concentrations gave incomplete reactions, and higher concentrations vitiated the violet color and changed it to yellow. When impure ferric chloride or an unfresh solution of it was employed, the addition of 2 drops of hydrogen peroxide (10 vol.) restored and potentiated the violet color. However, excess hydrogen peroxide was found unfavorable as the color changed to orange. The violet color, produced by the action of ferric chloride, develops within 2 min. and should be measured within 2–5 min. The color is not affected by light but is very sensitive to temperature.

Free chloramphenicol gave the same reaction as the salts, but the sensitivity was 5 times lower ($\leq$15 mg.) and the results were not reproducible. The addition of a carboxylic acid, however, was found to increase the sensitivity of the reaction. Many acids were tried, and succinic acid gave the highest sensitivity ($\leq$3 mg.). Although succinic acid alone gave negative reactions with the reagent, its presence affected the sensitivity of the reaction. Therefore, equal amounts of succinic acid were added to both test and standard preparations.

Due to the slight yellowish color produced by the action of sodium hydroxide on chloramphenicol or its esters, the blank experiment was done exactly like the experiment, omitting the addition of hydroxylamine hydrochloride reagent.

Data presented in Table I indicate the accuracy of the proposed method and the reproducibility of the results. The percentage of error with respect to the added and found amounts (Table I) ranges from $\pm$2.1 to 2.3 in chloramphenicol, from $\pm$1.7 to 1.9 in chloramphenicol palmitate, and from $\pm$2.7 to 3 in chloramphenicol succinate. The method was found applicable to different freshly formulated preparations, and the results (Table II) are comparable with those of the Aihara *et al.* method (4), the spectrophotometric method, and the α-naphthol method (6). The method of Aihara *et al.* (4), however, was not applicable to chloramphenicol esters. The comparison of the proposed method to the α-naphthol method and the spectrophotometric method on degraded samples of chloramphenicol and its palmitate produced varying results (Table III). The chloramphenicol analysis of the heated preparation decreased with an increase in temperature in the case of the proposed method and increased with the other two methods. This fact indicates that by means of the hydroxylamine method, chloramphenicol and its esters can be determined, while some of the degradation products interfere in the results of the other two methods. In addition, the color produced by the α-naphthol method was not the same in all cases; it ranged from greenish to bluish.

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Application of Absorbance Ratios to Analysis of Pharmaceuticals VI: Analysis of Binary Mixture Using a Reference Spectrum

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Abstract □ Binary mixtures may be resolved by using absorbance ratio values and a reference spectrum for one component in the mixture. The method is based on the differences between the absorbance values for the mixture at any two wavelengths and the values for a solution containing only one component in the mixture. Three constants are required to resolve the mixture, but only one of these is an absorptivity value. Unlike the absorbance ratio method described in the literature, this method does not depend on the use of a wavelength at which the two components in the mixture have identical absorptivity values.

Keyphrases □ Binary mixture analysis—reference spectrum □ Caffeine–Na benzoate—spectral characteristics □ Absorbance ratios—analysis □ UV spectrophotometry—analysis, absorbance ratios

The ratio of two absorbance values determined on the same solution at two different wavelengths is a constant. These ratios may be used to determine both the relative and absolute concentrations of the components in a binary mixture (1). However, absolute concentrations (w/v) cannot be determined unless one of the two wavelengths chosen for the analysis represents an isoabsorptive point.

Isoabsorptive points (those wavelengths at which the two components in a mixture have identical absorptivity values) are difficult to isolate. The mathematical derivations in the next section show that absolute concentration values can be obtained by analyzing the mixture at wavelengths that do not represent isoabsorptive points, and that the number and nature of the constants in the derived equations are the same as those associated with the absorbance ratio method of analysis (1). This method of analysis is based, therefore, on the use of two absorbance ratio values (Q values), one absorptivity value, and the differences, at two wave-

lengths, between the absorbance values for a solution and the values for a reference solution containing one of the two components in the mixture.

THEORY

Hypothetical spectrophotometric curves for X and Y are shown in Fig. 1 and will be used to clarify the derivations. It is assumed that Beer's law is obeyed at all wavelengths and at all spectrophotometrically significant concentrations.

The total absorbance of a mixture containing X and $Y(A_M)$ is equal to the sum of the absorbances due to $X(A_X)$ and $Y(A_Y)$. Therefore, at λ_4,

$$A_{M4} = a_6C_X + a_1C_Y \qquad \text{(Eq. 1)}$$

C_X and C_Y are the concentrations of X and Y in the mixture. Similarly, at λ_2,

$$A_{M2} = a_2C_X + a_5C_Y \qquad \text{(Eq. 2)}$$

The absorbance values, at λ_4 and λ_2, for a reference solution containing only X are:

$$A_{X4} = a_6C_{XR} \qquad \text{(Eq. 3)}$$

$$A_{X2} = a_2C_{XR} \qquad \text{(Eq. 4)}$$

C_{XR} is the concentration of X in the reference solution.

Subtracting Eq. 3 from Eq. 1 and rearranging yield:

$$a_1C_Y = D_4 - a_6C_X + a_6C_{XR} \qquad \text{(Eq. 5)}$$

D_4 is defined as $A_{M4} - A_{X4}$. Similarly, at λ_2,

$$a_5C_Y = D_2 - a_2C_X + a_2C_{XR} \qquad \text{(Eq. 6)}$$

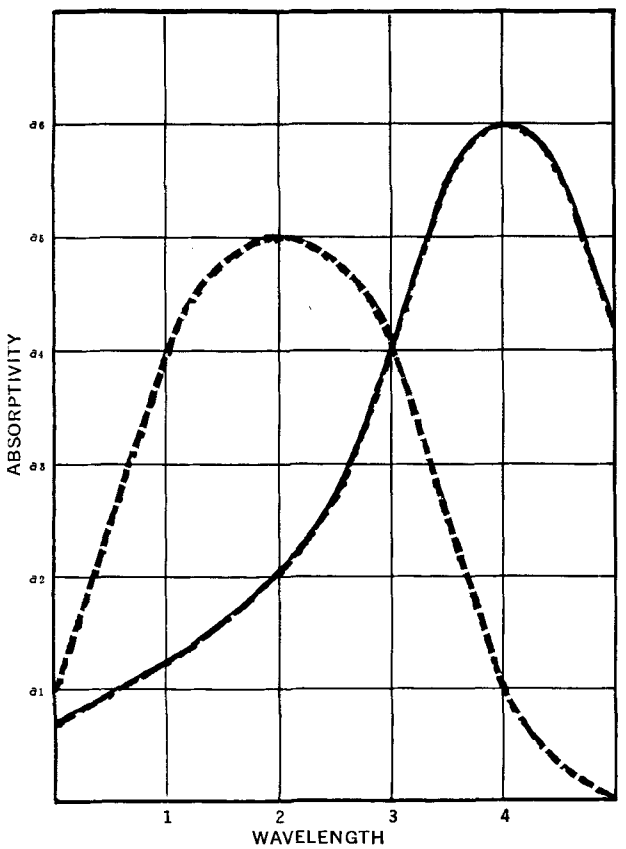

Figure 1—*Hypothetical spectrophotometric curves for* X (——) *and* Y (- - -).

Dividing Eq. 6 by Eq. 5 yields:

$$\frac{a_5C_Y}{a_1C_Y} = \frac{D_2 - a_2C_X + a_2C_{XR}}{D_4 - a_6C_X + a_6C_{XR}} \qquad \text{(Eq. 7)}$$

However, a_5/a_1 is equal to Q_Y, the absorbance ratio value for pure Y. Therefore:

$$Q_YD_4 - a_6Q_YC_X + a_6Q_YC_{XR} = D_2 - a_2C_X + a_2C_{XR} \qquad \text{(Eq. 8)}$$

Dividing both sides of Eq. 8 by a_6 and substituting Q_X, the absorbance ratio value for pure X, for a_2/a_6 yield:

$$\frac{Q_YD_4}{a_6} - Q_YC_X + Q_YC_{XR} = \frac{D_2}{a_6} - Q_XC_X + Q_XC_{XR} \qquad \text{(Eq. 9)}$$

After rearranging:

$$C_X = C_{XR} + \frac{D_2 - Q_YD_4}{a_6(Q_X - Q_Y)} \qquad \text{(Eq. 10)}$$

Therefore, the absolute concentration of $X(C_X)$ in a solution containing X and Y can be determined by measuring the differences, at two wavelengths, between the absorbance values for the mixture and the absorbance values for a reference solution containing only X. The constants, Q_X and Q_Y, are concentration independent and are easily determined by measuring absorbance values of solutions containing only X or Y.

The absorptivity value, a_6, is determined by measuring absorbance values, at the specified wavelength, of accurately prepared solutions containing only X.

The method described herein was checked experimentally by determining the absolute concentrations of sodium benzoate in solutions containing sodium benzoate and caffeine.

EXPERIMENTAL

Apparatus—A UV spectrophotometer[1,2] was used.

Reagents—Sodium benzoate USP and caffeine USP were used.

Spectral Characteristics of Sodium Benzoate and Caffeine—Caffeine and sodium benzoate (in water) absorb a maximum of radiant energy at 273 and 225 mμ, respectively. An examination of the sodium benzoate spectrum indicated that the analysis of this substance in the presence of caffeine could be carried out by determining absorbance values at 225 mμ (the absorption maximum for sodium benzoate) and at 235 mμ (a region in which the spectra have slight inflection points).

Two series of solutions were prepared, one containing caffeine and the other containing sodium benzoate. The absorbance value of each solution was determined at 225 and 235 mμ. The Q:235:225 values for caffeine and sodium benzoate were then calculated by dividing the absorbance at the first wavelength (235 mμ) by that at the second wavelength (225 mμ). Based on seven such solutions, the Q:235:225 value for caffeine was 0.721 ± 0.002; for sodium benzoate, it was 0.619 ± 0.003.

A series of solutions was prepared to contain from 5.0 to 14.0 mg. of sodium benzoate per liter of solution. The absorptivity value for sodium benzoate at 225 mμ was found to be 59.2 ± 0.3.

Procedure—Accurately dilute the solution containing caffeine and sodium benzoate. Determine absorbance values at 225 and 235 mμ.

Prepare a reference solution, by dilution, to contain a known quantity of sodium benzoate. (The reference solution used in these analyses contained 14 mg. of sodium benzoate per liter.) Determine absorbance values at 225 and 235 mμ.

Subtract the values for the reference solution from the values for the mixture. Substitute these D_2 and D_4 values into the numerical form of Eq. 10:

$$C_X \text{ (g./l.)} = 0.014 + \frac{D_2 - 0.721D_4}{59.2\,(0.619 - 0.721)} \qquad \text{(Eq. 11)}$$

Calculate the concentration of sodium benzoate in the solution.

[1] Beckman model DU-2.
[2] Bausch & Lomb Spectronic 505.

Table I—Analysis of Sodium Benzoate in Sodium Benzoate–Caffeine Mixtures

mg. Drug/l.		mg. Sodium Benzoate/l.
Sodium Benzoate	Caffeine	Recovered
4.5	7.5	4.54
5.0	7.0	5.06
5.0	5.0	5.02
6.0	6.0	6.05
6.5	5.5	6.32
7.0	5.0	7.11

Sixteen solutions were prepared to contain from 4.5 to 7.5 mg. of sodium benzoate per liter. Caffeine and sodium benzoate injection USP usually contains equal quantities of caffeine and sodium benzoate. However, to test the accuracy of the method, the solutions contained from three parts of sodium benzoate for every five parts of caffeine to five parts of sodium benzoate for every three parts of caffeine. To check the accuracy of the method further, a second set of six solutions was prepared and analyzed. The results for this set are shown in Table I. The percent recovery for the 22 solutions was 100.1%; the standard deviation value was ± 2.9%.

DISCUSSION

The caffeine–sodium benzoate mixture can be resolved by using simultaneous equations (2) or by the absorbance ratio method of analysis (1). If the former method is used, four absorptivity values must be determined. If the latter method is used, one absorptivity value and two absorbance ratio values must be determined. Absorbance ratio values are concentration independent; for this reason, the absorbance ratio method of analysis yields accurate results more quickly than does the method utilizing simultaneous equations. However, the absorbance ratio method of analysis yields absolute concentrations only if one of the two wavelengths chosen for the analysis is an isoabsorptive point. Such points are difficult to isolate, and the absorptivity values at such wavelengths cannot be determined with accuracy.

The method of analysis described here utilizes three constants. The absorbance ratio values are the same as those used in the absorbance ratio method of analysis. The absorptivity value, on the other hand, is determined at a wavelength at which one of the components absorbs a maximum of radiant energy. It is, therefore, not necessary to isolate an isoabsorptive point to calculate the absolute concentration of a drug in a mixture.

The method does require the use of a reference solution. However solutions must be prepared to determine constants, and one of these can be designated as the reference solution. This solution must, however, contain a quality of drug which will yield absorbance values somewhat greater than those observed for the mixture. If the difference between the absorbance values at the two specified wavelengths is small, the error in the analysis will be greater than necessary.

The results reported here indicate that the method is capable of yielding reasonably accurate results. The maximum percent recovery observed for the 22 solutions was 105.8%. The minimum percent recovery was 95.6%. A reexamination of these two solutions indicated that slight changes in absorbance readings can result in fairly substantial changes in percent recovery. This implies that each absorbance value should be determined twice (and the mean value used in the calculation) and that a stable, single-beam spectrophotometer should be used to measure absorbance values. Since the solutions were read in sequence, the values could not be rejected. Even so, the overall percent recovery for the 22 solutions was 100.1%. A standard deviation value of ±2.9% is acceptable if the speed and ease of analysis are taken into consideration.

The caffeine concentration in the solutions can be determined by using an equation similar to that given for sodium benzoate. Both equations, therefore, are based on absorbance differences. However, the equations may be derived by summing absorbance values. If this is done, the form of Eq. 10 is the same except that the C_{XR} term becomes negative.

Absorbance ratio values are used in both this method of analysis and that previously published (1). The appropriate references (1, 3) should, therefore, be consulted for a full discussion of the advantages and limitations of the use of such ratios in analysis.

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