

In Vitro Displacement of Bilirubin by Antibiotics and 2-Hydroxybenzoylglycine in Newborns

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Hyperbilirubinemia is frequently observed in neonates, and serious neurological complications such as kernicterus can be precipitated when the concentration of unconjugated bilirubin is abnormally increased. The administration of drugs which bind to albumin and compete with bilirubin can increase the possibility of such a complication. To test the bilirubin-displacing activity of pharmacological agents that are used with newborns, 52 antimicrobial agents were investigated *in vitro*. A glycine conjugate of salicylate, 2-hydroxybenzoylglycine, which is known to be present at elevated levels in newborns and has a potent bilirubin-displacing property, was used as a positive control agent. Pooled cord serum was used as a source of hyperbilirubinemic serum. A centrifugal ultrafiltration method with semipermeable cones was employed to determine the effects of potential bilirubin-displacing agents on the levels of total bilirubin. 2-Hydroxybenzoylglycine was demonstrated to be the most potent bilirubin-displacing agent. Antibiotics could be classified into four groups: high-level displacers (sulfisoxazole, sulfamethoxazole, dicloxacillin, cefoperazone, and ceftriaxone), intermediate-level displacers (moxalactam, nafcillin, and 14 others), low-level displacers (aztreonam, carbenicillin, and 11 others), and nondisplacers (mezlocillin, cefuroxime, kanamycin, and 15 others). It is concluded that the ultrafiltration method is a rapid and readily reproducible procedure for the determination of bilirubin displacement and that antibiotics with a tendency to displace bilirubin should be avoided in jaundiced newborns whenever appropriate alternatives are available.

It has been well established that a number of drugs as well as their metabolites can compete with bilirubin for binding to albumin and disrupt the equilibrium between bilirubin and albumin (4, 9, 13, 18). In newborns, in whom hyperbilirubinemia is frequently observed, any changes in the equilibrium that increase the levels of free bilirubin could enhance the possibility of a bilirubin-induced central neuropathy such as kernicterus.

A major metabolite of salicylate, 2-hydroxybenzoylglycine (HBG), has recently been demonstrated to be present at elevated levels in newborns and was further shown to be an effective binding inhibitor for a highly bound beta-lactam antibiotic, nafcillin (17). Although the exact mechanisms involved in the genesis of the compound have not been elucidated, HBG was demonstrated to be a potent displacing agent for bilirubin when added to pooled cord serum. Furthermore, when the bilirubin-displacing activity of HBG was compared with that of several other compounds which are known to displace bilirubin, HBG exhibited the highest activity among the agents tested. The compounds used in the comparative study included aspirin, 2-hydroxybenzoic acid, sulfisoxazole, and nafcillin (17).

Since HBG was shown to be such an effective bilirubin-displacing agent, it appeared that the compound could be used as an index substance in the measurement of bilirubin-displacing activities of other pharmacological agents. Therefore, several classes of antibiotics that could potentially be used in the management of infectious complications of neonates were surveyed for their activity in displacing bilirubin. Tetracyclines and quinolones were not tested, since their use in newborns is contraindicated.

In this paper, the relative bilirubin-displacing activities of 52 antibiotics and the potential clinical implications of their relative activities are discussed. The antibiotics that have

been tested in this study represent aminoglycosides, sulfonamides, penicillins, cephalosporins, monobactams, carbapenems, and several miscellaneous agents.

MATERIALS AND METHODS

Serum samples. Umbilical cord serum specimens were obtained from the Immunology Laboratory of Temple University Hospital, Philadelphia, Pa., as discards. Serum specimens obtained from individuals with nonreactive syphilis serology and from normal renal- and hepatic-function studies were pooled and used as the source of high-bilirubin-containing sera. The pooled sera were kept at -35°C until use. Two different pools of cord serum were used for this study.

Chemicals. Chemicals were acquired as follows: HBG, sulfadimethoxine, sulfamethazine, sulfaguanidine, sulfamethoxyypyridazine, sulfamerazine, cephalaxin, cephalothin, cephalixin, cefazolin, erythromycin, vancomycin, bilirubin standards, and human albumin (fraction V, fatty acid free) from Sigma Chemical Co., St. Louis, Mo.; cefatrizine, cefadroxil, ceforanide, oxacillin, amikacin, and kanamycin from Bristol-Myers Co., Evansville, Ind.; imipenem and cefoxitin from Merck Sharp & Dohme, Rahway, N.J.; cefuroxime and ceftazidime from Glaxo Inc., Research Triangle Park, N.C.; piperacillin from Lederle Laboratories, Pearl River, N.Y.; nafcillin, dicloxacillin, cyclacillin, and phenoxymethylpenicillin from Wyeth Laboratories, West Chester, Pa.; gentamicin, netilmicin, and sisomicin from Schering Corp., Bloomfield, N.J.; aztreonam, penicillin G, and cephradine from the Squibb Institute for Medical Research, Princeton, N.J.; amoxicillin, ampicillin, carbenicillin, and ticarcillin from Beecham Laboratories, Bristol, Tenn.; cefonicid from Smith Kline & French Laboratories, Philadelphia, Pa.; neomycin, streptomycin, and cefoperazone from Pfizer Inc., Groton, Conn.; sulfamethizole from Ayerst Laboratories, New York, N.Y.; cefotaxime from Hoechst-Roussel Pharmaceuticals Inc., Somerville, N.J.;

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mezlocillin from Miles Pharmaceuticals, West Haven, Conn.; ceftriaxone, sulfisoxazole, trimethoprim, and sulfamethoxazole from Hoffmann-La Roche Inc., Nutley, N.J.; cefamandole and moxalactam from Lilly Research Laboratories, Indianapolis, Ind.; clindamycin from The Upjohn Co., Kalamazoo, Mich.; and chloramphenicol from Warner-Lambert Co., Ann Arbor, Mich. Antibiotic solutions were constituted by following the recommendations of the manufacturer, and subsequent dilutions were made in Krebs-Ringer phosphate buffer (pH 7.4).

Assay procedures. Total protein and albumin concentrations in sera were determined by the methods of Gornall et al. (10) and Dumas et al. (7), respectively. The bilirubin concentrations were estimated by the method of Roth (14) with an Aminco-Bowman spectrofluorometer (American Instrument Co., Silver Spring, Md.), with excitation at 435 nm and emission at 500 nm. The HBG levels in pooled cord serum specimens were measured by high-performance liquid chromatography as previously described (17).

The degree of bilirubin displacement was measured by the ultrafiltration method of Singhvi et al. (15). Membrane ultrafiltration cones (CF50A Centriflo; Amicon Corp., Lexington, Mass.) were prepared as previously described (6). The pHs of the serum pools containing potential bilirubin-displacing agent at the desired concentrations were adjusted to 7.4, and 0.6-ml samples were placed in the cones and centrifuged for 5 min at $900 \times g$. The total bilirubin levels were measured before and after the centrifugation, and appropriate adjustments were made for changes in bilirubin concentration due to the ultrafiltrations. The controls were cord serum specimens treated the same way but without the displacing agent. The difference in total bilirubin concentrations before and after centrifugation was used as the measure of bilirubin displaced. Measurements of total bilirubin levels were made by the method of Roth (14) but with phosphoric acid (85%) extraction for 5 min instead of 1 min, as originally described by Roth. The total bilirubin levels of the pooled cord sera used in the study ranged from 3.5 to 5.5 mg/dl.

For each antibiotic studied, three concentrations (10, 50, and 100 $\mu\text{g/ml}$) were investigated. Experiments were performed a minimum of two times for each concentration and three to six times for most drugs. Values are presented as mean \pm standard error. The relative bilirubin concentration was calculated as the ratio of total bilirubin concentration in the presence of test drug to total bilirubin concentration in the absence of test drug (control).

Data analysis. Standard errors for ratios of bilirubin concentrations were calculated by the method of Hansen et al. (11). Simultaneous 95% confidence intervals (Bonferroni method) were calculated for differences in bilirubin concentrations in the presence and absence of a drug at each of the three concentrations (12). Displacement of bilirubin was considered significant if there was a significant difference in bilirubin level with the addition of a drug at any concentration. For drugs which showed significant bilirubin displacement, the effect of drug concentration was assessed by one-factor analysis of variance. Comparisons of the bilirubin-displacing ability of each of the antibiotics tested was accomplished by constructing simultaneous 95% confidence intervals for paired differences in means between all drugs; a modified Tukey procedure (12) was used because it is highly efficient when large numbers of comparisons are made. Linear regression and calculation of Pearson correlation coefficients were performed in accordance with established procedures. Parametric regression assumptions were verified by using plots of standardized residuals (12).

RESULTS

Effects of HBG on total bilirubin concentration. It has previously been demonstrated that HBG is present in cord sera at elevated levels ranging from 0 to 1.2 $\mu\text{g/ml}$, with an average value of approximately 0.3 to 0.4 $\mu\text{g/ml}$ (17). Two pools of cord serum that contained low levels of HBG ($<0.1 \mu\text{g/ml}$) were used in this study. HBG in the concentration range of 0 to 1.0 $\mu\text{g/ml}$ was tested for its ability to displace bilirubin in cord sera (Table 1). It was demonstrated that HBG was much more potent than any other agent tested. At approximately equimolar concentrations, HBG is at least 200 times more active than the most active antibiotic, sulfisoxazole.

Effects of antibiotics on total bilirubin concentration. A total of 52 antibiotics, including 7 sulfonamides, 12 penicillins, 18 cephalosporins, 7 aminoglycosides, 1 monobactam, 1 carbapenem, and 6 miscellaneous antimicrobial agents, were tested for their activities in displacing bilirubin in cord sera. The antibiotics tested showed various degrees of bilirubin-displacing activity. The antibiotics which did not exhibit significant bilirubin displacement activity were the sulfonamide sulfamethoxine; the penicillins mezlocillin, cyclacillin, and oxacillin; the cephalosporins cefuroxime, cefotaxime, cefatrizine, cefaclor, cephadrine, and cefadroxil; the aminoglycosides kanamycin, gentamicin, and streptomycin; and clindamycin, trimethoprim, rifampin, and chloramphenicol ($P > 0.05$). These antibiotics had relative bilirubin concentrations close to 1.0; with the exception of cyclacillin (maximum bilirubin displacement, 0.84), the relative bilirubin concentrations were all >0.88 at every antibiotic concentration. Each major antibiotic class is represented. Table 1 summarizes the various bilirubin-displacing activities of 34 antibiotics which demonstrated significant bilirubin displacement and of HBG. For 23 of the 34 drugs, there was a significant difference in bilirubin-displacing activity with the different drug concentrations. The relationship between antibiotic concentration and bilirubin displacement was not predictable; 15 of the 23 antibiotics showed significant increases in bilirubin displacement with increasing antibiotic concentrations, but 8 displayed other concentration-effect relationships.

The relative bilirubin-displacing activities of the 34 antibiotics listed in Table 1 were assessed by using simultaneous 95% confidence intervals for differences in means; the maximum bilirubin displacement for the three antibiotic concentrations was used (data not shown). The antibiotics could be classified into three groups on the basis of their displacement activities: high level (sulfisoxazole, sulfamethoxazole, dicloxacillin, cefoperazone, and ceftriaxone); intermediate level (moxalactam, nafcillin, sulfamerazine, sulfamethazine, sisomicin, ampicillin, cefonicid, sulfamethoxyppyridazine, cefoxitin, imipenem, vancomycin, cephalixin, sulfaguanidine, neomycin, erythromycin, and cephalirin); and low level (aztreonam, carbenicillin, ticarcillin, cefazolin, ceftazidime, piperacillin, phenoxymethylpenicillin, netilmicin, amoxicillin, cefuroxime, cefamandole, penicillin G, and amikacin). Results were unchanged when bilirubin displacement at a single drug concentration (100 $\mu\text{g/ml}$) was used. In general, sulfonamide antibiotics revealed a tendency to displace bilirubin, aminoglycoside and miscellaneous antibiotics showed little activity, and other classes showed varied activities.

The degree of protein binding of penicillin and cephalosporin antibiotics was compared with their bilirubin-displacing activities. A significant correlation was observed,

TABLE 1. Relative bilirubin-displacing activities of antibiotics and HBG in pooled cord sera

Antibiotic	Relative bilirubin concn ^a at test drug concn (μg/ml) of:		
	10	50	100
Sulfonamides			
Sulfaguanidine ^b	0.81 ± 0.01	0.91 ± 0.05	0.80 ± 0.02
Sulfamethoxypyridazine	0.84 ± 0.02	0.79 ± 0.06	0.77 ± 0.03
Sulfamethazine ^b	0.93 ± 0.04	0.88 ± 0.03	0.71 ± 0.03
Sulfamerazine ^b	0.99 ± 0.03	0.79 ± 0.04	0.70 ± 0.02
Sulfamethoxazole ^b	0.83 ± 0.02	0.60 ± 0.04	0.53 ± 0.02
Sulfisoxazole ^b	0.77 ± 0.09	0.63 ± 0.07	0.49 ± 0.06
Penicillins			
Penicillin G ^b	1.08 ± 0.01	1.02 ± 0.06	0.91 ± 0.07
Piperacillin ^b	0.89 ± 0.06	0.86 ± 0.05	1.07 ± 0.06
Phenoxymethylpenicillin G ^b	0.92 ± 0.05	0.86 ± 0.03	1.02 ± 0.04
Ticarcillin	0.83 ± 0.04	0.84 ± 0.04	0.90 ± 0.05
Amoxicillin ^b	0.90 ± 0.03	1.08 ± 0.05	0.88 ± 0.05
Carbencillin	0.83 ± 0.02	0.83 ± 0.04	0.88 ± 0.03
Ampicillin ^b	0.78 ± 0.02	0.75 ± 0.03	0.83 ± 0.03
Nafcillin ^b	0.82 ± 0.02	0.76 ± 0.03	0.70 ± 0.03
Dicloxacillin ^b	0.71 ± 0.02	0.75 ± 0.06	0.58 ± 0.05
Cephalosporins			
Cefamandole ^b	0.89 ± 0.03	0.91 ± 0.03	1.09 ± 0.04
Cefazolin ^b	0.84 ± 0.03	1.00 ± 0.05	0.93 ± 0.03
Ceforanide	0.93 ± 0.03	0.93 ± 0.03	0.88 ± 0.33
Ceftazidime ^b	1.02 ± 0.06	1.03 ± 0.04	0.85 ± 0.04
Cefonicid	0.80 ± 0.05	0.76 ± 0.03	0.84 ± 0.07
Cephapirin ^b	0.92 ± 0.03	0.98 ± 0.02	0.82 ± 0.02
Cephalexin	0.80 ± 0.04	0.87 ± 0.02	0.79 ± 0.04
Moxalactam ^b	0.93 ± 0.02	0.68 ± 0.02	0.78 ± 0.04
Cefoxitin	0.79 ± 0.06	0.80 ± 0.05	0.77 ± 0.05
Ceftriaxone ^b	0.77 ± 0.02	0.69 ± 0.03	0.60 ± 0.02
Cefoperazone	0.61 ± 0.03	0.67 ± 0.04	0.59 ± 0.04
Aminoglycosides			
Amikacin ^b	1.00 ± 0.01	1.15 ± 0.07	0.93 ± 0.05
Netilmicin	0.87 ± 0.03	0.88 ± 0.03	0.89 ± 0.04
Neomycin ^b	0.87 ± 0.03	0.88 ± 0.03	0.80 ± 0.04
Sisomicin	0.88 ± 0.05	0.80 ± 0.05	0.72 ± 0.07
Monobactam (aztreonam)	0.83 ± 0.05	0.87 ± 0.05	0.94 ± 0.05
Carbapenem (imipenem) ^b	0.93 ± 0.03	0.93 ± 0.05	0.78 ± 0.03
Miscellaneous			
Vancomycin ^b	0.97 ± 0.03	0.79 ± 0.05	0.81 ± 0.06
Erythromycin ^b	0.93 ± 0.04	1.06 ± 0.02	0.80 ± 0.04
HBG ^{b,c}	0.90 ± 0.02	0.42 ± 0.02	0.37 ± 0.03

^a Calculated as the ratio of total bilirubin concentration in the presence of test drug to total bilirubin concentration in the absence of test drug (control). Each value represents mean ± standard error calculated from experiments performed in triplicate for each drug concentration.

^b Agents for which there are significant differences in relative bilirubin concentration at different concentrations of test drug ($P < 0.05$).

^c The concentrations tested were 0.1, 0.5, and 1.0 μg/ml (100 times lower than those of the antibiotics).

with a correlation coefficient of 0.382 ($P = 0.045$). Figure 1 shows a plot of the amount of protein binding versus relative bilirubin concentrations of penicillins and cephalosporins, which are inversely related to bilirubin displacement activities. The fitted regression line was calculated as follows: relative bilirubin concentration = $-0.182 \times$ protein binding (%) + 0.975. Plots of standardized residuals confirmed the propriety of the linear parametric model. Thus, the more a protein bound an antibiotic, the greater the extent to which it caused bilirubin displacement.

The relative potency of HBG in displacing bilirubin is presented in Fig. 2, in which HBG bilirubin displacement activity is compared with that of several representative antibiotics. The concentration of HBG was 100 times lower than that of the antibiotics. It is well demonstrated that HBG

was the most potent bilirubin-displacing agent among those tested.

DISCUSSION

The ultrafiltration method described in this study used cord sera without dilution, as obtained from the umbilical cord blood. Therefore, any artifacts or erroneous results that could accompany the dilution process could be eliminated (5). Other methods previously reported for similar measurements require a dilution step(s) as an integral part of the procedure (1, 2, 19).

Since we have rediscovered HBG as a potent bilirubin-displacing agent and have been able to adapt a simple reproducible assay procedure to measure the degree of

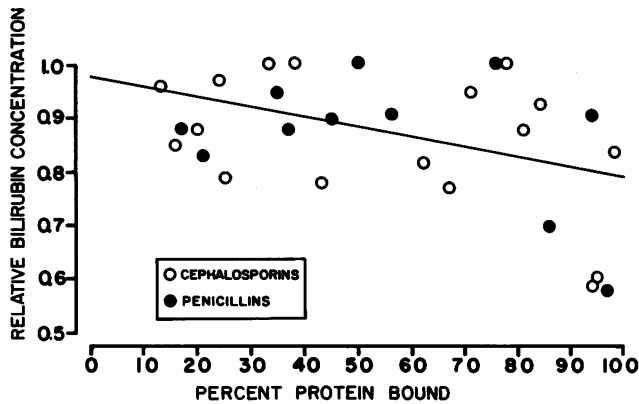


FIG. 1. Correlation between the extent of protein binding and relative bilirubin-displacing activity of penicillins and cephalosporins. Previously reported protein binding values (6) were used.

bilirubin displacement in cord sera, a total of 52 antibiotics were investigated for their relative potency in displacing bilirubin. They included 7 sulfonamides, 12 penicillins, 18 cephalosporins, 7 aminoglycosides, 1 monobactam, 1 carbapenem, and 6 miscellaneous antimicrobial agents. Tetracyclines and quinolone antibiotics were excluded since they were contraindicated in the patient population under investigation.

Of all the agents tested, HBG was again demonstrated to be the most potent bilirubin-displacing agent (by using the

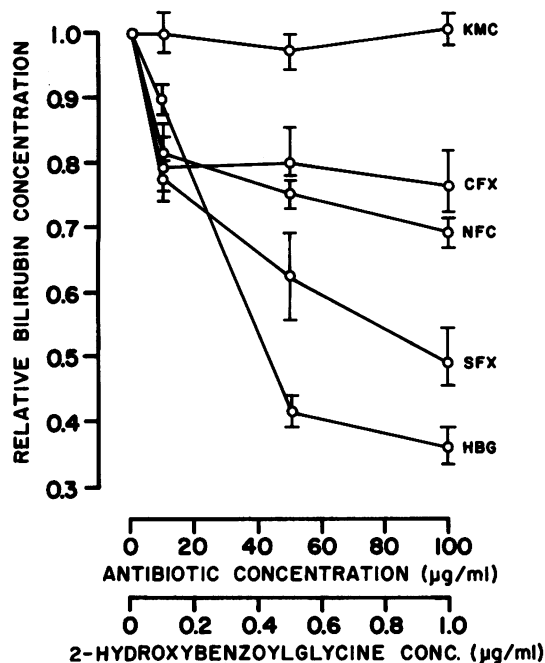


FIG. 2. Effects of antibiotics and HBG on total bilirubin concentration in pooled cord sera. The relative bilirubin concentration was calculated as the ratio of total bilirubin concentration in the presence of test drug to total bilirubin concentration in the absence of test drug (control). Each point represents mean \pm standard error calculated from experiments. Only a few representative antibiotics and HBG are presented. Abbreviations: KMC, kanamycin; CFX, cefoxitin; NFC, nafcillin; SFX, sulfisoxazole; HBG, 2-hydroxybenzoyleglycine.

maximum bilirubin displacement activities), followed by sulfisoxazole, sulfamethoxazole, dicloxacillin, cefoperazone, ceftriaxone, moxalactam, and nafcillin, in decreasing order of potency. These results are comparable to previously reported values for some of the antibiotics, including ceftriaxone (8), sulfisoxazole (3, 18), moxalactam, and cefoperazone (16). In general, almost every sulfonamide agent except sulfamethoxine revealed a strong tendency to displace bilirubin, while aminoglycosides showed minimal bilirubin-displacing activity. The miscellaneous antibiotics representing basic and neutral compounds showed little activity.

A significant difference in bilirubin displacement at different concentrations of each antibiotic was found in 23 of 34 bilirubin-displacing drugs. Although increasing antibiotic concentrations caused increased bilirubin displacement about two-thirds of the time, other relationships were also seen. This observation suggests that concentration-effect relationships may be complex; further study is needed on this point. Another factor affecting the propensity of an antibiotic to displace bilirubin was protein binding by the antibiotic. When this relationship was examined in penicillins and cephalosporins, there was a positive correlation.

It is concluded that whenever antimicrobial therapy is instituted in jaundiced newborns, antibiotics with a tendency to displace bilirubin should be avoided and appropriate alternative therapy should be chosen. However, when the bilirubin-displacing activity is not known for a potential therapeutic agent, the ultrafiltration method described here would be a useful tool for measuring the activity. In addition, it appears that HBG would be an excellent compound to use as a positive control agent.

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