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Effect of Sulfisoxazole on Pharmacokinetics of Free and Plasma Protein-Bound Bilirubin in Experimental Unconjugated Hyperbilirubinemia

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Abstract □ The effect of sulfisoxazole on the time course of free (unbound) bilirubin concentrations in plasma was studied. Normal adult rats were made hyperbilirubinemic by continuous intravenous infusion of bilirubin. Sulfisoxazole was administered by either rapid intravenous injection or slow intravenous infusion, and the plasma concentrations of free and total (free plus bound) unconjugated bilirubin were determined as a function of time. Rapid injection of sulfisoxazole caused a rapid and pronounced decrease of total bilirubin concentrations in plasma but had only a transient effect on the concentration of free bilirubin. Slow infusion of sulfisoxazole caused a gradual and eventually pronounced decrease of total bilirubin concentrations in plasma but had no apparent effect on the concentration of free bilirubin at any time. These results are consistent with recently developed pharmacokinetic theory according to which the plasma clearance of total bilirubin should increase upon administration of a displacing agent while the plasma clearance of free bilirubin should remain unchanged. Bilirubin-induced encephalopathy caused by sulfisoxazole or other displacing agents may be due to very transient elevations of free bilirubin concentrations in plasma of infants with elevated plasma concentrations of total bilirubin and the consequent redistribution of the pigment to extravascular sites, including the brain.

Keyphrases □ Sulfisoxazole—effect on pharmacokinetics of bilirubin in hyperbilirubinemic rats □ Pharmacokinetics—bilirubin in hyperbilirubinemic rats, effect of sulfisoxazole □ Bilirubin—pharmacokinetics in hyperbilirubinemic rats, effect of sulfisoxazole □ Antibacterials—sulfisoxazole, effect on pharmacokinetics of bilirubin in hyperbilirubinemic rats

Administration of sulfisoxazole to premature infants with neonatal jaundice has caused kernicterus (brain damage), often with fatal outcome (1, 2). Typically, this effect has been associated with a decrease of bilirubin concentrations in plasma. The same phenomenon was observed in rats with unconjugated hyperbilirubinemia (3). Sulfisoxazole is a potent displacer of plasma protein-bound bilirubin (4), and some of this displaced bilirubin is redistributed to extravascular sites, including the brain where it exerts its toxic effect (5).

Previous *in vivo* studies of the interaction between sulfisoxazole and bilirubin were limited by the lack of suitable methodology for the determination of free (unbound) unconjugated bilirubin in plasma. The more recently developed reaction rate method (6) permits determination of free bilirubin in undiluted plasma under clinically realistic conditions, *i.e.*, at bilirubin to albumin molar ratios of less than unity (6, 7). Therefore, an investigation was initiated to explore the kinetics of the interaction between sulfisoxazole and bilirubin in rats with experimental unconjugated hyperbilirubinemia, with emphasis on the temporal pattern of free and total (sum of free and protein-bound) unconjugated bilirubin concentrations in plasma and total unconjugated bilirubin concentrations in erythrocytes before and after intravenous injection or infusion of sulfisoxazole.

BACKGROUND

Bilirubin is eliminated almost entirely by conjugation in the liver and subsequent excretion of the conjugates in the bile and urine (8). The total plasma clearance of bilirubin is about 10% of the plasma perfusion rate of the liver (9), and the concentration of total bilirubin in erythrocytes is about 10% of the concentration in plasma of rats with experimental hyperbilirubinemia under otherwise normal physiological conditions (Ref. 10 and results of this study). Therefore, the following pharmacokinetic relationships may be expected to apply (11):

$$TC = f_p k'' \quad \text{(Eq. 1)}$$

$$C_\infty = R^0/TC \quad \text{(Eq. 2)}$$

$$f_p C_\infty = R^0/k'' \quad \text{(Eq. 3)}$$

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where TC is the total plasma clearance, k'' is the intrinsic clearance (reflecting the activity of the enzyme systems involved in the rate-determining steps of bilirubin conjugation), f_p is the free fraction of bilirubin in plasma (*i.e.*, the ratio of free to total bilirubin concentrations), C_∞ is the steady-state plasma concentration of total bilirubin, f_pC_∞ is the steady-state plasma concentration of free bilirubin, and R^0 is the sum of the production and infusion rates of bilirubin. When the infusion rate is sufficient to produce marked hyperbilirubinemia in normal animals (as in this study), $R^0 \simeq$ infusion rate.

Equation 1 was confirmed experimentally in rats (12). Accordingly, administration of a displacing agent, resulting in an increased f_p, should cause a decrease of C_∞ (Eqs. 1 and 2) but should have no effect on the *steady-state* plasma concentration of free bilirubin as long as the displacing agent has no effect on k'' (*i.e.*, on the activity of the enzyme systems responsible for the biotransformation of bilirubin). This prediction was confirmed with respect to salicylic acid (10). From a toxicologic point of view, pharmacokinetic studies of interactions between bilirubin and displacing agents should focus particularly on the temporary perturbations of bilirubin kinetics and not solely on the steady state before and after (or during continued) administration of the displacing agent. The investigation of the interaction between bilirubin and sulfisoxazole described here was designed on the basis of these considerations.

EXPERIMENTAL

Male Sprague–Dawley rats[1], 360–460 g, were maintained on a standard diet[2]. Two days before an experiment, a silicone rubber–polyethylene cannula was placed permanently in the right jugular vein to facilitate intravenous infusion, injection, and frequent withdrawal of blood samples (13, 14). On the day of an experiment, a solution of bilirubin and sodium taurocholate (1:1.1 weight ratio, in 0.7% sodium chloride adjusted to pH 7.4) was infused at a rate of 0.8 mg of bilirubin/kg/min for 15 min and then at 0.32 mg of bilirubin/kg/min for 105 min.

Half of the rats then received an intravenous injection of sulfisoxazole, 40 mg/kg, in 0.5 ml of pH 7.4 phosphate buffer, and the infusion was continued for an additional 120 min with bilirubin, 0.32 mg/kg/min, and sulfisoxazole, 0.20 mg/kg/min. The other rats received the same bilirubin infusion regimen for the first 2 hr, followed by an infusion of sulfisoxazole, 0.44 mg/kg/min, and bilirubin, 0.32 mg/kg/min, for the next 2 hr. About 1 week later, crossover experiments were performed. All infusion equipment, including syringes and cannulas, was covered with aluminum foil to protect bilirubin from light.

Heparinized blood samples (0.6 ml) were collected at 1, 1.5, and 2 hr and then again at 15, 30, 60, and 120 min after the sulfisoxazole injection or the start of the sulfisoxazole infusion. An additional blood sample was obtained 5 min after the sulfisoxazole injection. Considerable care was taken to obtain "mainstream" blood samples uncontaminated by the bilirubin infusion solution (14). Plasma and erythrocytes were separated by centrifugation and analyzed for free (4, 6) and total unconjugated bilirubin (15) and for sulfisoxazole (16). Whole blood concentrations of unconjugated bilirubin were calculated from the concentrations in plasma and erythrocytes and the fractional volumes of these phases in whole blood.

RESULTS

The bilirubin infusion schedule resulted in steady-state plasma concentrations of total bilirubin between 5.7 and 10.3 mg/100 ml until sulfisoxazole was administered. The total bilirubin concentration in plasma decreased rapidly and substantially from 7.43 ± 1.91 mg/100 ml (mean ± *SD*) immediately before sulfisoxazole injection to 4.10 ± 1.24 mg/100 ml 5 min after injection ($p < 0.02$) and then increased slightly as sulfisoxazole concentrations in plasma declined with time (Fig. 1). The concentration of free bilirubin increased about threefold immediately after sulfisoxazole injection but returned to the presulfisoxazole level soon thereafter. This temporary increase occurred in all four rats but differed significantly in magnitude among animals. Therefore, four additional animals were studied.

The average (± *SD*) concentration of free bilirubin in the plasma of all eight rats immediately before sulfisoxazole injection was 3.4 ± 0.8 μg/100 ml; it increased to 12.6 ± 10.2 μg/100 ml 5 min after sulfisoxazole injection ($p < 0.05$ by paired t-test; $p < 0.004$ by sign test). The concentration of total unconjugated bilirubin in whole blood decreased from

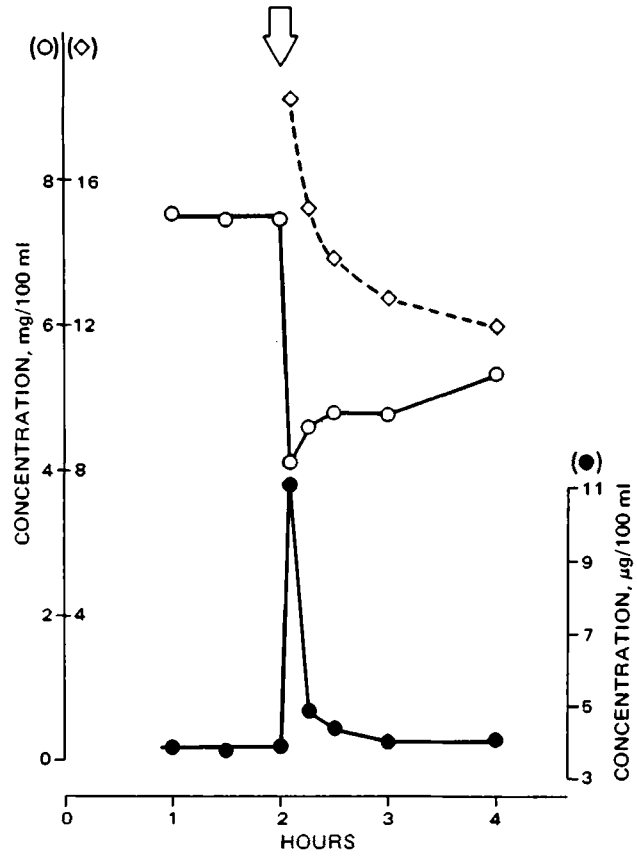

Figure 1—*Time course of free bilirubin (●), total bilirubin (○), and sulfisoxazole (◇) concentrations in plasma of four rats with experimental unconjugated hyperbilirubinemia. The arrow indicates the time of injection of sulfisoxazole, 40 mg/kg. Following the injection, sulfisoxazole was infused at a rate of 0.20 mg/kg/min.*

4.54 ± 1.06 immediately before to 3.12 ± 1.02 mg/100 ml 5 min after sulfisoxazole injection ($p < 0.05$).

Slow infusion of sulfisoxazole over 2 hr resulted in a gradual decrease in the total bilirubin concentration while the concentration of free bilirubin remained essentially constant at all times (Fig. 2), but blood was not sampled 5 min after the start of the sulfisoxazole infusion. The total bilirubin concentration in plasma at the end of the 2-hr sulfisoxazole infusion was significantly lower than immediately before the start of the infusion ($p < 0.005$). An examination of the data shows that a given sulfisoxazole concentration produces the same quantitative decrease in the concentration of total bilirubin in plasma whether sulfisoxazole concentrations were decreasing (after injection) or increasing (during infusion) with time (Fig. 3).

The total bilirubin concentrations in the erythrocytes during the sulfisoxazole injection and infusion experiments are listed in Table I. These concentrations increased upon administration of sulfisoxazole, indicating a shift of bilirubin from plasma to erythrocytes. Notably, the elevation of erythrocyte bilirubin concentrations persisted for the duration of the experiments.

DISCUSSION

The results of this investigation are consistent with and confirm the applicability to bilirubin of the pharmacokinetic relationships represented by Eqs. 1–3. The plasma concentration of total bilirubin decreased upon administration of sulfisoxazole. The magnitude of this effect increased with increasing concentrations of sulfisoxazole, consistent with the concentration-dependent displacing effect of this drug on plasma protein-bound bilirubin (4). On the other hand, the concentration of unbound bilirubin remained constant, except for a temporary increase immediately after injection pending reestablishment of the steady state. This finding indicates that sulfisoxazole had no effect on the intrinsic clearance, k'', of bilirubin under the conditions of this investigation.

Direct and indirect evidence indicates that the concentration of free rather than total bilirubin in plasma determines, among other factors,

[1] Blue Spruce Farms, Altamont, N.Y.
[2] Charles River Formula 4RF.

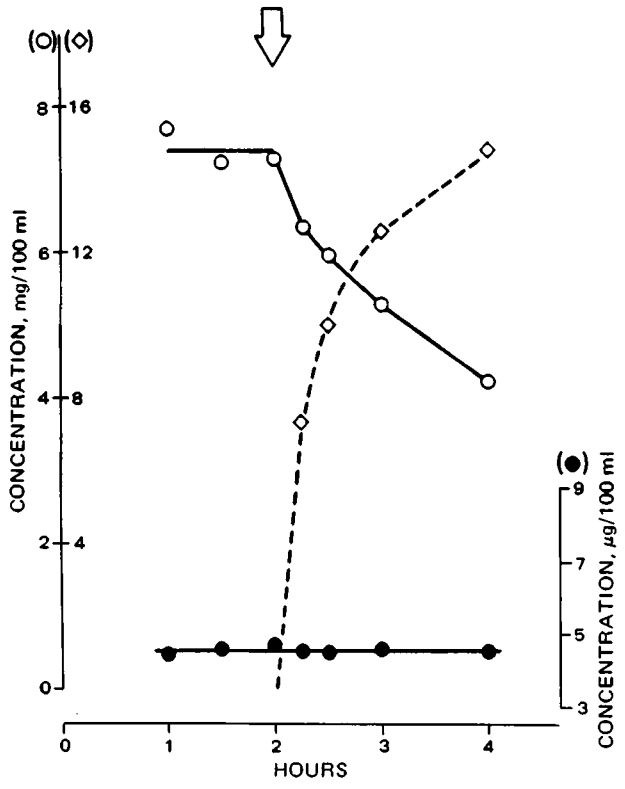

Figure 2—*Time course of free bilirubin (●), total bilirubin (○), and sulfisoxazole (◇) concentrations in plasma of four rats with experimental unconjugated hyperbilirubinemia. The arrow indicates the beginning of an infusion of sulfisoxazole at a rate of 0.44 mg/kg/min.*

the risk of brain damage in hyperbilirubinemic infants (17). Based on the results of this investigation, the bilirubin-induced encephalopathy caused by sulfisoxazole or other drugs capable of displacing bilirubin from plasma albumin is probably due to transient elevations of free bilirubin concentrations in plasma of jaundiced infants associated with perturbation of steady-state conditions and the consequent redistribution of bilirubin from plasma to other sites. These other sites include the brain (5) and erythrocytes.

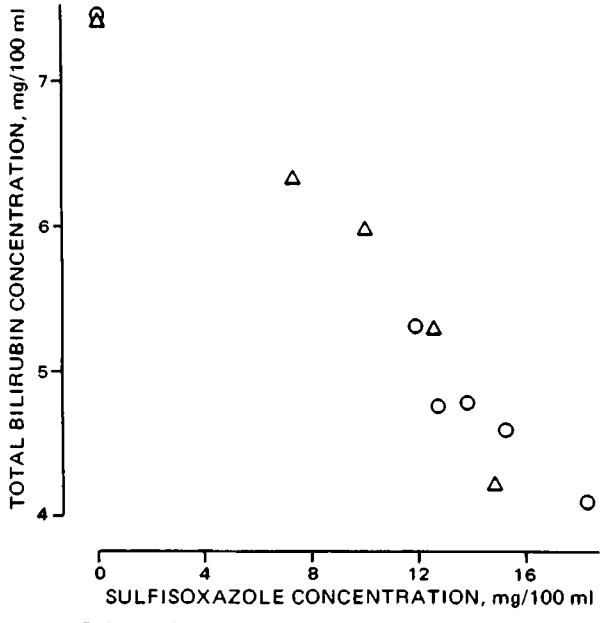

Figure 3—*Relationship between the plasma concentrations of total bilirubin and sulfisoxazole after rapid injection (○) and during infusion (△) of sulfisoxazole. Each data point represents the mean of four animals.*

Table I—Effect of Sulfisoxazole Injection or Infusion on Total Bilirubin Concentration in Erythrocytes of Rats with Experimental Unconjugated Hyperbilirubinemia

Hours	Concentration, mg/100 ml (mean ± *SD*, *n* = 4) Injection	Infusion
1.0	0.84 ± 0.13	0.80 ± 0.13
1.5	0.76 ± 0.07	0.74 ± 0.03
2.0	0.77 ± 0.08	0.82 ± 0.16
2.08	1.77 ± 0.70[a]	—[b]
2.25	1.58 ± 0.39[a]	1.31 ± 0.26[a]
2.5	1.57 ± 0.44[a]	1.62 ± 0.38[a]
3.0	1.48 ± 0.38[a]	1.42 ± 0.21[a]
4.0	1.47 ± 0.48[a]	1.41 ± 0.28[a]

[a] Statistically significantly different from control (1.0–2.0 hr) period, $p < 0.005$. [b] No blood sample was taken at this time.

As in a previous study with salicylate (10), the elevated concentrations of bilirubin in the erythrocytes persisted throughout the experiment (*i.e.*, for at least 2 hr), unlike the total bilirubin concentrations in plasma which reversed readily as drug concentrations declined (Figs. 1 and 3). Studies in hyperbilirubinemic rats and in human infants have shown persistence of bilirubin in the brain (18). Precipitation of the very poorly water-soluble pigment in the brain and erythrocytes may be responsible for this persistence; crystals of bilirubin have been found in both tissues (19, 20).

The results of this investigation suggest that clinical assessment of jaundiced infants may be facilitated by the determination of bilirubin concentrations in erythrocytes in addition to the determination of the free bilirubin concentration in plasma, particularly if the patients have been treated with drugs that can displace bilirubin from binding sites on plasma proteins. The potential usefulness of this procedure will have to be assessed in a prospective clinical trial.

The effects of salicylic acid (10) and sulfisoxazole on the plasma protein binding and pharmacokinetics of bilirubin are similar in most respects but strikingly different in one: rapid intravenous injection of salicylate was not associated with an elevation of free bilirubin concentrations in plasma 5 min later (10) while sulfisoxazole injection caused a pronounced, though temporary, rise in these concentrations under the same conditions (Fig. 1). The diffusion of free bilirubin from plasma to extravascular sites (but apparently not from plasma to erythrocytes) appears to be slower after injection of sulfisoxazole than after injection of salicylate. The relevance of this observation to the induction of encephalopathy by bilirubin and the mechanism of the apparent difference in the distribution kinetics of bilirubin in the presence of salicylate or sulfisoxazole remain to be determined.

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Spectrophotometric Determination of Theophylline Formulations

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Received May 23, 1977, from the *Faculty of Pharmacy, University of Alexandria, Alexandria, Egypt.* Accepted for publication February 8, 1978. *Present address: Department of Pharmacy, Faculty of Biological and Pharmaceutical Sciences, University of Nigeria, Nsukka, Nigeria.

Abstract □ Minophylline (theophylline ethanoate of piperazine) and aminophylline (theophylline ethylenediamine) were determined spectrophotometrically in dosage forms without interference from excipients and/or preservatives. A mixture of minophylline, in about 30-fold concentration, with phenobarbital was assayed for both components with good accuracy and high reproducibility.

Keyphrases □ Minophylline—spectrophotometric analysis in pharmaceutical formulations □ Aminophylline—spectrophotometric analysis in pharmaceutical formulations □ Spectrophotometry—analyses, minophylline and aminophylline in pharmaceutical formulations □ Diuretic-vasodilators—minophylline, spectrophotometric analysis in pharmaceutical formulations □ Relaxants, smooth muscle—aminophylline, spectrophotometric analysis in pharmaceutical formulations

The assay of binary mixtures in pharmaceutical formulations is challenging. One example is minophylline[1] and phenobarbital mixtures, especially when the latter component is present in small amounts. The interference of excipients and/or preservatives increases the severity of the problem.

BACKGROUND

The various methods dealing with the correction of interfering absorbances were reviewed (1, 2). The correction of linear interferance can be carried out graphically (3) or algebraically (4–7). By applying the algebraic version to the correction of linear impurity absorption, the concentration, C, can be determined from:

$$C = \frac{A_1(\lambda_2 - \lambda_3) - A_2(\lambda_1 - \lambda_3) + A_3(\lambda_1 - \lambda_2)}{E_1(\lambda_2 - \lambda_3) - E_2(\lambda_1 - \lambda_3) + E_3(\lambda_1 - \lambda_2)} \quad \text{(Eq. 1)}$$

in which A_1, A_2, and A_3 are the absorbances at λ_1, λ_2, and λ_3, respectively; E_1, E_2, and E_3 are the corresponding 1-cm path length absorbances of a 1% solution. Dividing both numerator and denominator by $(\lambda_1 - \lambda_3)$ and substituting h for $(\lambda_2 - \lambda_3)/(\lambda_1 - \lambda_3)$ give the following equation after simple rearrangement:

$$A_2 - hA_1 - (1 - h)A_3 = C[E_2 - hE_1 - (1 - h)E_3] \quad \text{(Eq. 2)}$$

Substitution of the left-hand term by corrected A (A_c) and the second term in the right-hand side by K yields:

$$A_c = CK \quad \text{(Eq. 3)}$$

A linear relationship is obtained by plotting A_c *versus* C.

Another method for the correction of interfering absorbances is Glenn's method of orthogonal function (8), in which absorbance A is replaced by the coefficient of the orthogonal function, p_j. This coefficient is proportional to concentration. To extract the coefficient of a given polynomial from an absorption curve, it is necessary to obtain absorbances at a number of equally spaced wavelengths. Thus, to extract the coefficient of the quadratic polynomial p_2, for example, six absorbance measurements at six equally spaced wavelengths are needed. By plotting the p_2 at different intervals *versus* λ_m (the mean set of wavelengths), a convoluted absorption curve is obtained (9).

The present paper reports the determination of minophylline in the presence of the tablet base, sweetening agent, coloring agent, and preservatives usually existing in pharmaceutical preparations; the determination of aminophylline in ampuls containing benzyl alcohol as a preservative; and an assay for a minophylline–phenobarbital mixture in syrup. Determination of phenobarbital in this mixture is difficult since it is present in a small amount.

EXPERIMENTAL

Materials—Minophylline[2] and aminophylline[3] standard solutions were at a concentration of 1 mg/ml in 0.1 *N* H_2SO_4. Phenobarbital sodium[4] standard solution was 1 mg/ml in water. Minophylline tablets[2], Batch 7, contained 250 mg/tablet; minophylline ampuls[2], Batch 29, contained 200 mg/2 ml.

Minophylline–phenobarbital[2], Batch 101,004, contained 2.0 g of minophylline and 0.06 g of phenobarbital/100 ml. Aminophylline ampuls[5], Batch S/52D, contained 500 mg of aminophylline/2 ml and 0.04 ml of benzyl alcohol as the preservative.

Reagents—Analytical grade 0.1 *N* H_2SO_4, 0.5 *N* NaOH, 0.25 *M* Na_2CO_3 (anhydrous), 0.25 *M* $NaHCO_3$, and alcohol were used.

Instruments—A photoelectric spectrophotometer[6] with 1-cm silica cells was used.

Procedures—*Standard Curves for Minophylline and Aminophylline Using* A_c *Method*—Different solutions containing 0.3, 0.6, 0.9, 1.2, 1.5, and 1.8 mg % minophylline were prepared by dilution with 0.1 *N* H_2SO_4. The absorbance of each solution was measured at λ_1 246 nm, λ_2 274 nm, and λ_3 295 nm.

For aminophylline, the concentrations prepared were 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, and 2.1 mg %; λ_1, λ_2, and λ_3 were 242, 270, and 287 nm, respectively. The A_c for each concentration of minophylline or aminophylline was calculated.

Standard Curve for Minophylline Using p_2 *Method*—The absorbances of the same solutions were measured at 266, 270, 274, 278, 282, and 286 nm. The coefficient p_2 for each concentration was calculated.

Standard Curve for Phenobarbital Applying ΔA *Method*—Two sets of solutions were prepared so that each contained 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5 mg % phenobarbital. One set was prepared in 0.1 *N* NaOH (Solution A), and the other was prepared in a mixture of 0.025 *M* Na_2CO_3 (anhydrous) and 0.025 *M* $NaHCO_3$ (Solution B). The absorbance of Solution B was measured at 238 nm using Solution A as a blank. Then Solution A was measured at 260 nm using Solution B as a blank. The $\Sigma \Delta A_{238}$ and ΔA_{260} for each concentration were calculated.

Assay for Pharmaceutical Preparations—*Minophylline Tablets*—From powdered tablets (10 tablets were powdered and mixed), an

[1] The theophylline ethanoate of piperazine. The International Nonproprietary Name is acefylline piperazine.

[2] Alexandria Company for Pharmaceutical and Chemical Industries.
[3] Boehringer Ingelheim, Germany.
[4] VEB Chemische Werk, Germany.
[5] Burroughs Wellcome and Co.
[6] Prolabo, Paris, France.

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