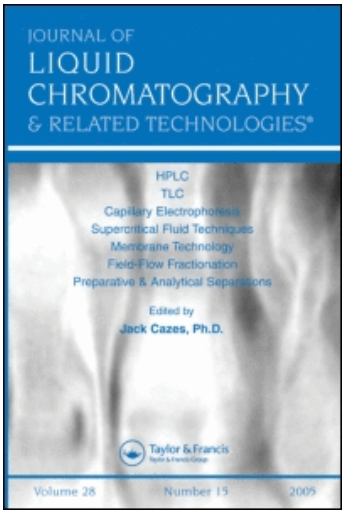

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Resolution of the Non-Specific Spectra of Barbiturates by UV-Photodiode Array Detection. II. Effects of Sample Concentration on Spectral Matching Accuracy

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RESOLUTION OF THE NON-SPECIFIC SPECTRA OF BARBITURATES BY UV-PHOTODIODE ARRAY DETECTION. II. EFFECTS OF SAMPLE CONCENTRATION ON SPECTRAL MATCHING ACCURACY

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ABSTRACT

The procedure described uses a photodiode array detector for the qualitative determination of peak identity. The object was to determine the capability of a photodiode array detector system to accurately discriminate among the non-specific spectra of barbiturates at concentrations down to 1 μg/ml. Five barbiturates, all of which possessed highly similar non-specific spectra were analyzed and spectrally compared. Standard spectra for each barbiturate were created using a concentration of approximately 0.5 mg/ml. Samples of each barbiturate were prepared at concentrations down to 1 μg/ml. Spectra from these samples are compared to the standard spectra library. The photodiode array detector system accurately identified all of the barbiturate samples at all concentrations tested.

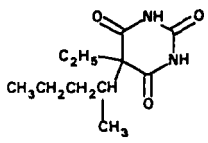
INTRODUCTION

In the previous investigation[1] the ability of a photodiode array detector system to accurately and reliably distinguish among the non-specific spectra of

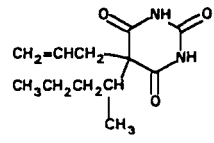
ten barbiturates samples was explored. Recalling those conditions necessary for the generation of ideal UV spectra by photodiode array:

- Very large signal to noise ratio
- Ideal baselines
- Very low UV cutoff
- Good resolution (chromatographic)
- Equivalent AU for all peaks
- Significant spectral differences

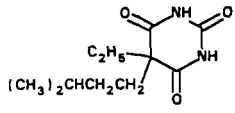
The first series of experiments were concerned primarily with the last point, how well could the photodiode array system distinguish among spectra which were not only highly similar, but also non-specific (absorption was limited to the extreme low wavelengths, 190 - 250 nm). The conclusion was that the system is clearly capable of performing this discrimination. However, when addressing the utility of the technique in a clinical sense the concentrations of the samples used (approximately 500 μg/ml) must be more closely examined. It is doubtful that barbiturate concentrations at these levels would ever be encountered in any clinical environment outside a post-mortem. The more important question that needs to be addressed (now that the system is proven to be capable of distinguishing among the barbiturate spectra) is that upon the establishment of an average standard spectra, could the photodiode array system accurately and reliably identify barbiturates at different concentrations down to 1 μg/ml, concentrations normally expected in the blood. The concentrations tested will range from therapeutic dose levels to those found normally after lethal doses. Three different "classes" of barbiturates will be tested; fast-acting/short duration (pentobarbital and secobarbital), intermediate-acting/intermediate duration (amobarbital and butabarbital), and slow-acting/long duration (phenobarbital). These barbiturate samples are particularly useful since 4 of the 5 were analyzed in the previous experiments. Butabarbital is the exception. Figure 1 lists the structures of these five barbiturates.



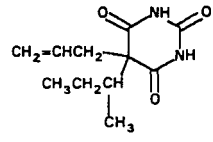
Pentobarbital



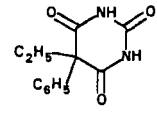
Secobarbital



Amobarbital



Butabarbital



Phenobarbital

FIGURE 1 Barbiturate structures.

MATERIALS AND METHODS

Materials and methods remained unchanged from the initial experiments with one important exception. A new deuterium lamp was installed in the photodiode array detector. This is actually a critical parameter to examine. Because of the similarity of the subject spectra, changes in the lamp performance, such as the intensity and the signal to noise ratio, could adversely affect sample spectra and result in spectral misidentification. The lamp used in this photodiode array detector (Model L1888, Hamamatsu) has an expected lifetime of approximately 1000 hours, a correlation (in this case) of about 1 year. If standard spectra generated using one lamp were incorrectly or inconsistently matched with sample spectra generated using a different lamp, this would represent a severe limitation in the technique, particularly if

extensive spectral libraries needed to be recreated on an annual basis. Following the manufacturers recommendation, an extended lamp equilibration period of about 18 hours was allowed to elapse before the analyses began.

System Suitability

The system suitability was determined by employing the technique recommended in the previous experiments. Standard solutions of pentobarbital, secobarbital, amobarbital and phenobarbital were prepared at about 500 μg/ml (butabarbital was excluded since there was no previous standard spectra for comparison). Standard spectra (average of seven spectra) were created and compared to the standard spectra for these barbiturates previously generated using the old lamp. These new standard spectra will have a twofold purpose - 1) To determine the suitability of the chromatographic system (baseline and detector drift effects on the spectra), and 2) Confirm that spectra generated with the "old" lamp are comparable to those generated using the "new" lamp. The lower concentration samples will be compared to a library made up of standard spectra generated during this study and to the standard spectra generated for the previous study. Accurate and consistent spectral matching will be another indicator of the ruggedness of the technique.

Sample Preparation

Stock standard solutions of the five barbiturate samples were prepared at concentrations of 400 to 500 μg/ml. These stock standard solutions were used to create the average standard spectra used for comparison. The following dilutions of each stock standard solution was performed:

- Pentobarbital and Secobarbital - 30 μg/ml, 20 μg/ml, 10 μg/ml, 5 μg/ml and 1 μg/ml.

TABLE 1

Concentration of Barbiturate in Blood Versus Degree of CNS Depression Blood Barbiturate Level in ppm (ug/ml)

Barbiturate	Onset/ Duration	Degree of Depression in Nontolerant Persons*				
		1	2	3	4	5
Pentobarbital	fast/short	≤2	0.5-3	10-15	12-25	15-40
Secobarbital	fast/short	≤2	0.5-5	10-15	15-25	15-40
Amobarbital	intermediate/intermediate	≤3	2-10	30-40	30-60	40-80
Butabarbital	intermediate/intermediate	≤5	3-25	40-60	50-80	60-100
Phenobarbital	slow/long	≤10	5-40	50-80	70-120	100-200

*Categories of degree of depression

1. Under the influence and appreciably impaired for purposes of driving a motor vehicle or performing tasks requiring alertness and unimpaired judgement and reaction times.
2. Sedated, theraputic range, calm, relaxed and easily aroused.
3. Comatose, difficult to arouse, significant depression of respiration.
4. Compatible with death in aged or ill persons or in the presence of obstructed airway, other toxic agents or exposure to cold.
5. Usual lethal level, the upper end of the range includes those who received some supportive treatment.

TABLE 2

RESULTS FROM SYSTEM SUITABILITY TESTING

Standard Spectra (New)	Best Match (Old Standards)	Similarity Dissimilarity	PuP nm (New) (Old)	% Deviation
Pentobarbital	Pentobarbital	0.99995 0.01208	210.185 210.267	0.04
Secobarbital	Secobarbital	0.99999 0.00477	210.248 210.270	0.01
Amobarbital	Amobarbital	0.99998 0.00626	210.051 210.088	0.02
Phenobarbital	Phenobarbital	0.99932 0.03681	210.059 210.454	0.19

- Butabarbital - 70 μg/ml, 50 μg/ml, 40 μg/ml, 10 μg/ml and 1 μg/ml.
- Amobarbital - 50 μg/ml, 45 μg/ml, 30 μg/ml, 5 μg/ml and 1 μg/ml.
- Phenobarbital - 100 μg/ml, 70 μg/ml, 50 μg/ml, 20 μg/ml and 5 μg/ml.

Individual injections of these sample dilutions will be compared to the spectral libraries. These concentrations were not arbitrarily chosen, but were selected from Table 1 which appears in the Physicians' Desk Reference[2].

RESULTS

Table 2 lists the results of the system suitability testing, standard spectra for pentobarbital, secobarbital, amobarbital and phenobarbital are compared to the previously generated standard spectra. Figure 2 is the spectral comparison of the two secobarbital standard spectra. Table 3 lists the results of the spectral matching of the diluted sample solutions with both spectral libraries. Figure 3 graphically compares the spectral dissimilarity with sample concentration using the "new" standard spectra. Figure 4 graphically compares the spectral dissimilarity with sample concentration using the "old" standard spectra. Table 4 compares the Purity Parameter^{TM_1} (abbreviated PuP) values of the new standards with those obtained for the samples. Table 5 compares the PuP values for the old standards with the values obtained for the samples.

DISCUSSION

Results from the system suitability testing (Table 2) were very encouraging. All of the standard spectra generated for these experiments using the new lamp were correctly matched with the previously generated standard spectra. The comparison of the phenobarbital standard spectra exhibited the

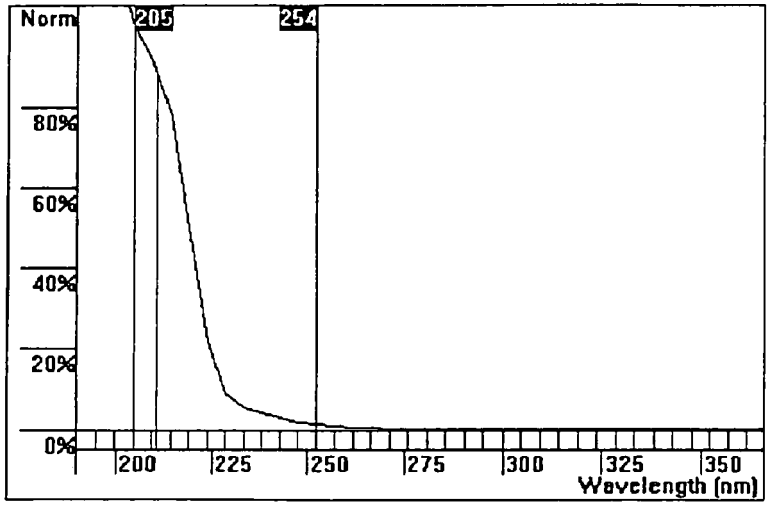
[1]This value is defined as the average wavelength of a spectrum weighted by the square of the absorbance at each wavelength of the spectrum over the range that defines that purity[3].

SEARCH SPECTRUM

Name:	Secobarbital Standard Spectra (generated 11 FEB 92)
PuP (205 - 254 nm):	210.270 nm
Spectra Type:	Spectral Sum

SPECTRUM OF POSSIBLE MATCH

Name:	Average Spectra of Secobarbital (generated 24 APR 92)
PuP (205 - 254 nm):	210.248 nm
Spectra Type:	Spectral Sum



SEARCH REPORT

Matches:	Sim.	Dissim.	PuP(nm)	tR(min)	Lib	#
AVERAGE SPECTRA OF SECOBARBI	0.99999	0.00477	210.270	0.000	A	2
AVERAGE SPECTRA OF TALBUTAL	0.99997	0.00713	210.281	0.000	A	10
AVERAGE SPECTRA OF APROBARBI	0.99986	0.01661	210.311	0.000	A	13
AVERAGE SPECTRA OF ALLOBARBI	0.99985	0.01741	210.345	0.000	A	1
AVERAGE SPECTRA OF BUTALBITA	0.99984	0.01796	210.118	0.000	A	4

Search Conditions
PuP Range: 205->254 nm PuP Interval: +/- 5.00 nm
Time Range: 0.000 - 1440.000 min

FIGURE 2 Secobarbital standard spectra comparison.

TABLE 3

SPECTRA MATCHING RESULTS
SAMPLES VS "NEW" AND "OLD" LIBRARIES

		"New" Library		"Old" Library	
SAMPLE	CONC. (µg/ml)	MATCH	DISSIM.	MATCH	DISSIM.
PENTOBARBITAL	30	pentobarbital	0.01464	pentobarbital	0.00899
	20	pentobarbital	0.01489	pentobarbital	0.00893
	10	pentobarbital	0.01546	pentobarbital	0.00851
	5	pentobarbital	0.01895	pentobarbital	0.01140
	1	pentobarbital	0.01753	pentobarbital	0.01517
SECOBARBITAL	30	secobarbital	0.01096	secobarbital	0.01055
	20	secobarbital	0.01136	secobarbital	0.01138
	10	secobarbital	0.01050	secobarbital	0.01060
	5	secobarbital	0.01299	secobarbital	0.01307
	1	secobarbital	0.01812	allobarbital	0.02016*
(200 - 254 nm)				secobarbital	0.01817
AMOBARBITAL	50	amobarbital	0.01663	amobarbital	0.01477
	45	amobarbital	0.01673	amobarbital	0.01499
	30	amobarbital	0.01722	amobarbital	0.01542
	5	amobarbital	0.01301	amobarbital	0.01352
	1	amobarbital	0.02334	barbital	0.02074**
(200 - 254 nm)				amobarbital	0.01764
PHENOBARBITAL	100	phenobarbital	0.00274	phenobarbital	0.03825
	70	phenobarbital	0.00293	phenobarbital	0.03794
	50	phenobarbital	0.00331	phenobarbital	0.03808
	20	phenobarbital	0.00446	phenobarbital	0.03875
	5	phenobarbital	0.00316	phenobarbital	0.03656
BUTABARBITAL	70	butabarbital	0.01375	-	-
	50	butabarbital	0.01475	-	-
	40	butabarbital	0.01509	-	-
	10	butabarbital	0.01674	-	-
	1	butabarbital	0.01892	-	-

*Although allobarbital was selected as the best match, the spectral dissimilarity is in reference to the secobarbital standard spectra.

**See above, dissimilarity is in reference to the amobarbital standard spectra.

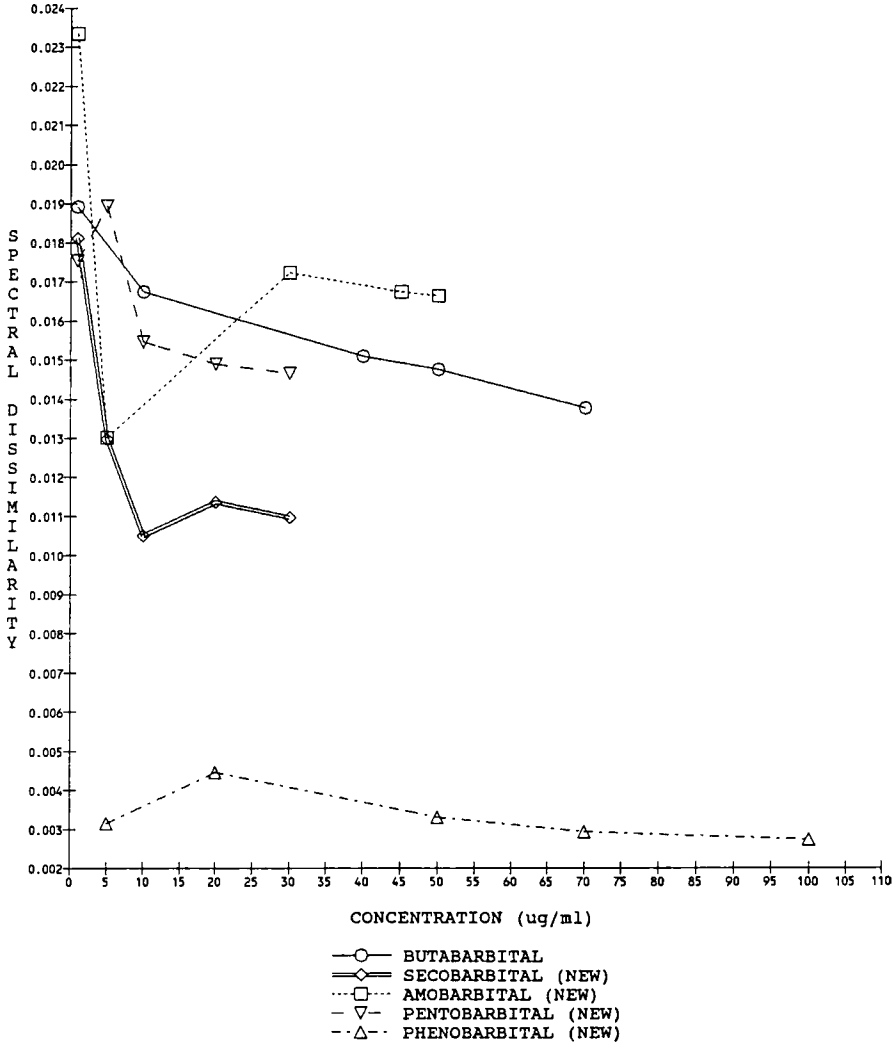


FIGURE 3 Comparison of spectral dissimilarity of barbiturate samples with new standard spectra.

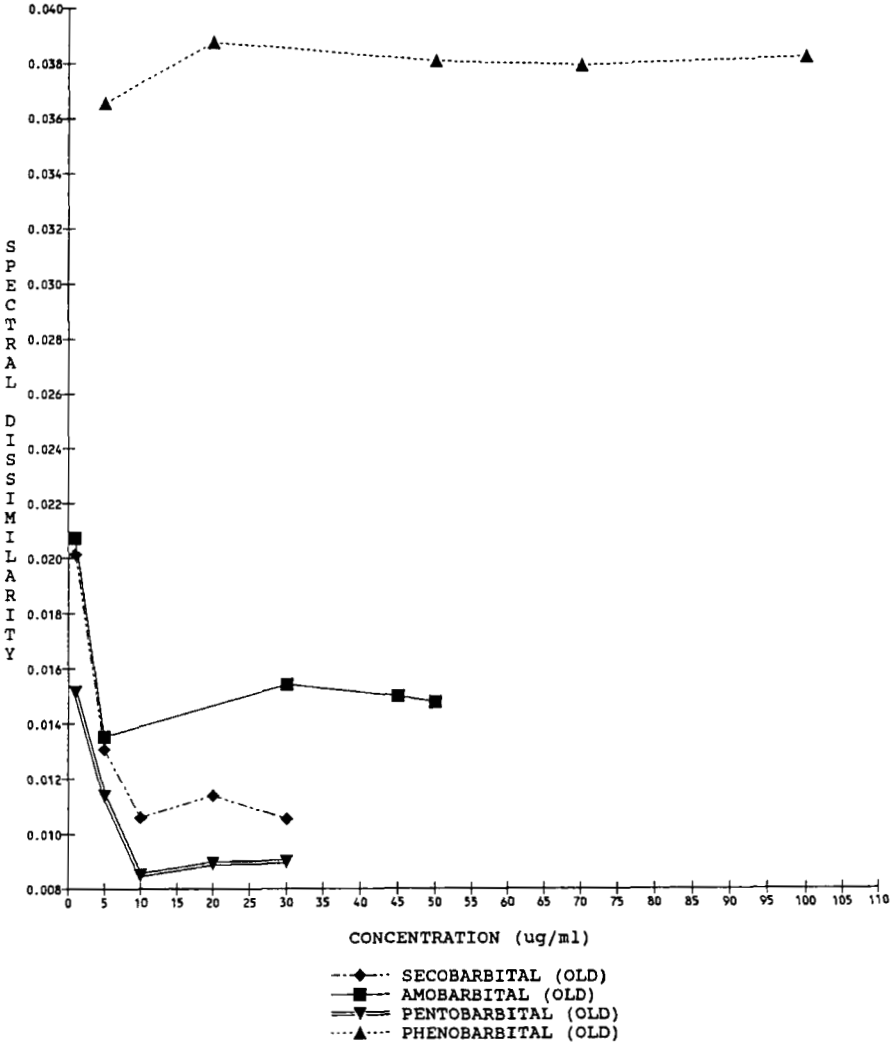


FIGURE 4 Comparison of spectral dissimilarity of barbiturate samples with old standard spectra.

TABLE 4

PuP VALUES: NEW STANDARDS VS SAMPLES

STANDARD	PuP (nm)	SAMPLE	PuP (nm)	%DEVIATION
PENTOBARBITAL	210.185	30 μg/ml	210.300	0.05
		20 μg/ml	210.301	0.05
		10 μg/ml	210.304	0.06
		5 μg/ml	210.354	0.08
		1 μg/ml	210.263	0.04
SECOBARBITAL	210.248	30 μg/ml	210.329	0.04
		20 μg/ml	210.339	0.04
		10 μg/ml	210.323	0.04
		5 μg/ml	210.363	0.05
		1 μg/ml	210.336	0.04
AMOBARBITAL	210.051	50 μg/ml	210.189	0.07
		45 μg/ml	210.189	0.07
		30 μg/ml	210.192	0.07
		5 μg/ml	210.106	0.03
		1 μg/ml	210.250	0.09
BUTABARBITAL	210.208	70 μg/ml	210.314	0.05
		50 μg/ml	210.321	0.05
		40 μg/ml	210.326	0.06
		10 μg/ml	210.335	0.06
		1 μg/ml	210.369	0.08
PHENOBARBITAL	210.059	100 μg/ml	210.032	0.01
		70 μg/ml	210.037	0.01
		50 μg/ml	210.034	0.01
		20 μg/ml	210.021	0.02
		5 μg/ml	210.067	<0.01

TABLE 5

PuP VALUES: OLD STANDARDS VS SAMPLES

STANDARD	PuP (nm)	SAMPLE	PuP (nm)	%DEVIATION
PENTOBARBITAL	210.267	30 µg/ml	210.300	0.02
		20 µg/ml	210.301	0.02
		10 µg/ml	210.304	0.02
		5 µg/ml	210.354	0.04
		1 µg/ml	210.263	< 0.01
SECOBARBITAL	210.270	30 µg/ml	210.329	0.03
		20 µg/ml	210.339	0.03
		10 µg/ml	210.323	0.03
		5 µg/ml	210.363	0.04
		1 µg/ml	210.336	0.03*
(200 - 254)	(206.244)		(206.088)	(0.07)
AMOBARBITAL	210.088	50 µg/ml	210.189	0.05
		45 µg/ml	210.189	0.05
		30 µg/ml	210.192	0.05
		5 µg/ml	210.106	0.01
		1 µg/ml	210.250	0.08*
(200 - 254)	(206.845)		(206.700)	(0.07)
PHENOBARBITAL	210.454	100 µg/ml	210.032	0.20
		70 µg/ml	210.037	0.20
		50 µg/ml	210.034	0.20
		20 µg/ml	210.021	0.21
		5 µg/ml	210.067	0.19

* Original match incorrect.

highest deviation. This is explained by the fact that the original standard spectra for phenobarbital was generated using sample concentrations that were out of the linear range of the detector (1.5 AU). Recalling that standard spectra generated from results outside the linear range of the detector cause a higher PuP value due to an incorrect weighting factor for the wavelengths and a higher imprecision in the averaged PuP values, it is not suprising that this was the "worst" match. Despite these conditions, both phenobarbital standard spectra were correctly matched. If an inaccurate match had been obtained, the old phenobarbital standard spectra could be re-created using a lower absorbance region of the peak within the detectors linear range. The reconstructed standard spectra would not exhibit any of the bias observed in the original standard spectra. This was not performed in these experiments since the standard to standard and sample to standard comparisons were accurate, although the degree of accuracy would probably be enhanced. In Figure 2 the normalized overlay of the two secobarbital standard spectra exhibit no readily visible differences.

In Table 3 the results of the comparisons of the diluted barbiturate samples to both spectral libraries is listed. Because these are all highly similar spectra, spectral dissimilarity provides a more visible variance when determining small differences in the compared spectra. Spectral dissimilarity is defined by the following expression:

$$Dissim(A,B) = \sin\theta = \sqrt{1-\cos^2} = \sqrt{1-Corr^2(\vec{A},\vec{B})}$$

Where θ is the angle between an n dimensional vector for the sample spectra as compared to the reference spectra. Like the system suitability, the matches of the sample spectra to the new standard spectra were all accurate. Generally, spectral dissimilarity increases as sample concentration decreases. The samples all showed about the same degree of variance to the standard, with the exception of phenobarbital, where spectral dissimilarity was extremely low for all five of the sample concentrations.

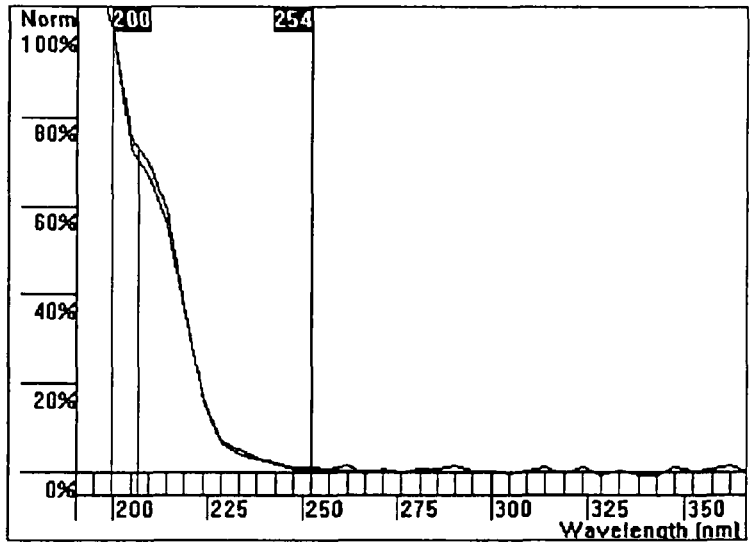
The comparison of the sample spectra to the old spectral library was nearly as successful. Two of the matches were inaccurate. Both of these samples represented the lowest concentration of their respective series. Note

SEARCH SPECTRUM

Name:	Secobarbital 1 µg/ml Sample
PuP (200 - 254 nm):	206.088 nm
Spectra Type:	Sample Spectra

SPECTRUM OF POSSIBLE MATCH

Name:	Secobarbital Standard
PuP (200 - 254 nm):	206.244 nm
Spectra Type:	Spectral Sum



SEARCH REPORT

Matches:	Sim.	Dissim.	PuP(nm)	tR(min)	Lib	#
AVERAGE SPECTRA OF SECOBARBI	0.99969	0.02481	206.244	0.000	A	2
AVERAGE SPECTRA OF TALBUTAL	0.99942	0.03400	206.353	0.000	A	10
AVERAGE SPECTRA OF PENTOBARB	0.99927	0.03816	206.274	0.000	A	5
AVERAGE SPECTRA OF BUTALBITA	0.99852	0.05438	206.436	0.000	A	4
AVERAGE SPECTRA OF ALLOBARBI	0.99796	0.06300	206.731	0.000	A	1

Search Conditions
PuP Range: 200->254 nm PuP Interval: +/- 5.00 nm
Time Range: 0.000 - 1440.000 min

FIGURE 5 Comparison of secobarbital 1 µg/ml sample to standard PuP range 200 -254 nm.

that neither of the suggested matches are barbiturates included in this series of experiments (allobarbital and barbital). In both cases, the correct spectra were the next most likely candidates for identification, meaning that if the old library had been edited down to just those barbiturates included in this study, a 100% correct match for both old and new libraries would have been achieved. However, this solution is counterproductive since the primary concern is the creation of a truly comprehensive spectral library. After observing that for most of the sample spectra, the dissimilarities resulting from the comparison had a high degree of correlation (note particularly those values for the secobarbital sample comparisons), the incorrect matches were investigated further. After review of the sample and standard spectra, a spectral inflection point was discovered in both the secobarbital and amobarbital spectra at 205 nm. For this reason, the range of the monitored wavelengths was increased to 200 - 254 nm. Using this new range, the sample spectra was re-compared to the standard spectra. In both cases, an accurate spectral match was obtained for both secobarbital and amobarbital. This data is also listed in Table 3. Figure 5 is the result of the secobarbital 1 μg/ml sample - standard comparison.

The phenobarbital samples generated the highest dissimilarity when compared to the old spectral library. These values were much in excess of those that gave incorrect matches, yet all were accurate. This can be explained by the slightly different UV spectra of phenobarbital, which is more absorbent in the 240 - 254 nm portion of the UV spectra. Recalling that significant spectral differences are necessary in the generation of ideal UV spectra by photodiode array, this minor change in absorbance in the phenobarbital sample spectra permits a much higher degree of variance while maintaining accuracy.

In Tables 4 and 5, the absolute deviation of the PuP values compared to the respective standard spectra are listed. Interestingly, the absolute deviations in the PuP values are smaller when the samples are compared to the old standards (with the exception of phenobarbital). This is further evidence as to the integrity of the spectral matches. Initially, attempts were made to correlate these deviations in the PuP values as a means to determine the lowest concentration at which an analyte could be accurately identified by UV photodiode array detection. While this was unsuccessful, it did yield one of the

most important findings of the entire study. The number of spectral library entries is inversely proportional to the magnitude of acceptable variance encountered in an accurate spectral match. In other words, as a spectral library becomes more comprehensive, increasingly smaller amounts of deviation among very similar spectra are tolerable before misidentification occurs. In libraries where significant spectral differences are present among the spectra, a large number of spectra may be stored without concern of inaccurate identification. In a library that contains similar spectra (like barbiturates), multiple libraries should be maintained in order to segregate the data to eliminate the possibility of incorrect matches with analytes that are not relevant to the study (as in this case, allobarbital and barbital). The more similar the spectra, the more limited the library should be.

CONCLUSION

The UV photodiode array detector system was able to accurately and reliably discriminate among the non-specific spectra of five barbiturates at concentrations down to 1 μg/ml. The integrity of the sample to standard match was reaffirmed by comparison of the sample spectra to both new and old standard spectra. Changing the UV lamp was also found not to effect the spectral matching accuracy, indicating that the technique is very rugged. A necessary refinement of the technique is the use of equivalent AU standard spectra for comparison to the sample spectra. This will probably provide a more accurate match and is the subject of further investigation It was interesting to note that although the old phenobarbital standard spectra was out of the detectors linear range, accurate matches with the phenobarbital sample spectra were attained. Although a more accurate equivalent spectra could have been created and used for comparison, the old spectra was retained to demonstrate the level of acceptable variance that even small spectral differences provide and to maintain the continuity of these experiments with those conducted previously[1]. An important observation was that the creation of a comprehensive spectral library for barbiturates should be approached by the use of multiple libraries that would include only those standard spectra immediately

relevant to the investigation. From a clinical perspective, one important variable has not yet been addressed. The standard and sample dilutions were mixed and prepared in the LC mobile phase. Consequently, the impact of a biological matrix on the accuracy of the spectral match is unknown. This impact should be negligible if the barbiturate peak is well resolved from other sample constituents. The applicability of this technique is not limited to the clinical environment. Because accurate spectral matches are easily achieved at concentrations down to 1 μg/ml, this becomes a viable means of identifying barbiturates that may occur as impurities/related materials in the chemical process environment. Since barbiturate spectra represent a "worst case" scenario (because of the high degree of similarity), identification of impurities/related materials in various other chemical processes should prove at least as accurate.

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