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Peak Homogeneity Determination for the Validation of High-Performance Liquid Chromatographic Assay Methods

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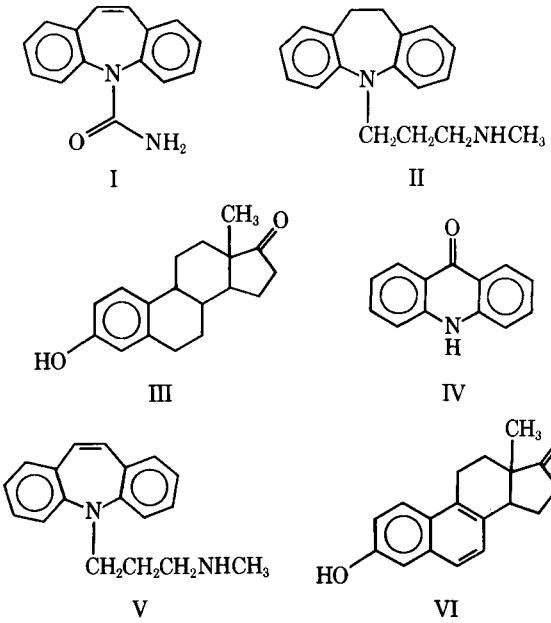
Abstract □ To validate high-performance liquid chromatographic assay procedures with regard to specificity, methods were developed to determine the homogeneity of the chromatographic peaks. These methods employed a rapid-scanning UV-visible spectrophotometer to monitor the chromatographic effluent. The absorption data were processed to nullify the signal due to the drug substance specifically, while allowing the detection of coincident impurities. Results from three model systems indicated the ability of these methods to detect as little as 0.1% of a coincident impurity.

Keyphrases □ High-performance liquid chromatography—validity, determination of peak homogeneity □ Impurities—high-performance liquid chromatography, determination by measurement of peak homogeneity □ UV spectrometry—use in determination of peak homogeneity of high-performance liquid chromatographic assays

The use of high-performance liquid chromatography (HPLC) for quantitative analyses of pharmaceuticals has been increasing rapidly (1). HPLC offers excellent sensitivity, accuracy, and precision, as well as convenience. Perhaps the most significant advantage of HPLC is the specificity obtained, since the drug substance is assayed following separation from any impurities. It is this specificity which has led to the acceptance of HPLC methods for stability-indicating assays. Naturally, the validity of such procedures is dependent on the homogeneity of the chromatographic peak of interest. In ordinary practice, a procedure is considered sound in this regard if the chromatographic peak representing the drug-substance is resolved from all known or theoretical synthetic impurities as well as decomposition products (2). Such indirect methods do not actually examine the homogeneity of the peak and are limited in scope to compounds previously identified as potential impurities. However, homogeneity, within specified limits, can be shown for any chromatographic technique, if it can be demonstrated that a critical physical property of the peak in question does not change with time. For example, GC peak homogeneity can be shown by using rapid-scanning mass spectrometers as specific detectors to demonstrate the constancy of the mass spectrum of the eluting peak with time (3, 4).

This report presents a similar method which evaluates the homogeneity of HPLC peaks directly, by monitoring the constancy of the UV–visible (UV/VIS) absorption spectrum of the moving eluting substance without the use of a stopped-flow apparatus, which examines only a small portion of the peak of interest. Specifically, it is the ratio between absorbances at specified wavelengths in the absorption spectrum of the eluted peak which is examined. Homogeneity is demonstrated by the fact that for sufficiently dilute solutions of a pure substance, the ratio of absorbances should remain constant, regardless of concentration throughout the chromatographic peak. To accomplish this, a rapid-scanning, microcomputer controlled UV/VIS spectrophotometer was employed to examine the chromatographic effluent. Several reports have appeared (5–9) concerning the use of rapid-scanning spectrophotometers as detectors for HPLC. This report represents the first application of these detectors for HPLC method validation in pharmaceutical analysis. Results obtained for three model systems are described; each of these systems contains a drug substance: carbamazepine (I), desipramine (II), or estrone (III), plus a representative impurity designed to coelute.



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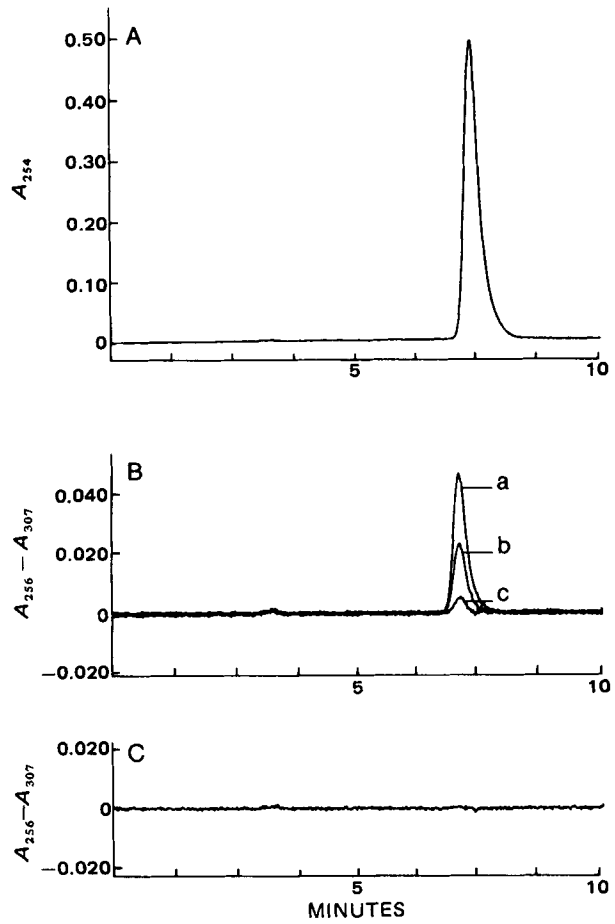

Figure 1—*A) Chromatogram of carbamazepine containing 1% acridone; B) chromatograms of carbamazepine containing (a) 1% acridone, (b) 0.5% acridone, and (c) 0.1% acridone; C) baseline chromatogram obtained for pure carbamazepine. Approximately 5 μg of carbamazepine was injected in 2.5 μl of mobile phase in all cases.*

EXPERIMENTAL

Materials—Carbamazepine[1] (I), acridone[1] (IV), desipramine[1] (II), 5-(3-methylaminopropyl)-5*H*-dibenz[*b*,*f*]azepine[1] (V), estrone[2] (III), and equilenin[2] (VI) were used in the model systems. Spectrophotometric grade solvents[3] were used in all mobile phases. The ion-pairing reagent was prepared by dissolving 5.5 g of sodium heptane sulfonate[4] in 50 ml of distilled water and diluting to 100 ml with glacial acetic acid. All mobile phases were passed through membrane filters[5] prior to use.

Apparatus—All analyses were performed using a high-performance liquid chromatograph[6] equipped with a standard injector, a 3.9 mm × 30-cm reversed-phase column and a fixed wavelength UV absorption detector. The outlet line from the detector was connected *via* a low dead-volume coupling to a quartz micro flow cell[7] (volume, 8 μl, light path, 1 cm) mounted on the optical bench of the rapid-scanning UV/VIS spectrophotometer[8]. The spectrophotometer's built-in microcomputer (16-bit word, 32K words of memory) processed the spectral data in real time. Full-range spectra (200–800 nm) were recorded *versus* air as the blank on the chromatographic effluent, computations were made, and the resultant data were plotted[9] every second to generate the chromatograms shown.

Peak Homogeneity—The methods employed to detect inhomogeneities in chromatographic peaks are designed to specifically nullify the signal due to the drug substance, while allowing the detection of other

[1] Ciba-Geigy Corp., Suffern, NY 10901.
[2] Sigma Chemical Co., St. Louis, MO 63178.
[3] Fisher Scientific Co., Fair Lawn, NJ 07410.
[4] Eastman Organic Chemicals, Rochester, NY 14650.
[5] Millipore Corp., Bedford, MA 01730.
[6] Model 6000A solvent delivery system, U6K injector, μ-Bondapak C_{18} column, and Model 440 detector, Waters Associates, Milford, MA 01757.
[7] Model 178.32, Hellma Cells, Inc., Jamaica, NY 11424.
[8] Model 8450A, Hewlett-Packard, Palo Alto, CA 94304.
[9] Model 7245A, Hewlett-Packard, San Diego, CA 92127.

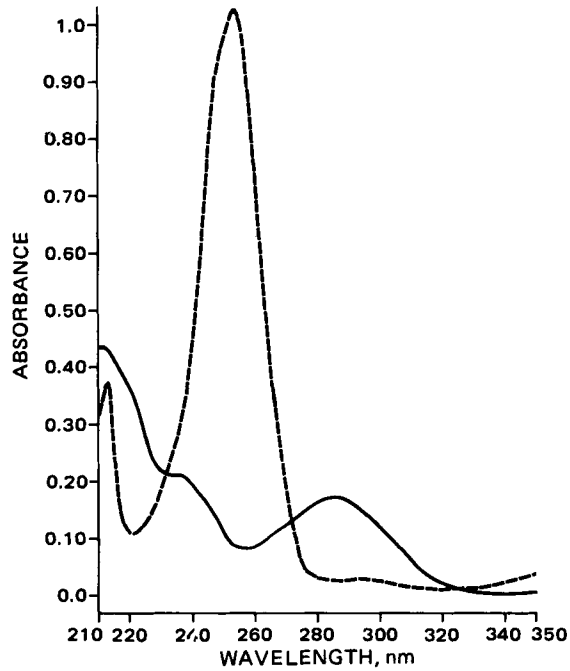

Figure 2—*UV absorption spectra of carbamazepine (—) and acridone (- - - -) obtained as 10 μg/ml solutions in mobile phase.*

compounds. The strict proportionality between absorbances at two wavelengths in the absorption spectrum of the pure drug substance was used, as given:

$$\frac{A_{\lambda_1}}{A_{\lambda_2}} = k_{1,2} \qquad \text{(Eq. 1)}$$

This equation can also be expressed as the null relationship of Eq. 2:

$$A_{\lambda_1} - k_{1,2}A_{\lambda_2} = 0 \qquad \text{(Eq. 2)}$$

which provides a basis for eliminating the response due to the drug-substance. By recording this difference *versus* retention time, a chromatogram which is void of any signal resulting from the elution of the drug substance is obtained. In the simplest case, two wavelengths of equal absorbance (*i.e.* when $k_{1,2} = 1.0$) have been chosen from the absorption spectrum of the drug substance.

In some cases it was advantageous to compare the average absorbance over two or more wavelengths with the absorbance at another single wavelength. The average absorbance over two wavelengths, λ_1 and λ_2, is related to the absorbance at a third wavelength, λ_3. The proportional relationships between the individual wavelengths obtained from Eq. 1 are:

$$A_{\lambda_1} = k_{1,3}A_{\lambda_3} \qquad \text{(Eq. 3)}$$

and

$$A_{\lambda_2} = k_{2,3}A_{\lambda_3} \qquad \text{(Eq. 4)}$$

Addition of these two equations and dividing by 2 gives an expression for the average absorbance at λ_1 and λ_2, $\overline{A}_{\lambda_{1,2}}$:

$$\overline{A}_{\lambda_{1,2}} = \frac{A_{\lambda_1} + A_{\lambda_2}}{2} = A_{\lambda_3}\frac{(k_{1,3} + k_{2,3})}{2} \qquad \text{(Eq. 5}a\text{)}$$

This is more simply written as:

$$\overline{A}_{\lambda_{1,2}} = k'A_{\lambda_3} \qquad \text{(Eq. 5}b\text{)}$$

where $k' = (k_{1,3} + k_{2,3})/2$. Equation 5*b* is then rearranged to give a null relationship similar to Eq. 2.

Comparison of average absorbances of two or more sets of wavelengths is a useful technique. In the simplest case, the average absorbance at λ_1 and λ_2, $\overline{A}_{\lambda_{1,2}}$, is related to the average absorbance at λ_3 and λ_4, $\overline{A}_{\lambda_{3,4}}$. Two equations relate $\overline{A}_{\lambda_{1,2}}$ to the absorbances at the individual wavelengths λ_3 and λ_4:

$$\overline{A}_{\lambda_{1,2}} = k'A_{\lambda_3} = k''A_{\lambda_4} \qquad \text{(Eq. 6)}$$

where $k'' = (k_{1,4} + k_{2,4})/2$. Addition of these equations and division by

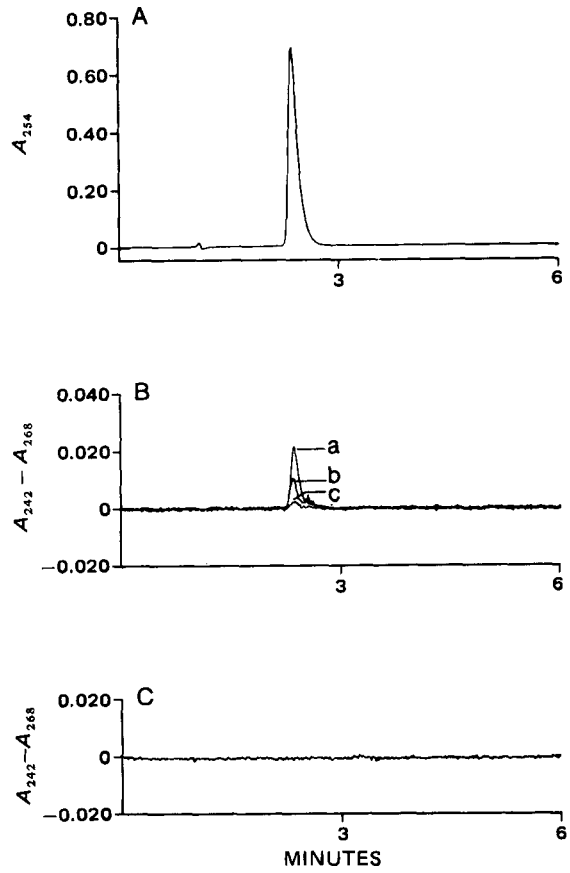

Figure 3—*A) Chromatogram of desipramine containing 1% V; B) chromatograms of desipramine containing (a) 1% V, (b) 0.5% V, and (c) 0.1% V; C) baseline chromatogram obtained for pure desipramine. Approximately 10 μg of desipramine was injected in 5 μl of mobile phase in all cases.*

2 gives Eq. 7, which expresses $\overline{A}_{\lambda_{1,2}}$ as a function of A_{λ_3} and A_{λ_4}:

$$\overline{A}_{\lambda_{1,2}} = \frac{k'A_{\lambda_3} + k''A_{\lambda_4}}{2} \qquad \text{(Eq. 7)}$$

As seen from Eq. 7, $\overline{A}_{\lambda_{1,2}}$ is directly proportional to $\overline{A}_{\lambda_{3,4}}$ only when $k' = k''$:

$$\overline{A}_{\lambda_{1,2}} = k'\frac{(A_{\lambda_3} + A_{\lambda_4})}{2} = k'\overline{A}_{\lambda_{3,4}} \qquad \text{(Eq. 8)}$$

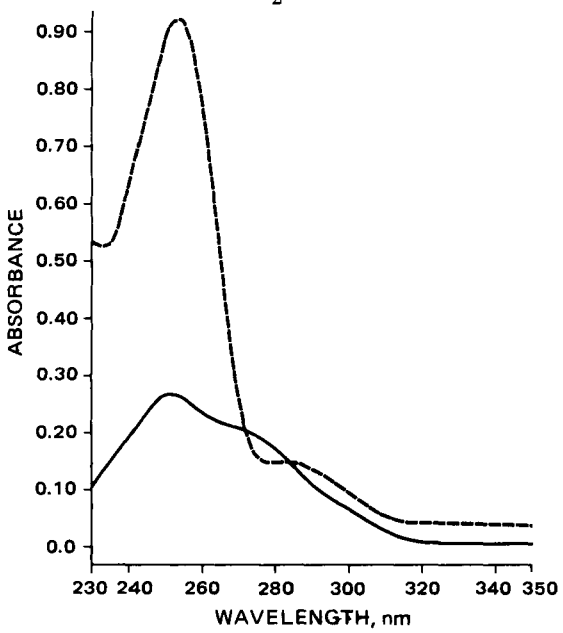

Figure 4—*UV absorption spectra of desipramine(—) and V (- - - -) obtained as 10 μg/ml solutions in mobile phase.*

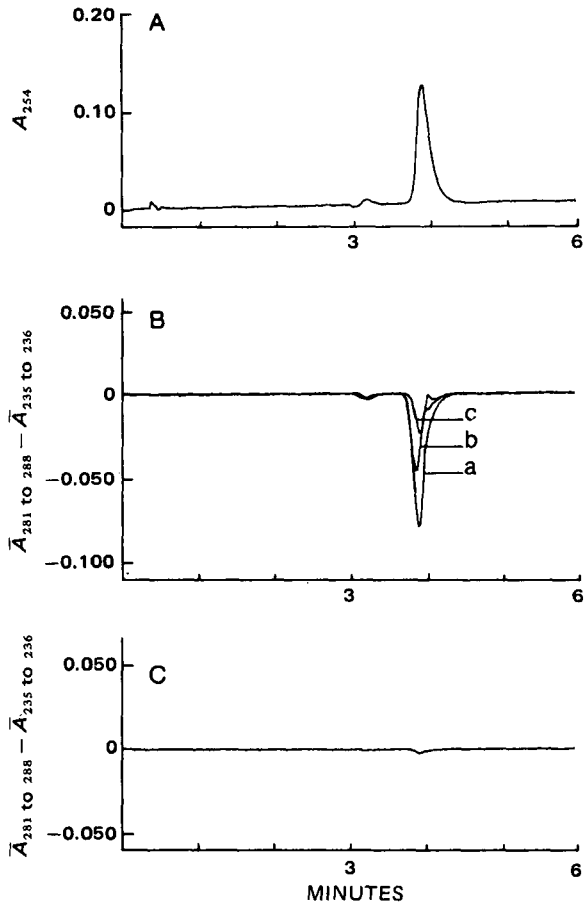

Figure 5—*A) Chromatogram of estrone containing 1% equilenin; B) chromatograms of estrone containing (a) 1% equilenin, (b) 0.5% equilenin, and (c) 0.1% equilenin; C) baseline chromatogram obtained for pure estrone. Approximately 20 μg of estrone was injected in 10 μl mobile phase in all cases.*

Rearrangement of this equation gives the null relationship:

$$\overline{A}_{\lambda_{1,2}} - k'\overline{A}_{\lambda_{3,4}} = 0 \qquad \text{(Eq. 9)}$$

when $k' = k''$. The implication of the requirement that $k' = k''$ in Eq. 9 is that the absorbances at λ_3 and λ_4 must be equal. In practice, this requirement is not a major limitation due to the broad nature of the UV/VIS absorption spectra of most organic compounds. Thus, a range of wavelengths at absorption maxima or minima have essentially equal absorbances, and on all but the steepest curves adjacent wavelengths (within 1–2 nm) have absorbances close enough to be considered equal in these comparisons.

When null relationships are applied to the absorption data obtained as the drug substance elutes from the column, inhomogeneities in the peak result in positive or negative deviations from the baseline. Homogeneity is indicated by a flat baseline.

RESULTS AND DISCUSSION

Carbamazepine—A standard chromatogram (absorbance at 254 nm *versus* time) of carbamazepine containing 1% (w/w) of acridone (IV), a potential impurity, is shown in Fig. 1A. The composition of the mobile phase [methanol–water (55:45); flow rate, 1.0 ml/min] was adjusted to cause the two materials to overlap at a retention time of 7.4 min, since they are readily separable in other mobile phases. Under these conditions, no indication of inhomogeneity in the carbamazepine peak was observed. To detect the acridone, the response derived from the carbamazepine was removed by manipulation of the spectral data. The UV spectrum of carbamazepine (Fig. 2) contains two points of equal absorbance at 256 and 307 nm. To nullify the signal of carbamazepine, the difference in absorbance at these two wavelengths is plotted *versus* time. The resulting chromatogram for pure carbamazepine is shown in Fig. 1C, which reveals a flat baseline. Examination of the UV spectrum of acridone (Fig. 2) shows a strong absorbance at 256 nm and essentially no absorbance at 307 nm,

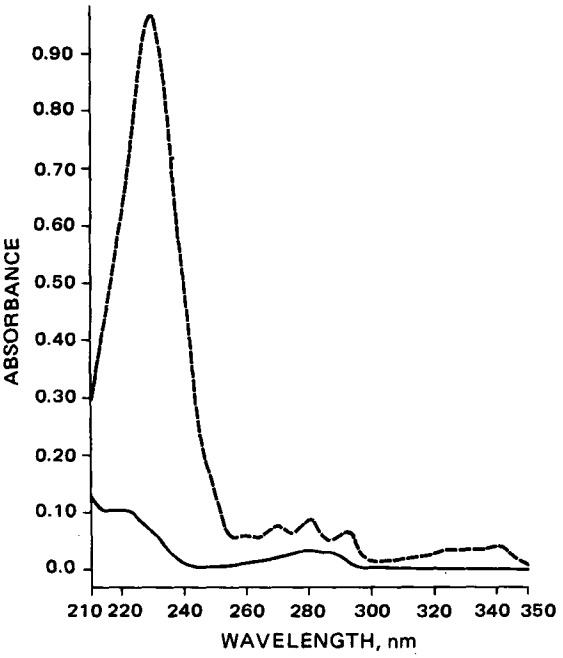

Figure 6—*UV absorption spectra of estrone (—) and equilenin (- - - -) obtained as 4 μg/ml solutions in mobile phase.*

which indicates a positive response should be observed for acridone when this absorbance difference is monitored. The 1% solution used to generate the chromatogram of Fig. 1A gives chromatogram *a* in Fig. 1B. Chromatograms *b* and *c* of Fig. 1B result from the analyses of carbamazepine solutions containing 0.5 and 0.1% acridone, respectively.

Desipramine—A completely analogous method was used to evaluate the homogeneity of the desipramine chromatographic peak in conjunction with its potential by-product (10) 5-(3-methylaminopropyl)-5*H*-dibenz[*b*,*f*]azepine (V). As shown in Fig. 3A, both materials chromatograph with a retention time of 2.5 min under the conditions chosen [mobile phase, acetonitrile/water/ion-pairing reagent/triethylamine (500:500:20:2); flow rate, 2.5 ml/min]. Referring to the UV spectrum of desipramine (Fig. 4), approximately equal absorbances were noted at 242 and 268 nm. Subtraction of the absorbances at these two wavelengths effectively eliminates the response from desipramine, as shown in the chromatogram of Fig. 3C. Samples of desipramine spiked with V at levels of 1.0, 0.5, and 0.1% gave the chromatograms shown in Fig. 3B. The positive signal results from the greater absorbance exhibited by V at 242 nm as compared with 268 nm (Fig. 4).

Estrone—Estrone preparations commonly contain the estrogen equilenin (VI) (11). Using a mobile phase of methanol and water (85:15) and a flow rate of 1.0 ml/min both substances elute at 3.9 min as shown in Fig. 5A. In this case, the relationship of Eq. 9 was applied, as the average absorbance from 235 to 236 nm was subtracted from the average absorbance between 281 and 288 nm (refer to the UV spectrum in Fig. 6) to give the baseline shown in Fig. 5C. When the spiked samples (1.0, 0.5, and 0.1% equilenin) are measured, negative peaks are observed (Fig. 5B), as equilenin has a greater absorbance in the low wavelength range (Fig. 6).

In each of the three systems, the lack of homogeneity created by the addition of low levels of a coeluting substance is apparent. Comparison of the chromatograms for the three 1% mixtures shown in Figs. 1B, 3B, and 5B reveals a range of responses from ~0.02*A* to 0.08*A*. These differences in intensity are related to the absorption characteristics of the particular impurity, the detection method employed and the amount of sample analyzed. The effect of the detection method can be appreciated by considering desipramine. Greater sensitivity for V could be obtained by using Eq. 10 as the null relationship since 255 nm is the absorption maximum of this impurity (Fig. 4):

$$A_{255\text{ nm}} - kA_{280\text{ nm}} = 0 \qquad \text{(Eq. 10)}$$

where:

$$k = \frac{A_{255\text{ nm}}\text{ (desipramine)}}{A_{280\text{ nm}}\text{ (desipramine)}} \qquad \text{(Eq. 11)}$$

The results obtained for these model systems illustrate the potential of the technique for the rapid determination of the homogeneity of HPLC

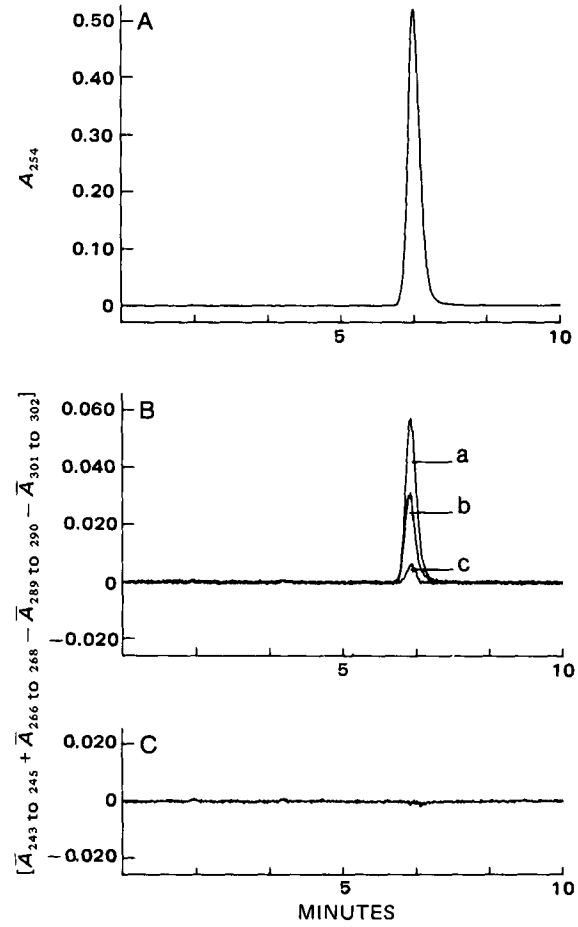

Figure 7—*A) Chromatogram of carbamazepine containing 1% acridone; B) chromatograms of carbamazepine containing (a) 1% acridone, (b) 0.5% acridone, and (c) 0.1% acridone; C) baseline chromatogram obtained for pure carbamazepine. Approximately 5 μg of carbamazepine was injected in 2.5 μl of mobile phase in all cases.*

peaks. Application of these principles to validate HPLC assay procedures is straightforward. The UV/VIS absorption spectrum of the drug substance is measured in the appropriate mobile phase in the flow cell as the material is chromatographed, to eliminate interference from any resolved contaminants. From these data, the nullifying relationship is constructed. The drug-substance is then rechromatographed and the null relationship is monitored *versus* retention time. Homogeneity is indicated if no significant deviation from zero is observed as the drug substance elutes. Hence, from the standpoint of specificity, the chromatographic method is valid.

An erroneous indication of peak homogeneity is possible in the described test if an exactly coincident impurity gives rise to a signal equal in half-width to that of the drug substance. In this case, the contribution from the impurity's absorbance to the total absorbance is proportionally the same throughout the peak. This situation is unlikely in pharmaceutical analysis. Although chromatographic theory (12) predicts equal band widths for substances of identical retention time, impurities typically are present in such small amounts relative to the drug substance (*i.e.*, less than 1%) that they have much narrower effective band widths. The presence of the narrow peak under the envelope of the drug substance is detected readily by these methods.

To bolster the initial indication of peak homogeneity, additional comparison studies should be done. These experiments use the null relationship generated on the original sample to examine the homogeneity of other samples of the drug substance containing increased levels of impurities (and those containing lower levels, if available). Such samples could be derived from purification steps (mother liquors, chromatographic fractions, *etc.*) or as products of accelerated decomposition experiments. These studies are particularly important for stability-indicating assays. Any deviation in the baseline as the drug substance elutes signifies a potential problem in the HPLC method. An invariant baseline represents further evidence of peak homogeneity and essentially eliminates the possibility of a coincident impurity of equal half-width in the

original sample.

Although the method outlined is an independent one, which does not require prior knowledge of possible interfering substances, care must be taken to ensure its generality. The inclusion of just two wavelengths in the detection method, as in Eq. 2, ignores absorbances from contaminants at all other wavelengths. A material which has the same ratio of absorbances at these two wavelengths as the drug-substance (or zero absorbance at each) would not be detected, regardless of the nature of its absorbances at other wavelengths. To enable the detection of the widest range of interfering substances, absorption values across the entire spectral region of interest should be compared. This can be accomplished by either sequential application of the simple relationship of Eq. 2, varying the wavelengths each time, or by using an extended expression which includes the absorbances at several wavelengths.

The latter approach is illustrated in Fig. 7 for the carbamazepine system. Figure 7A shows the unresolved chromatographic peak due to carbamazepine and acridone (1%). To detect the acridone, the multiwavelength expression of Eq. 12 was plotted *versus* retention time:

$$\overline{A}_{243\text{ nm}-245\text{ nm}} + \overline{A}_{266\text{ nm}-268\text{ nm}} - \overline{A}_{289\text{ nm}-290\text{ nm}} - \overline{A}_{301\text{ nm}-302\text{ nm}} = 0 \quad \text{(Eq. 12)}$$

For simplicity, wavelength ranges were chosen whose absorbance values will cancel without the use of proportionality constants. As shown in Fig. 7B, the results are comparable in terms of sensitivity to those given in Fig. 1, and the cancellation of the response due to pure carbamazepine is equally complete (Fig. 7C). The major benefit of this approach is in the generality obtained in one operation. A standard method, which includes sufficient wavelengths to be of wide scope, would be the use of absorbances at 20 nm intervals across a broad spectral region (~200 nm).

One concern is the degree of homogeneity indicated in these methods by a flat baseline. Two limitations imposed by the apparatus described under *Experimental*, are the experimentally determined response threshold of ±0.001*A* and the photometric linearity constraint. This latter restriction requires maximum absorbance values of ~1.0 *A* for wavelengths used in the null relationship, to preserve optimum photometric linearity. Thus, the maximum amount of drug substance analyzed, which governs the relative level of impurities found, is determined by this photometric limitation and by the chromatographic constraint against overloading the column. Given these instrumental restrictions, there are three principal factors which affect the response level of a given impurity: the intensity of its absorption (molar absorptivity), the degree of similarity between its absorption spectrum and that of the drug substance, and the detection method (null relationship) employed. In the case of unknown materials, absorption characteristics are unknown quantities, and the detection method cannot be tailored to attain the maximum response. Because of this, the use of multiple expressions containing data obtained at several wavelengths is recommended. An *a priori* detection limit cannot be stated for all possible impurities. However, these methods can ensure >99% homogeneity in most cases.

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Kinetics and Stability of a Multicomponent Organophosphate Antidote Formulation in Glass and Plastic

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Abstract □ An aqueous solution of trimedoxime bromide, atropine, and benactyzine hydrochloride was formulated to have maximum stability as an antidote in organophosphorus poisoning. The stability of the mixture in glass and plastic cartridges was determined. Glass cartridges were more desirable than plastic; there was less vapor loss, color formation, and anomolous reaction. Trimedoxime was stable, losing 1.4% of its potency after 1 year at 25° and atropine was more stable than trimedoxime. Considerable degradation of benactyzine occurred; 20% of its potency was lost after 1 year at 25°. Equations for predicting the shelf life of each ingredient at selected temperatures are presented.

Keyphrases □ Benactyzine—in formulation, kinetics and stability in glass and plastic □ Atropine—in formulation, kinetics and stability in glass and plastic □ Trimedoxime—in formulation, kinetics and stability in glass and plastic

The administration of atropine with and without oxime is a common therapy for poisoning by organophosphorus anticholinesterase pesticides such as parathion[1] and malathion[1], and for other organophosphorus compounds such as isopropylmethylphosphonofluoridate (sarin) and pinacolyl methylphosphonofluoridate (soman). Atropine is used to overcome cholinergic stimulation from the anticholinesterases, and oxime reactivates and restores the activity of the enzyme. A recent report claims that the effectiveness of a therapy could be enhanced significantly by simultaneous administration of the cholinolytic drugs,

[1] Parathion, *O,O*-diethyl-*O*-(4-nitrophenyl)phosphorothioate; malathion, *O,O*-dimethyl-*S*-(1,2-dicarbethoxyethyl)phosphorodithioate; trimedoxime bromide, pyridinium-1,1′-(1,3-propanediyl)bis(4-(hydroxyimino)methyl)-dibromide; and benactyzine, α-hydroxy-α-penylbenzeneacetic acid 2-(diethylamino)ethyl ester.

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