18-OH-DOC (4G), 18-OH-PROG (4E), and their metabolites. Statistical significance of this index was evaluated by using Student's t test against a hypothetical binding affinity of 0%. Both compounds were mineralocorticoid-like in their in vivo activity with M/DOCA ratios of 0.76 and 0.71, respectively. Binding of [^{3}H]aldosterone to the renal aldosterone receptor was identically inhibited with 18-OH-PROg (4E) and 18-OH-DOC (4G) by 34%.

Registry No. 1A, 60325-73-7; 1A acetate, 3020-10-8; 1B, 55388-46-0; 1D, 93716-45-1; 1E, 53620-26-1; 1F, 53803-17-1; 1G, 53512-61-1; 2A, 1238-51-3; 2A acetate, 2878-66-2; 2B, 3599-24-4; 2D, 93716-46-2; 2E, 38863-68-2; 2F, 93781-10-3; 2G, 81940-97-8; 3A, 93781-07-8; 3D, 93716-43-9; 3E, 1900-47-6; 3F, 86698-70-6; 3G, 31935-07-6; 4A, 3246-19-3; 4A ketal, 93716-38-2; 4B, 4813-43-8; 4C, 93716-39-3; 4D, 58210-92-7; 4E, 22618-71-9; 4F, 53512-59-7; 4G, 10385-97-4; 5A, 2858-27-7; 5A ketal, 26302-63-6; 5B, 26302-65-8; 5C, 93755-93-2; 5D, 93716-41-7; 5E, 14425-53-7; 5F, 86698-72-8; 5G, 86698-67-1; 6A, 93781-08-9; 6A ketal, 93859-83-7; 6B, 93781-09-0; 6C, 93716-40-6; 6D, 93716-42-8; 6E, 93716-47-3; 6F, 86698-68-2; 6G, 86698-69-3; ethylene glycol, 107-21-1; 18,20-dihydroxy-3α-tetrahydropyranyloxy-5β-pregnane, 93716-44-0.

Notes

Bile Acids with Cyclopropane-Containing Side Chain. 2.[1] Synthesis and Properties of 3α,7β-Dihydroxy-22,23-methylene-5β-cholan-24-oic Acid (2-Sulfoethyl)amide

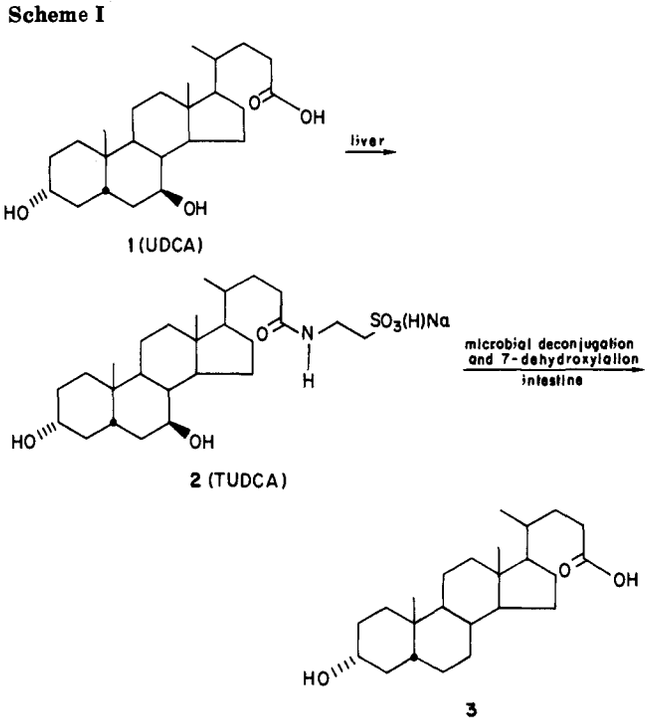
Roberto Pellicciari,*† Sergio Cecchetti,† Benedetto Natalini,† Aldo Roda,*‡ Brunella Grigolo,‡ and Adamo Fini‡

Istituto di Chimica Farmaceutica e Tecnica Farmaceutica, Università degli Studi, Perugia, and Istituto di Scienze Chimiche, Facoltà di Farmacia, Università degli Studi di Bologna, Bologna, Italy. *Received May 1, 1984*

The preparation of 3α,7β-dihydroxy-22,23-methylene-5β-cholan-24-oic acid (2-sulfoethyl)amide (5) by the one-step EEDQ-induced conjugation between ursodeoxycholic acid "cyclopropylog" (4) and taurine is described. The presence of a cyclopropyl ring adjacent to the amide bond is shown to make it resistant to degradation by intestinal bacteria. This new cyclopropylog is neither deconjugated at the C-24 amide bond nor 7-dehydroxylated when incubated with human stools in anaerobic conditions.

We have recently started a program aimed at the design of structurally modified bile acids (BA) in an attempt to increase the biological activity as well as to limit undesirable side effects exhibited by members of this class of compounds, such as ursodeoxycholic acid (UDCA),[2a] which is widely employed in several countries as cholesterol gallstone dissolving agent.[2a,b] Recent studies have established the conditions for successful therapy, such as the cholesterol nature of the gallstone crystalline structure and the high dose and length of treatment. The latter requirements, in particular, are motivated by the biotransformations which UDCA and the other natural BA undergo during enterohepatic cycling.[4] The fate of UDCA can be summarized as follows: after ingestion, it enters the small intestine and is passively absorbed throughout the intestine by the portal route. It has been estimated that the first-pass hepatic uptake for UDCA is 50%, with a resulting spillover into the systemic circulation of an analogous amount. Once in the hepatocyte, it is conjugated with glycine or taurine to form the corresponding N-acyl conjugate. The preferential conjugation with glycine can be explained by a reduced availability of taurine in the hepatocyte and has been considered negative since the taurine conjugate of UDCA is currently thought to have superior cholesterol-solubilizing properties. In order to overcome this problem, taurine, taurine jointly with UDCA, tauroursodeoxycholic acid (TUDCA 2), and TUDCA supplied with taurine have been administered to patients, and preliminary results seem to indicate a shift toward taurine in the UDCA conjugation pattern as a consequence of these approaches.[5,6]

Scheme I



A second problem arises once UDCA, as a conjugate, is secreted from the hepatocyte into the bile and with the

† Università degli Studi, Perugia.
‡ Università degli Studi di Bologna.

(1) See ref 11.
(2) (a) Danzinger, R. G.; Hofmann, A. F.; Schoenfield, L. J.; Thistle, J. L. *N. Engl. J. Med.* **1972**, 288. (b) Makino, I.; Shinozaki, K. J.; Yoshino, K.; Nakagawa, S. *Nippon Shokakibyo Gakkai Zasshi* **1975**, *72*, 690.

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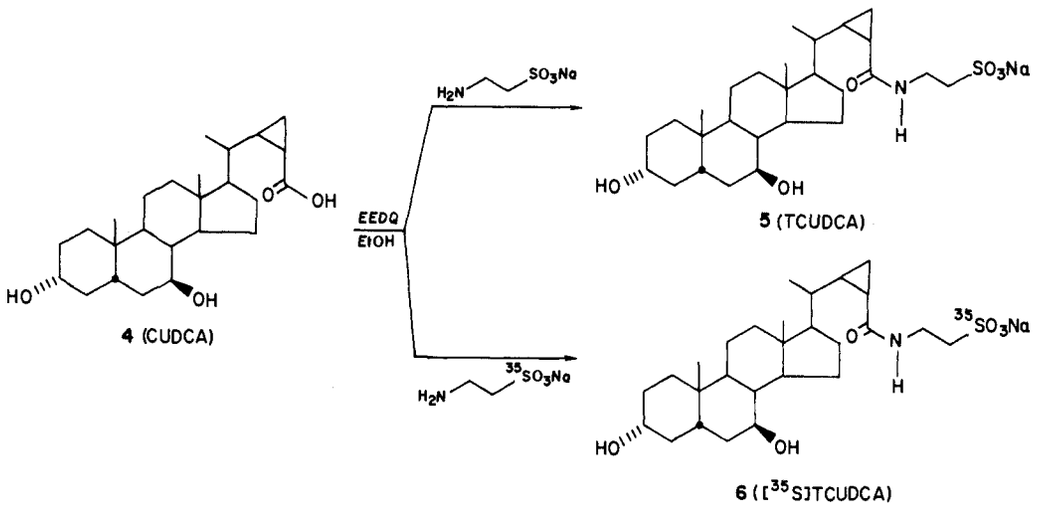
Scheme II



bile enters the intestine. In the distal small intestine and in the colon, the BA conjugate is converted by bacterial enzymes into the corresponding secondary BA by initial deconjugation, followed by 7-dehydroxylation. This process, which in the case of a 3,7-dihydroxylated BA leads to the formation of the hepatotoxic lithocholic acid (**3**),[7-10] is represented in Scheme I.

A TUDCA analogue with enhanced stability toward bacterial biotransformations, yet retaining or improving the biological properties of the parent compound, would therefore be of great interest in order to overcome the problems raised by the administration of UDCA. We have recently reported the synthesis as well as the physicochemical and preliminary biological properties of a side-chain "cyclopropylog" of ursodeoxycholic acid (**4**).[11] Both **4** and **1** exhibit very similar solubility and pK_a values, while **4** shows a lower critical micellar concentration (CMC) and, therefore, better detergent properties than those of **1**. Among the biological properties of **4**, two features are noteworthy: (a) **4** significantly increases the biliary secretion of BA and phospholipids, and (b) **4** is only partially conjugated (70%) by the liver. The latter property, in particular, can be explained by the presence in the side chain of **4** of a cyclopropyl moiety. By introduction of an additional steric parameter, this moiety can induce negative interactions for reactions in a key topographical area, such as the C-24 carboxyl group, while, at the same time, not affecting those positions which are known to be essential for biological activity, such as the hydroxy groups at C-3 and C-7. The above features shown by **4**, made this compound a suitable candidate for the task of designing a taurine conjugate UDCA analogue with improved properties in terms of (a) higher water solubility at low and high pH, (b) resistance to intestinal bacterial degradation, and (c) increased biological half-life time. The preparation and some relevant biological and physicochemical properties of the taurine conjugate of the cyclopropyl derivative of ursodeoxycholic acid (TCUDCA **5**) are the object of the present study.

Chemistry. The stereoisomeric mixture of 3α,7β-dihydroxy-22,23-methylene-5β-cholanoic acid (**4**) was prepared, as previously reported,[11] with use of the dirhodium tetraacetate catalyzed decomposition of ethyl diazoacetate in the presence of the Δ^{22}-norcholene derivative, followed by alkaline hydrolysis of the cyclopropyl ester thus obtained.

Direct conjugation of **4** with the sodium salt of taurine in aqueous ethanol in the presence of EEDQ[13] gave the sodium salt of tauro-3α,7β-dihydroxy-22,23-methylene-5β-cholanoic acid (**5**) in 75% yield (Scheme II). Analogously, the ^{35}S-labeled derivative **6** was obtained by conjugation of **4** with the sodium salt of [^{35}S]taurine.

Physicochemical Properties. Critical Micellar Concentration. The critical micellar concentration values (CMC) were determined by using a dye solubilization method as previously described.[12]

The CMC values are as follows: TCUDA (**5**) = 14.5 mM, TUDCA (**2**) = 18.0 mM, CUDCA (**4**) = 15.5 mM, UDCA (**1**) = 19.0 μM.

Comparison of the value of **5** with that of the corresponding unconjugated acid **4** shows that they are in the same order, suggesting that the positive effect on the CMC induced by the presence of the taurine (increased side chain length $C_{25} \rightarrow C_{27}$) is balanced by the introduction of the polar amide bond.[12]

When CMC data of **5** and **4** are compared with that of the natural conjugated analogue **2**, the former values are found to be lower and this can be attributed to the presence of one more C atom on the side chain.

Solubility. The aqueous solubility of compound **5** is extremely high both at low and high pH. This is explained by the presence of the terminal sulfonic acid group with low pK_a. This represents an improvement with respect to the corresponding unconjugated acid **4**, which at a pH

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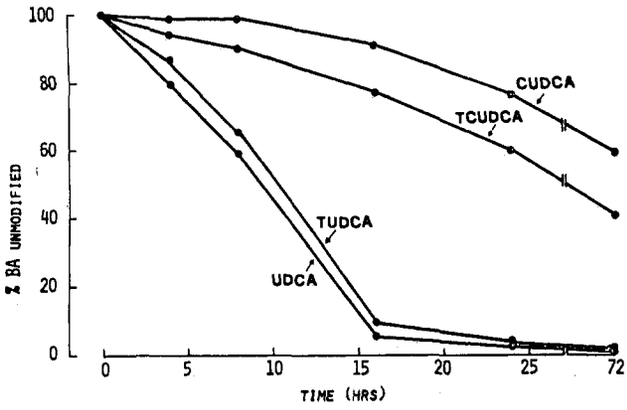

Figure 1. In vitro anaerobic fecal biotransformation of different ursodeoxycholic acid analogues.

below 5 precipitates as protonated acid.

The higher water solubility together with a similar CMC of **5**, when compared to **4** is advantageous when this compound is administered orally; at the low pH of the stomach (pH ~1), precipitation is prevented and consequently the bioavailability and the transport could be improved.

Biological Properties. The main hypothesis to be tested was the resistance of the amide bond at C-24 and of the hydroxy group at C-7 toward deconjugation and 7-dehydroxylation by bacterial enzymes, respectively.

Previous studies have shown that the deconjugation step is the initial reaction in the metabolism of BA by intestinal microflora[14] even though contradictory results have been reported.[15] For some naturally occurring BA, such as chenodeoxycholic acid, the substrate for the 7α-hydroxylase is the BA in its unconjugated form while the conjugate acid appears less actively dehydroxylated.[14-16] To test this hypothesis, two different studies were carried out.

Enzymatic Hydrolysis. Compound **5** was incubated at 37 °C for several hours at pH 5.6 in the presence of a choloylglycine hydrolase (EC 3.5.24) enzyme able to split the amide bond both in glycine and taurine physiological conjugated BA. TUDCA was used as a control. The latter was completely (98%) deconjugated in the medium after 15 min to form ursodeoxycholic acid, while TCUDCA was recovered unmodified even when the incubation time was prolonged to 72 h.

Anaerobic Biotransformations. A second study was carried out with human stools as substrate in order to evaluate both the side-chain deconjugation and the 7-dehydroxylation. It is well known that in anaerobic conditions and in culture broth, human feces have been shown to be a substrate simulating intestinal ecology.

Several intestinal bacteria, such as strains of the families Bacteroidaceae, Bifidobacterium, Lactobacillaceae, act on conjugated bile acids as deconjugating and dehydroxylating agents.

TCUDCA and TUDCA and the corresponding unconjugated CUDCA and UDCA were incubated for several periods of time with fresh stools in broth media for anaerobic cultures at pH 6.0. After the reaction was stopped by strong alkalinization, the acids were extracted by reverse-phase chromatography with use of a C18 Bond Elut column. The chemical analysis was carried out by high-performance liquid chromatography.

Tauroursodeoxycholic acid (**2**) was rapidly deconjugated with formation of ursodeoxycholic acid and then dehydroxylated with formation of 3-hydroxy-5-cholanoic acid (**3**).

To the contrary, TCUDCA is slowly metabolized; more than 70% of it remains unmodified after incubation for a long period of time (24 h) (Figure 1). The unconjugated "cyclopropylog" **4** is also stable and the percentage of this compound transformed is very low when compared with UDCA, which is quickly 7-dehydroxylated.

Experimental Section

Melting points were measured with a Kofler Micro hot stage apparatus and are uncorrected. ^{1}H NMR spectra were taken on a Varian EM 390 spectrometer. IR spectra were determined with a Perkin-Elmer 1320 spectrometer. Elemental analysis was performed with a Model 1102 automatic analyzer, Carlo Erba, Italy.

3α,7β-Dihydroxy-22,23-methylene-5β-cholan-24-oic Acid (2-Sulfoethyl)amide, Sodium Salt (5). A solution of *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ)[13] (3.02 g, 12.21 mmol) in 95% ethanol (123 mL) and a solution of taurine (1.09 g, 8.71 mmol) in 2 N NaOH (17.4 mL) were added to a stirring solution of 3α,7β-dihydroxy-22,23-methylene-5β-cholanoic acid (**4**) (3.52 g, 8.71 mmol) in 95% ethanol (176 mL), and the reaction mixture was kept at 40 °C with stirring for 18 h. The solvent was then evaporated under vacuum (35 °C) and the residue thus obtained was dissolved in methanol (40 mL). The resulting solution was then diluted with diethyl ether (225 mL) and the crystalline precipitate was collected after centrifugation and decantation of the supernatant, redissolved in methanol (45 mL), precipitated by addition of diethyl ether (225 mL), and separated by centrifugation. After this procedure was repeated three times under the above conditions, the crystalline residue obtained was dried under vacuum [120 °C (1 mmHg), 12 h] to give 3.42 g (75%) of **5**: mp 113–118 °C; IR (Nujol) ν 3340 (OH, NH), 1640, 1550 (CONHR), 1050 (CO) cm^{-1}, NMR (CD_3OD) δ 0.67 (3 H, s, C-18 Me), 0.95 (3 H, s, C-19 Me), 3.00 (2 H, t, J = 6 Hz, NCH_2CH_2S), 3.30–3.77 (2 H, m, C-3 and C-7 CHOH), 3.52 (2 H, t, J = 6 Hz, NCH_2CH_2S). Anal. Calcd for $C_{27}H_{44}O_6NNaS$: C, 60.76; H, 8.31; N, 2.62. Found: C, 59.08; H, 8.19; N, 2.32.

Treatment of the acid **4** (50 mg, 0.12 mmol) with [^{35}S]taurine (0.12 mmol) and EEDQ (40 mg, 0.16 mmol) in 95% ethanol and 2 N NaOH (240 μL) as above followed by preparative TLC gave pure [^{35}S]TCUDCA (**6**).

Critical Micellar Concentration. The CMC was measured with use of Orange OT, as a water-insoluble dye. Different bile salts solutions, ranging from 0.5 to 100 mM, were prepared, and the pH was adjusted to 8.0. Free crystals of the dye were added to the solution and the solutions left to equilibrate for a week. The solutions were filtered, and the absorbance was read at 492 nm. The absorbance was plotted against bile acid concentration and the CMC was calculated from the intersection of the line drawn below the CMC and when the solubilization began to increase.

Enzymatic Hydrolysis. Fifty milligrams of enzyme (activity = 100–200 units/mg of protein) was suspended in 5 mL of water and sonicated in an ultrasound bath. The enzymatic activity in the reaction mixture was ~50 units/mL.

The incubation mixture consisted of 200 μL of acetate buffer (0.3 M, pH 5.6), EDTA (0.2 M, 40 μL), mercaptoethanol (0.2%, 40 μL), enzyme (choloylglycine hydrolase, 50 μL), 10 μL of taurine conjugated acid (**5**) solution (10 mM) containing 0.5 μCi of the corresponding ^{35}S-labeled acid **6**.

After 10, 20, 30, 40, 50, 60 min and after 15 h the reaction was stopped by adding 200 μL of a 30% solution of KOH. Bile acids were isolated and analyzed by using the procedure described below.

Anaerobic Incubation of Stools. Immediately after evacuation fresh stools were homogenized with water (1/1, v/v) under a nitrogen stream, and 500 mg was transferred into sterile vials to which 5 mL of sterilized chopped-meat glucose medium (Scott Lab., Fiskeville, RI) was added. To this medium was added BA containing a corresponding ^{35}S-labeled BA (0.5 μCi) at a concentration ranging from 0.01 to 0.1 mM.

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All the experiments were carried out under a nitrogen stream on capped vials. The anaerobic conditions were maintained with a disposable anaerobic indicator (Gas Pac Becton Dickinson Co., Orangeburg, NY).

The incubations were carried out at 37 °C, and 4, 8, 16, 24, 72 h after the addition of the corresponding BA, the reaction was stopped with addition of 150 μL of a 30% solution of KOH.

Tubes were centrifuged at 3500 rpm for 10 min, and 2 mL of the supernatant was transferred into a tube to which 18 mL of 0.1 M NaOH solution was added. The solution was applied to a C_{18} Bond Elut cartridge, previously activated according to the manufacturer's instructions.

The solution was eluted at a flow rate of 1 mL/min and the cartridge washed with 10 mL of water and the BA collected with 4 mL of methanol. The elute was dried under a N_2 stream and reconstituted with 0.5 mL of CH_3OH. BA were separated by using TLC and HPLC techniques. Conjugated BA were separated from the free BA with use of silica gel 0.25-μm-thickness plates (Merck GRF) with a solvent system composed by $CHCl_3/CH_3OH/H_2O$ (75/25/3, v/v/v).

The qualitative–quantitative compositions were obtained by HPLC with a 5-μm C-18 reverse-phase column (Waters Associates). The mobile phase was composed of a mixture of CH_3OH/KH_2PO_4 (0.01 M, pH 5.8, 130/70 v/v).

The analysis was carried out in isocratic conditions at a flow rate of 0.3 mL/min with use of an UV detector at 200 nm.

After TLC separation the radioactivity of the different spots was measured by zonal scanning. The different fractions were transferred into scintillation vials, and 1 mL of ethanol/acetic acid (9:1, v/v) was added. Ten milliliters of Unisolve (Kooklight, England) as liquid scintillation cocktail was then added and the radioactivity measured in a β scintillation counter.

Conclusion

In conclusion, the in vitro experiments suggest that TCUDCA should be superior to TUDCA in vivo in therapy for gallstones owing to its much greater resistance to biotransformation to inactive or potentially toxic products.

Acknowledgment. We thank Professor Alan Hofmann for helpful discussion and suggestions. Two of us (B.G. and S.C.) acknowledge gratefully a fellowship from Gi-pharmex, Milano, Italy.

Registry No. 4, 93060-76-5; 5·Na, 93001-12-8; 6, 93038-89-2; NaOH, 1310-73-2; EEDQ, 16357-59-8; ethanol, 64-17-5; taurine, 107-35-7; ^{35}S-taurine, 2782-32-3.

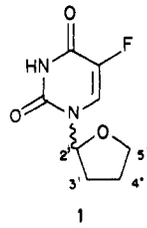
Synthesis and Antitumor Activity of a Series of Ftorafur Analogues: The Effect of Varying Electronegativity at the 1′-Position

Mark H. Holshouser,*† Annette M. Shipp, and Paul W. Ferguson

Divisions of Medicinal Chemistry and Pharmacology/Toxicology, School of Pharmacy, Northeast Louisiana University, Monroe, Louisiana 71209. *Received January 27, 1984*

To test the effect of changes in electronegativity within the alicyclic N-1 substituent of substituted 5-fluorouracil analogues on cytotoxic activity, a series of derivatives of ftorafur, 1-(2′-tetrahydrofuranyl)-5-fluorouracil, was synthesized and tested for antitumor activity in the P388 lymphocytic leukemia screen and cytotoxic activity in the L1210 cell culture screen. Two compounds of N-1 substituent with high electronegativity, the 2′-tetrahydrothiophene 1′-oxide and the 2′-tetrahydrothiophene 1′,1′-dioxide derivatives, demonstrated the highest in vitro L1210 cell inhibition (84.5% and 92.0%, respectively). Furthermore, against P388 lymphocytic leukemia in vivo, the 2′-tetrahydrothiophene 1′-oxide derivative showed significant activity (T/C = 143). Other compounds of similar or lower electronegativity within the N-1 cyclic substituent were inactive against P388 lymphocytic leukemia and less active against L1210 cells.

Ftorafur (1) [NSC 148958, 1-(2′-tetrahydrofuranyl)-5-fluorouracil] is under clinical investigation as a less toxic alternative to 5-fluorouracil (5-FU) (2) in the treatment of cancer. Although ftorafur offers the advantage of decreased myelosuppression and possible oral effectiveness, its role in cancer chemotherapy is probably limited to combination chemotherapy.[3] Some effectiveness in the treatment of adenocarcinoma and breast cancer has been demonstrated.[3]



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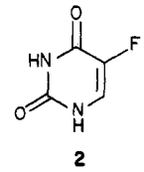

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It is generally accepted that ftorafur is a repository form of 5-FU. This conversion has been demonstrated in rat liver microsomes,[4] rat and mouse liver soluble fraction,[5] and human plasma and tumor tissue.[6,7]

† Present address: Analytical Development Division, R. P. Scherer, North America, Clearwater, FL 33518.

(1) This paper was presented in part at the APhA 130th Annual Meeting, Academy of Pharmaceutical Science Section, April 10–14, 1983, New Orleans, LA.

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