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Evaluation of a photodiode array detector for the verification of peak homogeneity in high-performance liquid chromatography

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Abstract: Photodiode array liquid chromatography detectors are claimed to have the ability of evaluating the homogeneity of chromatographic peaks and this could provide a very powerful tool in support of method development. However, in pharmaceutical analysis, for this to be of practical value it must be capable of detecting inhomogeneities at low levels. In this paper, a test has been devised to challenge the sensitivity of instruments to this application. The test makes use of mixtures of the similar benzodiazepines temazepam and lormetazepam in a chromatographic system which does not separate them. One instrument has demonstrated the ability to detect levels of just 0.5% w/w of one benzodiazepine in the other. Statistical *F*-tests and *t*-tests have been used to demonstrate that non-homogeneities have been detected with a high level of confidence. It is concluded that photodiode array detectors have the potential to evaluate the homogeneity of chromatographic peaks with a high degree of sensitivity. However, most instruments do not realize this potential because their software does not make proper use of all the data available.

Keywords: *Photodiode array detectors; HPLC; peak homogeneity; pharmaceutical analysis.*

Introduction

Rapid-scanning ultra-violet detectors for high-performance liquid chromatography (HPLC) based on the linear photodiode array have been available since the late 1970s. These instruments provide the chromatographer with an impressive potential for data manipulation. This is due to the vast amount of spectroscopic information that is collected "on the fly" during the chromatographic run and which may be interfaced with sophisticated computer facilities which are now available to process all this data. Many applications of these detectors have been well reviewed by Fell *et al.* [1] and include the following: chromatographic runs may be monitored at more than one wavelength; ultra-violet spectra of both zero and higher orders, of each component of a mixture, are readily available; spectroscopic and chromatographic data may be presented as three-dimensional projections or as contour plots; finally, the homogeneity of a chromatographic peak may be evaluated.

In the chromatographic analysis of pharmaceuticals, several of these functions are of value. Multiwavelength monitoring allows the analyst to determine the components of a mixture simultaneously and each at its optimum wavelength. The ready availability of ultra-violet spectra for each component, provides the analyst with data to assist in the identification of unknown impurities. The determination of peak homogeneity is a powerful tool in method development and validation.

During a recent evaluation of diode array detectors, these features were considered and it was concluded that for the authors' purposes the most important functions were to assist in identification of impurities and degradation products and to assess peak homogeneity. Since all instruments examined were capable of satisfying the first requirement, the latter became the major criterion.

Various approaches to establish peak homogeneity are available with diode array detectors, many of these being based on comparisons of spectroscopic features of different regions of a chromatographic peak, either with each other, or with the chromatographic peak of a reference standard. Differences between the observed features are then indicative of inhomogeneities. In some instruments, this evaluation depends on a subjective assessment of whether UV spectra taken from different regions of a peak after normalization can be overlaid on each other, or on that of a reference standard chromatographic peak.

Other instruments make use of a wavelength ratio approach [2–4]. Ratiograms are generated by monitoring elution profiles as the ratio of absorbances at two preselected wavelengths. For a pure component this results in a "square wave" but this is distorted if the peak is not homogeneous. This approach has been extended by Carter *et al.* [5]. The ratiogram of a known component is suppressed and any inhomogeneities lead to either positive or negative deviations from the baseline. This spectral suppression technique has subsequently been exploited by Fell *et al.* [6, 7].

Poile and Conlon developed an algorithm called the "absorbance index" which was originally made available for the Perkin Elmer LC 75, which is not a photodiode array instrument, operating in stopped flow mode [8]. Spectra of leading and tailing edges of a chromatographic peak are compared by dividing absorbance values at a number of wavelengths (at least nine) of one spectrum by those of the other. A "spectral discriminator" is then calculated as the quotient of the largest to the smallest difference and a value greater than 1.5 is considered to indicate a non-homogeneous peak. This approach has more recently been utilized in a diode array detector.

Alfredson and Sheehan introduced a further approach to evaluation of peak homogeneity which they termed "purity parameter" [9–11]. The purity parameter is defined as "the average wavelength of a spectrum weighted by the absorbance at each wavelength". Since the presence of an impurity can influence the value of a purity parameter, this may be used to evaluate the homogeneity of a chromatographic peak by comparing the values of purity parameters calculated for different regions of the peak either with each other or with that of a reference standard peak. This approach is the basis of the algorithm used by the Varian Polychrom 9060 detector.

HPLC is very widely used nowadays in pharmaceutical analysis. Applications include examination of active constituents to control levels of impurities arising from synthesis and monitoring of degradation in support of stability studies. Part of the validation of analytical procedures in both cases is to ensure that impurities of synthesis or products of degradation are separated from the main component and a peak homogeneity determination would appear to be a powerful tool in such an exercise. With most modern synthetic pharmaceutical active substances, it would be quite unusual for the level of an impurity to exceed 1% and so for a peak homogeneity routine to be of value to the pharmaceutical analyst it must be capable of detecting contaminants in main components at levels below this. Furthermore, it is quite possible that very similar chromophores are present in both components and therefore only small spectral differences may be expected.

The limitations of one diode array detector to assess peak purity using overlaid spectra and absorbance ratios have been previously recognized by Schieffer [12] in cases where components possessed very similar ultra-violet spectra. In this work a test has been devised to evaluate the sensitivity of peak homogeneity routines as supplied by the manufacturers of various diode array detectors and results are presented particularly when applied to a Varian Polychrom 9060.

Experimental

Reagents

The purities of temazepam and lormetazepam reference standards (Wyeth Laboratories, Havant, Hants, UK) were confirmed by IR, NMR, TLC and microanalysis.

HPLC grade methanol (Rathburn Chemicals Ltd, Walkerburn, UK) and deionized water were used throughout.

Apparatus

A Kratos Spectroflow 400 HPLC pump (Applied Biosystems Ltd, Warrington, Cheshire, UK) and a Varian Polychrom 9060 photodiode array detector with HP Thinkjet printer/plotter (Varian Associates Ltd, Walton-on-Thames, Surrey, UK) were used. Samples were injected automatically using a Perkin Elmer ISS-100 autosampler (Perkin Elmer Ltd, Beaconsfield, Bucks, UK) fitted with a 10-μl sample loop. The detector was linked to a Spectra Physics SP4270 integrator (Spectra Physics Ltd, Hemel Hempstead, Herts, UK). Ultra-violet spectra were recorded using a Lambda 7 spectrophotometer (Perkin Elmer Ltd, Beaconsfield, Bucks, UK).

Column and eluent

A Spherisorb S10 ODS 1 (25 cm × 4.6 mm i.d.) reversed-phase column (Phase Separations Ltd, Deeside, Clwyd, UK) was used. The eluent was composed of 70 vol of

methanol and 30 vol of water at a flow rate of 2.0 ml min^{-1} with sample injection volumes of 10 μl using the loop fill mode.

Preparation of solutions

Solutions of temazepam and lormetazepam were prepared by dissolving 20 and 100 mg of each reference standard in methanol and diluting to 100 ml. These solutions were then used to prepare a series of mixtures containing the combinations 99.5:0.5, 99.0:1.0 and 98.0:2.0, as detailed in Table 1.

Procedure

Ten-microlitre volumes of solutions 5 and 8 were chromatographed and peak purity parameter values collected at upslope, apex and downslope over the wavelength ranges 210–239, 210–302, 210–330, 239–302 and 239–330 nm to establish the optimum wavelength range.

To establish reproducibility, five replicate 10-μl injections of solution 1, 5, 9 and 13 were chromatographed on 2 separate days and peak purity parameters collected at upslope apex and downslope over the optimum wavelength range.

Five replicate 10-μl injections of each solution were then chromatographed and peak purity parameters similarly collected to assess peak homogeneities.

Chromatograms were prepared using a monitoring wavelength of 239 nm and ultraviolet spectra in 1 cm quartz cells were collected of solutions in mobile phase containing about 8 μg ml^{-1} of each benzodiazepine.

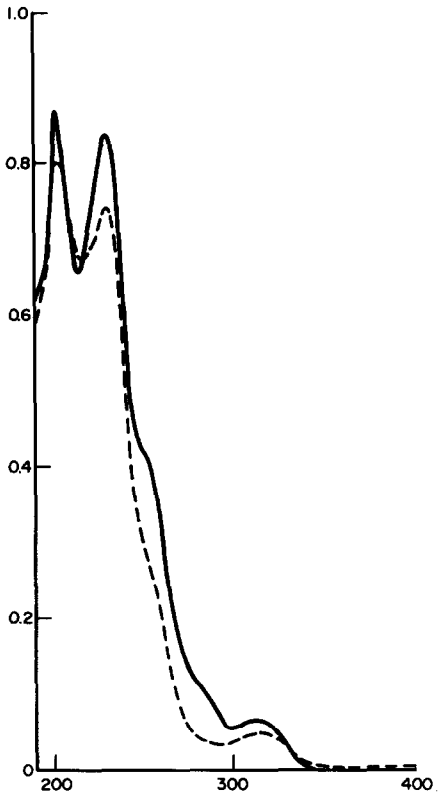

Figure 1
Ultra-violet spectra of temazepam 7.8 μg ml^{-1} (——) and lormetazepam 7.9 μg ml^{-1} (---) in mobile phase.

Results

The ultra-violet absorption spectra of temazepam and lormetazepam are presented in Fig. 1.

Chromatograms of 1.0 mg ml^{-1} solutions of lormetazepam, temazepam and a mixture containing 0.98 mg ml^{-1} lormetazepam and 0.02 mg ml^{-1} temazepam all monitored at 239 nm are presented in Fig. 2.

Purity parameters for a 1.0 mg ml^{-1} solution of lormetazepam and a solution containing 0.98 mg ml^{-1} lormetazepam and 0.02 mg ml^{-1} temazepam were established over five wavelength ranges in order to establish the optimum range. Table 2 presents the results of this optimization procedure.

The precision of the purity parameter determination was determined at upslope, apex and

Table 1
Concentrations of temazepam and lormetazepam mixtures

Solution number	Total Concentration 1 mg ml^{-1} Content of Tem–Lor (Solns 1–4) or Lor–Tem (Solns 5–8) (mg ml^{-1})	Solution number	Total Concentration 0.2 mg ml^{-1} Content of Tem–Lor (Solns 9–12) or Lor–Tem (Solns 13–16) (mg ml^{-1})
1 and 5	1.000:0.000	9 and 13	0.200:0.000
2 and 6	0.995:0.005	10 and 14	0.199:0.001
3 and 7	0.990:0.010	11 and 15	0.198:0.002
4 and 8	0.980:0.020	12 and 16	0.196:0.004

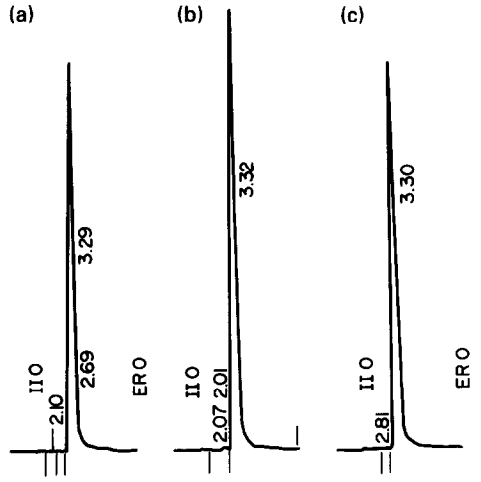

Figure 2
Chromatograms of (a) solution of lormetazepam 1.0 mg ml^{-1} in methanol, (b) solution of temazepam 1.0 mg ml^{-1} in methanol, and (c) solution of lormetazepam–temazepam 98.0:2.0, total concentration 1.0 mg ml^{-1} in methanol.

downslope of chromatographic peaks over the optimum wavelength range for solutions of temazepam and lormetazepam at concentrations of 0.2 and 1.0 mg ml^{-1} and the results are presented in Table 3.

Tables 4 and 5 show the values of purity parameters for a range of lormetazepam and temazepam mixtures with total concentrations of 0.2 and 1.0 mg ml^{-1}, respectively and containing levels of 0.5–2.0% of the minor component as detailed in Table 1.

Discussion

In order to challenge the sensitivity of a routine for the evaluation of peak homogeneity, two compounds were required with similar ultra-violet spectral characteristics. The two benzodiazepines temazepam and lormetazepam were selected because they satisfied this requirement and were readily available.

Table 2
Optimization of wavelength range for determination of peak purity parameters using lormetazepam–temazepam mixtures at 1.0 mg ml^{-1} total concentration

	Solution: lormetazepam–temazepam	Wavelength range (nm) 210–239	210–302	210–330	239–302	239–330
Upslope	100 :0.0	223.75	226.57	226.77	246.03	246.90
	98.8:2.0	223.75	226.58	226.78	246.05	246.94
	Difference	0.00	0.01	0.01	0.02	0.04
Apex	100 :0.0	223.73	226.56	226.75	246.07	246.92
	98.8:2.0	223.74	226.61	226.82	246.14	247.01
	Difference	0.01	0.05	0.07	0.07	0.09
Downslope	100 :0.0	223.74	226.56	226.76	246.04	246.90
	98.8:2.0	223.77	226.65	226.85	246.14	247.03
	Difference	0.03	0.09	0.09	0.10	0.13

Table 3
Precision of the peak purity parameter determination over the wavelength range 239–302 nm

Solution	Time	Standard Deviation % ($\nu_1 = \nu_2 = 4$; $P = 0.05$; $F = 6.39$) Upslope	Apex	Downslope
Temazepam (0.2 mg ml^{-1})	First day	0.707	0.548	1.304
	Second day	0.894	0.837	1.581
		$F = 1.60$	2.33	1.47
Lormetazepam (0.2 mg ml^{-1})	First day	0.837	0.548	1.095
	Second day	0.837	0.707	1.000
		$F = 1.00$	1.67	1.20
Temazepam (1.0 mg ml^{-1})	First day	0.707	0.548	0.548
	Second day	0.894	0.447	0.000
		$F = 1.60$	1.50	0.000
Lormetazepam (1.0 mg lm^{-1})	First day	0.000	0.000	0.447
	Second day	0.548	0.000	0.000
		$F = 0.00$	0.00	0.00

Table 4
Comparison of mean purity parameter values for lormetazepam–temazepam mixtures at 0.2 mg ml^{-1} total concentration

Solution: lormetazepam–temazepam	Mean purity parameter (239–302 nm) Upslope	Apex	Downslope
	(ν = 8; P = 0.05; t = 2.306)		
0.0:100	249.51	249.52	249.51
0.5:99.5	249.55	249.54	249.54
	t = 1.041	0.572	1.082
1.0:99.0	249.46	249.49	249.49
	t = 8.262*	9.731*	2.226
2.0:98.0	249.41	249.46	249.46
	t = 11.918*	18.890*	6.310*
100 : 0.0	245.97	245.98	245.97
99.5: 0.5	245.98	246.00	246.00
	t = 1.653	3.238*	3.505*
99.0: 1.0	245.99	246.02	246.02
	t = 3.305*	4.866*	5.262*
98.0: 2.0	246.00	246.06	246.07
	t = 4.958*	14.572*	11.918*

*Significant difference.

Table 5
Comparison of mean purity parameter values for lormetazepam–temazepam mixtures at 1.0 mg ml^{-1} total concentration

Solution: lormetazepam–temazepam	Mean purity parameter (239–302 nm) Upslope	Apex	Downslope
	(ν = 8; P = 0.05; t = 2.306)		
0.0:100	249.56	249.59	249.57
0.5:99.5	249.53	249.57	249.55
	t = 4.957*	2.644*	2.644*
1.0:99.0	249.50	249.56	249.54
	t = 9.254*	3.636*	5.262*
2.0:98.0	249.46	249.53	249.52
	t = 21.048*	11.333*	7.155*
100 : 0.0	246.03	246.08	246.04
99.5: 0.5	246.04	246.10	246.07
	t = 1.792	5.724*	3.584*
99.0: 1.0	246.05	246.11	246.10
	t = 2.164	4.957*	9.254*
98.0: 2.0	246.06	246.14	246.15
	t = 3.272*	17.173*	14.883*

*Significant difference.

The structures of these benzodiazepines are shown in Fig. 3 and their ultra-violet spectra in Fig. 1. The only structural difference is the presence of a chlorine atom at the 2-position of the 5-phenyl substituent in the case of lormetazepam which is absent in temazepam and the two spectra are therefore very similar.

The chromatographic system used was deliberately selected to ensure insufficient separation of a mixture of these compounds to be discernible. That this was accomplished is demonstrated in Fig. 2. Using the chromatographic system defined in the Experimental section the retention time of lormetazepam was typically 3.29 min and that of temazepam 3.32 min. Chromatograms of mixtures showed no evidence of resolution between these two components.

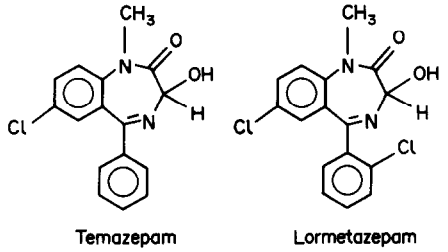

Figure 3
Structures of temazepam and lormetazepam.

Mixtures of temazepam and lormetazepam

were used in this way to challenge the peak homogeneity evaluation facilities of a number of diode array detectors on the market. In most cases, mixtures containing around 20% of one component and 80% of the other, were identified as inhomogeneous but at the 4% level, the software approaches used were unable to discern the inhomogeneity. The Polychrom 9060 peak purity parameter routine was rather more sensitive however and so the results obtained on this instrument are presented here in greater detail.

This routine includes a facility to select the wavelength range over which the purity parameter is determined and so in the first instance it was necessary to establish the optimum range for this benzodiazepine mixture. The result of this is shown in Table 2. The optimum range was taken as that over which, the greatest difference in peak purity parameter value was obtained between a peak obtained with a pure component and one obtained with a mixture (in this case containing 2.0% of a second component). The greatest differences were seen using the wavelength ranges 239–302 and 239–330 nm and since subsequently, better reproducibility was observed with the former range, this was selected for further study.

The homogeneity of a peak is evaluated using the purity parameters at different regions, i.e. upslope, apex and downslope. To establish whether differences in values were significant, it was necessary to subject the routine to statistical examination. In Table 3, the precisions have been determined for peak purity parameters at the above regions of chromatographic peaks for two different concentrations of temazepam and lormetazepam analysed on 2 different days using five replicate injections of each solution on each occasion. The standard deviations obtained demonstrate that the values obtained for purity parameters were highly reproducible and from which it followed that relatively small differences in these figures for different regions of a peak would indeed be significant.

Furthermore, application of an *F*-test to the standard deviations at the 95% confidence level showed no significant difference between the results obtained on 2 different days. It therefore followed that the sensitivity of the peak purity parameter to inhomogeneities could also be expected to show very good day-to-day reproducibility.

Purity parameters of solutions containing 0.0, 0.5, 1.0 and 2.0% of the minor component and of total concentrations 0.2 and 1.0 mg ml^{-1} are presented in Tables 3 and 4, respectively. Each purity parameter is the mean of five replicate injections and the values at upslope, apex and downslope for mixtures were compared with the corresponding values for the pure component. The significances of differences were established using Students *t*-test.

All samples containing 1.0 and 2.0% of minor component were shown to be significantly different from the appropriate pure component and it was noticeable that magnitudes of significance at different regions of peaks correlated with the order of elution of the components. In the 0.2 mg ml^{-1} solutions, no significant differences from a temazepam solution were detected at any of the regions on the peak of the solution containing 0.5% lormetazepam although for the corresponding comparison between a lormetazepam solution and one containing 0.5% temazepam, significant differences were detected at apex and downslope. In the 1.0 mg ml^{-1} solutions, the 0.5% level of lormetazepam was, however, detected.

From the results obtained in Tables 4 and 5, there was a correlation between the mean purity parameter and the level of impurity present. Calibration graphs of mean purity parameter from the upslope, apex and downslope versus the level of impurity present (%) in the lormetazepam/temazepam system at 0.2 and 1.0 mg ml^{-1} were rectilinear over the range 0–2.0%.

Conclusions

The results of this work demonstrate that detectors based on diode array optics have the potential to evaluate the homogeneity of chromatographic peaks with a high degree of sensitivity. The extent to which instruments realize this potential, however, is dependent on how they utilize the data collected during a chromatographic run and in the case of the Polychrom 9060 this is done efficiently. It is therefore considered that this instrument will be useful in the development and validation of stability indicating assays and of methods for determinations of impurities of synthesis and degradation products in pharmaceuticals.

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