Colorimetric Prednisolone Assay and Its Application to Dissolution Studies

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Abstract □ The colorimetric method of Porter and Silber, originally developed for assaying cortisone and related corticosteroids, was adapted to the assay of prednisolone in the presence and absence of a surfactant, octoxynol 9. Optimal conditions for the colorimetric reaction between prednisolone and acidified phenylhydrazine solution were 30 min at 50°C. The slight interference of octoxynol 9 with the absorbance of the yellow prednisolone–phenylhydrazine complex at 410 nm was corrected by adding an equal surfactant concentration to the blank. When freshly prepared acidified phenylhydrazine solutions were used, the colorimetric method had a sensitivity of 0.2 μg/mL and a precision of 0.1 μg/mL. Its averaged relative standard deviation was 3.7% in the prednisolone concentration range of 10–300 μg/mL.

Keyphrases □ Prednisolone—colorimetric assay, application to dissolution, effect of octoxynol 9 □ Colorimetry—assay of prednisolone, application to dissolution, effect of octoxynol 9 □ Dissolution—prednisolone, colorimetric assay, effect of octoxynol 9

Surfactants such as octoxynol 9 NF enhance the dissolution rate of prednisolone from tablets. To study the effects of surfactants on the *in vitro* dissolution of prednisolone from different tablet formulations, a sensitive analytical method was required to determine the amount of dissolved prednisolone in the dissolution medium as a function of time. In the present application, a tablet containing 25 mg of prednisolone was dissolved in 900 mL of a 0.1 M HCl dissolution medium. At 4 and 100% dissolution, the concentrations of dissolved prednisolone in the dissolution medium correspond to 1 and 28 μg/mL, respectively. The analytical method should have a minimum sensitivity of 1 μg/mL to afford an accurate assay of the amount of dissolved prednisolone in the dissolution medium even at the beginning of a dissolution run.

The colorimetric assay of Porter and Silber (1) for the determination of cortisone and related 17,21-dihydroxy-20-ketosteroids in adrenal extracts has a sensitivity of 1 μg/mL, suitable for the present study. It is based on the development of a yellow color by the reaction between the steroid and an acidified phenylhydrazine reagent in a methanolic medium. Peterson *et al.* (2) developed a procedure for assaying hydrocortisone based on the Porter–Silber method. Since prednisolone and hydrocortisone have similar chemical structures, D'Arcy *et al.* (3) and Isbister *et al.* (4) used the Peterson procedure for the bioassay of prednisolone in plasma. These investigators made no attempt to develop specific reaction conditions appropriate for prednisolone. The present investigation shows that, at least for prednisolone, the age of the acidified phenylhydrazine solution as well as reaction time and temperature are crucial to the rate and extent of color development. Improper conditions resulted in poor reproducibility and led to considerable errors.

Direct spectrophotometric determination of prednisolone by UV absorption (5, 6) is not sensitive enough for *in vitro* dissolution testing. Radioimmunoassay (5) is complicated and requires special handling. Because a fast and accurate assay was required for studying the release characteristics of prednisolone from tablets in the presence and absence of octoxynol 9, the Porter–Silber method was specifically adapted to prednisolone. Optimum conditions for the rate of color development, stability and intensity of color, and sensitivity and accuracy of the assay were investigated.

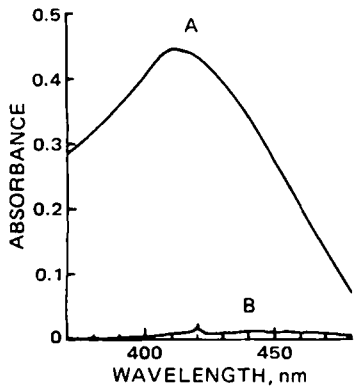

Figure 1—*Absorption spectra of the yellow prednisolone–phenylhydrazine complex (A) and of an octoxynol 9–phenylhydrazine solution (B).*

EXPERIMENTAL

Materials—The following tableting ingredients were employed. Two grades of prednisolone were used as received: anhydrous coarse powders[1] A and B, and anhydrous and micronized[2]. Most of the work, especially the tableting, dissolution, and deflocculation studies, was done with the anhydrous and micronized sample, and the other two coarser samples were merely included as additional sources of prednisolone to verify that the analytical method was independent of the origin of the steroid.

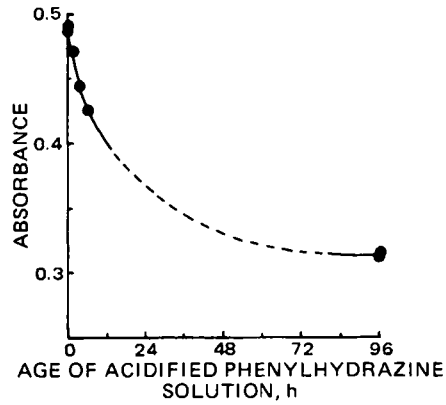

Figure 2—*Decrease in absorbance of the yellow prednisolone–phenylhydrazine complex at 410 nm with the age of the acidified phenylhydrazine solution. Prednisolone concentration = 150 μg/mL.*

[1] Coarse powder A supplied by Sigma Chemical Co., St. Louis, Mo., and coarse powder B supplied by The Upjohn Co., Kalamazoo, Mich.

[2] Schering Corp., Bloomfield, N.J.

0022-3549/84/0200-0157$01.00/0
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Table I—Formulations of Prednisolone Tablets

Ingredients[a]	Weight of Ingredients, mg Formulation I	Formulation II
Microcrystalline cellulose	75	75
Octoxynol 9	—	35
Spray-dried lactose	100	100
Prednisolone	25	25
Magnesium stearate	5	5
Spray-dried lactose	295	260

[a] Listed in the order of mixing.

Magnesium stearate[3] and spray-dried lactose[4] were USP grade. Octoxynol 9[5] and microcrystalline cellulose[6] were NF grade. The ACS-grade reagents used for the colorimetric assay were phenylhydrazine hydrochloride, anhydrous methanol, hydrochloric acid, and sulfuric acid. Double-distilled water was used throughout the experiments.

Equipment—Absorbances were measured with a double-beam grating spectrophotometer[7] equipped with a synchronized chart recorder[8] and 1-cm quartz cells. At an input voltage span of 1 mV, an absorbance value of 0.001 ± 0.0005 can be read directly from the chart. The temperatures for the colorimetric reaction were maintained within ±0.5°C.

Prednisolone tablets were prepared with a single-punch tablet press[9]. Membrane filters[10] were used to remove the undissolved tableting ingredients from the dissolution medium. The filters consisted of a mixture of cellulose nitrate and cellulose acetate and had two different porosities, 0.1 and 0.22 μm[11].

Reagents—Sulfuric acid at 68.7% w/w was prepared by diluting 1.63 parts by volume of concentrated sulfuric acid (93% w/w) with 1 part by volume of water. Porter and Silber investigated the effect of the sulfuric acid concentration on the cortisone acetate assay over a wide range and found that the concentration used for this study, 68.7% w/w, gave the best results. Acidified phenylhydrazine solution was prepared by dissolving 65 mg of phenylhydrazine hydrochloride in 100 mL of the 68.7% w/w sulfuric acid. Stock solutions of prednisolone were prepared in methanol at 10, 13.5, 15, 20, 28, 30, 60, 90, 150, 210, and 300 μg/mL. A stock solution of 0.4% w/v octoxynol 9 was prepared in 0.1 M HCl and diluted to 0.004, 0.01, and 0.05% w/v as required.

Prednisolone Tablets—Tablets containing 25 mg of prednisolone were prepared according to formulations I and II, as shown in Table I. Octoxynol 9 was excluded from the first formulation; 35 mg of octoxynol 9 was absorbed into the microcrystalline cellulose and incorporated into the second formulation. Several batches of 10 tablets each were prepared for each formulation using the following procedure. The tableting ingredients were triturated in a mortar and pestle. Compression at 10,000 psi on a single-punch tablet press yielded flat-faced cylindrical tablets with an average diameter of 12.8 mm, an average thickness of 2.8 mm, and an average tablet weight of 500 mg.

Dissolution—Individual prednisolone tablets were dissolved in 900 mL of 0.1 M HCl at 37°C using the USP XIX dissolution apparatus (7) and a 40-mesh metal basket rotated at 100 rpm. Five-milliliter aliquots of the dissolution medium containing dissolved prednisolone were withdrawn at appropriate time intervals and filtered through a 0.1-μm filter membrane that had been prewashed with 0.1 M HCl.

Prednisolone Assay—The accuracy of the Porter–Silber method applied to the assay of prednisolone was investigated as follows. The test solution consisted of 1 mL of a methanol solution containing a known amount of dissolved prednisolone and 8 mL of acidified phenylhydrazine solution placed in a stoppered test tube. The blank solution consisted of 1 mL of the dissolution medium and 8 mL of the acidified phenylhydrazine solution. Both solutions were heated for 20 min at 60°C (1), at which time the former developed a yellow color. After cooling to room temperature, the absorbance of the test solution was measured against the blank solution at 410 nm using 1-cm cells. Figure 1 shows a typical

[3] Impalpable powder; Mallinckrodt Inc., St. Louis, Mo.
[4] Manufactured by Foremost Dairies Inc. and supplied by SmithKline Beckman Corp., Philadelphia, Pa.
[5] Triton X-100; Rohm and Haas Co., Philadelphia, Pa.
[6] Avicel type PH-101; FMC Corp., Philadelphia, Pa.
[7] Coleman Model 124; Perkin-Elmer Corp., Norwalk, Conn.
[8] Coleman Model 165; Perkin-Elmer Corp., Norwalk, Conn.
[9] Carver Laboratory Press, Model C; Fred S. Carver Inc., Menomonee Falls, Wis.
[10] Fitted with Swinnex adaptors and Millipore membranes; Millipore Corp., Bedford, Mass.
[11] Type GSWP of 0.22-μm pore size and type VCWP of 0.1-μm pore size; Millipore Corp., Bedford, Mass.

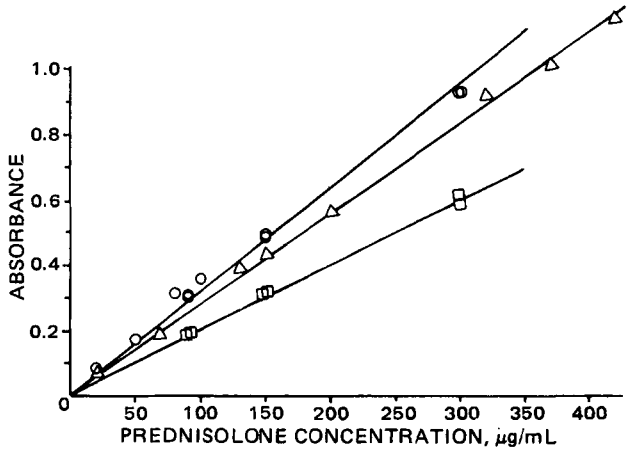

Figure 3—*Effect of aging the acidified phenylhydrazine solution on the relation between absorbance and prednisolone concentration. Key: (○) 0.5 h; (△) 6 h; (□) 96 h aging.*

absorption spectrum for the yellow prednisolone–phenylhydrazine complex, in which the maximum absorbance (A_1) occurred at 410 nm. This wavelength agrees well with the one reported by Porter and Silber for the phenylhydrazine complexes of cortisone and related compounds (1).

To correct for the absorbance due to the interaction between sulfuric acid and prednisolone in the absence of phenylhydrazine, a similar measurement was made omitting the phenylhydrazine solution from the reaction mixture. In this case, the absorbance (A_2) of a mixture of 1 mL of the methanol solution containing dissolved prednisolone and 8 mL of 68.7% w/w sulfuric acid was measured at 410 nm against a blank of 1 mL of the dissolution medium and 8 mL of the sulfuric acid solution. Both solutions were heated for 20 min at 60°C prior to the measurement. Subtracting the second absorbance (A_2) from the uncorrected absorbance (A_1) gave the correct absorbance for prednisolone solutions free from the interaction between sulfuric acid and prednisolone. Absorbance A_2 varied with each test sample and generally amounted to 5–15% of the uncorrected value.

RESULTS AND DISCUSSION

The adaptation of the Porter–Silber method to prednisolone and the optimization of the reaction conditions for maximum reproducibility and accuracy are discussed below.

Stability of the Acidified Phenylhydrazine Solution—Inconsistent results in preliminary experiments suggested that the acidified phenylhydrazine solution was unstable. The effect of aging of this solution on the absorbance of the colored complex with prednisolone was investigated.

A master batch of acidified phenylhydrazine solution was stored at

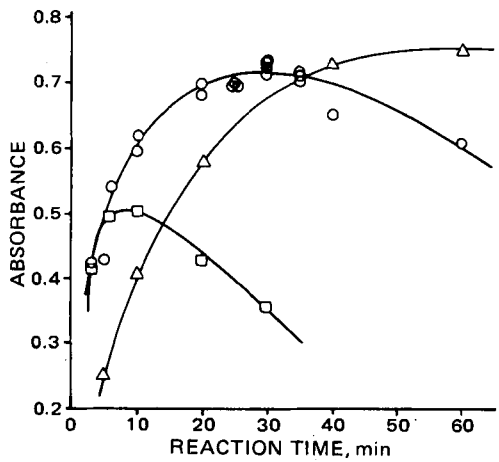

Figure 4—*Variation in absorbance of the prednisolone–phenylhydrazine complex at 410 nm with reaction time at different temperatures. Prednisolone concentration = 150 μg/mL. Key: (△) 40°C; (○) 50°C; (□) 60°C.*

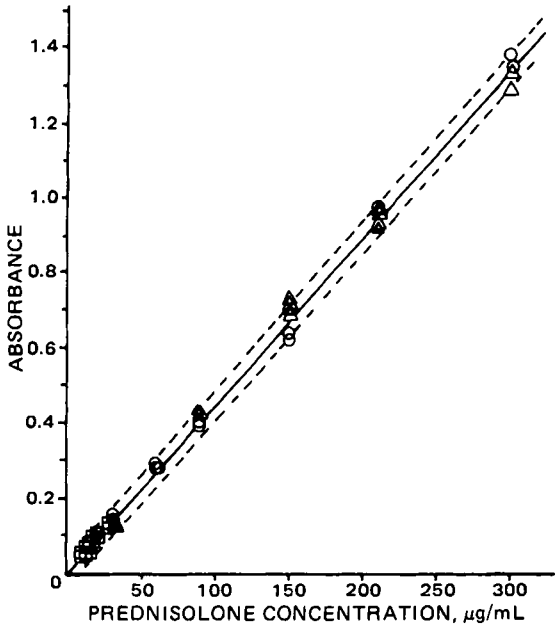

Figure 5—*Variation in absorbance of the prednisolone–phenylhydrazine complex with prednisolone concentration obtained with the standard procedure. Key: (○) anhydrous prednisolone coarse powder A; (△) anhydrous prednisolone coarse powder B; (□) micronized prednisolone. The broken lines represent the 95% confidence limits for the calculated absorbance.*

room temperature in the dark. Eight-milliliter aliquots were withdrawn immediately after the solution was prepared and after intervals of 0.5, 2, 4, 6, and 96 h. They were reacted with 1-mL volumes of prednisolone solution containing 150 μg/mL of dissolved prednisolone as described above. Corrected absorbance values at 410 nm are plotted against the age of the acidified phenylhydrazine solution in Fig. 2. A rapid initial decrease in absorbance is followed by a gradual leveling off.

Aging of the acidified phenylhydrazine solution for 96 h or more stabilized the absorbance of the yellow complex. However, it also reduced the molar absorptivity, or the slope of the Beer's-law plot, by ~40% (Fig. 3). The data of Fig. 3 were obtained by treating 1-mL aliquots of prednisolone solutions containing the indicated concentrations with 8-mL aliquots of acidified phenylhydrazine solutions aged 0, 6, and 24 h, respectively. Because of the high sensitivity required, subsequent prednisolone assays were performed within 1 h after the phenylhydrazine solution was prepared.

Dependence of Absorbance of the Yellow Complex on Reaction Time and Temperature—Porter and Silber reported that the absorbance values for the yellow complex of cortisone at 410 nm were reproducible when the colorimetric reaction was carried out at 60°C for 20 min. However, preliminary experiments suggested that the absorbance values for the prednisolone–phenylhydrazine complex were poorly reproducible under these reaction conditions. The influence of reaction time and temperature on the absorbance of the yellow complex was investigated.

One-milliliter aliquots of a 150-μg/mL prednisolone solution were reacted with 8-mL portions of freshly prepared acidified phenylhydrazine solutions at 40°C, 50°C, and 60°C. Absorbance values of the yellow complex were measured at 410 nm after 5, 10, 15, 20, 40, and 60 min for each temperature. The solutions were cooled to ambient temperature in a water bath before absorbance measurements. Figure 4 shows that the absorbance peaked at different reaction times for the three reaction temperatures. Lowering the reaction temperature reduced the reaction rate so that the peak value occurred after a longer reaction time. For example, as the reaction temperature was lowered from 60°C to 40°C, the time required to reach maximum absorbance increased from 10 to 40 min. The peak was broader at the lower temperatures, indicating that these absorbance values are more reproducible than those obtained at higher temperatures. While the maximum absorption occurred at 410 nm for the three temperatures, the absorbance at 410 nm was considerably stronger at the two lower temperatures.

Selection of Standard Reaction Conditions—Of the three experimental conditions required to attain maximum absorbance (50 min at 40°C, 30 min at 50°C, and 10 min at 60°C), the intermediate conditions were selected as the best compromise between reproducibility and reaction time. Subsequent colorimetric reactions were conducted at 50°C for 30 min.

Table II—Reproducibility of the Prednisolone Assay

Prednisolone Concentration, μg/mL	Average Absorbance	N^a	*SD*	*SE*	*RSD*, %
10.0	0.045	2	0.001	0.001	2.2
13.5	0.057	4	0.002	0.001	3.5
15.0	0.063	4	0.003	0.002	4.8
20.0	0.096	2	0.006	0.004	6.3
28.0	0.128	2	0.003	0.002	2.3
30.0	0.142	4	0.006	0.003	4.2
60.0	0.283	3	0.006	0.004	2.1
90.0	0.406	4	0.019	0.010	4.7
150.0	0.683	6	0.044	0.018	6.4
210.0	0.957	5	0.015	0.007	1.6
300.0	1.344	4	0.038	0.019	2.8

[a] Number of determinations.

Beer's Law Plot—The prednisolone assay for dissolution studies requires the use of a Beer's-law plot that correlates the absorbance of the prednisolone–phenylhydrazine complex with the prednisolone concentration. This plot was obtained by measuring the absorbance values of the yellow complex at 410 nm using prednisolone solutions of 11 different concentration levels in the range of 10–300 μg/mL. A total of 40 measurements were made, 14 with micronized prednisolone, 12 with the anhydrous prednisolone coarse powder A, and 14 with coarse powder B. Prednisolone samples from three suppliers were used to ensure that the results were independent of the source of the steroid.

The Beer's-law plot in Fig. 5 shows that the absorbance of the yellow complex is directly proportional to the prednisolone concentration up to at least 300 μg/mL. The absorbance values are independent of the source of prednisolone. The plot may be represented by the linear regression equation:

$$A = 0.0045C \qquad \text{(Eq. 1)}$$

where A is the absorbance at 410 nm, C is the prednisolone concentration in μg/mL, and 0.0045 is the slope. The absorptivity of prednisolone calculated from the slope is 0.0045 mL/μg·cm. It is equivalent to a molar absorptivity of 1622 L/mol·cm. The standard error for the slope of the regression line, SE_m, and the standard error of regression, SE_{AC}, were 0.00002 and 0.21, respectively (8, 9). The broken lines in Fig. 5 represent the 95% confidence limits for the calculated absorbance (9). The equations used for the linear regression analysis are given in the *Appendix*.

Reproducibility of the Prednisolone Assay—The standard deviation (*SD*), standard error (*SE*), and relative standard deviation (*RSD*) were calculated for each prednisolone concentration. Table II shows that the relative standard deviation varied randomly with the prednisolone concentration, ranging from 1.6 to 6.4% in the concentration range of 10–300 μg/mL. An average relative standard deviation ($\overline{RSD}$) was calculated for the entire concentration range using Eq. 2:

$$\overline{RSD} = \frac{\sum_{i=1}^{i=n} RSD}{n} \qquad \text{(Eq. 2)}$$

where RSD_i is the relative standard deviation at concentration C_i and n is the number of concentration levels used in the study. A value of 3.7% was obtained for $\overline{RSD}$ for concentrations ranging from 10 to 300 μg/mL. These statistics indicate that the measured absorbance is reproducible within ±4% for an entire dissolution run.

Sensitivity of the Prednisolone Assay—With an input voltage span of 1 mV for the recorder, an absorbance value of 0.001 ± 0.0005 can be determined directly from the chart. This is equivalent to a prednisolone concentration of 0.2 ± 0.1 μg/mL according to Eq. 1. The sensitivity of the assay is, therefore, at least 0.2 μg/mL, which is equivalent to 0.7% dissolution for a 25-mg tablet dissolving in 900 mL of dissolution medium. It far exceeds the required sensitivity of 1 μg/mL.

Dissolution Studies in the Absence of Octoxynol 9—When the dissolution experiment did not involve octoxynol 9, the test solution consisted of a 1-mL aliquot of the filtered dissolution sample and 8 mL of the freshly prepared acidified phenylhydrazine solution. The blank solution consisted of 1 mL of the dissolution medium and 8 mL of the phenylhydrazine solution. The absorbance of the prednisolone–phenylhydrazine complex was measured at 410 nm against the blank solution

Table III—Accuracy of the Prednisolone Assay in the Absence of Octoxynol 9

Tablet	Observed Prednisolone Content, mg/tablet[a]	
A	25.2	24.9
B	25.1	25.2
C	24.8	25.1
Mean	25.05	
N	6	
SE	0.07	
RSD	0.7%	

[a] Two determinations for each tablet.

using the standard reaction conditions and corrected for interference due to the interaction between prednisolone and sulfuric acid. The amount of dissolved prednisolone in the dissolution sample was calculated using Eq. 1. Results of the dissolution studies are reported in a separate publication (10).

Dissolution Studies in the Presence of Octoxynol 9—Preliminary experiments showed that 1 mL of a 0.0039% w/v octoxynol 9 solution and 8 mL of the acidified phenylhydrazine solution reacted at the standard conditions to develop a pale yellow color. The absorption spectrum for the reaction mixture is shown in Fig. 1. Three samples of the octoxynol 9–phenylhydrazine solution gave absorbance values at 410 nm ranging from 0.002 to 0.004.

Therefore, in dissolution studies where the surfactant was present in the dissolution medium the following procedure was employed to compensate for the slight interference of octoxynol 9 on the absorbance of the prednisolone–phenylhydrazine complex. The test solution consisted of a 1-mL aliquot of the filtered dissolution sample containing octoxynol 9 and the dissolved prednisolone plus an 8-mL portion of the acidified phenylhydrazine solution. The blank solution consisted of 1 mL of the 0.1 M HCl dissolution medium containing a quantity of the surfactant equal to the amount present in the test solution plus 8 mL of the acidified phenylhydrazine solution. The absorbance of the test solution was measured against this blank using the standard conditions and corrected for interference due to the prednisolone–sulfuric acid interaction.

In tablets containing octoxynol 9 (formulation II), the surfactant dissolved rapidly during prednisolone dissolution (10). Its concentration in the dissolution medium increased from 0 initially to a maximum of 0.0039% w/v at the complete release of the 35 mg of octoxynol 9 in the dissolution medium (900 mL). The faint yellow color produced by the interaction between octoxynol 9 in the filtered dissolution medium and phenylhydrazine remained essentially constant during an entire dissolution run: its absorbance was 0.003 at the time corresponding to the first prednisolone assay and 0.004 when all the prednisolone was dissolved.

In another experiment, conducted in the absence of prednisolone, an equal amount of the surfactant was added to the dissolution medium rather than incorporated into the tablets. Thus, the surfactant concentration remained constant at 0.0039% w/v throughout the entire dissolution run. The absorbance of this filtered dissolution medium when treated with the phenylhydrazine solution was 0.003 ± 0.001, comparable with the values obtained in the experiment where 35 mg of octoxynol 9 was incorporated into the tablets. Therefore, the absorbance due to the interference of octoxynol 9 with phenylhydrazine was essentially constant throughout a dissolution run regardless of whether the 35 mg of octoxynol 9 was originally incorporated into the tablet or added to the dissolution medium. A constant absorbance correction of 0.003 was made for both situations.

Accuracy of the Prednisolone Assay in Dissolution Studies—The accuracy of the prednisolone assay in the absence of octoxynol 9 was investigated by the following procedure. Three prednisolone tablets of formulation I were triturated individually with 10 mL of methanol to ensure the complete dissolution of the 25 mg of prednisolone incorporated into the tablet. Each mixture was diluted to 1 L with 0.1 M HCl, resulting in a final prednisolone concentration of 25 μg/mL. Each suspension was filtered individually through a 0.22-μm membrane that had been prewashed with a 5-mL portion of the 0.1 M HCl dissolution medium. Prednisolone assays were performed in duplicate for each filtrate as described above. The amount of dissolved prednisolone in the filtrate was calculated by means of Eq. 1.

The results of the prednisolone assays, summarized in Table III, shows that the experimental values of the drug content of the three tablets are in good agreement. The relative standard deviation was a mere 0.7%. The observed prednisolone contents are identical with the actual value of 25 mg per tablet. Therefore, the colorimetric assay has high accuracy and precision.

Nature of the Colored Complex—It is of interest to consider the chemical nature of the yellow color obtained by the reaction of prednisolone with phenylhydrazine. The reaction probably involves the following three steps:

1. The carbonyl group on C-3 reacts with one molecule of phenylhydrazine to form the phenylhydrazone.
2. The Mattox rearrangement involving the hydroxyl groups at C-17 and C-21 and the carbonyl group at C-20 produces the 17 β-glyoxal (C-20 and C-21 contain carbonyl groups, while the C-17 hydroxyl group is lost).
3. The carbonyl group at C-21 reacts with one molecule of phenylhydrazine to form a second phenylhydrazone.

The yellow pigment is thus the 20-keto-3,21-diphenylhydrazone (11).

APPENDIX

Slope m of the regression line is given by:

$$m = \frac{\sum_{i=1}^{i=n} C_i A_i}{\sum_{i=1}^{i=n} C_i^2} \qquad \text{(Eq. 3)}$$

where C_i is the prednisolone concentration in μg/mL, A_i is the absorbance of the yellow prednisolone–phenylhydrazine complex obtained at this concentration, and n is the number of concentration levels of prednisolone solution used for the regression analysis (8, 9).

The standard error of regression SE_{AC} is calculated by means of:

$$SE_{AC} = \left\{ \frac{\sum_{i=1}^{i=n} A_i^2 - \left[\left(\sum_{i=1}^{i=n} C_i A_i \right)^2 \Big/ \sum_{i=1}^{i=n} C_i^2 \right]}{n-1} \right\}^{1/2} \qquad \text{(Eq. 4)}$$

The standard error, SE_m, of the slope of the regression line is calculated by means of the equation:

$$SE_m = \frac{SE_{AC}}{\left(\sum_{i=1}^{i=n} C_i^2 \right)^{1/2}} \qquad \text{(Eq. 5)}$$

The linear regression equation (Eq. 1) is used to calculate the absorbance A_k for a known prednisolone concentration C_k. The 95% confidence limits for the calculated absorbance, A_k, at a specific value of prednisolone concentration, C_k, are calculated using:

$$\text{Absorbance} = A_k \pm t_{0.05}^{n-1} SE_{A,k} \qquad \text{(Eq. 6)}$$

where $t_{0.05}^{n-1}$ is the 5% point of a two-tailed t distribution with $n-1$ degrees of freedom, and $SE_{A,k}$ is a modified form of Eq. 6.12.1 in Ref. 9:

$$SE_{A,k} = SE_{AC} \left[1 + \frac{(\bar{C} - C_k)^2}{\sum_{i=1}^{i=n} (\bar{C} - C_i)^2} \right]^{1/2} \qquad \text{(Eq. 7)}$$

where $\bar{C}$ is the mean of all C_i values.

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ACKNOWLEDGMENTS

Adapted in part from a thesis submitted by L. Chong Kwan to Temple University in partial fulfillment of the Master of Science degree requirements.

Relationship Between Azo Dye Structure and Rat Hepatic Azoreductase Activity

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Received March 9, 1982, from the *College of Pharmacy and Allied Health Professions, Northeastern University, Boston, MA 02115.* Accepted for publication December 2, 1982. Present addresses: *Massachusetts College of Pharmacy and Allied Health Sciences, Boston, MA 02115 and ‡Birzeit University, P.O. Box 14, Birzeit, West Bank, Israel.

Abstract □ The rate of reduction was determined for a variety of azo dyes using the rat hepatic azoreductase enzyme system. In decreasing order, the rates of reduction for the azo dyes expressed as nmol of arylamine product formed/min/0.25 g of liver were amaranth (33.2), azosulfamide (32.5), orange G (12.4), 1,2-dimethyl-4-*p*-(carboxyphenylazo)-5-hydroxybenzene (CPA) (9.27), brilliant crystal scarlet (8.00), sulfachrysoidine (7.27), and Sudan I (1.03). A comparison of the partition coefficient with its rate of reduction indicated that the water-soluble azo dyes were reduced more rapidly than the lipid-soluble ones. Furthermore, higher rates of reduction were observed for those dyes containing electron-withdrawing groups on the aromatic rings.

Keyphrases □ Azoreductase—rat hepatic activity, relationship to azo dye structure, amaranth, azosulfamide, orange G, 1,2-dimethyl-4-*p*-(carboxyphenylazo)-5-hydroxybenzene, brilliant crystal scarlet, sulfachrysoidine, Sudan I □ Azo dyes—relationship between structure and rat hepatic azoreductase activity, amaranth, azosulfamide, orange G, 1,2-dimethyl-4-*p*-(carboxyphenylazo)-5-hydroxybenzene, brilliant crystal scarlet, sulfachrysoidine, Sudan I

The azo dyes and pigments form a large group of synthetic colorants used in many dye applications including foods, cosmetics, and pharmaceuticals. The chromophoric system consists of the azo group (N=N—) in association with one or more aromatic system. Monoazo, diazo, triazo, and polyazo dyes contain one, two, three, or more azo groups, respectively, yielding a large range of colors including reds, oranges, and purples (1).

In mammals, several enzyme systems are capable of reducing the azo bond including the liver (2–7) and the GI flora (4, 8–10). These azoreductase enzyme systems reduce a variety of azo dye substrates and do not appear to have a strict structural requirement other than the azo bond (2, 4, 10). A number of azo dyes, such as dimethylaminoazobenzene (butter yellow), amaranth, and others have carcinogenic or suspected carcinogenic activity (4). This toxic activity may be due to the parent compound, which contains the intact azo bond, or to the resulting arylamine. Although the mechanism of the reduction of the azo bond has been studied (2, 3, 5, 11, 12) only a few papers have compared chemical structure with the rates of reduction of the azo bond (2, 13). The objective of this study was to investigate the relationship of the structure of selected azo dyes to the rate of reduction to the corresponding amine by the rat hepatic azoreductase enzyme system. The rat hepatic azoreductase enzyme system obtained from the 10,000 × *g* supernatant fraction of liver homogenates was chosen since the mechanism of azo bond reduction by this enzyme preparation has been characterized by a number of investigators (2, 3, 5–7, 11, 12).

EXPERIMENTAL

Reagents and Chemicals—Azo dyes including brilliant crystal scarlet[1], orange G[1], Sudan I[1], amaranth[1], sulfachrysoidine[2], azosulfamide[2], and 1,2-dimethyl-4-(carboxyphenylazo)-5-hydroxybenzene[3] (CPA) were checked for chemical purity by either TLC on silica gel plates (*n*-butyl alcohol–acetic acid–water, 6:1:3) or ascending paper chromatography (*n*-butyl alcohol–acetic acid–water 12:3:5). Cation-exchange[4] and anion-exchange[4] resins were washed with several volumes of distilled water prior to use. NADP[5], glucose-6-phosphate[5], and nicotinamide[5], as well as other reagents and solvents, were used without further purification.

Partition Coefficient—A partition coefficient, K, in *n*-octanol–0.05 M phosphate buffer (pH 7.4) was determined for each azo dye in duplicate at room temperature. For water-soluble dyes, 1 mL of an aqueous dye solution (~10 μmol) was placed in an extraction tube containing 4 mL of 0.05 M phosphate buffer (pH 7.4). To this sample was added 5 mL of *n*-octanol previously saturated with distilled water. Lipid-soluble dyes were initially dissolved in the water-saturated 1-octanol and added to 5 mL of 0.05 M phosphate buffer (pH 7.4). Each sample was shaken, allowed to stand for a few minutes, and centrifuged. The distribution of the dye in each phase at equilibrium was obtained from absorbance of the dye in a spectrophotometer[6] at an appropriate wavelength. The partition coefficient for each dye was calculated with respect to a standard curve or from the absorbance of the aqueous or lipid phase before and after equilibration.

Preparation of Liver Homogenates—Male albino Charles River CD rats (180–220 g) were used for all studies. Rats were weighed and then sacrificed by decapitation. Each liver was excised and immediately rinsed with ice-cold 1.15% KCl (isotonic). The livers were trimmed, weighed, and homogenized in three volumes of 1.15% KCl using a polytef pestle and glass mortar. All subsequent procedures were performed at 4°C. The tissue homogenates were centrifuged at 10,000×*g* for 15 min in a refrigerated centrifuge. The 10,000×*g* liver supernatant containing the microsomal enzymes and soluble fraction was filtered through gauze. Approximately 15 mL of the 10,000×*g* liver supernatant was placed in 50-mL glass ampules and shell-frozen in dry ice–acetone. This enzyme preparation is stable at dry ice temperature for a week or more. Previous studies have shown that lyophilization of this preparation stabilized the enzymes for as long as 6 months with no apparent loss of activity (14).

[1] Aldrich Chemical Co., Milwaukee, Wis.
[2] Sterling-Winthrop Research Institute, Rensselaer, N.Y.
[3] ICN Nutritional Biochemics, Cleveland, Ohio.
[4] Cation exchange resin (AG50W-X8, 200-400 mesh) and anion exchange resin (AGL-X2, 200-400 mesh); Bio-Rad Labs, Richmond, Calif.
[5] Sigma Chemical Co., St. Louis, Mo.
[6] UV/Vis; Beckman Instruments, Inc., Irvine, Calif.

0022-3549/84/0200-0161$01.00/0
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