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Cholelithiasis Chemotherapy: An *In Vitro* Approach

Keyphrases □ Cholelithiasis chemotherapy—identification of cholesterol gallstone-dissolving agents, *in vitro* screening system □ Gallstones, cholesterol—dissolution, identification of drugs, *in vitro* screening system □ Cholesterol gallstones—dissolution, *in vitro* screening system to identify potential chemotherapeutic agents

To the Editor:

Cholelithiasis, a disease state resulting from deficiencies in cholesterol transport within the biliary system, is known to have afflicted humankind for centuries (1). Today, surgical removal of gallbladders and their stones is one of the most commonly performed operations with a third of a million cholecystectomies yearly in the United States alone (2, 3). Many treatments have been recommended including herbs, mineral waters, olive oil, and turpentine (4). More recent approaches to chemotherapy have generally been based on the feeding of endogenous components of bile, bile salts (5–8), or phospholipids (9). Clinical success with chenodeoxycholic acid administration was recently reported (8). Other studies have assessed the role of steroids (10), cod liver oil (11), and some drugs (12) on gallstone dissolution in rabbits fed a lithogenic diet.

The purpose of this research was to identify agents capable of bringing about the dissolution of cholesterol gallstones following oral administration. An *in vitro* screening system was developed which can monitor changes in the cholesterol-holding capacity of bile upon the addition of a test compound. The rationale for such a screening system is based on the physical–chemical properties of bile.

Bile salts will spontaneously aggregate into micelles above a critical temperature and concentration. Alone, these micelles have little ability to solubilize cholesterol; but in the presence of lecithin, the formation of mixed micelles enables the transport of large quantities of cholesterol (14). Small *et al.* (15) described this mixed micelle as a cylinder of bile salts, with hydrophilic sides facing the aqueous phase, surrounding an inner core of lecithin. The hydrophilic ends of the lecithin comprise the top and bottom of the cylindrical mixed micelle. Lecithin can interact with cholesterol to form liquid crystalline aggregates that are not soluble in aqueous media. Bile salts can engulf such aggregates to form mixed micelles and, in so doing, solubilize larger quantities of cholesterol. A complete description of these relationships may be found elsewhere (16).

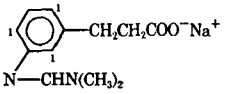
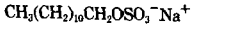
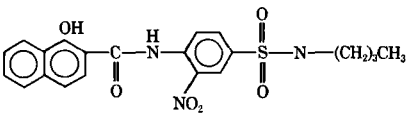
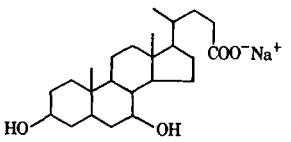
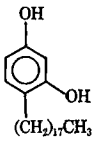
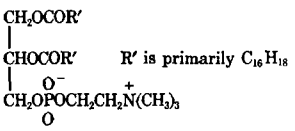
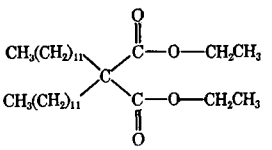
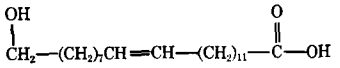
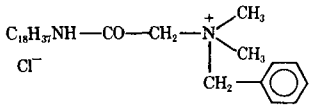
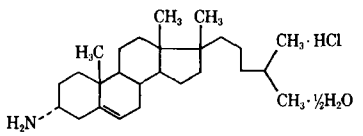
The solubility of cholesterol in these four-component systems (bile salt, lecithin, cholesterol, and water) has been extensively studied. However, it has been difficult to find agreement on the same solubility values. Recently, the factors responsible for the discrepancies were identified (17) and the basal solubility of cholesterol was found to be in the order of 5 mole % (17–19). With this equilibrium solubility value defined, it has become possible to design a screening system capable of identifying compounds that can modify the equilibrium solubility of cholesterol in bile; such compounds would be potentially useful as cholelitholytic agents (20).

The cholesterol-solubilizing potential of a homologous series of quaternary nicotinic acids was studied in an aqueous *in vitro* system devoid of biliary components (13). While such a procedure measures the inherent solubilizing characteristics of a compound, it fails to identify compounds that cannot form micelles or water-soluble complexes with cholesterol but can otherwise interact with the biliary transport system to increase its carrying capacity (lecithin, for example). There is also the possibility of a compound interfering with the structure and function of the biliary micelles. Thus, it was considered worthwhile to employ a primary *in vitro* screening procedure based upon the bile salt–lecithin system. Secondary tests could then consider the inherent solubilizing ability of active compounds to gain information on their mechanism of action. A secondary test evaluating the effect of an active compound on the kinetics of gallstone dissolution is warranted; recent studies have discovered barriers to cholesterol dissolution in undersaturated bile systems (21). The development of a primary screen and some preliminary solubility enhancement data are the subjects of this report.

Excess hydrated crystalline cholesterol spiked with cholesterol-4-^{14}C was added to a lecithin–bile salt solution containing 38 m*M* egg lecithin and 135 m*M* conjugated bovine bile salts in a 0.05 *M* phosphate buffer (pH 7.4), as previously described (22). The solubility of cholesterol at 37° in this milieu was followed until equilibrium was reached. Because the long incubation times and numerous samplings of the bulk cholesterol–lecithin–bile salt suspension permitted microbial alterations, it was necessary to develop methods for sterilization and storage of the system. An incubating–stirring apparatus was designed that allowed for storage and aseptic sampling of this suspension (23).

When the cholesterol reached its equilibrium solubility, homogeneous aliquots of the suspension were placed into prewarmed vials containing the carefully weighed drug candidates. The systems were incubated with shaking at 37° for 24 hr. The solubility of cholesterol was then measured by liquid scintillation counting of an aliquot of the filtrate obtained by membrane filtration. Efficient determination of cholesterol solubility and recording of data were abetted through the use of automated liquid scintillation counting instruments equipped with punch-tape outputs. The tape was fed into a time-share terminal and, with the use of appropriate programs, back-

Table I—Cholesterol Solubilizing Evaluation of Selected Compounds

Compound	Structure	Cholesterol Solubility Enhancement as Percent of Control
Sodium ipodate[a]		95
Sodium lauryl sulfate[b]		97
1-Hydroxy-2′-nitro-4′-(butylsulfamoyl)-2-naphthanilide[c]		99
Chenodeoxycholic acid sodium salt[d]		100
4-*n*-Octadecylresorcinol[e]		115
Egg lecithin		116
Bis(dodecyl) malonic acid diethyl ester[e]		117
Erucyl alcohol[f]		119
Benzyldimethyl[(octadecylcarbamoyl) methyl]ammonium chloride[g]		121
Cholesteryl-3-α-amine hydrochloride hydrate[h]		122

[a] Courtesy of E. R. Squibb & Sons, New Brunswick, N.J. [b] Fisher Scientific Co., Fairlawn, N.J. [c] Aldrich Chemical Co., Milwaukee, Wis. [d] Courtesy of A. F Hofmann, M.D., Rochester, Minn. [e] Alfred Bader Chemical Co., Milwaukee, Wis. [f] Koch-Light Laboratories Ltd., Colnbrook Buckinghamshire, England. [g] Smith Kline & French Laboratories, Philadelphia, Pa.

ground and quench effects were eliminated and the solubility of cholesterol in test vials was compared to that of the controls. The results were recorded as solubility enhancement as a percent of control.

For a compound to be active *in vivo* by physical enhancement, it would have to be excreted in the bile. Smith (24) discussed the physical–chemical factors responsible for directing a compound toward biliary excretion. Where feasible, these principles (polarity, molecular weight, and stereochemistry) were used to guide selection of screening candidates. Compounds from diverse chemical families were selected for initial screening to maximize the possibilities of identifying active moieties. Candidates for screening

were obtained from the indicated sources and used as received. Sufficient compound was carefully weighed into a glass vial so that a 10 m*M* concentration of drug resulted upon addition of 2 ml of stock suspension.

Some representative data appear in Table I. With the introduction of a drug candidate, lipid interactions in the lecithin–bile salt micelle become more difficult to explain than in the quaternary system. The purpose of the drug is to mimic the action of either bile salt or lecithin, with the result of an increased cholesterol-carrying capacity. A compound may interact with the micelle in such a way that the holding capacity is not increased or decreased. This would be dependent on the nature of the drug–micelle interaction: position of drug in micelle, nature of charge on drug, charge density, branching of drug, *etc.* The *in vitro* screen is designed to measure the result of the five-component interaction, *i.e.*, the solubility of cholesterol in the presence of a drug–lecithin–bile salt system.

A major class of drugs designed to be passed through the biliary tract is the cholecystographic agents. Archer *et al.* (25) demonstrated the importance of chain length in determining whether a potential cholecystographic agent would be excreted *via* the urinary or biliary system. It was shown that if the chain was too small, the compound was eliminated by the kidneys; if the chain was too large, the drug was not intestinally absorbed. Thus, a critical length for biliary excretion existed. A cholecystographic agent, sodium ipodate, was not found to influence the solubility of cholesterol in this screening system. The most active compounds appear to contain an alkyl chain and thus show more of a structural resemblance to lecithin than to bile salts.

A bile salt, chenodeoxycholic acid, has little activity in this system, which was not unexpected since bile salts alone are not known to be good cholesterol solubilizers. The addition of lecithin or other insoluble swelling amphiphiles (16) can swell the bile salt micelle and provide an environment capable of dissolving large quantities of cholesterol. Oral administration of chenodeoxycholic acid has been found to bring about remission of cholesterol gallstones in humans by making the bile undersaturated with respect to cholesterol (8); the biliary secretion of cholesterol is lowered in relation to the bile salt and lecithin output. The chenodeoxycholic acid would seem to be acting *more* by virtue of its physiological than physical characteristics.

A viable *in vitro* screening procedure capable of identifying compounds that significantly enhance the biliary holding capacity for cholesterol has been developed. Data are presented for some typical compounds, selected because of a particular discrete characteristic. Moreover, the application of physical–chemical principles to devise and evaluate a particular disease state has been accomplished. As we learn more about the complexities of the human body, certainly the utilization of *in vitro* model systems to screen potential compounds preliminarily should become commonplace in the search for potential drug products.

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