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A multiwavelength approach to the selection of absorbance ratios for the assessment of chromatographic peak purity*

J.B. CASTLEDINE,†‡ A.F. FELL,‡ R. MODIN§ and B. SELLBERG§

‡*Pharmaceutical Chemistry, University of Bradford, Bradford BD7 1DP, UK*
§*Analytical Chemistry, Kabi Pharmacia Therapeutics AB, Uppsala, Sweden*

Abstract: The routine use of diode-array detectors (DAD), based on the linear photodiode array device, has transformed the practice of UV–vis detection in liquid chromatography (LC). Multiwavelength detection is widely employed to generate absorbance ratios as a relatively non-specific method for characterizing peak purity in LC. If several wavelength pairs are selected the selectivity of the absorbance ratio method and its sensitivity to an interfering impurity can be increased, however, these attributes still depend on the selection of suitable pairs of wavelengths.

This paper presents a novel approach to the selection of absorbance ratios for the assessment of peak purity in LC, utilizing a matrix derived from all the spectral data collected. As with single absorbance ratios, the absorbance ratio matrix (ARM) generated (containing all possible finite absorbance ratios) is characteristic for the analyte and independent of the analyte concentration. Moreover, the ARM technique eliminates the need to select "appropriate wavelength pairs", for sensitive discrimination of small spectral differences, when used for peak purity assessment. The ARM is found to give comparably high sensitivity to the presence of co-eluting species, as compared with the use of the wavelength pair selected on the basis of the conventional optimization criteria.

Keywords: *Absorbance ratio; multiwavelength detection; peak purity.*

Introduction

Reliable and sensitive assessment of chromatographic peak purity is a major problem in liquid chromatography (LC)-method [1–14] development and validation. The potential for using UV-absorbance data, at a defined number of wavelengths, for peak deconvolution and solute identification was first recognized in a theoretical analysis by Ostojic [15]. Prior to the development of diode-array detectors (DAD), application of the theory required repeated analysis using two detectors in series or alternatively stop-flow conditions [2, 16]. Whilst the advent of DAD has eliminated many of the problems that affected the overall precision of using absorbance ratios, as discussed by the early workers [2], the sensitivity of the method remains largely dependent on the wavelengths chosen. In attempts to overcome this constraint, various workers have developed criteria for selecting three or more absorbance ratios to characterize a compound, as reviewed by Marr *et al.* [13]. While these approaches have been shown to be less sensitive to wavelength choice than the absorbance ratio method, their sensitivity still depends on the selection of suitable pairs of wavelengths.

This paper describes a new approach to the use of multiple absorbance ratios for the characterization of analytes and subsequently for the assessment of chromatographic peak purity. Whilst the calculation of a single absorbance ratio involves the division of the absorbance value at one wavelength by the absorbance value at a second wavelength, both from the same spectrum, the proposed technique computes all possible finite absorbance ratios that may be calculated from the spectrum collected. Each positive absorbance value of a UV–vis spectrum is divided by each positive absorbance value of the same spectrum to form a square matrix containing all finite absorbance ratios (Fig. 1). The absorbance ratio matrix (ARM) thus created is characteristic for the analyte, and independent of both the analyte concentration and the need to select "the most appropriate" wavelengths.

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†Author to whom correspondence should be addressed.

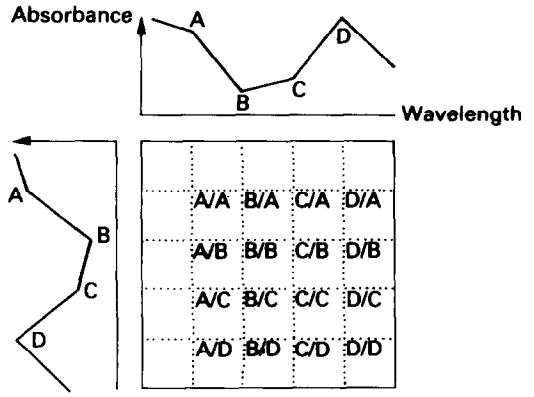

Figure 1
The ARM above contains 16 absorbance ratios, calculated as shown, of which 12 do not equal one and are characteristic of the spectrum A, B, C, D. The differences between this spectrum and a spectrum W, X, Y, Z may be assessed using a correlation coefficient which determines the difference between each pair of absorbance ratios (e.g. A/B and W/X). Key: A, B, C, D, absorbance values of spectrum; B/A, etc., absorbance ratio.

Marr *et al.* recently reported the use of a correlation coefficient to compare multiple absorbance ratio data (multiple absorbance ratio correlation: MARC) [13]. In analogous fashion, the ARM data created for the various analytes and mixtures examined, are compared using a similar correlation coefficient.

Experimental

Reagents

Methanol (HPLC grade, Rathburn Chemicals, UK), sodium dihydrogen phosphate monohydrate and sodium acetate anhydrous (Merck, Darmstadt, FRG) were used as received. All buffer salts were dissolved in distilled water and filtered using HVLP 0.45-μm filters (Waters, Millipore, Milford, MA, USA). Sulphasalazine (USP reference material, Batch 408641) and related compounds were from Kabi Pharmacia Therapeutics AB (Uppsala, Sweden).

Apparatus

The chromatographic system used consisted of a Series 400 Chromatograph with the SEC-4 Solvent Environment Controller, together with the ISS-101 Autosampler (all from Perkin Elmer, Norwalk, CT, USA) and the HP 1040A diode-array detector (Hewlett–Packard, Waldbrom, FRG). Data collection and evaluation were performed using the HP-85 computer, the HP-9000 Series Workstation, the HP-7470 plotter and a 9121 dual-disc drive (all from Hewlett–Packard, Waldbrom, FRG).

LC conditions

A stainless steel column (250 × 4.6 mm i.d.) packed with 7-μm Nucleosil C_{18} (Macherey–Nagel, Dueren, FRG) was used. The mobile phase, pumped at 1.0 ml min^{-1}, consisted of methanol–phosphate buffer (75:25, v/v), pH 4.8. Detection was effected using the diode-array at 320 nm.

Computation

A program was written, in BASIC, to generate and correlate the matrices using a microcomputer (Hewlett–Packard HP-85). The maximum size of the matrices allowed by memory limitations was 50 × 50 data points. Correlation coefficients were calculated using:

$$r = \frac{\Sigma A_{1i} \cdot A_{2i}}{\sqrt{(\Sigma A_{1i}^2 \cdot \Sigma A_{2i}^2)}}, \qquad [17]$$

where A_{1i} and A_{2i} are the absorbance values at i nm for spectra 1 and 2, respectively. In the present work, i varies from 250 to 450 nm with a step interval of 4 nm.

Since correlation coefficients are not normally distributed, the confidence limits were calculated after transformation of the data to give the normalized correlation, Z, using:

$$Z = 0.5 \ln [(1 + r)/(1 - r)].$$

The values of Z are *approximately* normally distributed [18]. Statistical evaluation was performed using Minitab Interactive Statistics (Version 81.1, Pen State University 1981).

Results and Discussion

An LC system was developed, using sulphasalazine and related compounds, such that one of the related compounds, "A", could be made to exactly co-elute with the sulphasalazine. Compound A is a potential related substance of sulphasalazine, consisting of an additional sulphapyridine attached to the sulphasalazine via the central benzene ring. Consequently, the UV–vis absorbance spectrum of compound A differs significantly from that of sulphasalazine (Fig. 2).

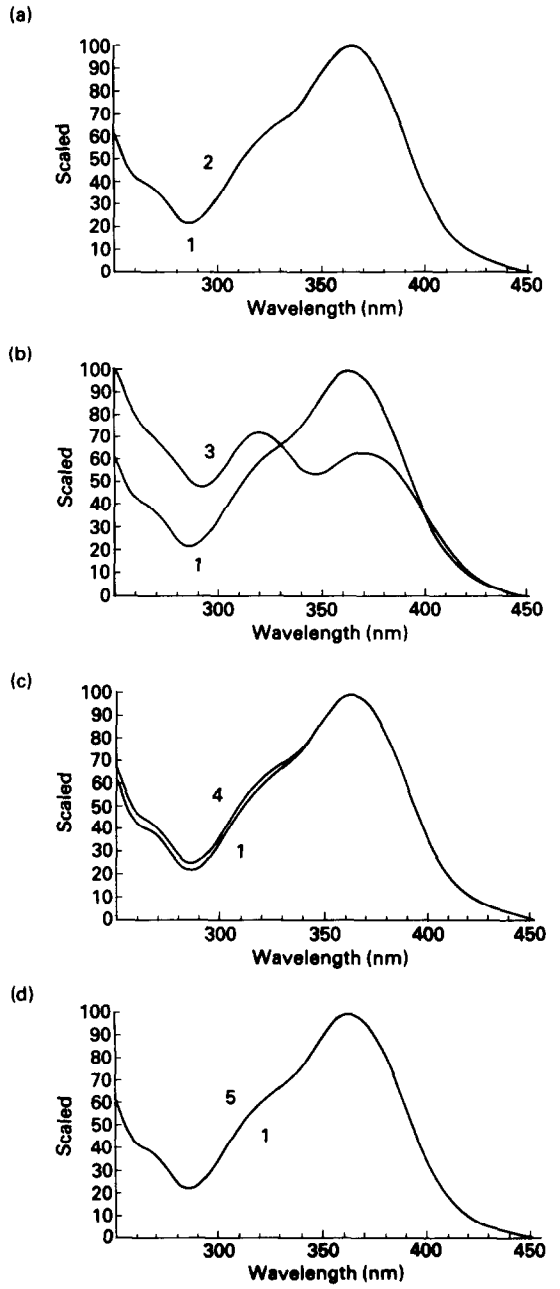

Figure 2
Overlaid normalized spectra: a comparison of the sulphasalazine chromatographic peak apex spectrum with the apex spectra of: (a) sulphasalazine; (b) compound A; (c) sulphasalazine + 10% A; and (d) sulphasalazine + 1% A. Key: spectra 1 and 2, sulphasalazine; spectrum 3, compound A; spectrum 4, sulphasalazine + 10% A; spectrum 5, sulphasalazine + 1% A.

Each of three solutions of sulphasalazine at different concentrations were spiked with 10, 1 and 0.1% of compound A, respectively. The chromatographic peak apex spectra obtained were compared with those obtained from unspiked sulphasalazine by:

(1) visual examination of the overlaid normalized spectra;
(2) single absorbance ratios (at several wavelength pairs, selected using various published criteria);
(3) correlation of the ARM.

Although compound A has two additional chromophores, (cf. sulphasalazine) resulting in significantly different absorbance spectra, the presence of a minor proportion of this related compound in a mixture with sulphasalazine results in a combined spectrum that closely resembles that of unadulterated sulphasalazine. Consequently, visual examination of the overlaid normalized apex spectra revealed detectable differences when 10% of compound A was added to the sample of sulphasalazine, but the presence of 1% or less of the related compound could not be detected (Fig. 2).

Tables 1 and 2 illustrate the dependence of the AR on the choice of wavelengths selected. When the spectrum of the co-eluting species *is* known, as in the model system described above, then the creation of the normalized difference spectra for the analyte and related compound identifies the wavelengths of greatest positive and negative difference, which give rise to "the most appropriate" absorbance ratio wavelength pair [14]. If these optimum wavelengths are selected (in this case, 280 and 360 nm) then it is apparent that the absorbance ratio method is more sensitive than the visual examination of normalized spectra (Table 1).

Since most assessments of peak purity are undertaken without prior knowledge of potentially co-eluting species, Yost *et al.* proposed that several wavelengths should be selected according to the following criteria: (a) absorbance maxima of the primary compound; (b) a general wavelength, where most compounds of interest have some absorption; and (c) a low wavelength, where most compounds have strong absorption [2]. An additional requirement is that these wavelengths should be well separated. The results obtained by choosing such wavelengths by these criteria, in this case (a) 364 nm, (b) 400 nm and (c) 250 nm, are shown in Table 2(a). In the present case, the above approach permits similar discrimination of impurity to that obtained by using the most suitable wavelength pair (through the selection of the wavelength pair, 250 and 364 nm).

It is also possible to demonstrate from the same data that inappropriate, and yet widely

Table 1
Spectral discrimination using "the most appropriate wavelength pair"

	Absorbance ratio of chromatographic peak apex spectra		
Wavelength pair (nm):	High conc. 10 μg on column 280/360	Medium conc. 2 μg on column 280/360	Low conc. 1 μg on column 280/360
Reference	0.313	0.313	0.310
sulphasalazine	0.314	0.314	0.311
	0.313	0.314	0.311
Sulphasalazine + 10% compound A	0.342	0.341	0.339
	0.342	0.340	0.340
	0.341	0.340	0.340
Sulphasalazine + 1% compound A	0.316	0.316	0.316
	0.316	0.316	0.316
	0.317	0.316	0.315
Sulphasalazine + 0.1% compound A	0.314	0.313	0.312
	0.313	0.314	0.312
	0.313	0.313	0.313

spaced, wavelength pairs can be selected when the absorbance characteristics of the potentially co-eluting impurities are unknown [Table 2(b)]. Such selection of the wavelength pairs, in this case 340 and 380 nm (also 420 and 340 nm, or 420 and 380 nm), results in the inability to detect even relatively high concentrations of compound A coeluting under the analyte peak.

The ARM technique was found to give comparably high sensitivity to the presence of co-eluting species, as that obtained by the use of "the most appropriate wavelength pair" (Table 3). This may be attributed to the use of a correlation coefficient for comparing the ARM data, since the presence, of the less-sensitive wavelength pairs in the matrices (e.g. in this case, 340 and 380 nm), do not significantly reduce the dissimilarity between the ARMs (caused by the presence, for example in this case, by such wavelength pairs as 250 and 362 nm) when they are compared in this manner.

Moreover, by correlating the ARMs of unknown samples with those of known purity, chromatographic separations repeated in triplicate give rise to nine correlation values, rather than three values for each absorbance ratio (as shown in the above data). Thus, while it is necessary to collect two sets of triplicate data for the sample of known purity, the ARM results (and their statistical analysis) assess the differences between the sets of spectra obtained for the known and unknown analytes, rather than the spread of possible values of the AR for the known and for the unknown.

In addition, this novel technique only requires the wavelength range of interest (and in this case, the data interval due to limitations in the computing power) to be selected by the operator. Thus the ARM technique increases the reliability of using ARs for analyte characterization and consequently for chromatographic peak purity determination.

Conclusion

Several different approaches to the selection of multiple absorbance ratio wavelength pairs have been published. However, these approaches do not always establish the most sensitive AR for distinguishing between a known analyte and the potential presence of trace levels of an unknown related species.

The ARM technique described above ensures that "the most appropriate" absorbance ratio is selected, in each case, because all finite values of absorbance ratio are generated within the matrix. Comparison of ARMs using a correlation coefficient leads to as sensitive a method as that using the single "most appropriate" wavelength pair, and directly assesses the differences between the data set of spectra for the compound of known purity and those of the unknown.

Table 2
Spectral discrimination using three wavelength pairs

	Absorbance ratio of chromatographic peak apex spectra								
	High conc. 10 μg on column			Medium conc. 2 μg on column			Low conc. 1 μg on column		
(a) Wavelength pair (nm):	250/364	400/364	400/250	250/364	400/364	400/250	250/364	400/364	400/250
Reference sulphasalazine	0.642	0.406	0.632	0.643	0.406	0.631	0.639	0.406	0.635
	0.644	0.408	0.634	0.644	0.406	0.630	0.640	0.405	0.632
	0.643	0.407	0.633	0.643	0.406	0.631	0.641	0.404	0.630
Sulphasalazine + 10% compound A	0.684	0.417	0.610	0.682	0.415	0.608	0.681	0.416	0.610
	0.684	0.417	0.610	0.682	0.415	0.609	0.681	0.415	0.610
	0.684	0.417	0.610	0.681	0.415	0.609	0.681	0.415	0.610
Sulphasalazine + 1% compound A	0.647	0.408	0.630	0.647	0.406	0.628	0.646	0.406	0.629
	0.648	0.408	0.630	0.648	0.407	0.628	0.646	0.406	0.629
	0.648	0.409	0.631	0.647	0.407	0.628	0.646	0.406	0.629
Sulphasalazine + 0.1% compound A	0.644	0.408	0.633	0.643	0.406	0.631	0.641	0.405	0.632
	0.643	0.407	0.632	0.643	0.406	0.631	0.641	0.406	0.632
	0.643	0.407	0.632	0.643	0.406	0.631	0.642	0.406	0.633
(b) Wavelength pair (nm):	340/380	420/340	420/380	340/380	420/340	420/380	340/380	420/340	420/380
Reference sulphasalazine	0.941	0.235	0.221	0.940	0.235	0.220	0.936	0.235	0.221
	0.940	0.235	0.221	0.940	0.235	0.220	0.936	0.236	0.221
	0.940	0.235	0.221	0.939	0.235	0.221	0.937	0.235	0.220
Sulphasalazine + 10% compound A	0.941	0.239	0.225	0.939	0.239	0.224	0.938	0.239	0.224
	0.941	0.239	0.225	0.940	0.239	0.225	0.937	0.240	0.225
	0.941	0.239	0.225	0.939	0.240	0.225	0.937	0.240	0.225
Sulphasalazine + 1% compound A	0.940	0.235	0.221	0.939	0.235	0.221	0.938	0.236	0.221
	0.941	0.235	0.222	0.938	0.235	0.221	0.938	0.237	0.222
	0.941	0.236	0.222	0.938	0.236	0.221	0.937	0.237	0.222
Sulphasalazine + 0.1% compound A	0.941	0.235	0.221	0.939	0.235	0.221	0.937	0.236	0.221
	0.941	0.235	0.221	0.939	0.235	0.221	0.937	0.236	0.221
	0.941	0.235	0.221	0.938	0.235	0.221	0.937	0.236	0.221

Table 3
Spectral discrimination using the ARM technique. Triplicate injections of each sample were correlated with triplicate injections of pure sulphasalazine. ARM correlation data were normalized using; $Z = 0.5 \ln [(1 + r)/(1 - r)]$: r = correlation coefficient

Normalized correlation coefficients of apex spectra ARM with those for sulphasalazine at similar comcentration, from 250 to 450 nm (at 4-nm intervals)

		High conc. 10 μg on column	Medium conc. 2 μg on column	Low conc. 1 μg on column
Reference	X:	7.590	6.805	6.192
sulphasalzaine	95%:	7.170–8.011	6.647–6.962	6.012–6.371
Sulphasalazine + 10% compound A	X:	4.147*	4.170*	4.136*
	95%:	4.136–4.158	4.157–4.183	4.119–4.153
Sulphasalazine + 1% compound A	X:	6.273*	6.223*	5.790*
	95%:	6.168–6.378	6.155–6.291	5.731–5.849
Sulphasalazine + 0.1% compound A	X:	7.572	6.768	6.289
	95%:	7.190–7.960	6.635–6.901	6.175–6.403

Key: X = mean; 95% = 95% confidence limits; $n = 9$; *co-eluting species can be reliably detected.

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