the analysis of eight binary mixtures by applying four basic equations in the final step of the procedure. All are based on the relationship between the absorbancy ratio value and the relative concentration of a binary mixture. The first of these, Eq. 1, may be discarded since Eqs. 3 and 4 yield the same type of results. Equation 2 may be used but the analysis is limited by the need of previously prepared Q curves. Although this may constitute good analytical practice, the use of graphs is often inconvenient. It is well to remember that portions of this type of curve may be used under certain circumstances. Assume that a 50–50 mixture is required in a particular granulation. The absorbancy ratio for such a mixture might be, for example, 2.00. Even though a plot of Q values *vs.* the relative concentration of the mixture does not constitute a straight line, limits of, for example, 1.98 to 2.02 can be established and, consequently, the mixture can be controlled. This was illustrated in the case of the analysis of sulfathiazole-sulfapyridine mixtures. Since the absorbancy ratio method requires no accurate weighings or dilutions, the uniformity of the granulation can be established rapidly.

If the initial weight of the mixture is known and an isoabsorptive point is used in the final measurement, then Eq. 3 can be used to provide the analyst with the absolute concentration of the individual components in the binary mixture. Once the weight of the mixture is established, however, no absolute dilutions are required, that is, graduated cylinders provide the necessary dilution accuracy. If, however, the weight of the initial mixture is not known or difficult to obtain (for example, the quantities obtained are small or the sample is processed in such a way that the mixture is never recovered in the crystalline form), then Eq. 4 must be used. For this type of analysis, accurate dilutions are required.

The absorbancy ratio method of analysis places no restrictions on the nature of the radiant energy used in the final measurements. In fact, the technique should be applicable to any case where a relationship similar to that existing between absorbancy and wavelength exists. The accuracy of the analysis will vary, of course, from region to region but this is due primarily to experimental and instrumental rather than to theoretical limitations.

CONCLUSIONS

The absorbancy ratio method of analysis is capable of yielding good results if used with caution and common sense. It should be of great importance to the analyst interested in the analysis of various products containing two active ingredients. This method is not a panacea but it does simplify and increase the speed of analysis of various mixtures of importance to the pharmaceutical industry. Part IV in this series of papers will show that absorbancy ratios can be used in the analysis of mixtures containing three active ingredients.

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Application of Absorbancy Ratios to the Analysis of Pharmaceuticals III

Simultaneous Analysis of Aminophylline and Phenobarbital

By F. YOKOYAMA and M. PERNAROWSKI

The principles inherent in the absorbancy ratio technique are applied to the analysis of binary mixtures containing aminophylline or theophylline and phenobarbital. The analysis is carried out without prior separation of the components of the mixture and is applicable to commercial preparations containing these substances.

THE DIURETIC PROPERTIES of theophylline were first observed over fifty years ago. Since that time, this drug and aminophylline have been used either alone or in combination with phenobarbital for the treatment of conditions such as hypertension, bronchial asthma, and cardiac and nephrotic edema. It is these combinations of theophylline or aminophylline and phenobarbital that must be subjected to anal-

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ysis by the control chemist. Even though several procedures have been published for such mixtures, the problems associated with such analyses have not been completely resolved. Hyatt (1) described a method that was later subjected to collaborative study (2). His method, which is based on spectrophotometric measurements and involves separation of the active ingredients by extraction, is the prescribed method of the A.O.A.C. (3). Griffenhagen and Brady (4), Bhattacharya and Banerjee (5), and David (6) proposed argentiometric and gravimetric procedures for aminophylline and phenobarbital combinations. Bartilucci and Discher (7) determined the active ingredients by differential titrimetry in either water or alcohol. Several of these methods, as well as a differential nonaqueous titrimetric procedure, were investigated in this laboratory. The spectrophotometric method described by Hyatt appeared to be the best but involved tedious extractions. This work indicated, however, that a simultaneous analysis of aminophylline and phenobarbital was possible if the techniques described by Pernarowski, Knevel, and Christian (8) were utilized.

The theory of this absorbancy ratio method of analysis is discussed in detail in the above paper. Briefly, a plot of absorbancy ratio values *versus* the fraction of one of the two components in a binary mixture is a straight line if one of the wavelengths used in the analysis represents an isoabsorptive point. The equation of this straight line is easily determined and provides the basis for the subsequent determination of the individual components in the binary mixture.

The terminology and symbology used in this paper shall be that suggested by Pernarowski, Knevel, and Christian (8). Thus, the term "Q value" is an abbreviation for "absorbancy ratio value." The ratio itself is determined by dividing the value of the measured absorbancy at one wavelength by that measured at another wavelength, the cell and the solution being the same for both measurements. A "Q curve" illustrates the relationship between the Q values of particular binary mixtures and the fraction of one of the two components in such mixtures. Other spectrophotometric terms and symbols used in this paper are those described in a letter circular published by the National Bureau of Standards (9).

EXPERIMENTAL

Apparatus.—Beckman model DU spectrophotometer; Shell precision dual titrometer; shielded glass electrode, Beckman No. 41262; calomel electrode, sleeve type, Beckman No. 43-908; Fisher-Johns melting point apparatus, calibrated against U. S. P. melting point standards.

Reagents.—Chloroform, reagent grade. Methanol, Analar grade. Nitric acid C.P. Aminophylline U. S. P.; the sample was assayed by the U. S. P. XVI method (10) and was found to contain, on the basis of five determinations, 82.3 ± 0.4% theophylline. Phenobarbital, recrystallized from aqueous ethanol; the sample melted between 174.5 and 175.5° (corrected) and assayed 99.7% by the method developed in this laboratory (11). Theophylline, recrystallized from ethanol; the sample melted between 270 and 271° (corrected) and assayed 101.1% by the method described in the U. S. P. XVI for aminophylline (10). Ferric ammonium sulfate T. S. Ammonia T. S. Hydrochloric acid, diluted, U. S. P. Silver nitrate, 0.1 *N*. Ammonium thiocyanate, 0.1 *N*. Potassium hydroxide, 0.1 *N* in anhydrous methanol. Thymol blue indicator, 0.5% in anhydrous methanol. Borate buffer, pH 9.5 (12); this solution was prepared by dissolving 6.185 Gm. of reagent grade boric acid and 7.455 Gm. of reagent grade potassium hydroxide in sufficient distilled water to make 500 ml., adding 350 ml. of carbonate-free 0.1 *N* sodium hydroxide, and then sufficient distilled water to make 1 L. of solution.

Spectral Characteristics of Phenobarbital.—Solutions of phenobarbital in borate buffer of pH 9.5 showed absorption maxima at 240 mμ and were stable for twenty-four hours as previously reported (12).

Spectral Characteristics of Aminophylline.—Solutions of aminophylline in borate buffer of pH 9.5 exhibited maxima at 274.5 mμ and were stable over a twenty-four-hour period. When the spectrum of aminophylline was compared with that of theophylline, it was evident that the ultraviolet absorption was due solely to the theophylline portion of the molecule (see Fig. 1). This fact was further substantiated when ethylenediamine itself failed to exhibit any absorption between 235 and 300 mμ as well as causing no alteration in the spectral characteristic of theophylline. The two curves, as shown in Fig. 1, were identical with respect to shape but failed to coincide completely because of the differences in the concentration of the ultraviolet absorbing component theophylline. However, when the weight of aminophylline was altered so as to contain an equivalent amount of theophylline, the two were then found to be completely superimposable.

Location of the Isoabsorptive Point.—The isoabsorptive point for phenobarbital and theophylline was located by comparing spectrophotometrically a 0.001% solution of phenobarbital (sample solution) with a 0.001% solution of theophylline (blank solution). On the basis of accumulated data, the isoabsorptive point for these compounds occurred at 252.5 mμ. This was further substantiated by determining the absorbancy index values of phenobarbital and theophylline at this wavelength. The former value, on the basis of 19 determinations, was equal to 23.7 ± 1.0 while the latter value, on the basis of 27 determinations, was found to be 23.6 ± 0.1. The details associated with the location of isoabsorptive points are given elsewhere (8).

Procedure.—Weigh and powder twenty tablets. Accurately weigh an aliquot representing approximately 100 mg. of aminophylline, add 100 ml. of

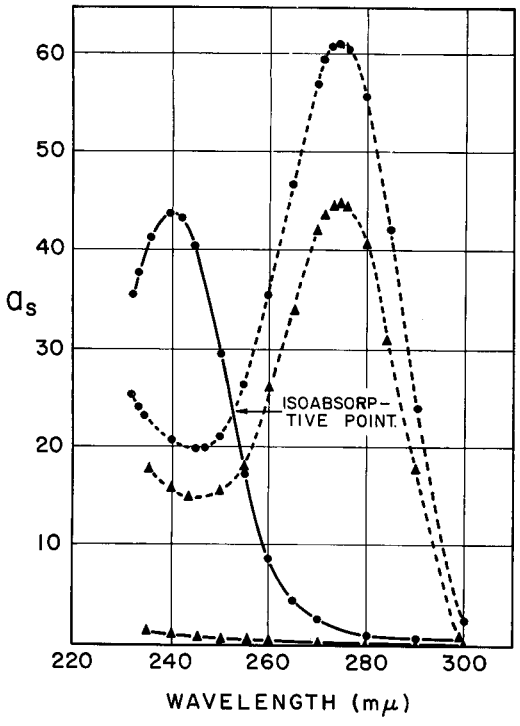

Fig. 1.—Spectrophotometric curves for phenobarbital (—●——●—), theophylline (- -●- -●- -), aminophylline (-▲- - -▲- -), and ethylenediamine (—▲——▲—).

borate buffer solution, and stir electromagnetically for thirty minutes. Filter through Whatman No. 1 filter paper into a 1,000-ml. volumetric flask, wash the residue with four additional 25-ml. portions of the buffer solution, and make up to volume with the same solvent. Take a 10-ml. aliquot of the solution and dilute to 100 ml. with the buffer. Measure the absorbancy of the solution at 252.5 and 274.5 mμ using borate buffer as the blank. Calculate the concentration of phenobarbital and theophylline by substituting the observed values into the following equation

$$\text{Gm. theophylline/L.} = \frac{Q{:}274.5{:}252.5 - 0.0451}{2.60} \times \frac{A_{252.5}}{a_{252.5}} \quad \text{(Eq. 1)}$$

Q:274.5:252.5 in the above equation is the absorbancy ratio value of the solution calculated by dividing the observed absorbancy at 274.5 mμ by that observed at 252.5 mμ. The absorbancy index value of both phenobarbital and theophylline at 252.5 mμ ($a_{252.5}$) is equal to 23.7 ± 0.7 on the basis of 46 determinations. The numerical values of the intercept and the slope of the Q curve are 0.0451 and 2.60, respectively. The concentration of phenobarbital is obtained by subtracting the weight of theophylline from the total weight of active components ($A_{252.5}/a_{252.5}$).

DISCUSSION

The spectral characteristics of phenobarbital, theophylline, ethylenediamine, and aminophylline are illustrated in Fig. 1. These characteristics indicated that binary mixtures containing phenobarbital and either aminophylline or theophylline could be analyzed by applying the principles described by Pernarowski, Knevel, and Christian (8). The first criterion cited by these authors for the use of the absorbancy ratio technique was the presence of an isoabsorptive point when the spectra of the substances being analyzed are superimposed. Such a point does in fact occur when the spectra of phenobarbital and theophylline are superimposed. This wavelength, 252.5 mμ, and the wavelength at which theophylline exhibits maximum absorption, 274.5 mμ, were chosen for the analysis. This choice of wavelengths results in optimum conditions, the slope value of the Q curve in this case being somewhat greater than if 240 mμ, the point at which phenobarbital exhibits maximum absorption, was chosen as the second wavelength. In addition to the above, ethylenediamine exhibits little absorption at 274.5 mμ. This implies that only the theophylline portion of aminophylline absorbs ultraviolet radiant energy. The proposed method of analysis results, therefore, in a direct measure of the therapeutically active moiety "theophylline" actually present in the labeled amount of aminophylline. The determined quantity of anhydrous theophylline can then be converted to an equivalent weight of aminophylline on the basis of established molecular relationships.

A plot of Q:274.5:252.5 values *vs.* the fraction of theophylline in various synthetic mixtures of phenobarbital and theophylline indicated that a linear relationship existed between the two variables over the entire concentration range of 0 to 100% theophylline (see Fig. 2). Although this Q curve is of theoretical interest, commercial preparations rarely contain less than 75% or more than 95% aminophylline, and consequently only that portion of the Q curve marked off as the area of significance and

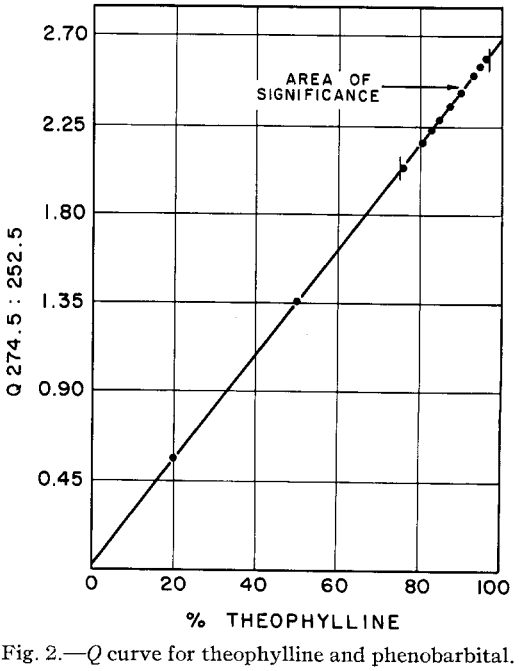

Fig. 2.—Q curve for theophylline and phenobarbital.

representing 74 to 97% theophylline was studied in detail. On the basis of 27 solutions containing both phenobarbital and theophylline, the equation of this portion of the Q curve was found to be

$$Q{:}274.5{:}252.5 = 2.60\ Ft + 0.0451 \quad \text{(Eq. 2)}$$

Ft, in the above equation is equal to the fraction of theophylline present in the binary mixture. This equation yields only relative values. If absolute values are required, Eq. 1 must be used.

To test the overall accuracy and precision of the proposed method of analysis, several synthetic mixtures of known composition were prepared and analyzed. The results of these analyses are shown in Tables I and II and indicate that the major component can be determined with a high degree of accuracy and precision. Phenobarbital, on the other hand, cannot be evaluated with the same degree of precision, the standard deviation being as high as 3% in certain instances. This, however, is a percentage figure and must be judged in relation to the speed and ease of anaylsis and to the quantity of substance being analyzed. The relative proportion of phenobarbital to major component is small and slight discrepancies in absolute recoveries will result in relatively large percentage differences. As an example, a variation of 0.05 mg. of phenobarbital from a mean value of 1.50 mg. will result in a 3.3% deviation from the mean. Absolute differences are, therefore, quite small and not particularly alarming.

TABLE I.—RESULTS OF THE ANALYSIS OF SYNTHETIC MIXTURES CONTAINING AMINOPHYLLINE AND PHENOBARBITAL

Mixture[a]	Aminophylline,[b] mg. Taken	Theophylline, mg. Found	Phenobarbital mg. Taken	Phenobarbital mg. Found
1	10.7	8.88	1.57	1.62
	10.7	8.88	1.57	1.62
2	10.2	8.41	1.51	1.55
	10.2	8.41	1.51	1.51
3	10.1	8.35	1.52	1.57
	10.1	8.36	1.52	1.56
4	10.1	8.39	1.53	1.61
	10.1	8.38	1.53	1.54
	Average % theophylline in aminophylline, 82.8 ± 0.2		Average % recovery, 102.6 ± 1.6	
5	10.7	8.86	3.14	3.24
	10.7	8.87	3.14	3.27
6	10.2	8.38	3.03	3.13
	10.2	8.38	3.03	3.17
7	10.1	8.41	3.03	3.02
	10.1	8.41	3.03	3.02
8	10.1	8.36	3.07	3.07
	10.1	8.36	3.07	3.07
	Average % theophylline in aminophylline, 82.8 ± 0.4		Average % recovery, 101.8 ± 2.2	

[a] Mixtures 1–4 simulate commercial preparations containing 1 1/2 gr. aminophylline and 1/4 gr. phenobarbital. Mixtures 5–8 are equivalent to commercial preparations containing 1 1/2 gr. aminophylline and 1/2 gr. phenobarbital. [b] The aminophylline used in the preparation of these mixtures analyzed 82.3 ± 0.4% by the U. S. P. XVI method.

Six samples of commercial tablets were analyzed by the proposed method and, where possible, by the procedure reported by David (6). The latter method involves a gravimetric determination of the phenobarbital in the mixture. Rather than resorting to such a technique, the method was modified so as to permit a titrimetric determination of the phenobarbital by the procedure described by Chatten (11). David's method tended to give slightly lower recoveries for the two components of the mixture, was operationally more difficult, and was not applicable to several of the commercial preparations analyzed. The slight discrepancies in recoveries between the two techniques may be due to a combination of several phenomena observed during various stages of the control method. It is inherently difficult to filter the ammoniacal solution of the tablet mass and this, in itself, may result in lower recoveries of both constituents. In certain cases, emulsions formed at the chloroform-water interface during the phenobarbital extraction step and may partly explain the lower recoveries of this substance. Silver theophyllinate itself is slightly soluble in ammoniacal solution and this factor may account for lower theophylline recoveries from mixtures (13).

TABLE II.—RESULTS OF THE ANALYSIS OF SYNTHETIC MIXTURES CONTAINING THEOPHYLLINE AND PHENOBARBITAL

Mixture[a]	Theophylline mg. Taken	Theophylline mg. Found	Phenobarbital mg. Taken	Phenobarbital mg. Found
1	8.01	7.87	1.50	1.46
	8.01	7.89	1.50	1.40
2	8.18	8.13	1.51	1.53
	8.18	8.16	1.51	1.55
3	8.01	7.99	1.55	1.55
	8.01	7.97	1.55	1.53
4	8.06	8.06	1.53	1.48
	8.06	8.06	1.53	1.48
	Average % recovery, 99.4 ± 0.7		Average % recovery, 98.3 ± 3.0	
5	8.01	7.89	3.00	2.82
	8.01	7.89	3.00	2.91
6	8.18	8.15	3.02	3.03
	8.18	8.16	3.02	3.02
7	8.01	8.02	3.09	3.07
	8.01	8.02	3.09	3.07
8	8.06	8.09	3.07	3.09
	8.06	8.09	3.07	3.09
	Average % recovery, 99.7 ± 0.8		Average % recovery, 98.9 ± 2.3	

[a] Mixtures 1–4 simulate commercial preparations containing 1 1/2 gr. aminophylline and 1/4 gr. phenobarbital. Mixtures 5–8 are equivalent to commercial preparations containing 1 1/2 gr. aminophylline and 1/2 gr. phenobarbital.

The results in Table III indicate that the proposed method is capable of yielding the same level of precision as that observed for synthetic mixtures. Tablet excipients such as talc, starch, lactose, and magnesium stearate do not interfere with the determination of the active ingredients. Compressed tablets (samples A, B, and C) were easily and quickly analyzed. Coated tablets, on the other hand, caused some difficulties because these contained water-soluble dyes in the outer coat (samples E and F). In these two cases, the method was modified slightly so as to allow for the removal of this outer coat by washing the tablets with distilled water.

The proposed method of analysis appears to be preferable to the methods described in the literature. It involves no solvent-solvent extractions, the manipulative techniques are easily mastered, and the accuracy and precision is perhaps greater than or at least equal to that obtainable by methods in current use.

TABLE III.—RESULTS OF THE ANALYSIS OF COMMERCIAL PREPARATIONS CONTAINING AMINOPHYLLINE AND PHENOBARBITAL

Sample	Aminophylline mg./Tablet Labeled	Theophylline mg./Tablet Found[a]	Theophylline mg./Tablet Found[b]	Phenobarbital mg./Tablet Labeled	Phenobarbital mg./Tablet Found[a]	Phenobarbital mg./Tablet Found[b]
A	97.2	77.6 ± 0.7	77.5	16.2	15.7 ± 0.3	15.6
B	97.2	71.2 ± 0.3	69.6	32.4	28.4 ± 0.4	28.1
C	97.2	77.3 ± 0.6	...	16.2	16.4 ± 0.7	...
D	97.2	73.1 ± 0.6	75.8	16.2	16.6 ± 0.2	...
E	97.2	80.0 ± 0.2	...	16.2	16.5 ± 0.4	...
F	194.4	160.8 ± 0.9	163.2	32.4	34.4 ± 0.9	34.0

[a] Each product was analyzed five times by the method described in the text. The averages and standard deviations of such analyses are reported in this column. [b] Where possible, comparative determinations were carried out using a modification of the method described by David (3). The averages of duplicate determinations are reported in this column.

SUMMARY

A spectrophotometric technique has been developed for the simultaneous determination of mixtures containing aminophylline and phenobarbital. This method is rapid, the manipulative techniques are simple, and the accuracy is equal to that inherent in published procedures.

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Pharmacology of Bunamiodyl I

Toxicity Studies

By IRVING LEVENSTEIN, ANNE WOLVEN, and ARNOLD URDANG†

The acute and chronic toxicity of a synthetic cholecystographic agent, bunamiodyl, 3-(3-butyrylamino-2,4,6-triiodophenyl)-2-ethyl sodium acrylate, was determined in rats and dogs. The LD_{50} was found to be 2.75 Gm. per Kg. of rat, and daily feedings of the material on the basis of 750 mg. per Kg. of rat caused no toxicity. Dogs receiving 1 Gm. of the test material per day for a two-week period showed no toxic symptoms and no damage to their kidneys.

IN THE SEARCH for synthetic cholecystographic agents, bunamiodyl,[1] 3-(3-butyrylamino-2,4,6-triiodophenyl)-2-ethyl sodium acrylate, was selected since it exhibited excellent opacity and a low incidence of side effects associated with these agents. A dose of 4.5 Gm. is necessary to obtain good gallbladder opacity in the human. It was necessary to carry out studies to indicate the safety of the compound in addition to determining its pharmacological fate following administration of this drug. This paper concerns itself with the investigation of the acute and chronic toxicity of bunamiodyl when administered orally to two species of animals. Studies dealing with the distribution and fate of bunamiodyl in animals and humans are presently being conducted and will be reported at a later date.

Toxicological studies performed in Europe (1) indicate that the LD_{50} of this material when administered intravenously to mice is 0.57 Gm. per Kg. The intravenous injection of bunamiodyl at a dose level of 0.2 Gm. per Kg. to a

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