A Study of the Physicochemical Interactions between Biliary Lipids and Chlorpromazine Hydrochloride

BILE-SALT PRECIPITATION AS A MECHANISM OF PHENOThIAZINE-INDUCED BILE SECRETORY FAILURE

By MARTIN C. CAREY,* PAUL C. HIROM† and DONALD M. SMALL
Division of Gastroenterology, Department of Medicine, Peter Bent Brigham Hospital and Harvard Medical School, and the Divisions of Biophysics and Gastroenterology, Department of Medicine, Boston University School of Medicine, Boston, MA, U.S.A.

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Since chlorpromazine hydrochloride [2-chloro-10-(3-dimethylaminopropyl)-phenothiazine hydrochloride] is commonly implicated in causing bile-secretory failure in man and is secreted into bile, we have studied the physicochemical interactions of the drug with the major components of bile in vitro. Chlorpromazine hydrochloride molecules are amphiphilic by virtue of possessing a polar tertiary amine group linked by a short paraffin chain to a tricyclic hydrophobic part. At pH values below the apparent pK (pK,' 7.4) the molecules are water-soluble cationic detergents. We show that bile salts in concentrations above their critical micellar concentrations are precipitated from solution by chlorpromazine hydrochloride as insoluble 1:1 salt complexes. In the case of mixed bile-salt/phosphatidylcholine micellar solutions, however, the degree of precipitation is inhibited by the phospholipid in proportion to its mole fraction. With increases in the concentration of chlorpromazine hydrochloride or bile salt, micellar solubilization of the precipitated complexes results. Sonicated dispersions of the negatively charged phospholipid phosphatidylserine were also precipitated, but dispersions of the zwitterionic phospholipid phosphatidylcholine were not. Chlorpromazine hydrochloride efficiently solubilized these membrane phospholipids as mixed micellar solutions when the drug:phospholipid molar ratio reached 4:1. Polarizing-microscopy and X-ray-diffraction studies revealed that the precipitated complexes were amorphous and potentiometric studies confirmed the presence of a salt bond. Some dissociation of the complex occurred in the case of the most polar bile salt (K0.365). As canicular bile-salt secretion determines much of bile-water flow, we propose that complexing and precipitation of bile salts by chlorpromazine hydrochloride and its metabolites may be physicochemically related to the reversible bile-secretory failure produced by this drug.

The phenothiazines, which are widely used in clinical medicine and psychiatry (Swazey, 1974), result in reversible bile-secretory failure (cholestasis) and liver injury in a large proportion of individuals (Sherlock, 1968; Popper, 1968). In about 50% of subjects treated with chlorpromazine hydrochloride (proprietary names: Thorazine, Largactil, Mega-phen), morphological and standard liver-function tests are consistent with mild subclinical cholestatic liver damage ( DeVore et al., 1956; Dickes et al., 1957; Bartholomew et al., 1958). Chlorpromazine hydrochloride and its metabolites are concentrated in the liver (Ragland, 1963), secreted into bile (Fyodorov, 1957; Flanagan et al., 1959, 1962; Hansson & Schmiterlöw, 1961) and undergo a limited enterohepatic circulation (Van Loon et al., 1964). The pathophysiological mechanisms responsible for this common side effect are, however, as yet unknown. In the past, chlorpromazine hydrochloride-associated cholestasis has been attributed to hepatic hypersensitivity reactions (Gutman, 1957; Zelman, 1959; Popper & Schaffner, 1959; Zimmerman, 1963), inhibition of bilirubin secretion (Har-geaves, 1965), alteration of bile viscosity (Sharma & Prasad, 1967), precipitation of biliary proteins (Clarke & Denborough, 1971) and spasm of the sphincter of Oddi (Moyer et al., 1954; Menguy et al., 1955), all without convincing pathophysiological proof. Although there is some direct experimental evidence
that inhibition of bile flow both in dogs (Stefko & Zbinden, 1963; Sharma & Prasad, 1967) and in perfused rat liver preparations (Clodi & Schnack, 1960; Kendler et al., 1971) occurs with chlorpromazine hydrochloride and is related to dose, no physicochemical studies have been carried out on the interactions of the drug with bile salts, the major organic anions in mammalian bile, and the substances responsible for much of bile-water flow (Wheeler, 1969; Erlinger, 1972). At physiological pH one would expect that bile salts (Plate 1), which are anionic detergent-like molecules (Carey & Small, 1972), and chlorpromazine hydrochloride (Plate 1), which is a cationic detergent (Scholtan, 1955; Attwood et al., 1974), would be incompatible in solution owing to coulombic interactions between their ionic groups. To test this hypothesis, we used a variety of biophysical techniques to study the interactions in vitro of chlorpromazine hydrochloride with bile salts and with other lipid and non-lipid components of bile individually and as mixed micellar solutions. We found that the common bile salts and other anionic lipids, including phosphatidylserine, were precipitated from either solution or liposomal dispersions by chlorpromazine hydrochloride as amorphous hydrophobic salts containing chlorpromazine and the anionic lipid in a 1:1 molar stoichiometry. The insoluble complexes were bonded primarily by electrostatic forces between the ionic polar head groups of the molecules and stabilized by secondary hydrophobic interactions.

Experimental

Materials

Crystalline chlorpromazine hydrochloride was obtained from Smith, Kline and French Laboratories, Philadelphia, PA, U.S.A., and was pure by t.l.c. (200µg spot in chloroform/methanol/water (18:8:1, by vol.)) and m.p. (197–199°C, uncorr., by hot-stage microscopy). The material was stored in actinic containers at 4°C under N₂. Sodium taurocholate (sodium salt of 3α,7α,12α-trihydroxy-5β-cholanoyltaurine), sodium glycocholate (sodium salt of 3α,7α,12α-trihydroxy-5β-cholanoylglycine), and sodium tauroliothocholate (sodium salt of 3α-hydroxy-5β-cholanoyltaurine) were obtained from Calbiochem (San Diego, CA, U.S.A.) and were at least 98% pure by t.l.c. (200µg spots in chloroform/methanol/ acetic acid (13:3:1, by vol.)) and were used as received. Sodium taurochenodeoxycholate (sodium salt of 3α,7α-dihydroxy-5β-cholanoyltaurine) was obtained from Maybridge Co. (Tintagel, Cornwall, U.K.) and was purified by thre washing a concentrated aqueous solution at pH 2.0 with dry diethyl ether to remove contaminating free bile acids and dried by freeze-drying (Carey & Small, 1969). Grade-1 egg-yolk phosphatidylcholine (1,2-diacyl-sn-glycero-3-phosphorylcholine) and the sodium salt of phosphatidylserine (1,2-diacyl-sn-glycero-3-phosphorylserine) from bovine spinal cord (Lipid Products, Redhill, Surrey, U.K.) and anhydrous cholesterol (Hormel Institute, Austin, MN, U.S.A.) were pure by t.l.c. [200µg spots in chloroform/methanol/water (18:8:1, by vol.)]. Long-chain saturated fatty acids (C₆-C₁₆) were purchased from the Hormel Institute and were found to be at least 99% pure by differential thermal analysis (5 mg samples) and potentiometric titration of 1g/dl solutions with 0.5M-HCl. The sodium salts were prepared by adding a stoichiometric amount of 1m-NaOH in 50% (v/v) ethanol and drying under reduced pressure. Gel-electrophoresis-grade sodium dodecyl sulphate was obtained from Bio-Rad Laboratories (San Diego, CA, U.S.A.) and was shown pure by potentiometric titration with 0.5M-HCl (1g/dl solution) and by surface-tension measurements. The material gave the expected titration curve over the pH range of 12–1 and precise equivalence was found between the numbers of mol of sodium dodecyl sulphate and the number of mol of HCl used to titrate it, indicating the absence of fatty acid and inorganic electrolyte impurities. The non-existence of a minimum in the surface-tension–log (sodium dodecyl sulphate concn.) curve in the vicinity of the critical micellar concentration indicated that dodecanol was not a significant contaminant (Elworthy & Myssels, 1966). Cetyltrimethylammonium bromide was obtained from Eastman Kodak Co. (Rochester, NY, U.S.A.) and was recrystallized thrice from ethanol (m.p. 239–241°C, uncorr.). Sodium fusidate (sodium salt of 3α,11α-dihydroxy-16β-acetoxyfusid-17(20)[16,21-cis],24-dien-20-oic acid; proprietary name, Fucidin) and its taurine and glycine conjugates were gifts from Dr. W. O. Godtfredsen (Leo Pharmaceutical Company, Ballerup, Denmark) and were at least 99% pure as described previously (Carey & Small, 1973). Cephalosporin P₁ (3α,7β-dihydroxy-6α,16β-diacetoxyfusida-17(20)[16,21-cis],24-dien-20-oic acid) was obtained from Dr. W. F. J. Cuthbertson (Glaxo Research Ltd., Stoke Poges, Bucks., U.K.) and was purified thrice in the cold (4°C) from hot (50°C) ethanol and the sodium salt was made as described above. Purified fat-free human serum albumin and serum α₂ glycoprotein were kindly supplied by Dr. Karl Schmid (Boston University School of Medicine, Boston, MA, U.S.A.). Formic acid, acetic acid, propionic acid and benzoic acid and inorganic salts were reagent grade (Fisher Scientific Co., Boston, MA, U.S.A.). Reagent-grade NaCl was roasted in air at 600°C for 4 h to oxidize and remove organic impurities. Water was doubly distilled from a Corning all-glass automatic still.
EXPLANATION OF PLATE I

Conventional chemical formulae (a), Stuart-Briegleb molecular models (b) and schematic drawings (c) of a molecule of chlorpromazine and a molecule of the bile salt sodium cholate.

Symbols + and − represent the charged cationic and anionic groups of the molecules and in (c) the black shading represents the hydroxyl groups of the bile salt.
EXPLANATION OF PLATE 2

X-ray powder patterns of chlorpromazine hydrochloride (a), sodium taurocholate (b) and dry chlorpromazine-taurocholate complex (c)

Bragg spacings (d) and diffraction intensities are tabulated in Table 2. The equatorially preferred narrow diffraction band in (c) was due to the Mylar windows of the sample holder. Powder X-ray diffractions were carried out at ambient temperature (approx. 23°C) with a camera fitted with toroidal mirror optics using CuKα X-rays from an Elliotrotati: anode generator. Sample-to-film distance was 61.85mm.

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(Corning Glass Works, Corning, NY, U.S.A.). All glassware was sequentially washed in 1 M-HNO₃ and 2 M-KOH/ethanol (1:1, v/v) and after rinsing with water and drying in a hot-air oven was silicone-treated by rapid immersion in a 1 g/dl aqueous silicone concentrate (Siliclad; Clay Adams Co., Parsippany, NJ, U.S.A.).

Methods

Preparation of mixtures and turbidity measurements. Appropriate amounts of chlorpromazine hydrochloride together with individual anionic lipids were weighed into actinic glass volumetric flasks and water (adjusted to pH 6.0 at 25°C with 1 M-HCl) was added to give the required volume. The samples were mixed vigorously on a Vortex shaker under N₂ for 3 min, and then transferred to 1 cm path-length cuvettes and examined by eye for turbidity or a Tyndall effect. Absorbance was read at 750 nm in a Beckman Acta III spectrophotometer at 25°C. Mixed micellar solutions of sodium taurocholate with phosphatidylcholine and of sodium taurocholate with phosphatidylcholine and cholesterol in water were prepared by standard methods (Small et al., 1966). Samples of hepatic bile were obtained from a female Rhesus monkey (Macaca mulatta) with a chronic biliary fistula and were assayed for total bile salts, phospholipids and cholesterol as described by Admirand & Small (1968). To avoid dilution of the mixed micellar solutions various amounts of dry chlorpromazine hydrochloride (2-140 mg) were added to cover a range of concentrations greater and less than the bile salt concentration. After vigorous vortex mixing for 3 min the absorbance was read at 750 nm in 1 mm path-length cuvettes. In other experiments 0.5 g/dl of either human serum albumin or α₂-glycoprotein was mixed with various concentrations of chlorpromazine hydrochloride and the absorbance determined as above. In these experiments, spectrophotometric absorbance was used to estimate the extent of precipitation from the decrease in the percentage of transmitted light. As an estimate of a precipitation reaction this technique is valid, even at absorbance values greater than 2.0, provided that no significant sedimentation occurs during the time of the procedure. In many preliminary experiments we found that readings taken 3-4 min after mixing gave reproducible values. To test whether the drug would precipitate or solubilize sonicated dispersions of phosphatidylserine, phosphatidylcholine, phosphatidylcholine/cholesteryl-ol mixtures (molar ratio 1:1) and phosphatidylcholine/sodium taurocholate mixtures (molar ratio 4:1), appropriate weights of these lipids were dissolved in chloroform/methanol (1:1, v/v), evaporated to dryness under N₂ and then in vacuo over P₂O₅ and sonicated in water under argon at 0°C for 30 min at 75 W (Branson Cell Disrupter, model W188) to give 6 mg/ml liposomal dispersions. Absorbance of these dispersions was read at 750 nm before and after the addition of various amounts of chlorpromazine hydrochloride (1 cm light-path, pH 6.0, 25°C). The effects of pH variation, temperature variation and the addition of NaCl on the physical state and turbidity of certain mixtures of chlorpromazine hydrochloride with other lipids were determined.

Hot-stage polarized light-microscopy. Mixtures were examined by direct and polarized light in order to determine the nature of the insoluble phases and to document the microscopic texture of the liquid-crystalline phases. In this manner the physical state of phospholipid liposome/chlorpromazine hydrochloride mixtures was determined both before and after freezing (−20°C) and then thawing at room temperature (23°C). Mixed micellar solutions are optically clear (isotropic) and form spontaneously without the need for an external energy source. They contain aggregates, which have hydrodynamic radii generally less than 5 nm. These solutions are thermodynamically stable in spite of drastic variations of temperature and remain isotropic by both direct and polarized light microscopy. Liposomal dispersions of low turbidity, on the other hand, require an external energy source (sonication) for their formation, and the lipid aggregates, which are usually greater than 100 nm in diameter, exhibit a characteristic birefringence when observed by polarized-light microscopy. These dispersions are thermodynamically unstable and aggregate with an increase in turbidity over a period of several days to weeks. This process can be accelerated by freezing and thawing. Precipitation of lipid mixtures was also observed microscopically by allowing water to flow between a few mg of chlorpromazine hydrochloride placed side by side on a glass slide with a few mg of an anionic lipid shown in the bulk state to form precipitates. At the interface where the two lipids come together a water-insoluble precipitate was seen. The effects of varying the aqueous pH and temperature on the physical state of lipids and insoluble complex were documented. Estimates of viscosity were obtained by pressing on the cover slip and observing the deformation of the sample. Birefringence was determined under crossed polarizers and where detected, the effects of increasing the hot-stage temperature (1-2°C/min) were studied.

Potentiometric titrations. Individual samples of chlorpromazine hydrochloride (10, 20 and 40 mm) and a 20 mm-chlorpromazine hydrochloride/sodium taurocholate (molar ratio 1:1) mixture in 5 ml portions were titrated with 0.5 M-HCl and back-titrated with 0.5 M-NaOH in a Radiometer titration assembly equipped with an H⁺ glass electrode and a micro-stirring apparatus at 25±1°C. The titration
assembly was kept in the dark after the addition of each portion of acid or base until the mixtures were equilibrated. A Tyndall cone was looked for periodically under illumination with a high-intensity lamp. Calculations of the solubility of chlorpromazine base and the apparent pK of chlorpromazine hydrochloride were carried out as described previously (Back & Steenberg, 1950; Small, 1971).

X-ray diffraction. X-ray-diffraction studies on dry chlorpromazine hydrochloride, sodium taurocholate and the chlorpromazine-taurocholate molecular complex were performed with focusing cameras fitted with toroidal mirror optics and using nickel-filtered CuKα radiation from an Elliot GX6 rotating anode generator operating at 40mA and 40mV. Specimens were contained in Lindemann glass tubes or by Mylar windows in sealed copper sample holders. The sample-to-film distance was 61.85 mm.

Results

Turbidity measurements

The plots in Fig. 1 show the turbidity of 20 mM mixtures of chlorpromazine hydrochloride and sodium taurocholate and of chlorpromazine hydrochloride and sodium dodecyl sulphate as a function of composition at 25°C. Broad absorbance maxima are observed, which reach a peak at mole fractions of 0.4 (sodium dodecyl sulphate) and 0.5 (sodium taurocholate). The turbidity of chlorpromazine hydrochloride and sodium taurocholate mixtures gradually cleared on heating, and the temperatures of total clearing varied with the sodium taurocholate mole fraction (Fig. 1, inset). The turbidity of the chlorpromazine hydrochloride/sodium dodecyl sulphate mixtures did not clear over the temperature range 25–95°C. All taurine- and glycine-conjugated bile salts, sodium fusidate and its glycine and taurine conjugates, and the sodium salt of cephalosporin P1 (steroid detergents structurally resembling the bile salts; Carey & Small, 1973; Carey et al., 1975) formed similar precipitates with chlorpromazine hydrochloride. Sodium salts of C6–C18 carboxylic acids were also precipitated with the drug over the temperature range 25–90°C, but formate, acetate, propionate and benzoate or inorganic salts (Na2SO4, K2SO4) produced no changes in turbidity compared with the control solutions. Significant differences were found between the turbidity of mixtures of chlorpromazine hydrochloride with glycine- and taurine-conjugated bile salts when the pH was varied. At pH 13.0 all 20 mM mixtures of chlorpro-

![Fig. 1. Absorbance of mixtures (final lipid concentration 20 mM) of chlorpromazine hydrochloride and sodium dodecyl sulphate (△) and of chlorpromazine hydrochloride and sodium taurocholate (■) versus mole fraction of sodium dodecyl sulphate and sodium taurocholate in water at 25°C](image)

The inset shows the temperatures of total clearing (E<0.05) of chlorpromazine hydrochloride/sodium taurocholate mixtures (■) plotted as a function of the mole fraction of sodium taurocholate. The components were mixed in the dry state in silicone-treated flasks and water was added. E750 values were recorded 4 min after mixing. Light-path length was 1 cm and pH was 6.0. The absorbance of aqueous mixtures of other bile salts or analogous steroid detergents with chlorpromazine hydrochloride gave similar curves.
Mixtures were of: sodium tauroliothocholate and chlorpromazine hydrochloride (∆); sodium taurochenodeoxycholate and chlorpromazine hydrochloride (○); sodium taurocholate and chlorpromazine hydrochloride (□). Dried components of the mixtures were weighed into volumetric flasks and water, adjusted to pH 6.0, was added to the required volume. After vortex mixing for 3 min the E250 was read at about 4 min in 1 cm light-path cells at 25°C. Absorbance becomes significantly different from zero at concentrations that approximate to the critical micellar concentration of the bile salts. Critical micellar concentrations of sodium lithocholate, sodium taurodeoxycholate and sodium taurocholate at 20°C in water, measured by the spectral-shift method, are 0.2, 1.5 and 2.8 mM respectively (Small, 1971; Carey & Small, 1969).

Mazine hydrochloride and sodium glycocholate and of chlorpromazine hydrochloride and sodium taurocholate were turbid and the absorbance increased in proportion to the concentration of the drug. At pH 2.0, the turbidity plot of mixtures of chlorpromazine hydrochloride and sodium taurocholate was similar to that at pH 6.0 (Fig. 1), but with mixtures of chlorpromazine hydrochloride and sodium glycocholate only those mixtures with a mole fraction of 0.8 sodium glycocholate or more were turbid. The absorbance of mixtures (molar ratio 1:1) of the drug and taurine-conjugated mono-, di- and tri-hydroxy bile salts is shown in Fig. 2 as a function of total concentration of bile salt and chlorpromazine hydrochloride. The turbidity curves show that mixtures of chlorpromazine hydrochloride and sodium tauroliothocholate precipitate at a lower total lipid concentration than mixtures of chlorpromazine hydrochloride and sodium taurochenodeoxycholate, which in turn precipitate at a concentration less than that of the mixtures of chlorpromazine hydrochloride and sodium taurocholate. In all cases the turbidity increases significantly with concentration. The maximum concentration of sodium tauroliothocholate studied (0.5 mM) is close to the limits of its aqueous solubility at 25°C (Small & Admirand, 1969). The concentration of each bile salt corresponding to the total concentration where the absorbance becomes significantly different from zero approximates to the critical micellar concentration of the bile salts (Carey & Small, 1969; Small, 1971).

The effects of added NaCl on the turbidity of 10 mM solutions of chlorpromazine hydrochloride and sodium taurocholate in a 1:1 molar ratio were studied at 25°C and 80°C. At 25°C a sharp decrease in turbidity occurred (absorbance change 1.4) with small additions of NaCl (final concn. 0.01–0.06M); however, the turbidity increased sharply to approximate to the initial values in water (1.8–2.0) on further additions of NaCl. The turbidity of the mixtures cleared (absorbance less than 0.05) at 80°C in the presence of 0.3 M-NaCl or less, whereas in electrolyte concentrations greater than 1 M-NaCl no significant change in turbidity occurred. These effects of temperature were reversible by cooling to 25°C and no metastable supersaturation was observed.

Mixed micellar solutions and liposomal dispersions

Turbidities of native monkey bile (11.9 mM-bile salts, 1.3 mM-phosphatidylcholine and 0.14 mM-cholesterol) and artificially prepared mixed micellar solutions of sodium taurocholate and phosphatidylcholine (all containing 10 mM-sodium taurocholate) are shown plotted semilogarithmically as a function of the final concentration of added chlorpromazine hydrochloride in Fig. 3. All turbidities reach a peak between a molar ratio of 1:1 and 2:1 chlorpromazine
hydrochloride and sodium taurocholate. As the proportion of phosphatidylcholine to bile salt is increased, the turbidity is significantly decreased and mixtures containing a phosphatidylcholine mole fraction of 0.4 or greater did not precipitate. In the monkey bile experiment, the peak turbidity decreased to the baseline in a large excess of chlorpromazine hydrochloride, but 24h was required for complete dissolution. Spectrophotometric absorbance experiments in which chlorpromazine hydrochloride (3 mg/ml) was added to human serum albumin, α2 glycoprotein, sonicated phosphatidylcholine, phosphatidylcholine/cholesterol mixtures or phosphatidylcholine/taurocholate liposomes (6 mg/ml) to give approximately equimolar ratios showed that turbidities with the added drug were not significantly different from blanked controls at 3 min, 24h, or 48h. However, in the presence of equimolar concentrations of chlorpromazine hydrochloride, precipitation occurred over 12–24h from both sonicated and unsonicated dispersions of phosphatidylserine at pH 6.2 (Table 1), and these precipitates could not be redispersed by sonication. In addition, when added in fourfold excess on a molar basis, chlorpromazine hydrochloride solubilized both phosphatidylcholine and phosphatidylserine as mixed micellar solutions (Table 1). Many runs such as those shown in Figs. 1–3 and Table 1 were made to check the reproducibility, and several were duplicated by using freshly prepared solutions. Excellent agreement was found in all cases. Mixed micellar solutions remained clear after repeated freezing to −20°C and thawing at room temperature, indicating their thermodynamic stability. Liposomes of phosphatidylcholine mixed with chlorpromazine hydrochloride also remained dispersed; however, those of chlorpromazine hydrochloride and phosphatidylserine with molar ratios of 1:1 and 1:7:1 precipitated completely from solution after similar thermal manipulations.

Polarized light-microscopy and analysis of precipitates and supernatants

Crystalline chlorpromazine hydrochloride melted sharply at 197–199°C to an isotropic liquid and no birefringence was noted. When water (pH 6.0) was allowed to penetrate a few grains of chlorpromazine hydrochloride on a slide, the drug rapidly dissolved with a narrow rim of smectic-textured birefringence at the solid/water boundary, indicating a liquid-crystalline phase at low water content (determined

Table 1. Physicochemical interactions of chlorpromazine hydrochloride with sonicated dispersions of phosphatidylserine and phosphatidylcholine

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at 0.8–0.9 mole fraction of chlorpromazine hydrochloride. This liquid-crystalline phase disappeared on heating the sample from ambient temperature (23°C) to 30°C. When water was allowed to flow between a few grains of chlorpromazine hydrochloride and sodium taurocholate or sodium dodecyl sulphate on a slide, an oily isotropic precipitate formed between the hydrating crystals. During co-hydration of sodium dodecyl sulphate and the drug a smectic liquid-crystalline phase was observed between the isotropic precipitate and the hydrating sodium dodecyl sulphate. Comparable phenomena were also observed when sodium dodecyl sulphate and the cationic detergent cetyltrimethylammonium bromide were co-hydrated with water.

Polarizing and direct light-microscopic examination of precipitated complexes (molar ratio 1:1) of chlorpromazine hydrochloride and sodium taurocholate and of chlorpromazine hydrochloride and sodium dodecyl sulphate after desiccation revealed a single isotropic phase composed of a firm non-birefringent glass. With the chlorpromazine–taurocholate complex, slight swelling but no evidence of dissolution occurred on the addition of water. However, partial dissolution resulted from the addition of acid (HCl, pH 2.0) and base (NaOH, pH 13.0) during the 10–15 min of observation. The lyotropic behaviour of the chlorpromazine–dodecyl sulphate complex was similar to the above, except that on the addition of NaOH the complex grew myelin figures. Polarized light-microscopy of the precipitates from mixed micellar solutions revealed characteristic birefringent droplets of phosphatidylcholine together with isotropic oily complexes. The precipitates of phosphatidylserine and the drug formed solid isotropic particles containing scattered birefringent Maltese Crosses with a positive sign of birefringence. Analysis by t.l.c. revealed that precipitates (at pH 6.0), irrespective of the initial bile-salt:chlorpromazine hydrochloride ratio contained equimolar amounts of chlorpromazine and the bile-salt anion, with the component in excess being found in the supernatant phase. With bile salt/chlorpromazine hydrochloride mixtures (molar ratio 1:1), small amounts of uncomplexed lipids were found in the supernatants. With mixtures of the drug and sodium glycocholate, the precipitates were shown by t.l.c. to be mainly the protonated glycocholic acid at pH 2.0 and chlorpromazine base at pH 13.0. Phosphatidylcholine and cholesterol, in addition to the insoluble chlorpromazine–bile-salt complex, were found in the pellet when mixed micelles and monkey bile were precipitated by the drug.

Potentiometric titrations

The forward and back equilibrium curves of 10 mM-chlorpromazine hydrochloride and 10 mM-chlorpromazine hydrochloride plus 10 mM-sodium taurocholate are shown in Figs. 4(a) and 4(b) respectively, and the forward titration curves for 20 and 40 mM concentrations of the drug are shown in Figs. 5(a) and 5(b) respectively. The natural pH of chlorpromazine hydrochloride in water was approx. 5.4. This pH was adjusted to about 2.0 with 1 M-HCl and the forward titration carried out with 0.5 M NaOH, plotted in µl on the abscissa (Figs. 4a, 5a and 5b). In the titration of 10 mM-chlorpromazine hydrochloride (Fig. 4a) the solution was clear up to pH 6.4, when a slight Tyndall effect was observed. Further additions of even small quantities of NaOH progressively increased the turbidity. At the inflexion point X, the concave slope of the curves becomes convex, indicating the first equivalence point where the reaction given in eqn. (1) starts. The plots then curve abruptly, and after Y (the pH of precipitation) further additions of NaOH lead to a plateau in the curves. Towards the end of the titrations this plateau portion changes to a steep concave up-sweep, and finally the reaction is complete at Z, where each curve shows a second inflexion point (final equivalence point).

This chemical reaction may be written:

\[
\text{Chlorpromazine hydrochloride} + \text{NaOH} \rightarrow \text{chlorpromazine base} + \text{NaCl} + \text{H}_2\text{O} \quad (1)
\]

where chlorpromazine hydrochloride is the fully ionized salt and chlorpromazine base is the insoluble form. The backward reaction with HCl reverses the forward titration curve and may be written:

\[
\text{Chlorpromazine base} + \text{HCl} \rightarrow \text{chlorpromazine hydrochloride} \quad (2)
\]

In the titration of the mixture of 10 mM-sodium taurocholate and 10 mM-chlorpromazine hydrochloride (Fig. 4b) two phases are present at all pH values and the turbidity increases from pH 2.0 to 10.2. The effects were reversible in the back titration with HCl (Fig. 4b). As both lipids are added in equimolar concentrations the initial reaction may be written:

\[
\text{Chlorpromazine hydrochloride} + \text{sodium taurocholate} \rightleftharpoons \text{chlorpromazine–taurocholate complex} + \text{NaCl} \quad (3)
\]

Calculations

The pK₄ of 10 mM-chlorpromazine hydrochloride in water (25°C), calculated from the curve in Fig. 4(a) (Back & Steenberg, 1950; Small, 1971), is 7.44. Similar calculations from the titration curves in Figs. 5(a) and 5(b) give apparent pK values of 7.37 and 6.89 for 20 and 40 mM-chlorpromazine hydrochloride respectively. The solubility of the chlorpromazine base can be calculated from the amount of
Fig. 4. Potentiometric titration curves of chlorpromazine hydrochloride and a chlorpromazine hydrochloride/sodium taurocholate mixture (molar ratio 1:1)

(a) Titration curve of 10 mM-chlorpromazine hydrochloride in 5 ml of aqueous solution with 0.5 M-NaOH (-----) and back-titration curve with 0.5 M-HCl (-----). Line X is drawn through the first equivalence point where the titration of the drug commences; the arrow at Y denotes the pH of precipitation (appearance of a Tyndall effect). Line Z is drawn through the final equivalence point where the titration of the drug is complete. (b) Titration curves of 10 mM-chlorpromazine hydrochloride mixed with 10 mM-sodium taurocholate in water: -----, the forward-titration curve with 0.5 M-NaOH; ---------, the back-titration curve with 0.5 M-HCl. Lines at X and Z have the same meanings as in (a). The system was turbid over the entire pH range. Lipids were weighed into volumetric flasks, diluted with water and adjusted to 5 ml after mixing. Titrations were carried out in a closed Radiometer titration assembly under N₂. Equilibrium pH values were recorded. Tyndall cones were observed under high-intensity illumination.

NaOH added between X and Y. In 10 and 20 mM-chlorpromazine hydrochloride, the solubilities of the chlorpromazine base calculated from the titration curves are 0.2 mM and 0.4 mM respectively. A similar calculation for the solubility of the free base in 40 mM-chlorpromazine hydrochloride (Fig. 5b) gives a value of 2.65 mM. As the critical micellar concentration of chlorpromazine hydrochloride is in the vicinity of 20 mM under these conditions (Scholtan, 1955), the micellar solubility of chlorpromazine base is therefore 2.25 mM (2.65 - 0.4). However, as the 40 mM micellar solution of the drug contains 20 mM of monomers, the ratio of the number of molecules of chlorpromazine base to chlorpromazine hydrochloride in the micelles is therefore 2.25:20. This indicates that 8 molecules of chlorpromazine
Fig. 5. Potentiometric titration curve of 20 mM-chlorpromazine hydrochloride (a) and 40 mM-chlorpromazine hydrochloride (b) titrated with 0.5 M-NaOH in water (25°C).

Letters X, Y, and Z have the same meanings as in Fig. 4(a). The concentration of chlorpromazine hydrochloride equivalent to the number of μl of 0.5 M-NaOH added between the first equivalence points (X) and the precipitation pH values (Y) is the solubility of free chlorpromazine base in each system. Note the increased solubility of chlorpromazine base above the critical micellar concentration (b) as compared with its solubility at the critical micellar concentration (a).

Lipids were weighed into 5 ml volumetric flasks and diluted with water to the required volume. Titrations were carried out in a closed Radiometer titration assembly under N₂. Equilibrium pH values were recorded. Tyndall effects were observed with high-intensity lamp illumination.

As an amount of taurocholate equivalent to 3.7 mM must also be in uncomplexed form, 6.3 mM of each component must be complexed. From these values $K_s = 0.345$.

This $K_s$ value indicates that at equilibrium the reaction has proceeded significantly to the right, i.e. in favour of the complex, but appreciable dissociation of the complex also occurs.

**X-ray diffraction**

The chlorpromazine hydrochloride and sodium taurocholate powder patterns give several diffraction lines of varying intensity in the low-angle and wide-angle (0.2–2 nm) regions (Plates 2a and 2b). The

$$K_s = \frac{k_2}{k_1} = \frac{[\text{chlorpromazine hydrochloride}][\text{sodium taurocholate}]}{[\text{chlorpromazine-taurocholate complex}][\text{NaCl}]}$$
Table 2. X-ray powder pattern measurements: Bragg spacings \((d)\) and diffraction intensities of chlorpromazine hydrochloride, sodium taurocholate and chlorpromazine–taurocholate complex

<table>
<thead>
<tr>
<th>Chlorpromazine hydrochloride</th>
<th>Sodium taurocholate</th>
</tr>
</thead>
<tbody>
<tr>
<td>(d) (nm)</td>
<td>Intensity</td>
</tr>
<tr>
<td>1.42</td>
<td>Medium</td>
</tr>
<tr>
<td>1.05</td>
<td>Strong</td>
</tr>
<tr>
<td>0.89</td>
<td>Medium</td>
</tr>
<tr>
<td>0.70</td>
<td>Very weak</td>
</tr>
<tr>
<td>0.60</td>
<td>Weak</td>
</tr>
<tr>
<td>0.56</td>
<td>Strong</td>
</tr>
<tr>
<td>0.52</td>
<td>Strong</td>
</tr>
<tr>
<td>0.49</td>
<td>Medium</td>
</tr>
<tr>
<td>0.47</td>
<td>Very strong</td>
</tr>
<tr>
<td>0.44</td>
<td>Medium</td>
</tr>
<tr>
<td>0.42</td>
<td>Medium</td>
</tr>
<tr>
<td>0.40</td>
<td>Weak</td>
</tr>
<tr>
<td>0.39</td>
<td>Strong</td>
</tr>
<tr>
<td>0.36</td>
<td>Medium</td>
</tr>
<tr>
<td>0.44</td>
<td>Medium</td>
</tr>
<tr>
<td>0.33</td>
<td>Weak</td>
</tr>
<tr>
<td>0.32</td>
<td>Weak</td>
</tr>
<tr>
<td>0.31</td>
<td>Weak</td>
</tr>
<tr>
<td>0.29</td>
<td>Weak</td>
</tr>
<tr>
<td>0.28</td>
<td>Weak</td>
</tr>
<tr>
<td>0.27</td>
<td>Weak</td>
</tr>
</tbody>
</table>

The diffraction patterns of dry chlorpromazine hydrochloride, sodium taurocholate and chlorpromazine–taurocholate complex (precipitated from water at \(pH\) 6.0) were performed on focusing cameras fitted with toroidal mirror optics. Nickel-filtered CuK\(\alpha\) X-radiation was from an Elliot GX6 rotating-anode generator operating at 40 mA and 40 MV. Specimens were contained in Lindeman glass tubes or by Mylar windows in sealed copper sample holders at \(23 \pm 1^\circ\text{C}\). The sample-to-film distance was 61.85 nm. The only Bragg spacing for the chlorpromazine–taurocholate complex is 0.54 nm (medium).

The chlorpromazine–taurocholate complex (molar ratio 1:1) gave a diffuse scattering profile in the low-angle region, with a single diffraction band showing preferred orientation (Plate 2c). In Table 2, the Bragg spacings \((d)\) and the intensities of the lines are tabulated. The preferred oriented diffraction from the chlorpromazine–taurocholate complex at 0.54 nm (Table 2) was due to the Mylar windows of the sample holder.

Discussion

These studies indicate that bile salts and chlorpromazine hydrochloride molecules interact electrostatically and precipitate from solution as a complex composed of one molecule of the drug and one molecule of bile salt, with the probable exclusion of a molecule of NaCl. As the aqueous solubilities of both chlorpromazine hydrochloride and bile salts depend on ionized polar groups (Scholtan, 1955; Small, 1971), the most likely primary bonding is that of a salt linkage. This possibility is supported by the fact that the addition of acid (HCl) or of small amounts of NaCl and an elevation of temperature dissociates the precipitated complex. The observations that simple univalent inorganic salts and short-paraffin-chain carboxylic acid anions fail to induce precipitation, and that the more hydrophobic bile salts lower the concentration of lipids necessary for the appearance of measurable turbidity, suggest that secondary hydrophobic interactions are important in formation and perhaps stability of the complexes. The stability of the chlorpromazine–dodecyl sulphate complexes at high temperatures suggests that the contributions of the hydrophobic interactions are dominant with long-chain aliphatic detergents. Concentrations of NaCl in excess of 1 M prevent thermal dissociation of the chlorpromazine–taurocholate complexes, possibly by preventing the solubility of the individual ions owing to dielectric saturation of the aqueous medium. The decrease in turbidity in the presence of a considerable excess of either chlorpromazine hydrochloride and sodium taurocholate or sodium dodecyl sulphate is consistent with solubilization of the complexes (molar ratio 1:1) by the detergent in excess as mixed micelles. The shape of the turbidity curves (Fig. 1) indicates that sodium dodecyl sulphate micelles are much more efficient solubilizers than bile-salt micelles and that the micelles of the drug poorly solubilize the chlorpromazine–dodecyl sulphate complex compared with the chlorpromazine–taurocholate complex. The poor mixed-micellar solubility of the former complex also suggests that hydrophobic interactions between the non-polar part of sodium dodecyl sulphate and the two phenyl rings of the chlorpromazine molecule result in weak mutual interactions between the chlorpromazine in the complex and chlorpromazine hydrochloride molecules in mixed micelles.

The ability of small quantities of micellized phosphatidylcholine to diminish the degree of turbidity in proportion to increases in the phosphatidylcholine:bile-salt ratio and eventually to inhibit precipitation completely suggests that bile-salt–phosphatidylcholine mixed micelles of a certain size solubilize the complexes formed. Alternatively, the added chlorpromazine hydrochloride could interact with the phosphatidylcholine molecules in the micelles by interdigitation between the phospholipid molecules in a similar fashion to what has been suggested for phenothiazine–lecithin interactions in monolayers and bilayers (Zografi & Auslander, 1965; Sears & Brandes, 1969–72; Chapman, 1975). The fact that at molar ratios of 4:1 or higher, chlorpromazine

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hydrochloride and membrane phospholipids form mixed micelles suggests that significant hydrophobic interactions occur between the hydrocarbon chains of phospholipid bilayers and the aromatic rings of the drug molecules. Provided that the critical micellar concentration of the system is exceeded and sufficient phospholipid is present, these hydrophobic interactions possibly prevent ionic interactions between the cationic groups of chlorpromazine hydrochloride molecules and the anionic groups of the bile salt molecules on the surface of the micelles. These interactions between chlorpromazine hydrochloride and phosphatidylcholine may be facilitated by the orientation of the planes of the two phenyl rings of the drug which in the crystal adopt an angle of 139° with each other (McDowell, 1969).

As suggested by the polarized light-microscopic observations and as proved by X-ray diffraction, the insoluble complexes are amorphous in nature in the dry state and in water over a wide range of temperatures. This may be due to the inability of the bile salt in the complex to pack closely into a crystalline or liquid-crystalline lattice owing to the alignment of hydrated hydroxyl groups on the bile-salt molecules (Plate 1). This contrasts with insoluble cholesterol esters of long-chain fatty acids, which also have two bulky hydrophobic ends linked by a polar group. However, cholesterol esters, in contrast with chlorpromazine–taurocholate complexes, exhibit crystalline structures and thermotropic liquid-crystalline behaviour in the dry state (Small, 1970).

Typical long-chain anionic and cationic detergents are well known to form insoluble hydrophobic salts in solution, and this observation led to one of the classical quantitative assays for cationic detergents (Cross, 1970). Further, the admixture of non-ionic surfactants interfered with end points detected by turbidity or prevented their occurrence (Cross, 1970), suggesting a general analogy with the effects of phosphatidylcholine in inhibiting the precipitation of bile salts by chlorpromazine hydrochloride. Scowen & Leja (1967) showed that precipitates obtained by mixing anionic and cationic detergents were stoichiometrically composed as reciprocal salt pairs and confirmed their ionic character by u.v.- and i.r.-spectral analysis.

Similar conclusions were reached by other investigators from phase equilibria and partition coefficient data on typical anionic–cationic detergent systems (Matalon et al., 1951; Boffey et al., 1959; Zografi et al., 1964; Scott, 1968; Chen & Hall, 1973). Electrostatic interactions between the phenothiazines, including chlorpromazine hydrochloride and various other important anionic biological materials, have been described previously. For example, chlorpromazine hydrochloride added to solutions of organic polyphosphate (Hele, 1964), ATP (Moriguchi et al., 1972), caffeine (Nakano, 1971), riboflavin (Yagi et al., 1959; Nakano, 1971), gangliosides (Harris & Saifer, 1962), chondroitin sulphate (Harris et al., 1960) and DNA (Kantesaria & Marfev, 1975) leads to precipitation of insoluble complexes. In these studies turbidity was also maximal to a 1:1 molar stoichiometry of chlorpromazine hydrochloride per charged group of the soluble anionic compound. Increases of temperature and suppression of ionization at extremes of pH or by the addition of a neutral inorganic salt in these studies also either inhibited hydrophobic salt formation or caused dissolution of the precipitates.

The fact that chlorpromazine hydrochloride induces a decreased bile flow in animals and in isolated rat liver preparations (Stefko & Zbinden, 1963; Sharma & Prasad, 1967; Kendler et al., 1971), and the reports that almost 50% of patients taking the drug for long periods develop evidence of hepatic dysfunction (DeVore et al., 1956; Bartholomew et al., 1958; Dickes et al., 1957), strongly suggest that chlorpromazine hydrochloride may have an intrinsic toxic effect on the liver. In view of the fact that the drug and its major metabolites are cationic molecules and are secreted into bile, it is logical to suggest from the present studies that ionic binding of bile salts in the liver by chlorpromazine hydrochloride or by its metabolites could be the initial step in compromising bile-salt output and bile flow during the administration of these drugs.

Even though conspicuous 'bile plugs' have been described in patients with chlorpromazine-induced cholestatic jaundice (Bartholomew et al., 1958; Popper, 1968) no information is available on their composition. Even if one speculates that precipitation of bile salts might occur within hepatic cells, canaliculor membranes or canaliculi, such hydrophobic precipitates would in all probability be removed by organic solvents during the graded dehydrated procedures used in the preparation of tissue sections for conventional light- and electron-microscopy. As an alternative, freeze–fracture electron microscopy suggests itself as a valid alternative procedure for morphological studies of the liver in chlorpromazine hydrochloride-induced cholestasis.

It is also puzzling why mild cholestatic liver damage is observed in only 50% of individuals treated with chlorpromazine hydrochloride. One explanation is that routine tests of liver function are not subtle enough to detect mild degrees of functional liver impairment in man. The development of an intravenous bile-salt-tolerance test (LaRusso et al., 1975) which measures the plasma disappearance rate of a conjugated bile salt by radioimmunoassay may be a more specific clinical test for detecting minimal bile-secretory failure in this condition.

We have studied the effects of intravenous infusions of 14C-labelled chlorpromazine hydrochloride on biliary lipid outputs and bile secretion (Ros et al.,
1975) in a Rhesus monkey model prepared with a chronic biliary fistula (Small et al., 1972). Our preliminary data indicate a high cumulative secretion of radioactivity in bile associated with a diminution in bile flow, bile-salt output and other biliary lipid outputs, the degree of which increased with increases in the pharmacological dose of administered chlorpromazine hydrochloride (Ros et al., 1975).

Impairment of bile flow was due to inhibition of both bile-salt-dependent and bile-salt-independent flow, suggesting that bile salts are complexed in vivo by chlorpromazine hydrochloride if it is not the only mechanism for bile-secretory failure in the primate. Therefore in addition it is tempting to postulate that complexing or micellar solubilization of certain important membrane lipids by chlorpromazine hydrochloride as demonstrated in the present work may jeopardize canalicular membrane Na+K+-dependent adenosine triphosphatase activity, which is thought to be responsible for the membrane sodium pump related to bile-salt-independent canalicular bile flow (Erlinger & Dhumeaux, 1974).

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