample was recrystallized from methanol to give yellow needles, which crystallized as a methanolate, mp 65–75°, reset 148–149°; IR ($CHCl_3$): 3500, 3420 (NH_2), 1740, and 1634 (2 C=O) cm^{-1}.

Anal.—Calc. for $C_{15}H_{10}ClF_3N_2O_2 \cdot CH_3OH$: C, 51.28; H, 3.77; N, 7.48. Found: C, 51.19; H, 3.71; N, 7.35.

2-[4-(2-Amino-5-chlorobenzoyl)phenoxy]acetic Acid (VI)—A solution of 30 g (0.12 mole) of IV in 75 ml of dry dimethylformamide under nitrogen was cooled in an ice bath, and 5.5 g (1.3 moles) of 57% sodium hydride in mineral oil was added with stirring. After 20 min, 24.5 g (0.16 mole) of methyl bromoacetate was added dropwise. The reaction mixture was allowed to stand at room temperature for 18 hr and then was poured into ice water. The precipitate was collected, dried, and recrystallized from methylene chloride–hexane to give VI as yellow needles (25.1 g, 65%), mp 144–147°; IR ($CHCl_3$): 3500, 3375 (NH_2), 1760, and 1638 (2 C=O) cm^{-1}.

Anal.—Calc. for $C_{16}H_{14}ClNO_4$: C, 60.10; H, 4.41; N, 4.38; Found: C, 60.03; H, 4.38; N, 4.28.

5-Chloro-4'-hydroxy-2-methylaminobenzophenone (VII) (16)—The 4.5 g of the XV–XVI mixture described under XV was heated on a steam bath for 2 hr with 70 ml of 48% hydrobromic acid and then at reflux for 1 hr. The reaction mixture was cooled, made basic with 3 *N* NaOH, and washed with 100 ml of ether. The water layer was acidified with hydrochloric acid and extracted with methylene chloride (2 × 100 ml). The methylene chloride extract was dried and evaporated. The residue was crystallized and recrystallized from aqueous methanol to give 2.9 g of VII as yellow rods, mp 163–164°.

Anal.—Calc. for $C_{14}H_{12}ClNO_2$: C, 64.25; H, 4.62; Cl, 13.55. Found: C, 64.03; H, 4.37; Cl, 13.50.

2-Bromo-4'-chloro-2'-(4-hydroxybenzoyl)acetanilide (VIII)[3]—A solution of 41 g (0.165 mole) of IV in 800 ml of ether and 200 ml of water was cooled to 5°. The mixture was stirred while 20 ml (0.277 mole) of bromoacetyl bromide and a 20% solution of sodium carbonate were added alternately, keeping the solution slightly basic. After 20 min, the reaction mixture was filtered to collect 60 g (~100%) of VIII, mp 201–202°. Recrystallization from ethanol gave colorless prisms, mp 201–203°.

Anal.—Calc. for $C_{15}H_{11}BrClNO_3$: C, 48.87; H, 3.01, total halides, 31.30. Found: C, 49.17; H, 3.34; total halides, 30.84.

2-(Bromoacetamido) - 5-chloro-4'-(2,2,2-trifluoroacetamido)-benzophenone (IX)—Following the procedure described for VIII but with sodium bicarbonate instead of sodium carbonate, V afforded IX in a 96% yield. Recrystallization of IX from methylene chloride–methanol–petroleum ether gave off-white rods, mp 198–201°; IR (KBr): 1750, 1663, and 1640 (3 C=O) cm^{-1}.

Anal.—Calc. for $C_{17}H_{11}BrClF_3N_2O_3$: C, 44.03; H, 2.39; N, 6.04. Found: C, 44.22; H, 2.56; N, 6.03.

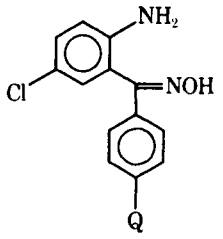
2-Bromo - 4'-chloro-2'-(4-methoxycarbonylmethoxybenzoyl)-acetanilide (X)—To a stirring solution of 16 g (0.05 mole) of VI in 350 ml of methylene chloride was added 6.3 g (0.075 mole) of sodium bicarbonate, followed dropwise by 12.1 g (0.06 mole) of bromoacetyl bromide. After 18 hr, 125 ml of water was added; the organic layer was separated, dried, and evaporated. Crystallization of the residue from methylene chloride–methanol afforded 19 g (86%) of X as colorless needles, mp 124–126°; IR ($CHCl_3$): 3300 (NH), 1760, 1682, and 1640 (3 C=O) cm^{-1}.

Anal.—Calc. for $C_{18}H_{15}BrClNO_5$: C, 49.06; H, 3.43; N, 3.18. Found: C, 48.96; H, 3.31; N, 3.05.

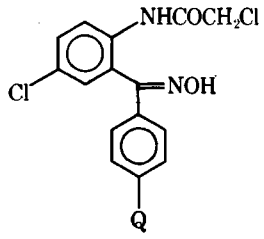
2-Bromo-4'-chloro-2'-(*p*-hydroxybenzoyl)-*N*-methylacetanilide (XI)[3]—To a solution of 2.9 g (0.011 mole) of VII in 100 ml of methylene chloride were added 2.4 g (0.012 mole) of bromoacetyl bromide, 1 g (0.012 mole) of sodium bicarbonate, and 25 ml of water. The reaction was stirred for 18 hr. The water layer was separated and extracted with 50 ml of methylene chloride. The combined organic layers were dried and evaporated. Crystallization of the residue from methanol and recrystallization from acetone–hexane gave 2.2 g (52%) of XI as colorless prisms, mp 187–189°.

[3] This compound was first prepared by Dr. A. Stempel of these laboratories.

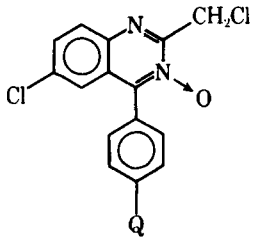
Structural Group B



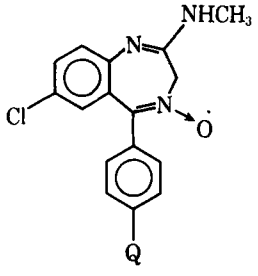
XXV: Q = OH
XXVI: Q = NHCOCF$_3$



XXVII: Q = OH
XXVIII: Q = NHCOCF$_3$



XXIX: Q = OH
XXX: Q = NHCOCF$_3$



XXXI: Q = OH
XXXII: Q = NH_2
XXXIII: Q = $OCH_2CO_2CH_3$
XXXIV: Q = $OCH_2CONHNH_2$
XXXV: Q = $O(CH_2)_3NHCO_2CH_2C_6H_5$
XXXVI: Q = $O(CH_2)_3NH_2$

synthetic sequences

IV, XXV, XXVII, XXIX, XXXI, XXXIII, and XXXIV
XXXI, XXXV, and XXXVI
V, XXVI, XXVIII, XXX, and XXXII

Anal.—Calc. for $C_{16}H_{13}BrClNO_3$: C, 50.22; H, 3.42. Found: C, 50.09; H, 3.73.

7-Chloro - 1,3-dihydro-5-(4-hydroxyphenyl)-2*H*-1,4-benzodiazepin-2-one (XII)—To 350 ml of liquid ammonia was added 60 g (0.163 mole) of VIII. After refluxing for 3 hr, the ammonia was allowed to evaporate. The residue was heated to reflux in 400 ml of ethanol for 3 hr. Ethanol was evaporated, and 800 ml of acetone was added. This suspension was heated and filtered to remove inorganic salts. Concentration of the acetone filtrate gave 31.5 g (67%) of XII, mp 271–272°.

Anal.—Calc. for $C_{15}H_{11}ClN_2O_2$: C, 62.84; H, 3.87; N, 9.77. Found: C, 62.52; H, 4.13; N, 9.92.

7-Chloro - 1,3-dihydro-5-[4-(2,2,2-trifluoroacetamidophenyl)]-2*H*-1,4-benzodiazepin-2-one (XIII)—A solution of 90 g (0.194 mole) of IX in a mixture of 900 ml of tetrahydrofuran and 900 ml of ethyl acetate was stirred in an ice bath and saturated with a stream of ammonia gas over 1 hr. The mixture was allowed to stand overnight at room temperature and then filtered. The filtrate was evaporated, and the residue was partitioned between methylene chloride and water.

The product mixture from evaporation of the methylene chloride layer was heated to reflux in 400 ml of ethanol for 3 hr. The solution was concentrated, cooled, and filtered to give 32 g (43%) of XIII. A sample was recrystallized from methylene chloride–ether to give colorless prisms, which reset on heating to form needles, mp 273–276°; IR (KBr): 1730 and 1678 (2 C=O) cm^{-1}.

Anal.—Calc. for $C_{17}H_{11}ClF_3N_3O_2$: C, 53.49; H, 2.90; N, 11.00. Found: C, 53.63; H, 2.92; N, 10.85.

7-Chloro - 1,3-dihydro-5-(4-methoxycarbonylmethoxyphenyl)-2*H*-1,4-benzodiazepin-2-one (XIV) and 5-(4-Carbamoylmethoxyphenyl) -7- chloro -1,3- dihydro-2*H*-1,4-benzodiazepin -2- one (XVII)—A solution of 14.3 g (32.5 mmoles) of X in 100 ml of dioxane and 150 ml of methanol was added to 150 ml of a saturated solution of ammonia in methanol. After 5 hr, the reaction mixture was neutralized with acetic acid and evaporated to dryness. The residue was partitioned between 150 ml of methylene chloride and 100 ml of dilute ammonium hydroxide. After a few minutes, filtration gave 4.5 g (41%) of XVII. A sample was recrystallized from chloroform–ethanol to give off-white prisms, mp 272–275°; IR (KBr): 3440, 3210 (NH_2), and 1695 (2 C=O) cm^{-1}.

Anal.—Calc. for $C_{17}H_{14}ClN_3O_3$: C, 59.40; H, 4.11; N, 12.22. Found: C, 59.06; H, 3.99; N, 11.89.

The methylene chloride layer was separated, dried, and filtered through activated magnesium silicate. Elution with ether and then ethyl acetate and recrystallization of these two fractions gave 2.2 g (19%) of XIV as pale-yellow prisms, mp 235–243°; IR (KBr): 1762 and 1680 (2 C=O) cm^{-1}.

Anal.—Calc. for $C_{18}H_{15}ClN_2O_4$: C, 60.26; H, 4.21; N, 7.81. Found: C, 60.39; H, 4.36; N, 7.83.

7-Chloro - 1,3-dihydro-5-(4-hydroxyphenyl)-1-methyl-2*H*-1,4-benzodiazepin-2-one (XV)[3] and 7-Chloro-1,3-dihydro-5-(4-methoxyphenyl)-1-methyl-2*H*-1,4-benzodiazepin-2-one (XVI)—To a solution of 6.5 g (0.022 mole) of XII in 40 ml of dimethylformamide was added 11.6 ml (0.055 mole) of 4.74 *M* sodium methoxide in methanol. After 90 min, the mixture was cooled in an ice bath and 9.4 g (0.066 mole) of methyl iodide was added dropwise with stirring. After 18 hr at room temperature, the reaction mixture was acidified with acetic acid and partitioned between 100 ml of methylene chloride and 100 ml of water.

The water layer was extracted once more with methylene chloride. The combined extracts were evaporated to dryness. The residual product mixture was separated on an activated magnesium silicate column. The column was eluted with methylene chloride, ether, and then ethyl acetate. The ethyl acetate fraction was evaporated and crystallized from methanol to give 0.40 g (6%) of XV as colorless prisms, mp 255–259°.

Anal.—Calc. for $C_{16}H_{13}ClN_2O_2$: C, 63.90; H, 4.35; N, 9.31. Found: C, 63.89; H, 4.29; N, 9.46.

The filtrates and the other fractions from the column were combined and evaporated to give 4.5 g of a XV–XVI mixture.

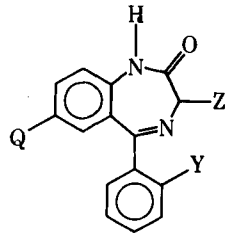
Compound XV from XI—A solution of 2.2 g (5.7 mmoles) of XI in a mixture of 90 ml of methylene chloride and 20 ml of tetrahydrofuran was added to 175 ml of liquid ammonia. After 18 hr, 100 ml of methylene chloride was added and the reaction mixture was dried and evaporated to dryness. The residual oil was crystallized from methanol to give 1.45 g (85%) of XV as colorless prisms, mp 254–260°.

7-Chloro -1,3- dihydro -5- (4-hydrazinocarbonylmethoxyphenyl)-2*H*-1,4-benzodiazepin-2-one Hemitetrahydrofuranate (XVIII)—To a solution of 0.50 g (1.3 mmoles) of XIV in a mixture of 25 ml of methanol and 30 ml of tetrahydrofuran was added 5 ml of hydrazine hydrate (85% in water). After 1 hr, the solution was evaporated under reduced pressure and 25 ml of methylene chloride and 20 ml of water were added. After standing overnight, the solution was filtered and the solid was recrystallized from tetrahydrofuran–hexane to give 0.30 g (59%) of colorless prisms, mp 180–185°, resets 210–220°; IR (KBr): 1700 and 1680 (C=O) cm^{-1}.

Anal.—Calc. for $C_{17}H_{15}ClN_4O_3 \cdot 0.5\ C_4H_8O$: C, 57.80; H, 4.88; N, 14.19. Found: C, 57.59; H, 4.79; N, 14.10.

7-Chloro - 1,3-dihydro-5-[4-(2,2,2-trifluoroacetamido)phenyl]-2*H*-1,4-benzodiazepin-2-one 4-Oxide (XIX)—To a mixture of 31.5 g (0.0825 mole) of XIII in 1.8 liters of methylene chloride was added, with stirring, 30 g (0.147 mole) of 85% *m*-chloroperbenzoic acid. After 18 hr, 500 ml of cold water was added and the solution was made basic with ammonium hydroxide. The crystalline XIX was collected on a filter. The

Structural Group C



XXXVII: Q = NO_2, Y = H, Z = OH
XXXVIII: Q = NO_2, Y = H, Z = $OCOCH_2CH_2CO_2H$
XXXIX: Q = NH_2, Y = H, Z = $OCOCH_2CH_2CO_2H$
XL: Q = NO_2, Y = Cl, Z = OH
XLI: Q = NO_2, Y = Cl, Z = $OCOCH_2CH_2CO_2H$
XLII: Q = NH_2, Y = Cl, Z = $OCOCH_2CH_2CO_2H$

synthetic sequences

XXXVII, XXXVIII, XXXIX; XL, XLI, and XLII

methylene chloride was concentrated for a second crop, giving a total yield of 21.5 g (66%). Recrystallization from methylene chloride–methanol gave off-white rods, mp 295–297° dec.; IR (KBr): 1698 (broad 2 C=O) cm^{-1}.

Anal.—Calc. for $C_{17}H_{11}ClF_3N_3O_3$: C, 51.33; H, 2.79; N, 10.57. Found: C, 51.32; H, 2.57; N, 10.44.

5-(4-Aminophenyl) - 7-chloro-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one 4-Oxide (XX)—To a solution of 1.0 g (2.51 mmoles) of XIX in 25 ml of methanol was added 20 ml (20 mmoles) of 1 *N* NaOH. After 15 min, 20 ml of water was added. The reaction mixture was allowed to stand for 3 hr and was then acidified with acetic acid; methanol was removed *in vacuo*. The mixture was made basic with ammonium hydroxide and filtered. Recrystallization from tetrahydrofuran–methanol gave 0.30 g (39%) of pale-yellow rods, mp 256–258°; IR (KBr): 3415, 3350, 3245 (NH_2, NH), and 1700 (C=O) cm^{-1}; mass spectrum: *m/e* 301 (M^+).

Anal.—Calc. for $C_{15}H_{12}ClN_3O_2$: C, 59.71; H, 4.10; N, 13.93. Found: C, 59.52; H, 4.02; N, 13.73.

3-Acetoxy - 7-chloro-1,3-dihydro-5-[4-(2,2,2-trifluoroacetamido)phenyl]-2*H*-1,4-benzodiazepin-2-one (XXI)—A mixture of 14 g (0.0352 mole) of XIX in 200 ml of tetrahydrofuran and 400 ml of acetic anhydride was heated on the steam bath for 2 hr. The mixture was evaporated under reduced pressure. Crystallization of the residue from methanol afforded 8.3 g of XXI. A second crop of 5.3 g was obtained from ether–hexane, giving a total yield of 13.5 g (87.7%).

Recrystallization from a mixture of tetrahydrofuran, methylene chloride, and hexane gave white prisms, mp 257–262°; IR (KBr): 1740, 1720, and 1688 (3 C=O) cm^{-1}; mass spectrum: *m/e* 439 (M^+).

Anal.—Calc. for $C_{19}H_{13}ClF_3N_3O_4$: C, 51.89; H, 2.98; N, 9.56. Found: C, 51.36; H, 2.99; N, 9.23.

5-(4-Aminophenyl) -7- chloro-1,3-dihydro -3- hydroxy-2*H*-1,4-benzodiazepin-2-one (XXII)—To a solution of 0.60 g (1.3 mmoles) of XXI in 25 ml of methanol was added 20 ml of 1 *N* NaOH, followed by the addition of 20 ml of water after 1 hr. The reaction mixture was filtered after 3 hr. The filtrate was acidified with acetic acid and then made slightly basic with ammonium hydroxide. It was extracted with methylene chloride (2 × 100 ml), which was dried and concentrated to a small volume.

Compound XXII, which crystallized, was collected. Recrystallization from tetrahydrofuran–methanol afforded 0.20 g (50%) of yellow prisms, mp >350°; IR (KBr): 3455, 3375 (NH_2, NH), 3225, 3160 (OH), 1713, and 1698 (split C=O) cm^{-1}; NMR (dimethyl sulfoxide-d_6): δ 10.60 (s, 1H, NH), 6.02 (d, 1H, OH), 5.58 (s, 2H, NH_2), and 4.64 (d, 1H, CH); mass spectrum: *m/e* 301 (M^+).

Anal.—Calc. for $C_{15}H_{12}ClN_3O_2$: C, 59.71; H, 4.00; N, 13.93. Found: C, 59.33; H, 4.46; N, 14.03.

5-[3-(4-Acetamidophenylazo)-4 - hydroxyphenyl]-7-chloro-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one (XXIII)—To a mixture of 1.2 ml (15 mmoles) of concentrated hydrochloric acid and 5 g of ice was added 0.90 g (6 mmoles) of *p*-aminoacetanilide, followed by the dropwise addition of a solution of 0.45 g (6.2 mmoles) of sodium nitrite in 5 ml of water. To a separate solution of 0.5 g (1.67 mmoles) of XV in 25 ml of tetrahydrofuran were added 1 ml (12 mmoles) of 1 *N* NaOH and 20 g of ice. The diazonium salt solution was added rapidly with stirring to the basic solution.

The mixture was refrigerated at 5° for 2.5 days. The precipitate was collected and recrystallized from methylene chloride–methanol to give 0.60 g (70%) of XXIII as red prisms. Recrystallization from methylene chloride–petroleum ether gave yellow rods, mp 276–278°; IR (KBr): broad 1700 (2 C=O) cm^{-1}.

Anal.—Calc. for $C_{24}H_{20}ClN_5O_3$: C, 62.41; H, 4.36; N, 15.16. Found: C, 62.24; H, 4.31; N, 14.97.

5-[3-(4-Aminophenylazo)-4-hydroxyphenyl]-7-chloro-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one (XXIV)—A solution of 0.60 g (1.3 mmoles) of XXIII in 50 ml of methanol and 10 ml of concentrated hydrochloric acid was heated for 10 min on a steam bath. After 2 hr at room temperature, the methanol was evaporated. The solution was made basic with ammonium hydroxide and extracted with 50 ml of methylene chloride. The organic layer was dried and evaporated. Crystallization of the residue from methanol and recrystallization from methylene chloride–methanol gave 0.25 g (45%) of brown prisms, mp 262–268°; IR (KBr): 3450, 3350 (NH_2), and 1682 (C=O) cm^{-1}.

Anal.—Calc. for $C_{22}H_{18}ClN_5O_2$: C, 62.93; H, 4.32; N, 16.68. Found: C, 62.96; H, 4.49; N, 16.47.

2-Amino-5-chloro-4'-hydroxybenzophenone Oxime (XXV)—The preparation of XXV from IV was described in Ref. 17.

2-Amino-5-chloro-4'-(2,2,2-trifluoroacetamido)benzophenone Oxime (XXVI)—A mixture of 3.1 g (8.27 mmoles) of V and 2.5 g (36 mmoles) of hydroxylamine hydrochloride in 100 ml of ethanol was heated under reflux for 66 hr and then concentrated. The residue was partitioned between 100 ml of methylene chloride and 75 ml of water. The organic layer was concentrated, cooled, and filtered to give 1.0 g of XXVI. An additional 0.6 g was obtained from the filtrates for a yield of 1.6 g (53%). Recrystallization from methylene chloride–hexane gave off-white prisms, mp 179–183°; IR (KBr): 3440, 3340, and 3280 (NH, NH_2, OH) cm^{-1}.

Anal.—Calc. for $C_{15}H_{11}ClF_3N_3O_2$: C, 50.37; H, 3.10; N, 11.75. Found: C, 50.31; H, 2.93; N, 11.72.

2,4' - Dichloro-2' - (4-hydroxybenzoyl)acetanilide Oxide (XXVII)—See Ref. 17.

2,4' - Dichloro-2'-(4-trifluoroacetamido-α-hydroxyiminobenzyl)acetanilide (XXVIII)—To a solution of 1.6 g (4.4 mmoles) of XXVI in 50 ml of ether was added 20 ml of water. The mixture was cooled to 5°, and 0.54 g (4.8 mmoles) of chloroacetyl chloride and a 5% solution of sodium bicarbonate were added alternately, keeping the solution slightly basic. After 1 hr, the ether layer was separated and the water layer was extracted with 50 ml of ether.

The combined ether layers were dried and evaporated. The residue was crystallized from methylene chloride–hexane to give 1.8 g (95%) of colorless prisms. A sample recrystallized for analysis from ether–methylene chloride melted at 210–214°; IR (KBr): 3420, 3300, 3200 (NH, OH), and 1700 (2 C=O) cm^{-1}.

Anal.—Calc. for $C_{17}H_{12}Cl_2F_3N_3O_3$: C, 47.03; H, 2.78; N, 9.58. Found: C, 46.94; H, 2.70; N, 9.48.

6-Chloro-2-chloromethyl-4-(4-hydroxyphenyl)quinazoline 3-Oxide (XXIX)[3]—A solution of 40.5 g (0.12 mole) of XXVII in 750 ml of dry dioxane and 40.5 ml (0.329 mole) of boron trifluoride etherate was heated to reflux. After 5 hr, it was cooled and 200 ml of water was added. The dioxane was removed by distillation, and the reaction mixture was made basic with a 5% sodium bicarbonate solution. The solid was collected, heated in methanol at reflux for a few minutes, cooled, and collected to give, in two crops, 21.3 g (56%) of XXIX. An analytical sample recrystallized from methanol melted at 226–227° dec.

Anal.—Calc. for $C_{15}H_{10}Cl_2N_2O_2$: C, 56.10; H, 3.14; N, 8.72. Found: C, 56.26; H, 2.81; N, 9.02.

6-Chloro - 2-chloromethyl-4-[4-(2,2,2-trifluoroacetamido)phenyl]quinazoline 3-Oxide (XXX)—Following the procedure described for XXIX, XXVIII was isolated by methylene chloride extraction. The residue from evaporation was crystallized from methanol and recrystallized from methylene chloride–methanol to give 1.0 g (62%) of pale-yellow rods, mp 244–248°. A good elemental analysis could not be obtained because of solvents in the crystals; IR (KBr): 1730 (C=O) cm^{-1}; mass spectrum: *m/e* 415 (M^+).

7-Chloro - 5-(4-hydroxyphenyl)-2-methylamino-3*H*-1,4-benzodiazepine 4-Oxide (XXXI)[3]—To a saturated methylamine solution in 600 ml of methanol in an ice bath was added 19.2 g (0.60 mole) of XXIX. The solution was stirred for 7 hr in an ice bath and for 10 hr at room temperature and then was evaporated under reduced pressure. The residue was acidified with dilute acetic acid, stirred with ether, and filtered to give 19 g of product. Recrystallization for analysis from ethyl acetate gave colorless plates, mp 278–279° dec.

Anal.—Calc. for $C_{16}H_{14}ClN_3O_2$: C, 60.86; H, 4.47; N, 13.30. Found: C, 61.23; H, 4.74; N, 13.14.

5-(4-Aminophenyl)-7 - chloro-2-methylamino-3*H*-1,4-benzodiazepine 4-Oxide (XXXII)—To 100 ml of methanol saturated with methylamine in an ice bath was added 1.6 g (3.8 mmoles) of XXX. After 18 hr at room temperature, the solution was evaporated to dryness. Crystallization of the residue from methanol followed by recrystallization from methylene chloride–methanol gave yellow prisms, mp 180–185°, reset 288–290°.

The filtrates were partitioned between 1 *N* HCl and ether. The acid layer was made basic with sodium hydroxide and filtered. The solids collected were recrystallized from methylene chloride–methanol to give 0.60 g (50%) of XXXII. A good elemental analysis was not obtained due to occlusion of solvents in the crystal; IR (KBr): 3250 (broad) and 1625 cm^{-1}; mass spectrum: *m/e* 314 (M^+).

7-Chloro - 5-(*p*-methoxycarbonylmethoxyphenyl)-2-methylamino-3*H*-1,4-benzodiazepine 4-Oxide (XXXIII)—A solution of 5.00 g (0.0158 mole) of XXXI in 25 ml of dry dimethylformamide under nitrogen was stirred and cooled in an ice bath. To the reaction was added 0.75 g (0.0174 mole) of 57% sodium hydride in mineral oil; after 45 min, 3.0 g (0.205 mole) of methyl bromoacetate was added dropwise. After 64 hr at room temperature, the mixture was poured into ice water.

The precipitate was collected and dissolved in 150 ml of methylene chloride, which was washed with water, dried, and evaporated. Crystal-

lization from methylene chloride–hexane gave 3.6 g (59%) of colorless prisms, mp 106–110°; IR ($CHCl_3$): 3460 (NH) and 1760 (C=O) cm^{-1}. An additional 1.6 g of crude XXXIII was obtained from the filtrates.

Anal.—Calc. for $C_{19}H_{18}ClN_3O_4$: C, 58.84; H, 4.68; N, 10.84. Found: C, 58.61; H, 4.56; N, 10.96.

7-Chloro - 5-(4-hydrazinocarbonylmethoxyphenyl)-2-methylamino-3*H*-1,4-benzodiazepine 4-Oxide (XXXIV)—A solution of 1.0 g (2.6 mmoles) of XXXIII in 125 ml of methanol was treated with 6 ml of 85% hydrazine hydrate. After stirring for 2 hr, the solution was concentrated to a small volume under reduced pressure and cooled. The precipitate was collected, triturated with ethanol, filtered, and recrystallized from dimethylformamide–methanol–water to give 0.90 g (90%) of colorless prisms, mp 254–256°; IR (KBr): 3300, 3240 (NH, NH_2), 1668 (C=O), 1635 (C=N), and 1250 (ether) cm^{-1}.

Anal.—Calc. for $C_{18}H_{18}ClN_5O_3$: C, 55.75; H, 4.68; N, 18.06. Found: C, 55.62; H, 4.58; N, 18.28.

7-Chloro -2- methylamino-5-[4-[(3-benzyloxycarbonylamino)-propoxy]phenyl]-3*H*-1,4-benzodiazepine 4-Oxide (XXXV)—A solution of 0.50 g (1.58 mmoles) of XXXI in 5 ml of dry dimethylformamide under nitrogen was treated with 0.080 g (1.9 mmoles) of 54.7% sodium hydride in mineral oil. After 20 min, 0.54 g (2 mmoles) of *N*-carbobenzyloxy-3-bromopropylamine and 0.10 g (0.29 mmole) of tetrabutylammonium hydrogen sulfate were added with stirring. After 18 hr, the reaction mixture was stirred with ice water, which was then decanted.

The remaining oil was dissolved in 25 ml of methylene chloride, washed with 15 ml of water, dried, and filtered over a pad of activated magnesium silicate. The pad was eluted with methylene chloride, ether, and then ethyl acetate. The ethyl acetate fraction was evaporated and purified by preparative TLC using five silica gel plates (20 cm × 20 cm × 1.5 mm), which were developed in benzene–acetone (1:1). The material having R_f 0.4–0.5 was eluted from the silica gel with methanol. Crystallization from methylene chloride–ether gave 0.15 g (19%) of colorless prisms, mp 103–106°; IR ($CHCl_3$): 3460 (NH) and 1720 (C=O) cm^{-1}; mass spectrum: *m/e* 506 (M^+).

Anal.—Calc. for $C_{27}H_{27}ClN_4O_4$: C, 63.97; H, 5.37; N, 11.05. Found: C, 63.87; H, 5.42; N, 10.80.

7-Chloro - 2-methylamino-5-[4-(3-amino)propoxy]phenyl-3*H*-1,4-benzodiazepine 4-Oxide Dihydrochloride (XXXVI)—To a stirring solution of 4 ml of 30% hydrogen bromide in acetic acid was added 0.10 g (0.197 mmole) of XXXV. After 40 min, 20 ml of ether was added. The precipitate was collected and partitioned between 30 ml of methylene chloride and 10 ml of dilute ammonium hydroxide. The organic layer was dried and evaporated, and excess ethanolic hydrogen chloride was added. After concentration, the oil was crystallized from isopropanol and recrystallized from a mixture of methanol and isopropanol to give 70 mg (80%) of colorless prisms, mp 211–215° dec.; IR (KBr): 3400 (broad NH, NH_2) and 1688 (C=O) cm^{-1}.

Anal.—Calc. for $C_{19}H_{21}ClN_4O_2 \cdot 2HCl$: C, 51.19; H, 5.20; N, 12.57. Found: C, 50.85; H, 5.49; N, 12.24.

1,3-Dihydro-3-hemisuccinyloxy - 7-nitro-5-phenyl-2*H*-1,4-benzodiazepin-2-one (XXXVIII) (18)—To a suspension of 6.60 g (22 mmoles) of 1,3-dihydro-3-hydroxy-7-nitro-5-phenyl-2*H*-1,4-benzodiazepin-2-one (XXXVII) (19) in 400 ml of tetrahydrofuran (dried by passing over a bed of activated alumina) was added 1.2 g of a 50% suspension of sodium hydride in mineral oil (27 mmoles hydride). After stirring for 0.5 hr at room temperature under a nitrogen atmosphere, a clear solution formed. Succinic anhydride (3.35 g, 33 mmoles) was added in one portion, and stirring was continued. After 1 hr, tetrahydrofuran was evaporated *in vacuo*.

The residual mixture was slurried with 200 ml of water, acidified with acetic acid, and extracted with 300 ml of methylene chloride. The acidification and extraction were performed rapidly enough so that the product did not crystallize out of solution. After drying over anhydrous sodium sulfate, the solution was concentrated to half of its volume. Hexane addition afforded 8.3 g of XXXVIII, mp 187–190° dec. After recrystallization from tetrahydrofuran–methylene chloride–hexane, 5.6 g (64%) of colorless needles was obtained, mp 183–185° dec.

An analytical sample obtained from recrystallization with methylene chloride–hexane melted at 187–190° dec.; IR (KBr): unresolved carbonyl bands at 1690–1760 cm^{-1}; UV λ_{max} (2-propanol): 219 (ε 24,500), 264 (17,100), and 305 (10,900) nm; NMR (dimethyl sulfoxide-d_6): δ 2.51–2.65 (m, 2, CH_2), 2.69–2.83 (m, 2, CH_2), 5.89 (s, 1, 3-H), 7.45–7.62 (m, 6, C_6H_5 and 9-H), 8.06 (d, *J* = 2.5 Hz, 1,6-H), and 8.48 (q, *J* = 2.5 and 9.0 Hz, 1, 8-H) ppm.

Anal.—Calc. for $C_{19}H_{15}N_3O_7$: C, 57.43; H, 3.81; N, 10.58. Found: C, 57.47; H, 3.87; N, 10.30.

7-Amino-1,3-dihydro-3-hemisuccinyloxy-5-phenyl-2*H*-1,4-benzodiazepin-2-one (XXXIX)—A solution of 397 mg (1.0 mmole) of XXXVIII in 25 ml of tetrahydrofuran containing 500 mg of Raney nickel was hydrogenated under a hydrogen atmosphere at room temperature for 2 hr. Removal of the catalyst and solvent followed by recyrstallizations from tetrahydrofuran–methylene chloride–hexane afforded 175 mg (48%) of XXXIX as yellow microprisms, melting point indefinite, gradually shrinking above 190°; UV λ_{max} (2-propanol): 243 (ε 31,100) and 354 (2700) nm; NMR (dimethylsulfoxide-d_6): δ 6.44 (d, *J* = 2.5 Hz, 1, 6-H), 6.80 (dd, *J* = 2.5 and 9.0 Hz, 1,8-H), and 6.94 (d, *J* = 9.0 Hz, 1,9-H) ppm.

Anal.—Calc. for $C_{19}H_{17}N_3O_5$: C, 62.12; H, 4.66; N, 11.44. Found: C, 62.20; H, 4.96; N, 11.17.

5-(2-Chlorophenyl)-1,3-dihydro-3-hydroxy - 7-nitro-2*H*-1,4-benzodiazepin-2-one (XL) (20)—A solution of 27 g (81 mmoles) of 5-(2-chlorophenyl)-1,3-dihydro-7-nitro-2*H*-1,4-benzodiazepin-2-one 4-oxide [prepared in 64% from clonazepam (V), the desoxy precursor, by oxidation with *m*-chloroperbenzoic acid and obtained from ethanol as colorless flakes, mp 254° dec.] in 300 ml of acetic anhydride was heated on a steam bath for 2 hr. After acetic anhydride was removed *in vacuo*, the residue was crystallized from benzene to afford 14 g of 3-acetoxy-5-(2-chlorophenyl)-1,3-dihydro-7-nitro-2*H*-1,4-benzodiazepin-2-one, mp 279–281°.

To a solution of the 3-acetoxy compound (14 g) in 400 ml of dioxane was added 160 ml of 1 *N* NaOH. The mixture was stirred at room temperature for 0.5 hr and then neutralized with 1 *N* HCl. Water (2 liters) was added, and the mixture was extracted with methylene chloride. The organic layer was washed with water, dried, and evaporated to dryness. Crystallization from acetone afforded XL, which, on recrystallization from acetone, yielded 8.7 g (30% overall) of a hemiacetonate of XL as colorless prisms, mp 159–160°; IR (KBr): 3200, 3140, 1700 (broad), 1530, and 1345 cm^{-1}.

Anal.—Calc. for $C_{15}H_{10}ClN_3O_4 \cdot 0.5C_3H_6O$: C, 54.93; H, 3.63; N, 11.65. Found: C, 55.14; H, 3.70; N, 11.47.

5-(2-Chlorophenyl)-1,3-dihydro-3 - hemisuccinyloxy-7-nitro-2*H*-1,4-benzodiazepin-2-one (XLI)—The 3-hydroxy compound (XL) was succinylated, as in the preparation of XXXVIII, in a 67% yield. Colorless prisms (tetrahydrofuran–methylene chloride–heptane) were obtained, mp 172–180° dec.; IR (KBr): 1770–1700 (strong unresolved), 1530, and 1350 cm^{-1}; UV λ_{max} (2-propanol): 215 (ε 32,800), 248 (16,500), and 308 (12,000) nm.

Anal.—Calc. for $C_{19}H_{14}ClN_3O_7$: C, 52.75; H, 3.08; N, 9.75. Found: C, 52.85; H, 3.27; N, 9.73.

7-Amino-5-(2-chlorophenyl)-1,3-dihydro - 3-hemisuccinyloxy-2*H*-1,4-benzodiazepin-2-one (XLII)—Catalytic hydrogenation of XLI in the manner described in the preparation of XXXIX afforded XLII in a 70% yield as a light-yellow amorphous solid from tetrahydrofuran–methylene chloride–hexane, melting point indefinite, beginning at 130°; IR (KBr): 3500–2800 and 1680–1760 (unresolved), 1620, 1505, and 1160 cm^{-1}.

Anal.—Calc. for $C_{19}H_{16}ClN_3O_5$: C, 56.80; H, 4.01; Cl, 8.82. Found: C, 57.06; H, 4.34; Cl, 8.54.

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Benoxaprofen, a New Anti-Inflammatory Agent: Particle-Size Effect on Dissolution Rate and Oral Absorption in Humans

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Abstract □ The particle-size effect of benoxaprofen, a new nonsteroidal anti-inflammatory agent, on the *in vitro* dissolution rate and oral absorption in humans was evaluated. Ten normal subjects participated in a randomized crossover-designed absorption study with two sieved particle-size formulations: one with crystals larger than 60 mesh (mean equivalent spherical diameter = 640 μm) and the other with crystals smaller than 100 mesh (mean equivalent spherical diameter = 67 μm). Plasma drug concentrations and urinary drug excretion were used to determine the relative absorption of the two formulations. The standard USP procedure was used for the dissolution study. Particle size had a dramatic effect on both the *in vitro* drug dissolution and its oral absorption in humans. *In vitro*, the smaller crystals dissolved more rapidly and more efficiently than the larger crystals. *In vivo*, the smaller crystals produced higher plasma concentrations, more rapid peak concentration attainment, and more drug excreted in the urine.

Keyphrases □ Benoxaprofen—effect of particle size on dissolution rate and oral absorption in humans □ Dissolution rate—benoxaprofen, effect of particle size □ Absorption, oral—benoxaprofen, effect of particle size □ Anti-inflammatory agents—benoxaprofen, effect of particle size on dissolution rate and oral absorption in humans

The particle size of sparingly soluble drugs (drugs that are practically insoluble in aqueous fluids at physiological pH) can affect their dissolution rate *in vitro* (1–7). Similarly, the rate and extent of drug absorption can be reduced *in vivo* when dissolution in GI fluids is limited by particle size (2, 8–11). In several cases, rank correlations were obtained between sparingly soluble drug dissolution *in vitro* and absorption *in vivo* (12). In several instances, drug dissolution improved when particle size was reduced, probably because an increased surface area was available for dissolution.

Certain physicochemical properties of the experimental anti-inflammatory compound benoxaprofen, *dl*-2-(4-chlorophenyl)-α-methyl-5-benzoxazoleacetic acid, indicate that its dissolution *in vitro* and its absorption *in vivo* might be affected by altering its crystal particle size. It is a crystalline solid at room temperature. The pKa in 66% dimethylformamide is 6.9, and its solubilities at 25° in phosphate buffer at pH 5.0, 6.0, 7.0, and 7.6 are 4.4, 21, 207, and 835 μg/ml, respectively[1].

Because of benoxaprofen's low aqueous solubility and its largely unionized, lipoid-soluble form in the GI tract, a rate-limiting step in oral absorption could be drug dissolution in the GI fluids. This report describes the relationship between drug particle sizes and dissolution rates *in vitro* and the effect of particle sizes on oral bioavailability in humans.

EXPERIMENTAL

Dosage Preparation—GLC showed benoxaprofen[2] to be 99.9% pure. Two particle-size fractions were isolated using U.S. standard mesh sieves. One fraction contained particles larger than 60 mesh (mean diameter = 640 μm), and one contained particles less than 100 mesh (mean diameter = 67 μm). The individual fractions were mixed with corn starch USP in a laboratory blender[3]. The blends were hand filled into size 2 gelatin capsules, each containing 100 mg of benoxaprofen and 180 mg of starch.

Dissolution Study—The assembly and conditions used to study the dissolution rate and extent of the two drug particle sizes are described in USP XIX (13). The dissolution assembly consisted of four variable-speed stirring motors attached to four basket and shaft assemblies with a three-blade stainless steel propeller mounted on each basket shaft immediately above each basket, four 3-liter beakers, and a water bath at 37 ± 0.5°. The dissolution medium for each determination was 2 liters of pH 7.6 phosphate buffer. One capsule was placed in each basket and rotated at 100 rpm. Four capsules from each formulation were tested.

Five-milliliter aliquots were withdrawn at 20, 60, and 120 min using a pipet fitted with a suitable filter. Aliquots withdrawn were not replaced with corresponding volumes of the dissolution medium[4]. After the 120-min sample withdrawal, the basket contents were quantitatively transferred to the remaining dissolution medium, a stirring bar was added, and the mixture was magnetically stirred (at >500 rpm) for ~1 hr. A final aliquot was then taken. The benoxaprofen content in each specimen was measured spectrophotometrically.

Clinical Study—In humans, the benoxaprofen availability from the dosage forms containing the two different particle sizes was compared on the basis of plasma unchanged drug concentration and of urinary excretion of the unchanged drug and its glucuronide conjugate.

Twelve healthy males participated in the study after being informed of the objectives, potential risks, and procedures. The subjects were 21–33 years and within an acceptable weight range (14). According to a randomized crossover design, each subject received a single oral 100-mg drug capsule. Two weeks elapsed between the single doses of the two parti-

[1] The pKa and solubilities of benoxaprofen were determined at the Lilly Research Laboratories, Indianapolis, Ind., by Dr. R. F. Childers, Jr. (unpublished data).

[2] Synthesized at the Lilly Research Centre in England.
[3] Twin-Shell.
[4] Corrections were made for changes in dissolution volume in calculations of the percent of drug released at each period.

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