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Comparison of Serum Bilirubin Levels in Humans and Two Monogastric Animal Species After a Single Administration of Sulfisoxazole

ROBERT L. SUBER*§x, JOHN C. GUDAT‡, and GEORGE T. EDDS*

Received January 27, 1981, from the *Department of Preventive Medicine and Toxicology, and the ‡Department of Pathology, Clinical Chemistry, University of Florida, Gainesville, FL 32610. §Present address: National Center for Toxicological Research, Pathology Services Project, Jefferson, AR 72079. Accepted for publication May 29, 1981.

Abstract □ Administration of sulfonamides during periods of hepatobiliary failure or hepatic immaturity increases the toxic potential of unconjugated or indirect bilirubin. A small but statistically significant increase of indirect, or unconjugated bilirubin was noted in dogs after oral administration of sulfisoxazole (100 mg/kg). A similar increase was not observed in swine after oral or intravenous administration of sulfisoxazole (100 mg/kg) or in humans (~28 mg/kg) after oral administration or in dogs (100 mg/kg) after intravenous administration. Total and conjugated bilirubin showed small but statistically significant increases and were significantly correlated in dogs after oral and intravenous administration of sulfisoxazole (100 mg/kg) and in swine after oral administration of sulfisoxazole (100 mg/kg). There was a significant negative correlation between conjugated and indirect bilirubin, while total bilirubin increased in dogs after oral and intravenous administration of sulfisoxazole. These data illustrate a difference in species and administration route when attempting to assess the potential toxicity of bilirubin.

Keyphrases □ Sulfisoxazole—comparison of serum bilirubin levels after a single administration, dogs, pigs, humans □ Bilirubin—serum levels after a single administration of sulfisoxazole, dogs, pigs, humans □ Toxicity—potential, indirect bilirubin serum levels after a single administration of sulfisoxazole

Sulfisoxazole [4-amino-*N*-(3,4-dimethyl-5-isoxazolyl)]-benzenesulfonamide is a white-yellowish, odorless, slightly bitter, crystalline powder with a pK of 4.9 (1). It is distributed in the extracellular fluid and fails to enter cells (2–5) resulting in a plasma concentration which is three times higher than that produced by an equal quantity of sulfanilamide (4). Sulfonamides, as a class of chemotherapeutic agents, are considered to be toxic since they may precipitate in the kidney, producing crystalluria (5). The infrequency of renal toxicosis (crystalluria) with sulfisoxazole is due to the exceptionally high water solubility of the free and conjugated (acetyl) fractions within the physiological pH range (6, 7).

Clinical toxicities have been induced by sulfisoxazole competing for the same binding sites as warfarin (8) and furosemide (9, 10), inducing hemolytic anemia due to glucose-6-phosphate dehydrogenase deficiency (11), inhibition of anticoagulant factor VIII (12), hypersensitivity (13), anorexia (14), agranulocytosis (14), and aplastic anemia (14). A case of myocarditis, myositis, and vasculitis associated with severe eosinophilia following sulfisoxazole therapy has been reported (15). Kernicterus has been reported in premature infants with increased levels of serum bilirubin after treatment with sulfisoxazole (4, 16, 17).

Kernicterus occurred when unconjugated or indirect bilirubin was less than 20 mg% in 24 infants, less than 17 mg% in 15 infants, and less than 15 mg% in 11 infants. These occurrences were enhanced by prior acidosis, hypercapnia, and hypothermia (4).

Plasma samples from six adult patients showed that sulfisoxazole concentrations above 5 mg/100 ml had a significant displacing effect on bilirubin *in vitro* (18). When comparing the displacing effects of salicylic acid, salicyluric acid, and aspirin at sulfisoxazole concentrations of 10 mg/100 ml, the most pronounced effect was observed when sulfisoxazole displaced bilirubin from plasma sam-

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Table I—Mean Control Values of Serum Bilirubin in Humans, Dogs, and Swine [a]

	Total Bilirubin, mg/dl	Conjugated Bilirubin, mg/dl	Indirect Bilirubin, mg/dl
Humans	0.54 ± 0.10	0.41 ± 0.05	0.13 ± 0.02
Dogs	0.21 ± 0.06	0.08 ± 0.04	0.13 ± 0.03
Swine	0.35 ± 0.04	0.19 ± 0.06	0.15 ± 0.05

[a] The mean value ± 1 *SD* of eight samples before oral and intravenous administration of sulfisoxazole in six dogs and six swine. The mean value ± 1 *SD* for humans are calculated from time zero samples for six human subjects.

ples of six adults and 13 infants *in vitro* (18). The displacement of bilirubin is due to competition for similar binding sites on the albumin molecule (19).

Considering these observations and the importance of sulfisoxazole as an antibiotic in treating urinary tract infections, this study was initiated to define a non-rodent animal model which could be used in drug-safety screening of sulfonamides.

EXPERIMENTAL

Model—The trial consisted of six male human volunteers 25–30 years old, weighing 70–80 kg; three female dogs ~2 years old, weighing 20.0 ± 1.0 kg; and six female pigs ~3 months old, weighing 20.0 ± 1.0 kg. The human volunteers were administered 2.0 g of sulfisoxazole in a single oral dose and blood samples were taken at 0, 1, 2, 4, 6, and 8 hr after administration. The dogs were administered 100 mg/kg by intravenous and oral routes in two replicates for each route with a 30-day rest period between each administration. The pigs also were administered 100 mg/kg by intravenous and oral routes with a 21-day rest period between administration. Blood samples were taken at 0.5, 1, 2, 3, 4.5, 6, 9, 12, 22, 32, 44, 56, and 72 hr after intravenous administration and 0, 1, 2, 4, 6, 8, 10, 12, 14, 23, 32, 44, 56, 76, and 96 hr after oral administration in dogs and swine. All animals were maintained on a commercial diet[1,2] with *ad libitum* access to water and were housed in metabolism cages.

Materials—A 12.5% solution of sulfisoxazole[3] was prepared with lithium hydroxide. The solution was filtered and placed in sterile 50-ml ampuls[4]. This solution was used for both the oral and intravenous administration of the drug in dogs and swine and for oral administration to the human subjects. Due to the potential toxicity of intravenous sulfisoxazole administration and its removal from the drug market, intravenous administration of the drug to humans was not allowed[5].

Blood samples were taken from the cephalic vein in dogs and humans and from the anterior vena cava in swine. Human samples were drawn directly into 7-ml sterile silicone-coated tubules[6]. Ten-milliliter samples were taken by a sterile syringe with a 20-gauge needle from the dogs and pigs and transferred to sterile silicone-coated tubes[6]. The samples were centrifuged (2000×*g*) for 10 min, the serum extracted, protected from light, refrigerated, and analyzed within 30 min for total and conjugated (glucuronidated) bilirubin.

Serum Bilirubin—Serum was analyzed for total and conjugated (direct or glucuronidated) bilirubin on a discrete automatic analyzer[7] using a commercial standard[8].

The conjugated method is a modification of the Van den Bergh diazo reaction (20). Under acidic conditions, *p*-nitrobenzenediazonium tetrafluoroborate is coupled to the glucuronidated bilirubin which is measured as an end point reaction at 540 and 600 nm. The normal range in humans is considered to be 0.00 to 0.36 mg/dl[8].

The total bilirubin (the conjugated and unconjugated fractions) was determined also using a derivation of the Van den Bergh reaction (20). A surfactant[9] was used to solubilize the unconjugated (free) bilirubin, which along with the water-soluble glucuronidated bilirubin reacts with *p*-nitrobenzenediazonium tetrafluoroborate in an acid medium to measure an end point reaction at 540 and 600 nm. The normal total bilirubin concentration in humans is <1.5 mg/dl[8]; in dogs, the normal level is <0.5 mg/dl (17); and in swine is <0.6 mg/dl (21).

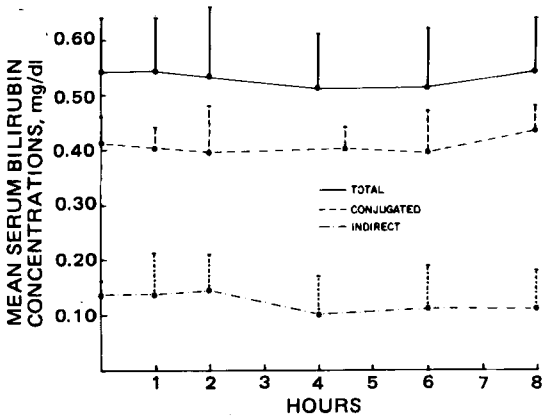

Figure 1—*Serum bilirubin concentrations in humans after oral administration of sulfisoxazole (mean ± 1* SD).

Interferences in these methods are due to hemolyzed samples and light degradation of the bilirubin. All analyses were conducted within 30 min of sampling on serum which was kept in a cool, dark environment.

Total, conjugated, and indirect bilirubin were analyzed for statistical differences[10]. The ANOVA program also produced a correlation coefficient (*R*) for parameters with a significant correlation.

RESULTS

Due to the lack of metabolic heterogenicity of humans, dogs, and swine, each species was used as its own control. The mean values for serum bilirubin were then used as initial or baseline values (Table I).

Humans, Oral Administration—An analysis of variance evaluation after oral administration of sulfisoxazole resulted in no statistical difference in mean total, conjugated, and indirect bilirubin levels (Fig. 1). The lack of significance could be due to the low therapeutic dose (2.0 g, ~28 mg/kg) administered to the human subjects, the fact that humans are able to reduce the *in vivo* effects of sulfisoxazole on the hepato-biliary system, or the sampling period being too short, and additional samples should have been taken over a longer trial period.

Dogs, Intravenous Administration—The mean total bilirubin concentration in six dogs administered sulfisoxazole intravenously exhibited statistically significant ($p < 0.01$) linear increases at 4.5 ($p < 0.05$), 6.0 ($p < 0.1$), 9.0 ($p < 0.01$), and 12.0 ($p < 0.01$) hr with the maximum levels occurring at 6 and 12 hr (Fig. 2). By the next sampling period (22 hr), the mean total bilirubin concentration had decreased to levels which were not significantly different from control values until the 56-hr sampling period ($p < 0.01$).

The mean conjugated bilirubin levels also showed statistically significant increases at 4 hr ($p < 0.01$) and continued throughout the sampling period ($p < 0.01$). There was a significant ($p < 0.01$) maximum mean

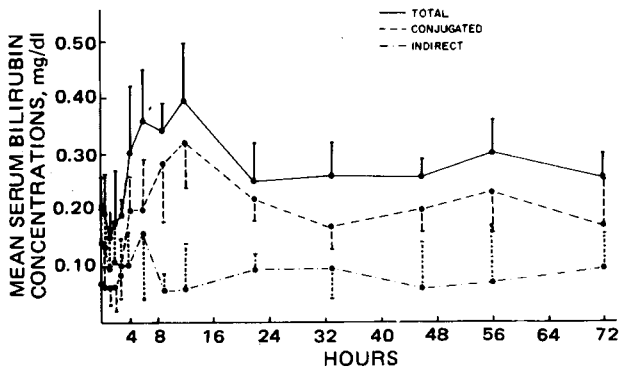

Figure 2—*Serum bilirubin concentrations in dogs after intravenous administration of sulfisoxazole (mean ± 1* SD).

[1] Purina Dog Chow, Ralston Purina, St. Louis, Mo.
[2] Swine Feed 18% protein, University of Florida, Gainesville, Fla.
[3] Hoffmann-LaRoche, Nutley, N.J.
[4] Wheaton, Scientific Products, Ocala, Fla.
[5] Human Research Committee of the University of Florida.
[6] Vacutainer, Becton-Dickinson, Rutherford, N.J.
[7] ACA, Dupont Instruments, Wilmington, Del.
[8] Dade Division, American Hospital Supply, Miami, Fla.
[9] Tween 20, Dupont Instruments, Wilmington, Del.
[10] ANOVA program (22), Northeast Regional Computer Center, University of Florida.

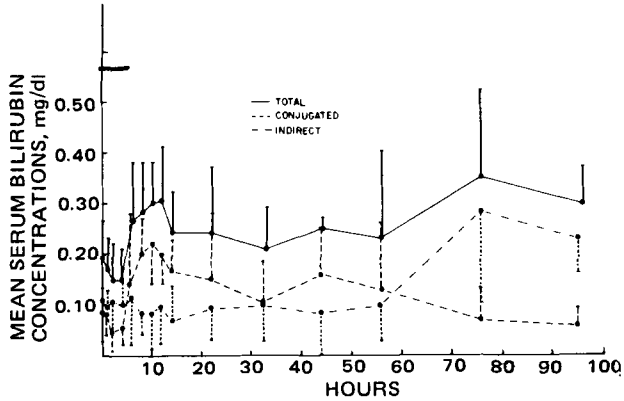

Figure 3—*Serum bilirubin concentrations in dogs after oral administration of sulfisoxazole (mean ± 1 SD).*

conjugated bilirubin concentration at 12 hr, which coincided with the maximum mean total bilirubin concentration (Fig. 2). A second increase was observed at 56 hr ($p < 0.01$) which coincided with the second increase in total bilirubin (Fig. 2). The mean indirect bilirubin concentration (Fig. 2) was not significantly different from the control level throughout the sampling period.

The mean total bilirubin was significantly correlated with mean conjugated bilirubin ($R = 0.75, p = 0.0001$) and with mean indirect bilirubin ($R = 0.31, p = 0.004$) after intravenous administration. The significant correlation of total and conjugated bilirubin is undoubtedly due to the concomitant increase in glucuronidation activity as total bilirubin levels increased. If the glucuronidation enzyme system is not capacity limited, the possibility of kernicterus due to increased indirect bilirubin is minimized after intravenous administration of sulfisoxazole in the dog. This enzyme system acts to maintain a reduced or limited indirect bilirubin concentration and reduces the toxicity of displaced protein-bound bilirubin or increased heme degradation. This was further emphasized by the significant negative correlation of conjugated and indirect bilirubin ($R = -0.39, p = 0.0002$). As the level of conjugated bilirubin increased, the potentially toxic indirect bilirubin level decreased.

A second explanation would be that sulfisoxazole alters the function of the normal hepatocyte to increase conjugated bilirubin regurgitation into the general circulation, instead of being excreted into the bile. The resulting increase in total bilirubin would be the result of the increased level of regurgitated, conjugated bilirubin, along with the normal level of indirect bilirubin.

Dogs, Oral Administration—When sulfisoxazole was administered orally to dogs, there was a significant linear increase in mean total ($p < 0.01$), conjugated ($p < 0.001$), and indirect ($p < 0.0001$) bilirubin (Fig. 3). Total bilirubin reached its first significant peak at 12 hr ($p < 0.01$) and a higher concentration at 76 hr ($p < 0.01$) (Fig. 3). The mean total bilirubin levels were significantly increased at 8 ($p < 0.05$), 10 ($p < 0.01$), and 12 ($p < 0.01$) hr. A second higher peak occurred at 76 hr ($p < 0.01$) and remained greater than the control levels at the end of the sampling period, 96 hr ($p < 0.01$) (Fig. 3). The mean conjugated bilirubin levels also increased linearly ($p < 0.001$) during the sampling period. Significant increases ($p < 0.05$) were observed at 8, 10, and 12 hr (Fig. 3), the same

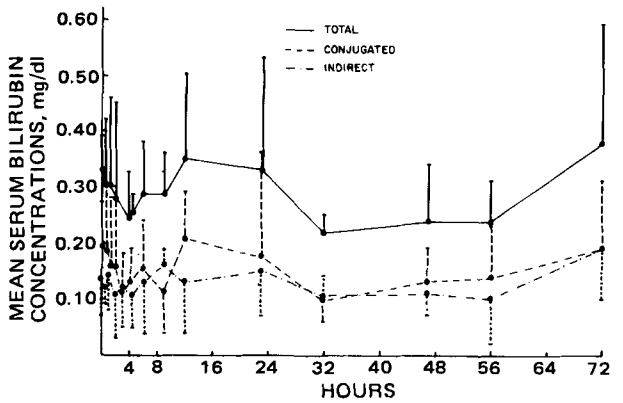

Figure 4—*Serum bilirubin concentrations in swine after intravenous administration of sulfisoxazole (mean ± 1 SD).*

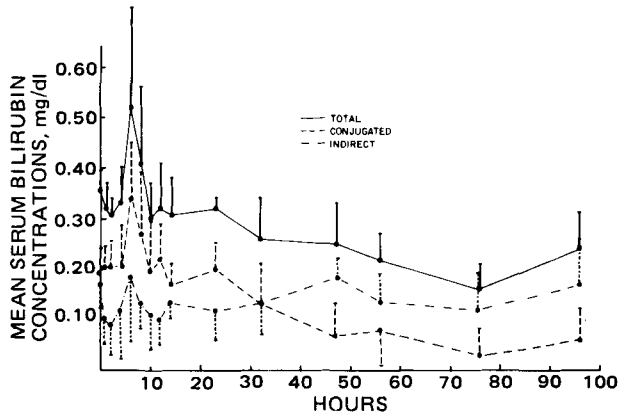

Figure 5—*Serum bilirubin concentrations in swine after oral administration of sulfisoxazole (mean ± 1 SD).*

periods in which total bilirubin was significantly increased. After the 12-hr period, the mean conjugated bilirubin had returned to levels which were not significantly different from control levels. The mean conjugated bilirubin reached its maximum concentration (0.22 mg/dl) at 10 hr, while the maximum total bilirubin (0.31 mg/dl) occurred at the 12-hr sampling period (Fig. 3). The mean indirect bilirubin was not significantly different from control levels until the 76- and 96-hr periods ($p < 0.01$). Mean indirect bilirubin reached a maximum of 0.28 mg/dl at 76 hr but had begun to decline by the end of the trial.

Total bilirubin was significantly correlated with conjugated bilirubin ($R = 0.06, p = 0.0001$) and with indirect bilirubin ($R = 0.56, p = 0.00001$) while conjugated bilirubin was also negatively correlated with indirect bilirubin ($R = -0.31, p = 0.003$). The significant correlation of total and conjugated bilirubin is explained by an increase in glucuronidation as increased heme degradation or increased indirect bilirubin occurs. It may also be due to regurgitation of conjugated bilirubin into the general circulation. The significant correlation of indirect and total bilirubin illustrated that the glucuronidation activity could not account for conjugation of all the bilirubin present. The negative correlation of indirect and conjugated bilirubin is supportive evidence that toxic, indirect bilirubin levels can be reduced to prevent kernicterus, if glucuronidation activity can be stimulated.

Swine, Intravenous Administration—Analysis of variance using mean bilirubin concentrations revealed no statistical difference among the six pigs or during time intervals for total, conjugated, or indirect serum bilirubin after intravenous administration of sulfisoxazole. The mean total conjugated and indirect bilirubin were increasing at the end of the trial but were not significantly different from control levels (Fig. 4).

Total bilirubin was significantly correlated with conjugated bilirubin ($R = 0.86, p = 0.0001$) and with indirect bilirubin ($R = 0.61, p = 0.0001$) after intravenous administration of sulfisoxazole.

Swine, Oral Administration—After oral administration of sulfisoxazole, mean total bilirubin was significantly increased ($p < 0.01$) to 0.52 mg/dl at 6 hr (Fig. 5). A parallel significant ($p < 0.01$) increase was also observed in mean conjugated bilirubin at 6 hr, 0.34 mg/dl (Fig. 5). A significant ($p < 0.0001$) linear decrease occurred over the trial period in conjugated bilirubin. Mean indirect bilirubin was not significantly different from control values at any time after oral administration of sulfisoxazole. However, indirect bilirubin levels did exceed conjugated levels after the 32-hr sampling period and remained at an increased level throughout the remainder of the trial (Fig. 5).

Total bilirubin was significantly correlated with conjugated bilirubin ($R = 0.81, p = 0.0001$) and with indirect bilirubin ($R = 0.42, p = 0.0001$) after oral administration of sulfisoxazole.

Comparison of Bilirubin Levels in Dogs, Swine, and Humans—Control values for total and conjugated bilirubin were higher in humans than in swine and lowest in dogs (Table I). The indirect bilirubin levels in all three species were similar.

A significant increase in total bilirubin occurred in dogs at 12 hr (Fig. 2) after intravenous administration of sulfisoxazole but did not occur in swine (Fig. 4) after administration of the same dose. After oral administration of sulfisoxazole, a significant increase in total bilirubin occurred in dogs (Fig. 3) and swine (Fig. 5) at 6 and 12 hr, respectively, with another increase in dogs at 76 hr. In humans, this increase was not observed over the same time period, probably due to the difference in the oral dosage (Fig. 1).

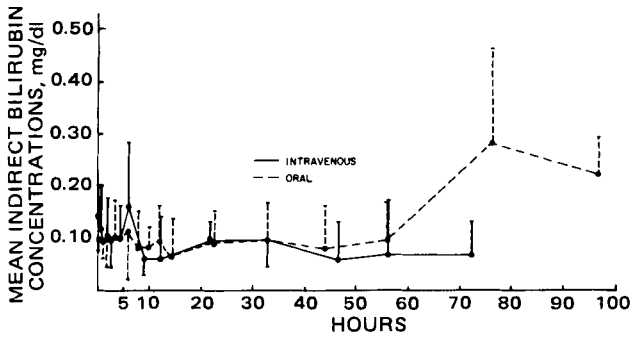

Figure 6.—*Comparison of the indirect bilirubin concentrations in dogs after intravenous and oral administration of sulfisoxazole (mean ± 1* SD).

Initially, total bilirubin levels were higher in swine than in dogs (Table I) but after intravenous administration of sulfisoxazole, total bilirubin was significantly ($p < 0.01$) higher in dogs between 3 and 15 hr (Fig. 2). Total bilirubin levels after oral administration were higher in swine than in dogs until the 56 hr period when the levels in dogs were significantly increased ($p = < 0.01$) (Fig. 3). Even after administration of sulfisoxazole, the total bilirubin increases in dogs and swine did not reach the control or treated levels in humans.

Conjugated bilirubin levels were not significantly increased in swine after intravenous administration of sulfisoxazole even though the control levels were greater in swine than in dogs. Conjugated bilirubin levels were significantly increased in dogs after intravenous administration of sulfisoxazole, exceeded the levels in swine after 3 hr, and reached a maximum at 12 hr after administration. After oral administration of sulfisoxazole, conjugated bilirubin levels were elevated in swine at 6 hr and showed a statistically significant increase in dogs at 10 hr after administration of the drug. The conjugated bilirubin levels in dogs and swine after intravenous or oral administration of sulfisoxazole did not reach the control or treated conjugated bilirubin levels in humans at any of the sampling periods.

Potentially toxic indirect bilirubin showed a statistically significant, but clinically small, increase after oral administration of sulfisoxazole in dogs (Fig. 6). There were no statistically significant increases in indirect bilirubin after oral administration in humans or swine or after intravenous administration of sulfisoxazole to dogs or swine.

DISCUSSION

Small but statistically significant increases in total and conjugated bilirubin occurred when sulfisoxazole was administered orally to dogs and swine but only when administered intravenously to dogs. The maximum increases occurred at different times, depending on species and route of administration. This species variation is further reflected by the indirect bilirubin exceeding conjugated bilirubin levels in dogs and swine when sulfisoxazole was administered orally. While toxic levels of bilirubin have not been established, these results show that the oral route of administration does increase the potentially toxic, indirect bilirubin concentration at a time far removed from the initial single dose in dogs and swine.

The small but statistically significant increase of total and conjugated bilirubin, after oral administration of sulfisoxazole, suggests that the absorption of the oral dosage form may cause the hepatocytes to increase the normal rate of heme degradation or to increase conjugated bilirubin regurgitation into the general circulation. This suggestion is supported by the intravenous dose given to dogs increasing only the total and conjugated bilirubin and not significantly affecting the indirect bilirubin level.

The lack of significant effects in humans and the lack of correlation between humans and dogs or swine could be due to the shorter sampling period in humans, the reduced dosage given to humans, or the species variation which exists even though all are monogastric.

When comparing the correlation coefficient of total and conjugated bilirubin in dogs ($R = 0.75, p = 0.0001$) and swine ($R = 0.85, p = 0.0001$) after intravenous administration, these parameters are more closely correlated in the pig. This is also true for the significant correlation of indirect and total bilirubin in dogs ($R = 0.31, p = 0.004$) and swine ($R = 0.61, p = 0.0001$) after intravenous administration of the drug. When sulfisoxazole is administered orally, the total and conjugated bilirubin levels are more closely correlated in swine ($R = 0.81, p = 0.0001$) than in dogs ($R = 0.60, p = 0.0001$) but indirect bilirubin is more closely correlated to total bilirubin in dogs ($R = 0.56, p = 0.0001$) than in swine ($R = 0.42, p = 0.0001$). In addition, conjugated and indirect bilirubin are negatively correlated after intravenous ($R = -0.39, p = 0.0002$) and oral ($R = -0.31, p = 0.003$) administration only in the dog.

It is evident that the route of sulfisoxazole administration affects the bilirubin level differently in these three species of animals. The potentially toxic indirect bilirubin level is significantly increased only after oral administration in one of the three species (dogs). This observation would be consistent with the first-pass effect (23) which states that oral administration of drugs results in peripheral venous concentrations exclusively *via* the hepatic portal system. This phenomenon, coupled with the reduced or impaired metabolic capability of the dog, would explain the increase in unconjugated bilirubin in this one-animal model. As a non-rodent model, the dog presents an acceptable model to be used in the preliminary evaluation of potentially-toxic, drug-induced effects on bilirubin metabolism after oral administration of the compound.

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