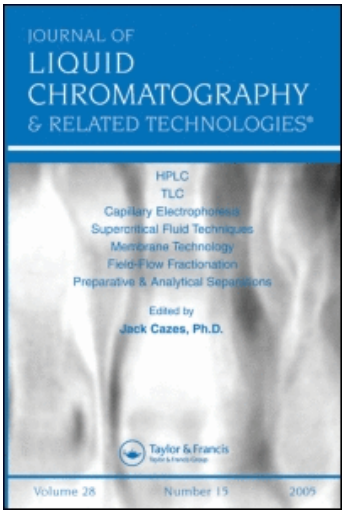

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ANALYSIS OF BARBITURATE MIXTURES USING HPLC WITH DIODE ARRAY DETECTION

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ABSTRACT

A study has been conducted on the HPLC analysis of barbiturate mixtures in which coeluting components with similar UV spectra could be distinguished by diode array detection. Mixtures containing phenobarbital and barbital in combination from about 0.25 to 0.00025 mg/mL each were measured using the spectral overlay, absorbance ratio, peak maximum absorbance and purity parameter techniques. The most sensitive of the standard methods available was absorbance ratio plotting in which the presence of 0.0125 mg/mL barbital could be distinguished from phenobarbital at 0.2375 mg/mL.

INTRODUCTION

Chromatographic separations of barbiturates including phenobarbital, 5-ethyl-5-phenyl-2,4,6(1H,3H,5H)-pyrimidinetrione, and barbital, 5,5-diethyl-2 ,4,6(1H,3H,5H)-pyrimidinetrione, have

been frequently discussed in the literature because of these drugs' common usage, interesting combination therapy available and potential for abuse with forensic implications. Several reviews of current chromatographic methods have been published for barbiturates (1-4) including applications in biological fluids monitoring, forensic science and with electrochemical detection. A complete review of phenobarbital methods of analysis and physical-chemical properties has also been written (5).

More recent studies have dealt with capillary gc (6) and TLC (7) separations of barbiturates as well as analysis in pharmaceutical dosage forms (8-9). Major interest has continued to focus on analysis of barbiturates in plasma and serum (10-12) and tissues (13).

Multicomponent mixtures of the barbiturates or barbiturates with other active constituents as well as degradation products and metabolites have been separated in several of the above studies. The possibility exists, however, that under certain conditions a known or previously unknown degradation product or metabolite might coelute with the primary analyte resulting in incorrect quantitation and possible failure in component detection.

The application of diode array detection to problems of multicomponent analysis and peak purity determination is well established and has been reviewed (14-26). In the present study mixtures of phenobarbital and barbital, as a representative chemical analog, were made to coelute and were analyzed by diode array detection in order to establish limits of detection for coeluting peaks using only the standard equipment and software available in commercial instruments.

EXPERIMENTAL

Chemicals and Reagents

Phenobarbital (USP) was from J.T. Baker and barbital (USP) was from Sterling Drug Inc. Water and methanol were HPLC grade from Fisher Scientific (Fair Lawn, NJ).

Apparatus

Modular HPLC systems (A and B) were used. System A consisted of a Varian 5020 ternary gradient pump (Varian Instrument, Walnut Cr., CA) run isocratically at 1.0 mL/min with a Rheodyne 7125 manual 20 μL loop injector (Rheodyne, Cotati, CA). The diode array detector was a Varian 9060 Polychrom with a deuterium lamp, 4.7 μL flow cell and an externally scanned array of 38 diodes. Its internal microcomputer is based on the Motorola MC68000 16/32 bit microprocessor and was connected to a 640kb IBM-XT (IBM, Boca Raton, FL) using Varian Polysoc version 2.7

software. In addition, a 10 mv Fisher Recordall 5000 strip chart recorder (Fisher Instruments, Pittsburgh, PA) and a Hewlett Packard Think Jet printer (Hewlett Packard, Avondale, PA) were connected.

System B consisted of a Beckman 110A pump (Beckman Berkeley, CA) operated at 1.0 mL/min with a Rheodyne 7125 20 μL loop injector. The diode array detector was a Hewlett Packard 1040A (Hewlett Packard, Palo Alto, CA) using software A-2243 with a deuterium lamps, 4.5 μL flow cell and a self-scanned array of 211 photodiodes. This detector was connected to an HP-85 microcomputer, an HP-7470A plotter, an HP-82901M dual disc drive and a Easterline Angus strip chart recorder (Rainin Instruments, Woburn, MA).

The same column and mobile phase were used with each system. These were a deteriorated Partisil PXS 10/25 PAC column (25 cm x 4.6 mm, 10 μ particle size) (Whatman Inc., Clifton, NJ) and water:methanol (300:700).

Sample Preparation

Solutions of phenobarbital and barbital were accurately prepared at about 0.25 mg/mL in methanol and diluted with each other to give phenobarbital: barbital (1:1), (90:10), (95:5), (99:1) and (10:90), (5:95), (1:99) and (0.1:99.9).

RESULTS AND DISCUSSION

Methods used for extracting information from coeluting peaks employing photodiode array detection include spectral overlays, absorbance ratios, mathematical deconvolution and spectral suppression. Several studies have been presented in the past involving extensive data manipulation to show peak purity while only two investigations used commercially available instrumentation (HP 1040A) with no mathematical manipulations other than those built into the system software. One group found that as little as 3% impurity could be detected using either the spectral overlay or the absorbance ratio technique for two coeluting components with differing spectra (27). Another investigator using the same instrumentation found no difference in spectral overlays or absorbance ratios from 7-22% impurity for two coeluting peaks of coincident UV spectra (28).

Chromatograms of the components phenobarbital and barbital in the present study at about 0.25 mg/mL are shown in Figure 1A and 1B respectively while a 1:1 mixture of those components gave the chromatogram in Figure 1C. These were obtained at 215 nm using the Varian Polychrom of system A. An approximate 1:1 ratio of peak heights was found under these conditions which gave molar absorptivities of 374 for barbital and 516 for phenobarbital at this wavelength. This peak height and therefore, absorbance ratio agreed well with that obtained from individual spectral plots for each pure component.

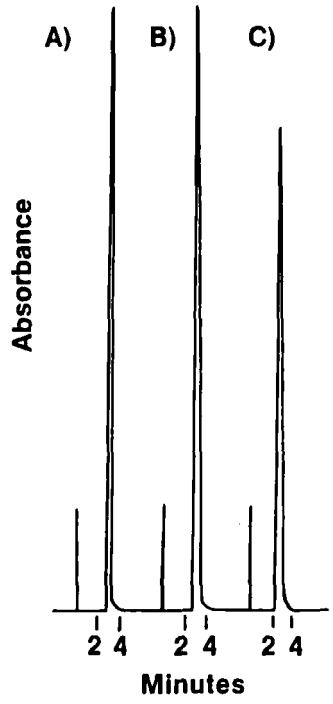

Figure 1. Chromatograms of phenobarbital (A) and barbital (B) at about 0.25 mg/mL in methanol and (1:1) mixture of phenobarbital: barbital in methanol (C) obtained using conditions described in the text with system A.

Whereas the peaks in the 1:1 mixture in Figure 1C appear to coelute perfectly, when the time axis was elongated, as shown in Figure 2, a signal plot of the 1:1 mixture from system B, it is clear that this was not the actual case. In fact retention times of 3.1 and 3.3 minutes were found for barbital and phenobarbital respectively with a resolution factor of about 0.3 obtained from

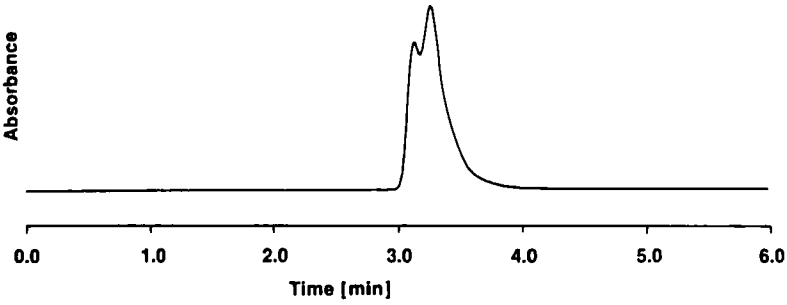

Figure 2. Signal plot (chromatogram) of the (1:1) mixture of phenobarbital:barbital obtained using system B showing the retention times of 3.1 minutes for barbital and 3.3 minutes for phenobarbital.

this plot. In addition, the individual component spectra shown in Figure 3 recorded from system B show an imperfect congruence with a shoulder seen in the barbital spectrum at 210 nm. Aside from this aspect the spectra are similar and the two peaks could be considered coeluting under a normal chart speed of 0.1 in. per min.

Normalized spectra obtained at the upslope, apex and downslope of the (50:50) mixture of barbital to phenobarbital were overlaid on a peak apex spectrum of the pure barbital standard using system B with results shown in Figure 4. The apex and downslope spectra clearly depart from that of barbital while the upslope did not. Such deviations of 5-10% absorbance constitute

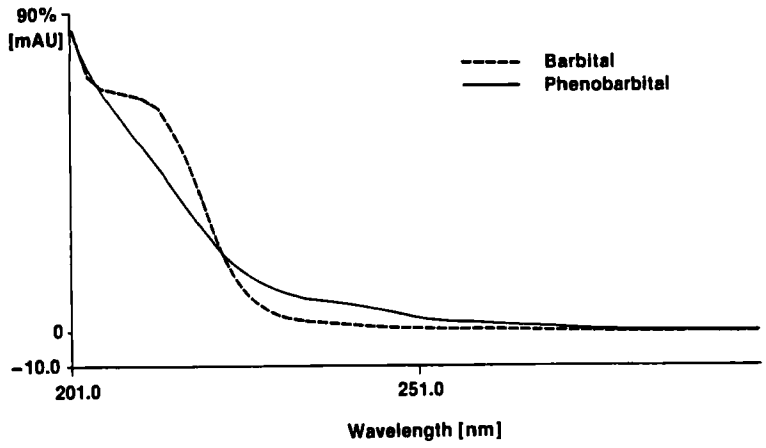

Figure 3. Normalized ultraviolet spectra of barbital and phenobarbital obtained at the apex of each peak when the pure compounds were chromatographed using system B.

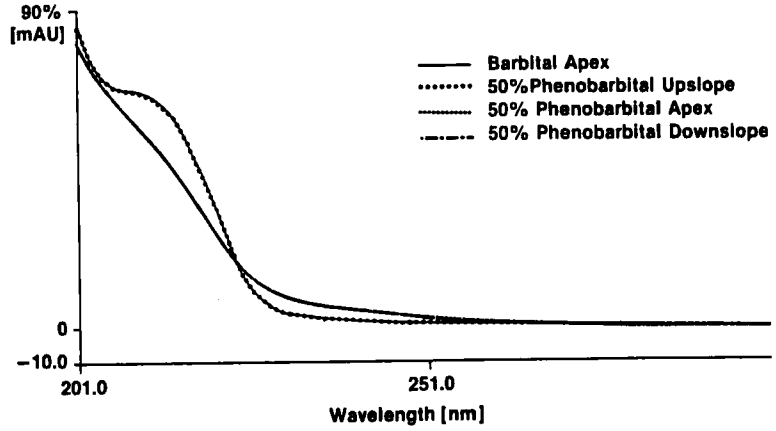

Figure 4. Overlays of normalized spectra at the barbital peak apex along with those taken at the upslope, apex and downslope of the (1:1) mixture of barbital:phenobarbital obtained on system B.

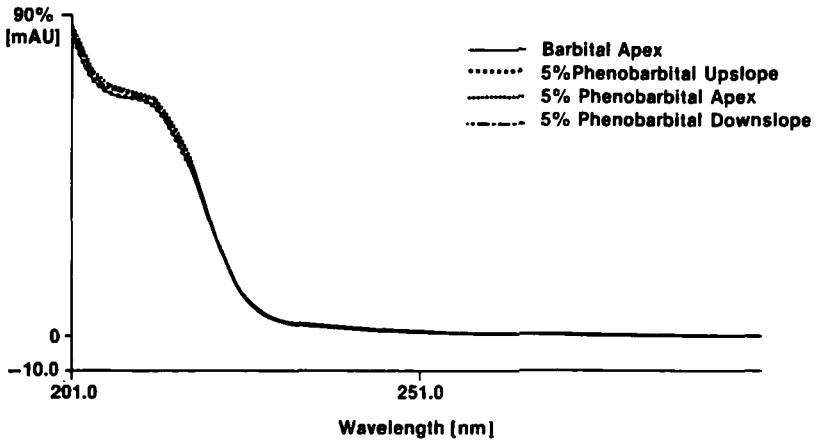

Figure 5. Overlays of normalized spectra at the barbital peak apex along with those taken at the upslope, apex and downslope of the mixture containing 5% phenobarbital obtained on system B.

readily differentiable spectra from which even a novice could conclude the peak was impure. Figure 5 by contrast shows the spectral overlays of the solution containing 5% phenobarbital over the barbital peak apex spectrum from which a conclusion on the presence of an impurity can not be made. The 10% phenobarbital solution gave spectra which might be interpreted as indicating peak inhomogeneity although this was not nearly as clear as in Figure 4.

Application of the absorbance ratio technique provides best results when the spectral characteristics of each component of

the coeluting mixture is known from which the ratio wavelengths are chosen. Normally the spectrum of only the standard substance would be available, however. In the present study the wavelengths 215 nm and 254 nm were chosen because of their applicability to fixed wavelength UV detectors and because of the shoulder seen in the barbital spectrum (Figure 3). Accordingly, the results obtained by applying the ratio technique could be somewhat better than they should have been if both compounds had characterless spectra and the same wavelengths were chosen or if only the spectrum of one component was known and the wavelengths were chosen at random.

The absorbance ratio at 215 nm/254 nm recorded over the peak width for the barbital standard using system B is shown in Figure 6A while that for phenobarbital is shown in Figure 6B. These plots display the usual difficulties of interpretation such as not showing an absolutely flat top in 6A and the noise spike in 6B. The lowest concentration that could be detected as a coeluting component under these conditions was 5% phenobarbital shown in Figure 6C. The highest concentration of coeluting component that could not be detected in the present study by the absorbance ratio technique was 1% phenobarbital shown in Figure 6D. Identical results were obtained with system A where pure barbital, pure phenobarbital, 5% phenobarbital and 1% phenobarbital absorbance ratios are shown in Figure 7A, B, C and D

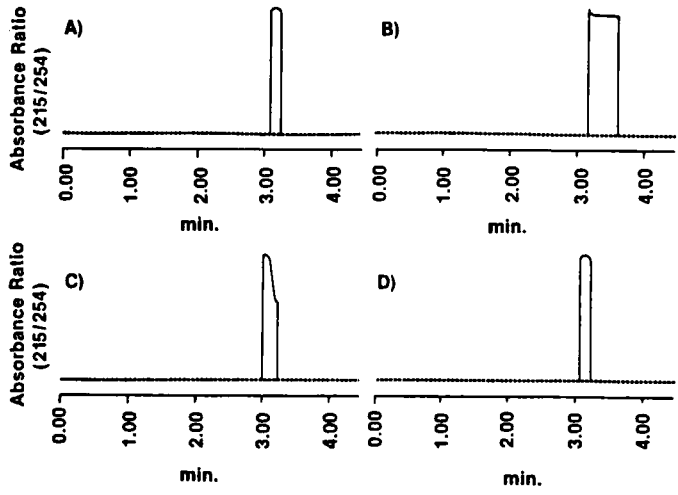

Figure 6. Absorbance ratio plots (215 nm/254 nm) of peaks representing 100% barbital (A), 100% phenobarbital (B), barbital:phenobarbital (95:5) solution (C) and barbital:phenobarbital (99:1) solution (D) using system B.

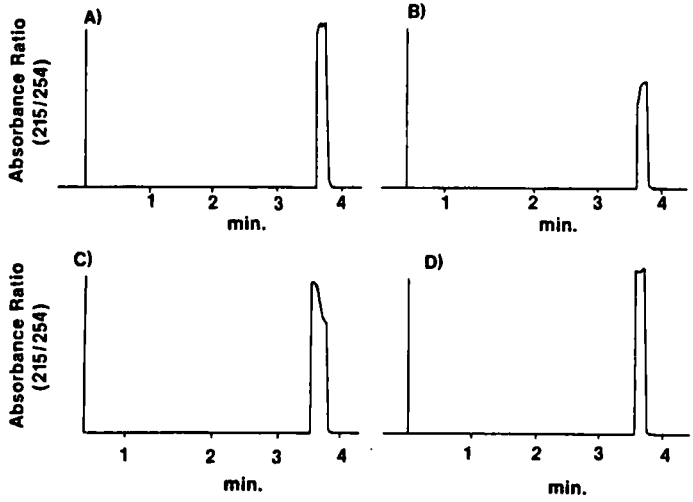

Figure 7. Absorbance ratio plots (215 nm/254 nm) of peaks representing 100% barbital (A), 100% phenobarbital (B), barbital:phenobarbital (95:5) solution (C) and barbital: phenobarbital (99:1) solution (D) using system A.

respectively. As a further check on the influence of the wavelengths selected for ratioing, the combinations 215 nm/230 nm and 230 nm/254 nm were also run on system B. The results of absorbance ratios obtained with these two additional combinations were exactly the same as for 215 nm/254 nm. That is, the 5% phenobarbital mixture could be easily distinguished as consisting of two components while the 1% could not. This indicates that even when wavelengths are chosen in nondescript regions of the components' spectra the absorbance ratio technique is useful and probably more sensitive than the spectral overlay method in distinguishing coeluting peaks.

One tabulated result available on system A is the purity parameter. This number displayed to six significant figures is an absorbance-weighted mean wavelength of the spectrum taken at designated times during a chromatographic run. Peak apex purity parameters which were calculated between 200 and 300 nm for each mixture and for pure barbital and phenobarbital solutions are shown in Figure 8. These curves indicate that the 1:1 barbital: phenobarbital mixture shows a distinctive difference from either pure component. The solutions containing 10% of either component, however, gave purity parameters of only 1-3% different from those of pure barbital or phenobarbital so the distinction could not be made at this level.

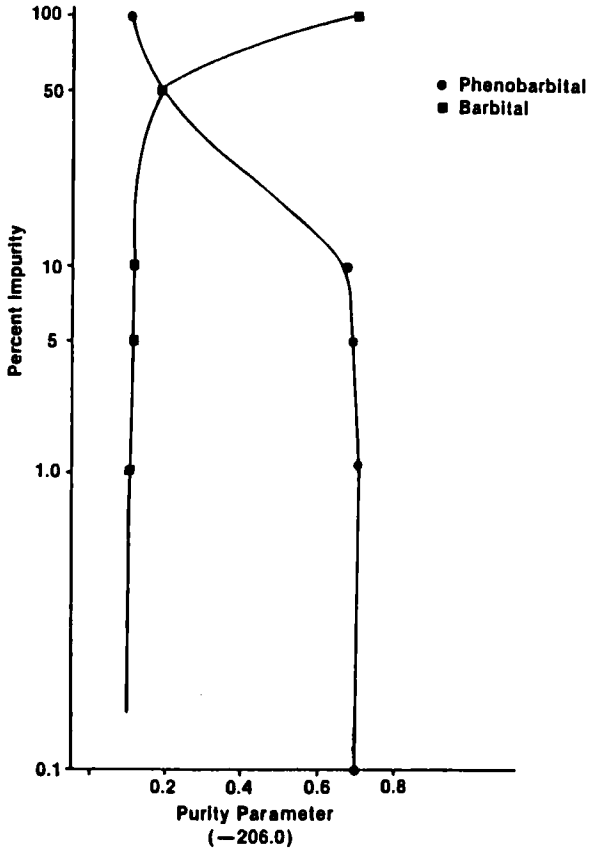

Figure 8. Plots of peak apex purity parameters calculated between 200 and 300 nm for mixtures of 0.1-100% phenobarbital and 1-100% barbital versus the percent impurity obtained on system A.

A final parameter available on diode array detectors is the peak maximum absorbance. While use of this parameter has seldom been recommended for assessing peak homogeniety, Hill and Langner have offered a variant of this concept called fraction of total

absorbance (FTA) for a chromatographic peak which is the absorbance at a particular wavelength divided by the area under the spectral curve. This value in addition to retention times in two different HPLC systems was used to help identify unknown drugs with a library search routine (29).

The simplicity of the net absorbance measurement is initially appealing but its applicability would probably be limited to components of dissimilar spectra or dissimilar absorptivities. If in an extreme example a coeluting impurity had an absorptivity of 50% of the main component's and if the impurity was present at a 10% level, the net absorbance (peak height) would be diminished by 5% which could easily be distinguished. Likewise, if an impurity had an absorptivity of twice the main component's at a particular wavelength and it was present at a 10% level the net absorbance would be readily distinguishable from the pure main component with a 10% increase.

These consequences are obviously based on the assumptions of additivity of component absorbances to give total solution absorbance, ideal solution behavior and linearity of detector response/concentration relationships for each component.

When peak apex absorbances at 200 nm obtained from the normalized spectra were plotted against log percent impurity for

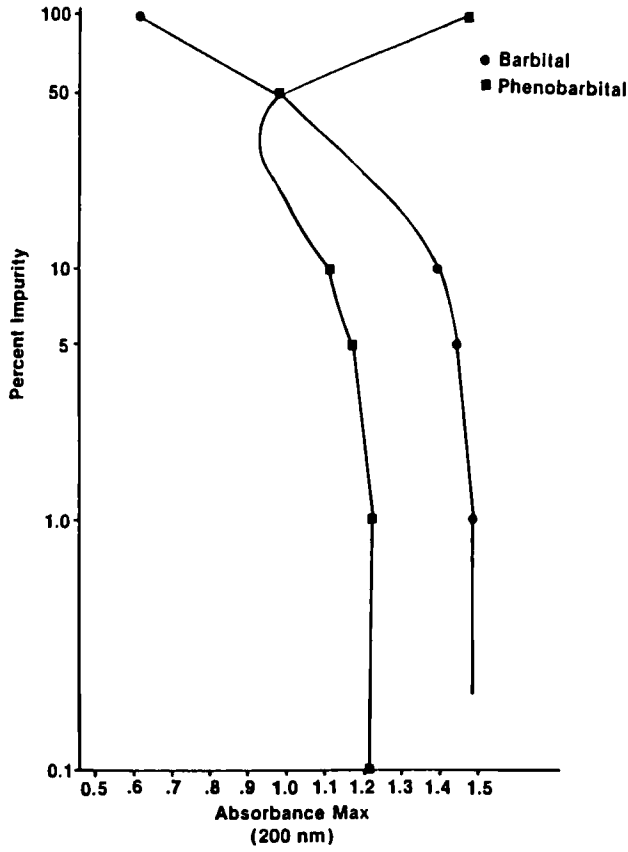

Figure 9. Plots of peak apex absorbance maximum values at 200 nm for mixtures of 0.1-100% phenobarbital and 1-100% barbital versus the percent impurity obtained on system B.

system B, Figure 9 resulted. The barbital curve followed a normal asymptotic behavior showing that 10% and probably even 5% could be distinguished from pure phenobarbital, whereas the phenobarbital curve did not show this behavior. Instead the

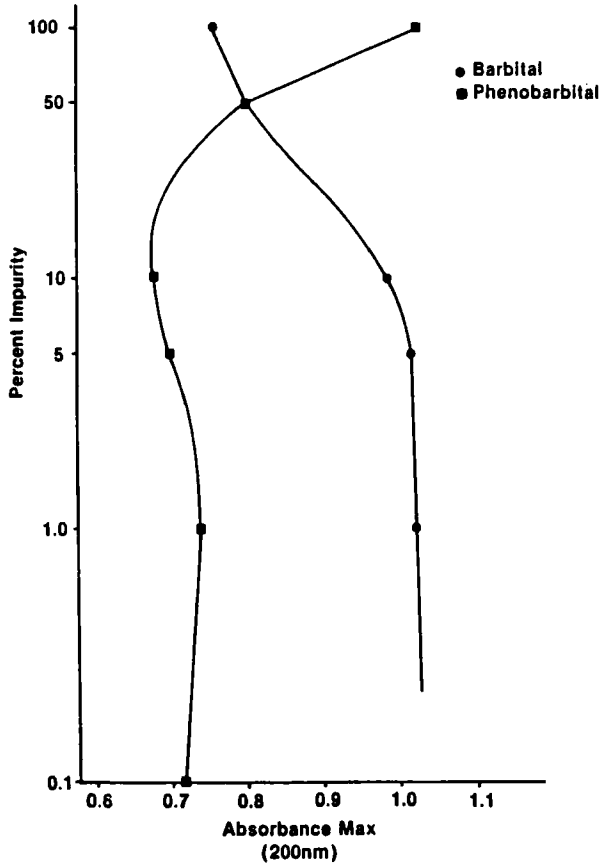

Figure 10. Plots of peak apex absorbance maximum values at 200 nm for mixtures of 0.1-100% phenobarbital and 1-100% barbital versus the percent impurity obtained on system A.

absorbance deceased for the 50:50 mixture but then increased again for lower percentages of phenobarbital indicating nonideal behavior. This was also found with system A shown in Figure 10 where the phenobarbital absorbance decreased and then increased at lower concentrations. The cause of the nonideal behavior of phenobarbital in this regard was further investigated by obtaining spectra on system B at various concentrations from 0.03 to 10 mg/mL in methanol. With a mobile phase of water: methanol: 85% phosphoric acid (500:500:3) on an Alltech ODS 5/25 column the peak apex spectra for these solutions shown in Figure 11 were obtained. This unusual shift in spectra with changing concentration should not have been related to solution pH and degree of ionization since all were obtained in the acidic mobile phase although it could have been a consequence of detector overload resulting in nonlinear response. This shift could also conceivably be related to the phenobarbital results in Figures 9 and 10 where at lower concentrations the absorbance at 200 nm increased. These results point to the problems in this approach which relies on ideal behavior although in certain cases valuable information on homogeneity may be obtained from the peak maximum absorbance.

The applicabilty of photodiode array detection to quantitation of phenobarbital in the presence of the structural analog, barbital, or vice versa, has been examined with the finding of

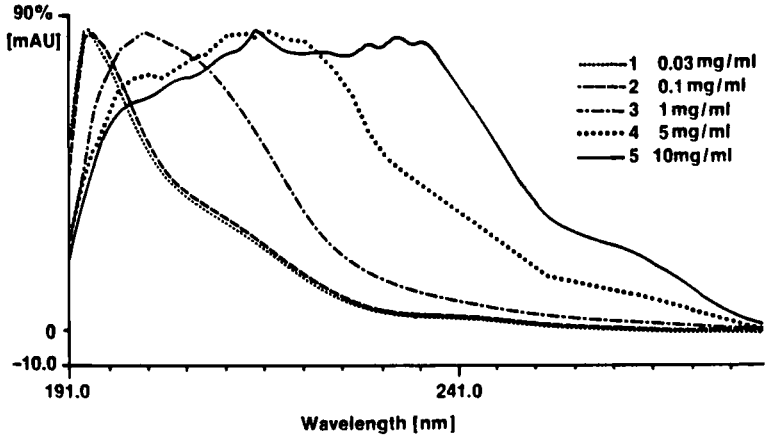

Figure 11. Peak apex spectra for solutions containing 0.03 to 10.0 mg/mL phenobarbital obtained on system B using a water: methanol:85% phosphoric acid (500:500:3) mobile phase with an Alltech 5/25 ODS column.

usefulness for the method within certain limits. These include lower detection limits for coeluting, similar spectra impurities using the spectral overlay, absorbance ratio, purity parameter and peak absorbance maximum methods of about 10, 5, 10-50 and 10 % respectively. While use of diode array detection for multi-component barbiturate mixtures may be restricted to these limits, it does provide an adjunct to separation development and may indicate the presence of otherwise undetected compounds.

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