Determination of Drugs in Dosage Forms by Difference Spectrophotometry

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Abstract □ The technique of difference spectrophotometry was applied to the rapid assay of numerous pharmaceuticals, without prior separation from other materials. Contributions to absorbance by interferences were automatically canceled; characteristic isosbestic points provided proof of this. Factors affecting selection of optimum pH were investigated, and reactions such as hydrolysis or formation of Schiff bases were utilized to produce spectral shifts. These and other aspects of the technique are discussed in detail.

Keyphrases □ Drugs—analysis in dosage forms, difference spectrophotometry, pH selection, hydrolysis, and Schiff-base formation considered, examples □ Spectrophotometry, difference—analysis of drugs in dosage forms, pH selection, hydrolysis, and Schiff-base formation considered, examples □ Difference spectrophotometry—analysis of drugs in dosage forms, pH selection, hydrolysis, and Schiff-base formation considered, examples □ Analysis—drugs in dosage forms, difference spectrophotometry

Electronic absorption spectrophotometry is the technique most extensively used in official methods as the measurement step for the quantitative assay of drugs in dosage forms. Although this use is in part a result of the rapidity and simplicity of the method, these advantages are somewhat negated by the requirement (1) that the actual determinative step be preceded by an isolation procedure, since tablet excipients, a syrup medium, or other substances may contribute to the total absorbance. It has been shown, for example, that simply dissolving trifluoperazine tablets and reading the absorbance of the solution gave errors of up to 5% due to background absorbance of excipients (2). Errors of such magnitude may not always be detected by simple inspection of the spectrum.

As an alternative to preliminary separation, the authors explored the use of difference spectrophotometry[1]; this technique provides for automatic cancellation of interferences while retaining the inherent convenience of direct reading. The method is applicable to all substances that can be made to undergo reproducible spectral changes.

The method is not new; notable examples of its application to drug analysis include assays for estradiol (3), morphine (4), caffeine (5), and corticosteroids (6, 7). Some elegant procedures for drug mixtures that undergo multiple spectral changes have been described (8, 9). However, the technique has not yet received widespread application nor understanding and is frequently misapplied.

This paper reports the difference spectrophotometric assay of numerous acidic, basic, and amphoteric drug substances. It is shown how detailed knowledge of ionization behavior is essential for utilization of pH-induced spectral shifts or how, alternatively, shifts may be effected without change in pH. It is demonstrated how isosbestic points, characteristic of most difference spectra, provide information concerning cancellation of interferences, purity of ionization or reaction processes, and location of optimum wavelengths for analysis of mixtures.

EXPERIMENTAL

All spectra were obtained on a recording spectrophotometer[2], using 1-cm silica cells.

Stock solutions were prepared by direct dissolution of the dosage form to give a concentration of, typically, from two to 10 times the final desired concentration. Water was the preferred solvent, but alcohol or dimethyl sulfoxide was used for preliminary solution, when required, without introducing any complication.

Duplicate aliquots of the stock solution were treated, each in a different manner, by addition of, *e.g.*, acid to one aliquot and base, buffer, other reagent, or nothing to the other. Both aliquots were then diluted to an identical final concentration, giving two sample solutions, A and B. Duplicate aliquots of a standard stock solution were similarly treated. When the buffer or other reagent had a detectable absorbance at wavelengths of interest, blank Solutions A and B were also prepared.

The zero point of the recording spectrophotometer was raised manually to permit recording of minima. The baseline was obtained by reading blank against blank. Sample Solution A was placed in the sample compartment, sample Solution B was placed in the reference compartment (or vice versa), and the difference spectrum was recorded. The standard difference spectrum was likewise scanned, superimposed on the sample spectrum to facilitate observance of isosbestic points.

Calculations of sample concentration were then made as in conventional spectrophotometry, using the recorded absorbance of the maximum, minimum, or amplitude.

RESULTS AND DISCUSSION

General Considerations—In double-beam spectrophotometry, a reference solution is scanned simultaneously with the sample solution to compensate for any opacity of cell, solvent, or added reagents. Ideally, the reference and sample solutions should be identical in all respects except for the presence of the analyte. In practice, when analyzing pharmaceutical samples, there is always uncertainty concerning interfering materials which may have accompanied the sample and which remain uncompensated for by the reference solution.

[1] The technique has been variously known as the $\Delta\epsilon$ method, differential spectrophotometry, and (incorrectly) derivative spectrophotometry. Difference spectrophotometry has also been used and is favored here since it best connotes the simple subtractive nature of the instrumental process.

[2] Cary 15 or 118, Cary Instruments, Monrovia, CA 91016

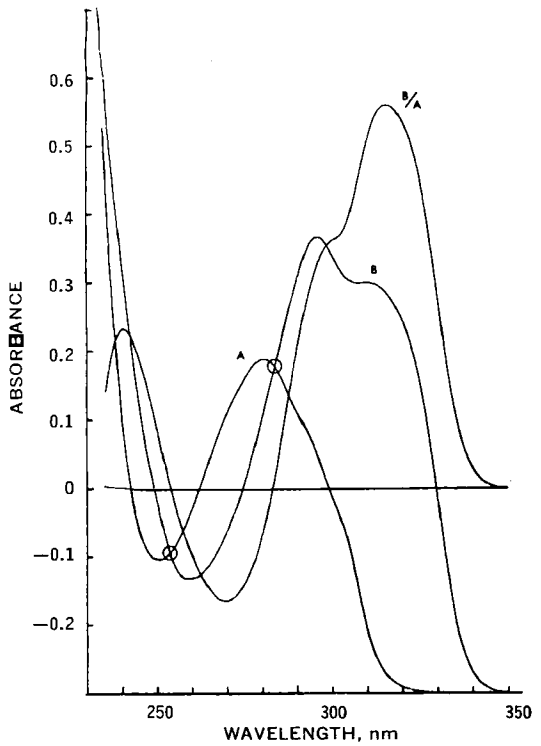


Figure 1—*Conventional and difference spectra of benzthiazide (about 15 μg/ml). Key: A, acidic solution; B, basic solution; and B/A, difference spectrum of basic* versus *acidic solutions. Note the isosbestic points (circled) at 287 and 255 nm, corresponding to intersection of the difference spectrum with the baseline.*

Difference spectrophotometry provides an approximation of the ideal reference solution by employing an aliquot of the sample solution itself as reference, adjusted by change in pH or other parameter but containing both the substance being analyzed and all extraneous substances at exactly the same concentrations as the sample. If the pH or other variation causes an alteration in the spectrum of the sample, the instrument records this as a characteristic difference spectrum. If other materials present are unaffected by the change in conditions, their contribution to the total absorbance of each solution will be identical and their effect will be exactly canceled.

Figure 1 shows the conventional spectra of the acidic drug benzthiazide in aqueous acid and in aqueous base, together with the difference spectrum recorded as base *versus* acid. Here and elsewhere, the zero point of the difference spectrum has been manually raised, so that minima as well as maxima may be observed. The choice of sample and reference solutions is essentially arbitrary, making the distinction between maximum and minimum meaningless. The difference in absorbance between an adjacent maximum and minimum may be termed the amplitude.

Since both the basic and acidic solutions obey Beer's law at all wavelengths, it is evident that the difference of these two spectra does likewise. This difference is precisely what the instrument records automatically; the amplitude may therefore be used for quantitative determinations in exactly the same manner that simple absorbance is used in conventional spectra.

Figure 2 shows the difference spectra of standard benzthiazide at various concentrations. The 313-nm maximum, the 271-nm minimum, and the amplitude between these peaks all gave linear absorbance–concentration plots, as expected. It is usually more convenient and always more sensitive to employ the amplitude rather than either of the individual peaks. However, when a buffer or other reagent added to one of the two solutions has a detectable, uncompensated absorbance over the wavelength interval involved, as blank readings will demonstrate, it is simpler to take readings of a single peak relative to the baseline rather than to make the necessary corrections for calculation of the amplitude.

Spectral changes that are apparently minor may, nevertheless, produce useful difference spectra. Figure 3 shows the conventional spectra of hydrochlorothiazide in acid and base. The maxima are shifted by only 6 nm, and the absorbances at these maxima differ by only 12%. However, the difference spectrum, superimposed on the figure and determined at the same concentration, gives an amplitude that is fully 53% as sensitive as the acid spectrum. Most of the amplitude is due to the peak at 339 nm, which occurs in a region where the absorbances of both acid and base spectra are rapidly increasing. In spite of this, operation of the spectrophotometer at normal scan speeds gave reproducible results, identical with those obtained at slow scan.

Isosbestic Points—The principal advantage of difference spectrophotometry lies in its potential for the cancellation of interferences; therefore, it is necessary to have some indication that this has been accomplished in a given case. The isosbestic points usually present in difference spectra provide a useful test for such cancellation.

In Fig. 1, for benzthiazide, the conventional acidic and basic spectra intersect at 287 and 255 nm, indicating that the two forms have identical absorbance at these isosbestic wavelengths. In the difference spectrum, the net absorbance at these wavelengths will be zero; this will be true for any concentration of the drug (Fig. 2). Therefore, sample and standard curves may be checked for maintenance of isosbestic points even if they are not at the same, or even similar, concentrations. This provides a powerful test for spectral (but not physical) purity.

If a sample difference spectrum does intersect the baseline at all points predicted by the standard, then this confirms that interferences, if present, have canceled at these points and strongly suggests that this is true throughout the spectrum. It does not constitute absolute proof. An extraneous substance could exhibit a zero difference absorbance at the indicated wavelengths but a positive absorbance at the wavelength of measurement. This would be improbable, however. Failure to maintain one or more points conclusively demonstrates that sample and standard solutions differ in some respect (in addition to any concentration difference, which does not affect the points). Some other substance that also undergoes spectral shifts must be present, necessitating a preliminary isolation or alternative assay procedure.

When it is desired to use this criterion as a test for spectral purity, it is convenient to record baseline, standard, and sample curves superimposed. Maintenance of the points may then be seen at a glance, as shown in Fig. 4.

Isosbestic points also find many uses in the development and selection of optimum analytical conditions, as discussed in succeeding sections.

Choice of pH—In most examples studied, the spectral changes are induced by simple reversible ionization of groups directly con-

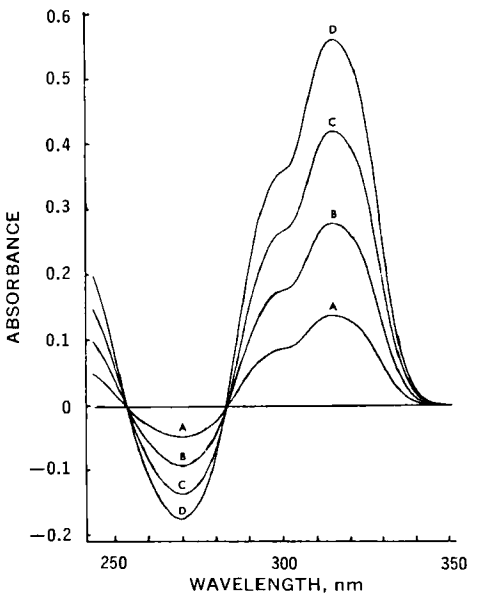


Figure 2—*Difference spectra used to verify Beer's law relation for benzthiazide (basic* versus *acidic solution for concentrations of: A, 5; B, 10; C, 15; and D, 20 μg/ml).*

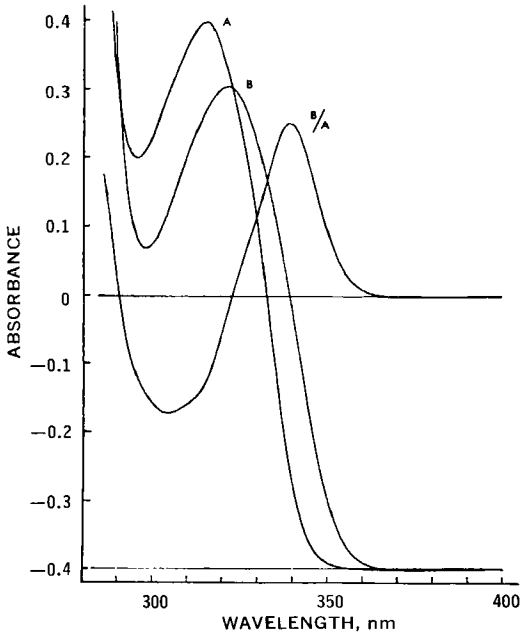


Figure 3—*Conventional and difference spectra of hydrochlorothiazide (about 20 μg/ml). Key: A, acidic solution; B, basic solution; and B/A, difference spectrum of basic* versus *acidic solutions.*

jugated to a chromophore. It is important that the acid and base solutions be selected so that both are at least two pH units removed from the pKa, on opposite sides of this value. For monofunctional compounds, this will ensure that each solution represents a single species of at least 99% spectral purity. At pH values closer to the pKa, small pH changes may result in appreciable changes in the difference spectrum.

Williams and Zak (10) described a difference spectra method for the analysis of barbiturates which employs solutions at pH 13.5 and 10.5. This method depends on the ionization of the monoanion to the dianion as the pH is raised. However, at pH 13.5, the dianion is present to an extent of less than 90% for most barbiturates; small differences in pH between standard and sample solutions would thus lead to serious errors.

For routine assay, the authors prefer to utilize the spectral change between the free barbiturate (read in dilute hydrochloric acid) and the monoanion (read at pH 10.5). The species so obtained are at least 99% pure. The use of these conditions does have

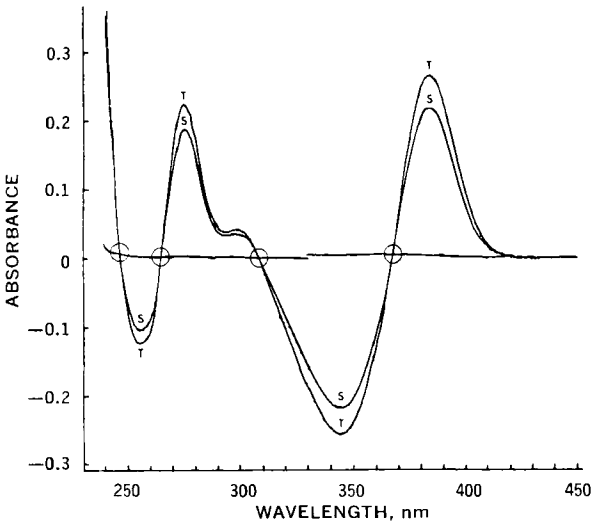


Figure 4—*Difference spectrum of triamterene, basic* versus *acidic solutions (about 2.5 μg/ml in aqueous alcohol). Key: S, standard solution; and T, direct dissolution of ground tablets. Note the presence of four isosbestic points.*

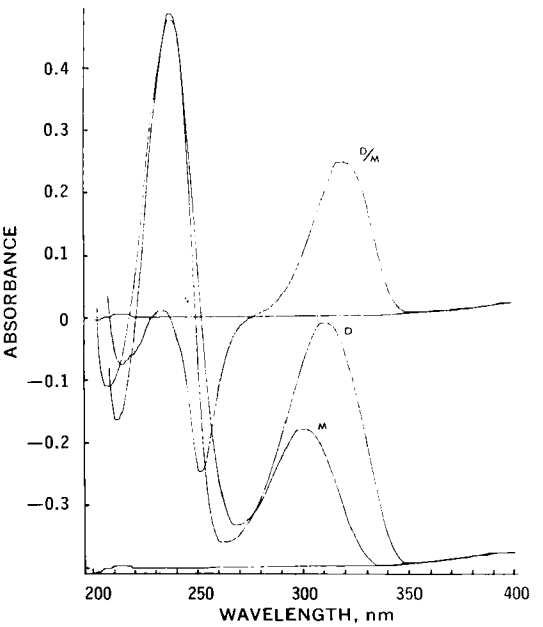


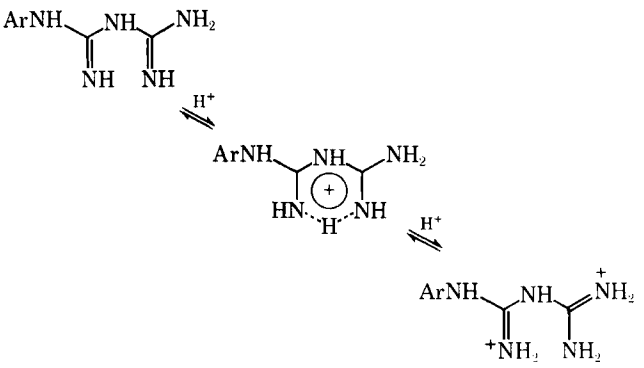
Figure 5—*Conventional and difference spectra of methapyrilene (about 25 μg/ml). Key: M, monocationic species at pH ~7; and D, dication at pH ~1. The pH's were chosen to utilize the ionization on the 2-aminopyridine ring of methapyrilene.*

a drawback in that the acid spectrum is essentially featureless so that no isosbestic points are observed and the difference spectrum is primarily that of the monoanion. However, the desired cancellation of interferences is still expected, making the procedure superior to simple reading against a blank. On the other hand, additives such as parabens, which may be present, undergo spectral changes due to ionization of the phenolic hydroxyl (pKa ~10), interfering with the assay by either of the discussed variations.

For polyfunctional compounds, a detailed knowledge of the ionization constants may be necessary to select optimum conditions. Goodman *et al.* (11) determined methapyrilene by a difference spectra method utilizing measurements at pH 10.6 and 7.45. This pH range includes the ionization of the aliphatic amine function of methapyrilene (pKa_1 = 8.9); ionization of this group produces a usable but relatively small change in the spectrum of the remote 2-aminopyridine portion of the molecule.

In contrast, the authors determined methapyrilene in hydrochloric acid and neutral solutions (Fig. 5), taking advantage of the ionization of the pyridine ring itself (pKa_2 of methapyrilene = 3.8) and thus obtaining a sensitivity six times greater than that reported by Goodman *et al.* (11).

Phenformin hydrochloride in neutral solution displays an intense peak at 233 nm, due apparently to a delocalized pseudo six-membered ring of the protonated biguanidine group (Scheme I). This group is so basic ($pKa_1 \sim 13$) that solutions with an effective pH > 15 would be required to obtain the pure free phenformin species. By contrast, a second proton is readily accepted in acid so-



Scheme I

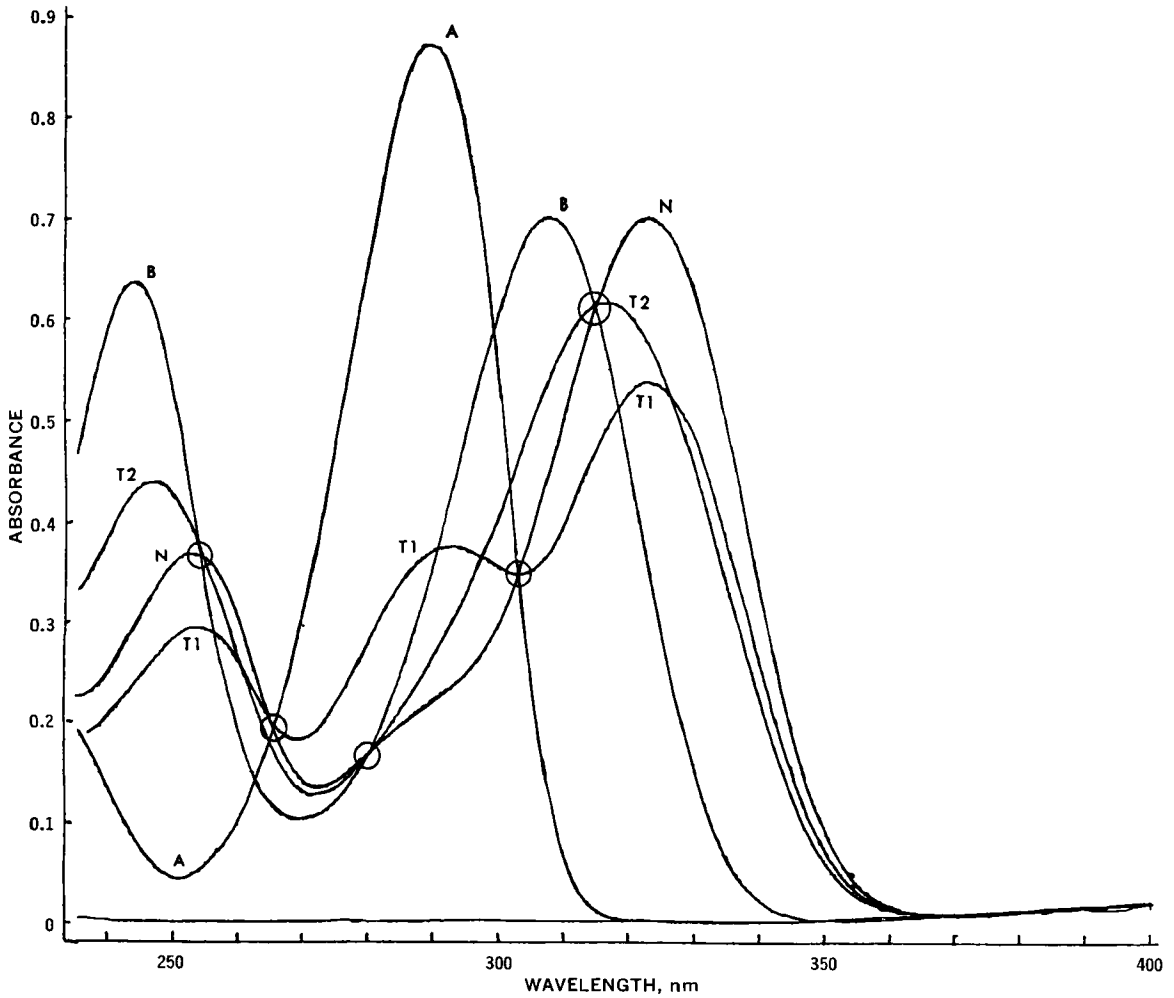


Figure 6—*Conventional spectra showing transformations due to multiple ionization of the amphoteric drug pyridoxine. All solutions are at identical concentrations of about 20 μg/ml. Key: A, pH 1; T1, pH 5; N, pH 6.6; T2, pH 9; and B, pH 13. The isosbestic points (circled) at 303 and 266 nm correspond to the transformation of the cation to neutral species; those at 315, 280, and 255 nm are associated with the further ionization to the anion.*

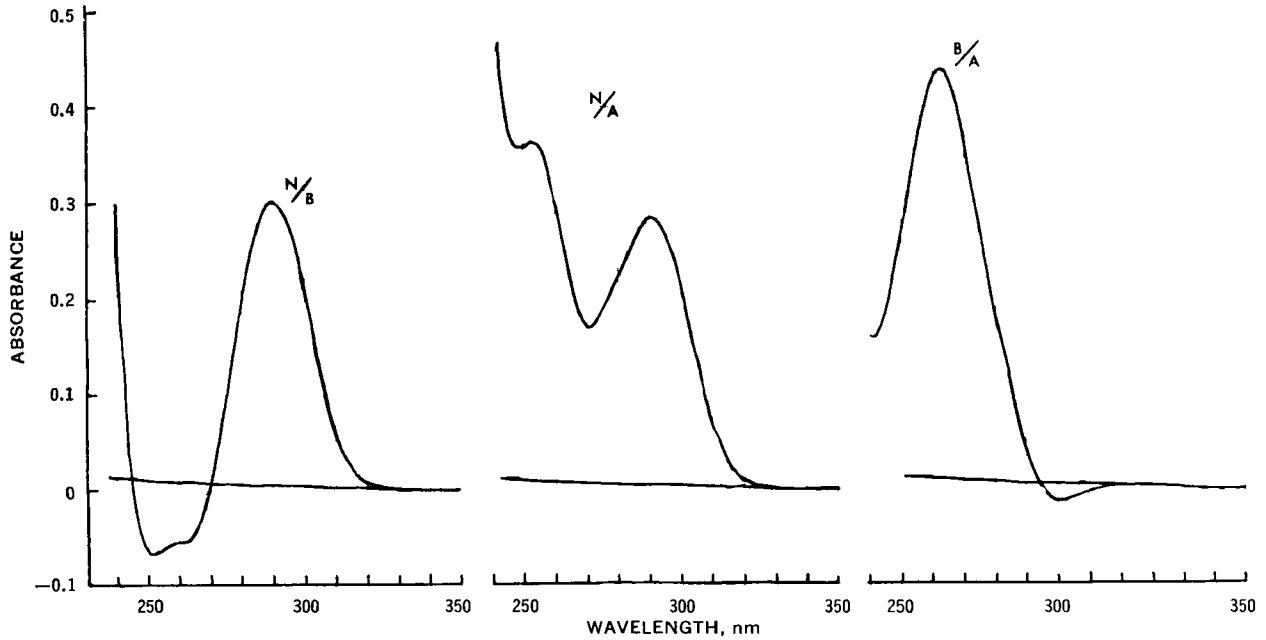


Figure 7—*Three difference spectra for sulfathiazole in aqueous alcohol. Key: A, acid; B, base; and N, neutral. All solutions are at identical concentrations of about 10 μg/ml.*

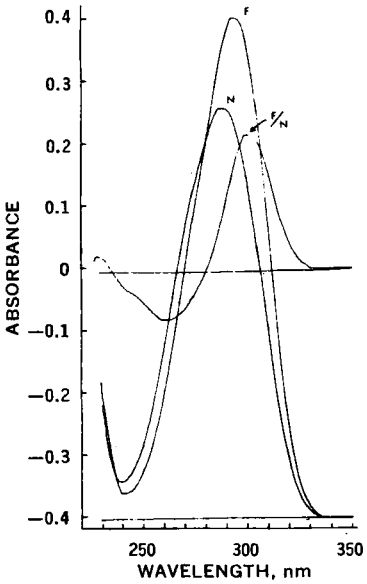


Figure 8—*Conventional and difference spectra of procaine, utilizing Schiff-base formation. All solutions are at identical pH (neutral) and concentration (about 10 μg/ml). Key: N, no reagent; and F, formaldehyde added.*

lution ($pKa_2 \sim 3$) with loss of absorbance; a neutral solution read against a solution with pH < 1 provides an effective assay method.

The amphoteric drug pyridoxine is highly soluble in water at all pH values and cannot be extracted by organic solvents. Figure 6 shows the complex spectral changes this compound undergoes as a function of pH. Simple ionization of the basic nitrogen and of the acidic aromatic hydroxyl fully accounts for these changes. This is demonstrated by the maintenance of isosbestic points at 303 and 266 nm in going from pH 1 to 6.6, together with the loss of these points and the appearance of a second set of points at 315, 280, and 255 nm as the pH is further increased from 6.6 to 13.

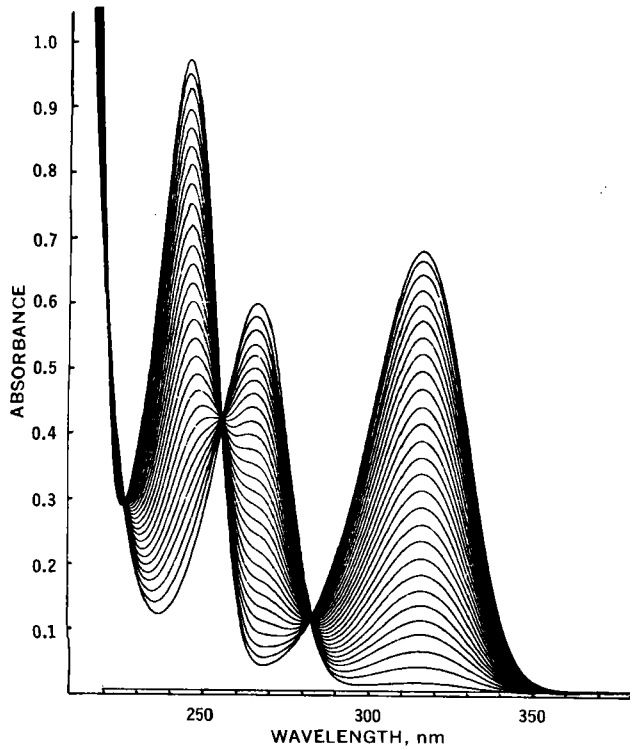


Figure 9—*Hydrolysis of pyridostigmine bromide in dilute alkali. Twenty-eight conventional scans were taken over 2 hr from the inception to the completion of the reaction, during which time the starting material (peak at 266 nm) was gradually replaced by the product (peaks at 246 and 316 nm). Note the isosbestic points which demonstrate lack of complicating side reactions. Rate of hydrolysis is controlled by the concentration of alkali.*

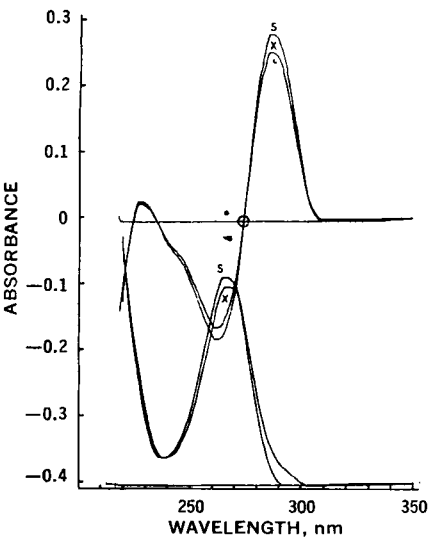


Figure 10—*Analysis of a partially decomposed sample of pyridostigmine bromide. Key: X, sample with 10% decomposition; and S, standard solution. The lower curves show the conventional spectra; the upper curves show the difference spectra using the hydrolytic method (see text).*

Recording the curves and determining the pH of each of the five solutions in Fig. 6 provided sufficient data to estimate the ionization constants of pyridoxine; pKa_1 was found to be 4.85 for the basic nitrogen, and pKa_2 was calculated as 9.07 for the acidic hydroxyl. Thus, the difference spectrum taken in the usual hydrochloric acid and sodium hydroxide solutions is known to result from pure cationic and anionic species and may, therefore, be used with confidence despite the complexity of the intermediate transformations.

Sulfathiazole is representative of a large class of amphoteric sulfonamides which may be analyzed by this method. The ionization constants are generally available in the literature; therefore, the pH's required to obtain pure species may be ascertained beforehand. Most of these compounds are relatively insoluble in water; preparation of the stock solution in 50% alcohol avoids this problem.

Figure 7 shows the difference curves obtained by the three possible pairings of cationic, neutral, and anionic species of sulfathia-

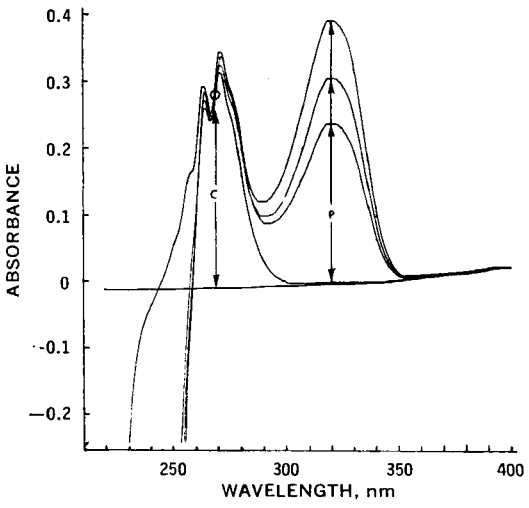


Figure 11—*Difference spectra analysis of the mixture of chlorpheniramine (C) and pyrilamine (P). Four solutions with constant concentrations of chlorpheniramine and varying concentrations of pyrilamine are shown scanned as acid* versus *neutral. The constant absorbance at 268 nm provides a measure of chlorpheniramine; pyrilamine is measured at 320 nm.*

zole. All gave useful spectra of comparable sensitivity; the neutral *versus* acid curve lacks an isosbestic point and is unsuited for the detection of interferences.

Reaction Methods—While most instances of difference spectra involve pH effects, spectral changes may also result from factors other than ionization. These permit sample and reference solutions to be prepared at an identical pH, with improved likelihood for cancellation of interferences. For example, the chromophore in the A ring of corticosteroids was reduced with sodium borohydride, and the intact drug was scanned against the reduction product (6, 7).

The authors found that the reaction of formaldehyde with primary aromatic amines, which affords Schiff bases, proceeds rapidly and reproducibly at room temperature and alters the spectra of such pharmaceutical substances as many of the local anesthetics and sulfa drugs. Figure 8 shows a typical result for procaine. Tetracaine, often combined with procaine, is a secondary amine; it does not react and thus cancels out of the difference spectrum so that no separation is required to assay for procaine in this mixture.

The most suitable reactions are rapid, clean, and complete and employ mild reagents that are transparent over the spectral region of interest. If both the drug and its reaction product have distinct spectra of comparable intensity, then isosbestic points should be produced. A reaction illustrating all of these desirable features is the alkaline hydrolysis of pyridostigmine bromide. Figure 9 shows consecutive spectra obtained during hydrolysis in dilute sodium hydroxide. The isosbestic points at 285, 258, and 228 nm demonstrate that the reaction is clean; pseudo-first-order kinetics computed from the consecutive scans were used to establish the completeness of reaction.

The hydrolysis product in alkaline solution can be read against the intact neutral drug, but a superior procedure is to adjust the reaction solution back to neutral after complete hydrolysis. The spectrum shifts again but remains distinct from that of the original compound. One solution is prepared by mixing drug, sodium hydroxide, and then excess acid after reaction. The other, unreacted, solution is prepared by mixing the same amounts of sodium hydroxide, excess acid, and then drug. Although the only difference is the order of addition of the reagents, a pronounced, reproducible difference spectrum results.

The assay of a sample of pyridostigmine bromide containing 10% hydrolyzed material by this procedure is shown in Fig. 10. Also shown is the conventional spectrum of the same mixture. The theoretical result is that the decomposed portion will not be detected by the difference method, as demonstrated by the maintenance of isosbestic points in Fig. 10, so that the true assay for intact drug alone is obtained.

On the other hand, the analyst would remain unaware of the presence of the decomposition product. It could be detected by comparison of the conventional spectrum with a standard, as shown. This conventional spectrum alone would, however, give a false assay value. The methods thus complement each other.

Mixtures of Drugs—Assay of mixtures of drugs by difference spectrophotometry, where more than one drug undergoes spectral shifts, constitutes a special challenge which can sometimes be met. The analysis of analgesics (8, 9) established the guidelines for such problems. Figure 11 illustrates an example from this laboratory for the antihistamine combination of chlorpheniramine and pyrilamine. Both have second ionization constants (pKa_2) of about 4.

The difference spectrum of pyrilamine exhibits an isosbestic point at 268 nm; this is the wavelength of choice for the analysis of the other component, chlorpheniramine, since the contribution of pyrilamine to the net absorbance at this wavelength is always zero. Chlorpheniramine has a difference curve with maximum at 272 nm. Readings are taken not at this maximum but at 268 nm on a slope and are best done with the instrument set at fixed wavelength at 268 nm.

In general, such methods for mixtures require meticulous technique and depend on the fortuitous juxtaposition of an isosbestic point of one compound with a maximum of another. By contrast, difference spectra methods for single-component dosage forms are usually simple and rugged.

CONCLUSIONS

Although only slightly more elaborate than direct dissolve-and-read methods, difference spectrophotometry has the advantages of potential cancellation of interferences and of an automatic test for such cancellation. Although it is not meant to supplant traditional extraction or chromatographic procedures, it can be argued that it achieves spectrophotometric rather than physical isolation of the analyzed substance. It is recommended especially for rapid routine assay of drugs with solubility characteristics that make preliminary separation difficult or impractical.

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