Journal of Chromatography, 592 (1992) 27–36
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2448

Assessment of chromatographic peak purity by means of multi-wavelength detection and correlation-based algorithms

J. B. Castledine and A. F. Fell*

Pharmaceutical Chemistry, University of Bradford, Bradford BD7 1DP (UK)

R. Modin and B. Sellberg

Analytical Chemistry, Kabi Pharmacia AB, Therapeutics, Uppsala (Sweden)

ABSTRACT

Dual-wavelength UV detection is widely employed to generate absorbance ratios as a relatively non-specific method for characterizing peak purity in liquid chromatography. The sensitivity of this method is largely dependent on the pair of wavelengths chosen. An alternative technique, correlation of the spectral information, proposed primarily to overcome this constraint, has been investigated. The implications for the application of correlation coefficients to peak purity and homogeneity determination are examined. Particular attention is focused on the successful use of correlating multiple chromatographic peak-area data (peak areas calculated from the absorbance values at various detection wavelengths) for the reliable assessment of both peak identity and peak homogeneity. Using a model system, 1% of a putative related compound of sulphasalazine in a mixture with sulphasalazine could be detected regardless of the chromatographic resolution between the two compounds.

INTRODUCTION

One of the key requirements for the validation of chromatographic methods is to determine the purity of the analyte peaks. The establishment of the purity of a chromatographic peak is in essence a negative concept, requiring that the absence of impurity be demonstrated and as such is not directly measurable [1]. Consequently, the identity and purity of a pharmaceutical compound are often determined by comparison of the results obtained from several tests with those acquired under similar conditions from a standard of known and accepted quality [2,3].

In addition to comparing the chromatographic retention characteristics of the compound of interest with a reference sample, it was first recognized in a theoretical analysis by Ostojic [4] that multiple sets of detection data could be used to enhance solute identification and facilitate peak deconvolution. Prior to the development of the now widely used diode-array detectors (DAD) [5–21], application of this theory based on UV absorbance data required repeated analysis using two detectors in series; alternatively, stopped-flow conditions were used [6,7]. The identity or purity of individual peaks can be confirmed when absorbance ratios (usually calculated from the chromatographic peak apex spectrum, as proposed by White and Catterick [17]) are compared with those from reference samples. Moreover, by comparing absorbance ratios calculated at points through the chromatographic peak, it is possible in principle to determine the presence of partially overlapping species, provided that there are sufficient differences between the spectra involved and their respective elution times.

Although the advent of DAD has reduced many of the problems that affected the overall precision of absorbance ratio measurements, as discussed by

0021-9673/92/$05.00

early workers [6], the sensitivity of the method remains largely dependent on the wavelengths chosen and the consequent differences in absorptivity of the overlapping solutes. If several wavelength pairs are selected, as is possible with the DAD, the selectivity of the absorbance ratio method and its sensitivity to interfering impurities can be increased [6,9,16,18]. Multiple absorbance ratio correlation (MARC) is an alternative approach to enhancing peak purity detection, as proposed by Marr *et al.* [22], based on the correlation between five-point absorbance ratio vectors at points through the chromatographic peak. A competitive approach, recently advocated for the assessment of protein and peptide purity [23,24], is based on calculating the correlation coefficient directly between pairs of UV–visible absorption spectra.

This paper examines the use of correlation coefficients to compare spectral data for the assessment of peak purity, as data at all wavelengths of detection can be utilized for the comparison. Moreover, mathematical transformation of the correlation coefficients obtained is possible, giving rise to an approximately normal distribution of results. This makes the data generally applicable for Student's *t*-test evaluation, rather than relying on an empirically based descriminatory factor [18].

Monitoring an absorbance ratio continually through a chromatographic peak can provide two separate pieces of information. The appearance of the function as a square waveform may give an indication of peak homogeneity, within the constraints applied due to noise, resolution, peak tailing and wavelength choice. Second, the average value of the square waveform may be used to establish the peak's purity and, if pure, its identity. In analogous fashion, correlation coefficients may be used to assess peak homogeneity and identity [22].

Although most work reported using absorbance ratios utilizes the ratio between absorbance values at two wavelengths at a given time point in the chromatographic peak [5–21], it is possible to generate such ratios from a comparison of chromatographic peak areas, calculated from integrating the chromatographic peaks generated at different wavelengths of detection [17,25]. The principles and the relative advantages and disadvantages of this method are similar to those involved in ratioing single absorbance values. White *et al.* [17] found that, for peak identification, better precision was obtained with ratios derived from peak-height measurements. This may be attributed, at least in part, to the integration algorithm employed.

An advantage of ratios calculated from peak areas is that they assess peak homogeneity in addition to peak identity, giving a single figure assessment of purity. Given that the sensitivity of this method is dependent on the wavelength pair used, this paper re-examines this technique using correlation of the chromatographic peak areas at several detection wavelengths.

EXPERIMENTAL

Reagents

Methanol [high-performance liquid chromatographic (HPLC) grade, Rathburn Chemicals, Walkerburn, UK], sodium dihydrogenphosphate monohydrate and anhydrous sodium acetate (Merck, Darmstadt, Germany) 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 potential related compounds were obtained from Kabi Pharmacia Therapeutics (Uppsala, Sweden).

Apparatus

The chromatographic system used consisted of a Series 400 chromatograph with an SEC-4 solvent environment controller and an ISS-101 autosampler (all from Perklin-Elmer, Norwalk, CT, USA), and an HP 1040A diode-array detector (Hewlett-Packard, Waldbron, Germany). Data collection and evaluation were performed using an HP-85 computer, HP-9000 Series Workstation (with HPLC Chemstation software), HP-7470 plotter and a Model 9121 dual-disk drive (all from Hewlett-Packard). The original HP-85A specifications were enhanced through the addition of read-only memory modules for input–output, printer–plotter communications and available memory (expanded by 16K) (Hewlett-Packard).

LC conditions

A stainless steel column (250 mm × 4.6 mm I.D.) packed with 7-μm Nucleosil C_{18} (Macherey, Nagel & Co., Düren, Germany) was used. The

mobile phase, pumped at 1.0 ml/min, was a mixture of methanol and phosphate–acetate buffer.

Computation

Programs were written in BASIC to correlate the chromatographic peak apex spectra and also the sets of peak-area data using an HP-85 microcomputer (Hewlett-Packard). Correlation coefficients were calculated using the equation [26]

$$r = \frac{\sum A_{1i} \cdot A_{2i}}{(\sum A_{1i}^2 \cdot \sum A_{2i}^2)^{\frac{1}{2}}}$$

where A_{1i} and A_{2i} are the absorbance values or peak areas at i nm for spectra or chromatograms 1 and 2, respectively.

As 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 [27].

Statistical evaluation was performed using Student's t-test. As it was found that the sample standard deviations varied significantly and were dependent, in part, on the absolute value of the correlation coefficient, it was considered that the population standard deviations could not be assumed to be equal. Hence the appropriate equation used was [28]

$$t = (x_1 - x_2)/(s_1^2/n_1 + s_2^2/n_2)^{\frac{1}{2}}$$

where x_1 = mean, s_1 = standard deviation and n_1 = number of normalized correlation coefficients calculated between sample and reference data and x_2 = mean, s_2 = standard deviation and n_2 = number of normalized correlation coefficients calculated between two sets of reference data.

The number of degrees of freedom (*d.f.*) was calculated thus [28]:

$$d.f. = \left[\frac{(s_1^2 n_1 + s_2^2/n_2)^2}{\frac{(s_1^2/n_1)^2}{n_1 + 1} + \frac{(s_2^2/n_2)^2}{n_2 + 1}} \right] - 2$$

the results being rounded to the nearest whole number.

RESULTS AND DISCUSSION

An LC system was developed, as described above, using sulphasalazine and potential-related compounds, such that one of these compounds, A, could be made to elute simultaneously with sulphasalazine ($R_s = 0$). By varying the composition of the mobile phase, three different degrees of chromatographic peak overlap between sulphasalazine (10 μg on-column) and compound A were obtained and investigated. In addition to the simultaneous elution conditions ($R_s = 0$), separations of the respective retention times for sulphasalazine and compound A of +0.25 min and +0.45 min were established. This corresponds to R_s values of *ca.* 0.2 and 0.3, when calculated with reference to sulphasalazine (10 μg on-column) and compound A (1 μg on-column), chromatographed individually.

Visual examination of spectra

Sulphasalazine is prepared by diazotizing sulphapyridine and coupling the diazotized intermediate with salicylic acid [29]. Compound A contains an additional sulphapyridine moiety attached to the sulphasalazine molecule via the central benzene ring. Consequently, the UV–visible absorption spectrum of compound A (which thus contains two additional chromophores) differs significantly from that of sulphasalazine (Fig. 1b). Nevertheless, the presence of a minor proportion of this potential-related compound in a mixture with sulphasalazine results in a combined spectrum that closely resembles that of the unadulterated sulphasalazine. Although visual examination of the overlaid normalized apex spectra, obtained using $R_s = 0$ data, revealed detectable differences for 10% of compound A added to the sample of sulphasalazine, the presence of 1% or less of related compound A could not be detected in this way (Fig. 1).

Evaluation of correlation-based methods

Solutions of sulphasalazine (0.5 mg/ml), in the linear response range of the LC system (measured at 320 nm), were spiked with 10%, 1% and 0.1% of compound A. The chromatographic peak apex spectra obtained for $R_s = 0$ conditions were compared with those acquired for the USP reference sulphasalazine by (i) single absorbance ratios and (ii) correlation of the UV–visible absorption spectra.

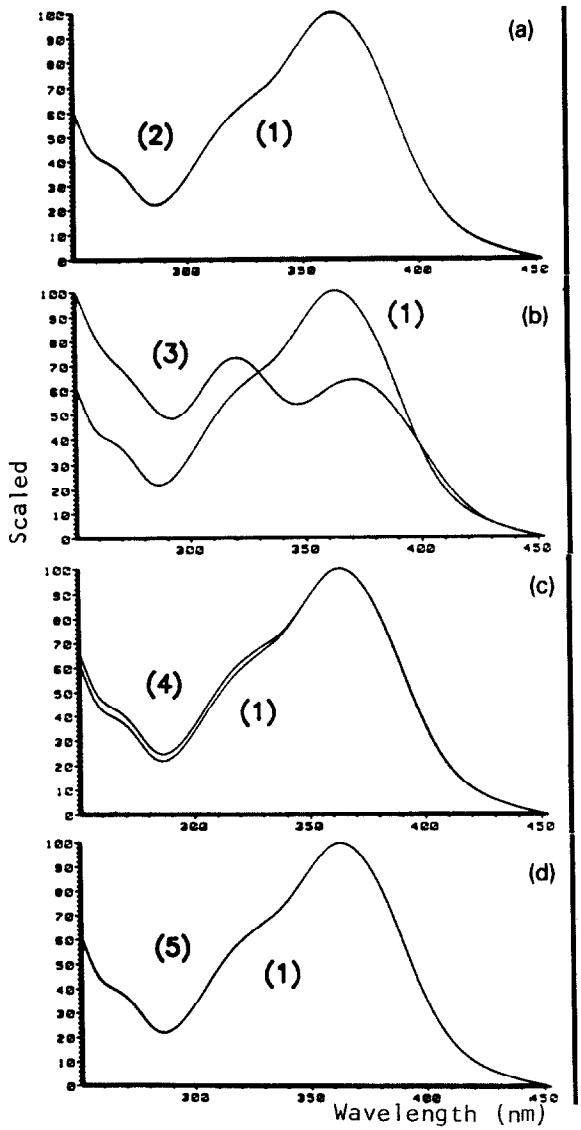

Fig. 1. Overlaid normalized spectra: comparison of the sulphasalazine chromatographic peak apex spectrum with the apex spectra of (a) sulphasalazine, (b) compound A, (c) sulphasalazine + 10% A and (d) sulphasalazine + 1% A. Spectra: 1 and 2 = sulphasalazine; 3 = compound A; 4 = sulphasalazine + 10% A;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

From the data in Table I, the sensitivity of the absorbance ratio (*AR*) algorithm and its dependence on the wavelength pair selected can be observed. For the model system described, it was found possible to detect 1% of the simultaneously eluted compound A in a mixture with sulphasalazine when the chromatographic peak apex *AR*, calculated from data at 280 and 360 nm, was compared with a similar *AR* for sulphasalazine alone. These results, when compared with the visual examination of overlaid normalized spectra (Fig. 1), illustrate the potentially high sensitivity that the *AR* technique can provide in the assessment of peak purity under well defined conditions. Moreover, the dependence of the *AR* method on the wavelength pair selected is illustrated by the ratios generated using data at 340 and 380 nm, as the presence of relatively high concentrations of the related compound cannot be detected.

The comparable sensitivity of the correlation coefficient to that of a well chosen *AR* and the application of Student's *t*-test to the data generated are shown in Table I.

The ability of multi-wavelength LC detectors to generate large amounts of data necessitates a reduction of the data set interrogated for efficient application of peak-purity algorithms. Data reduction may be effected in the wavelength and/or time domain.

In the case of non-continuous use of the time data set, as used by the Hewlett-Packard Chemstation Data Editor peak-homogeneity correlation-based assessment algorithm [25,30], when the partially overlapping "impurity" peak elutes at a retention time that corresponds closely with a sampling point in the time domain (*e.g.*, downslope inflection), the presence of a heterogeneous peak may be reliably detected. Thus, in the system described above with an interval of 0.25 min between the retention times, corresponding to elution of compound A coincident with the downslope inflection of the sulphasalazine peak, the sensitivity of detection of compound A is at a maximum (Table II). If this is not the case, then such algorithms can fail, as also shown in Table II.

Further experiments were therefore initiated to examine the alternative approach of reducing the number of data points in the wavelength domain.

Fig. 2 illustrates graphically the relationship between the spectral correlation coefficient (*SCC*) and the number of data points used. By comparing spectra collected at a similar time point on the downslope of the chromatographic peak, obtained from the injection of *ca.* 1 μg of the samples, the signal-to-noise ratio of the data selected was such that 1% of compound A mixed with sulphasalazine could only be detected after careful choice of the

TABLE I

COMPARISON OF CHROMATOGRAPHIC PEAK APEX SPECTRA USING ABSORBANCE RATIOS AND THE CORRELATION BETWEEN SAMPLE AND REFERENCE SOLUTES APEX SPECTRA

Triplicate injections (10 μg on-column) of each sample. Spectral correlations calculated from 250 to 450 nm at 4-nm intervals.

Sample	Absorbance ratio		Normalized spectral correlation		
	280/360 nm	340/380 nm	Mean ($n = 9$)	t-Value	Tabulated t ($P = 0.01$ one-tailed)
Reference sulphasalazine	0.313	0.941			
	0.314	0.940	7.92		
	0.313	0.940			
Sulphasalazine + 10% A	0.342[a]	0.941			
	0.342[a]	0.941	4.40	23.4[a]	2.90
	0.341[a]	0.941			
Sulphasalazine + 1% A	0.316[a]	0.940			
	0.316[a]	0.941	6.55	8.24[a]	2.68
	0.317[a]	0.941			
Sulphasalazine + 0.1% A	0.314	0.941			
	0.313	0.941	7.92	0	2.55
	0.313	0.941			

[a] Denotes reliable detection of simultaneously eluted impurity.

TABLE II

ASSESSMENT OF PEAK HOMOGENEITY USING THE HEWLETT-PACKARD CHEMSTATION, DATA EDITOR, PEAK-PURITY ALGORITHM

Triplicate injections (10 μg on-column) of each sample.

Sample	Retention of compound A relative to sulphasalazine	
	+0.25 min.	+0.45 min
Reference sulphasalazine	1000	1000
	1000	1000
	1000	1000
Sulphasalazine + 10% A	989[a]	1000
	996[a]	998[a]
	997[a]	1000
Sulphasalazine + 1% A	1000	1000
	1000	1000
	1000	1000
Sulphasalazine + 0.1% A	1000	1000
	1000	1000
	1000	1000

[a] Denotes that the presence of the impurity could be detected.

number of data points used in the correlation.

Surprisingly, the data show that, in this instance, as few as four spectral data points (distributed equally through the spectra) are sufficient to give a reliable correlation coefficient. This may be explained by observations from overlaying the normalized spectra (Fig. 1). These show that the spectra differ by a small amount over a wide range of wavelengths, rather than display large differences over a very limited number of wavelengths. This, in turn, may be explained by the nature of UV–visible absorption, as electronic transitions are also accompanied by vibrational and rotational transitions within the molecules. The additive effect of these transitions cause UV–visible absorption bands to be broader than if only electrons were involved.

While the data presented are for one pair of related compounds only, a similarly small number of data points (six) was found to be necessary by Marr *et al.* [22] in order to characterize the difference between theophylline and the spectrally similar paraxanthine. This is further supported by recent work investigating protein purity [23]. Examination

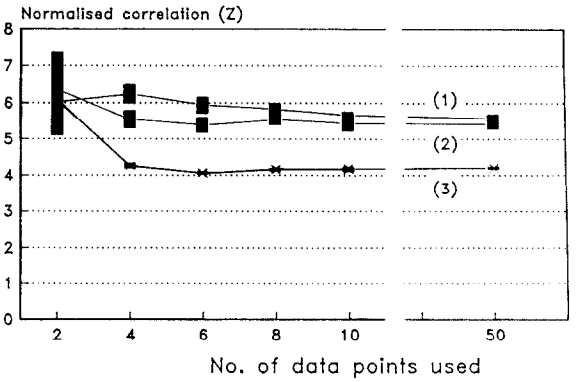

Fig. 2. Spectral correlation: comparison of the number of data points used in the correlation and the ability to identify the presence of a simultaneously eluted compound. 1 = Sulphasalazine; 2 = sulphasalazine + 1% A; 3 = sulphasalazine + 10% A. ■ = 95% confidence interval; × = mean (n = 9).

of the literature reveals that small spectral differences over a wide wavelength range are common for closely related compounds [31]. Hence it may be postulated that simultaneously eluted "impurities" will be efficiently detected using the *SCC* with a relatively small number of data points.

Fig. 2 also demonstrates that the number of data points in excess of this critical threshold figure determines, in part, the sensitivity of the correlation methods. This may be attributed to the nature of the correlation algorithm, as all variations (both positive and negative) between both of the two spectra compared will reduce the degree of correlation between them. Thus every data point (each incorporating experimental error, *i.e.*, signal noise), in excess of the threshold figure, will reduce the degree of correlation between spectra from the same sample and thus affect the discriminatory ability of the algorithm.

Peak identity and homogeneity assessment

The above observations gives rise to several implications for the use of correlation coefficients to assess the identity and homogeneity of a chromatographic peak. To examine this further, the LC model system was used. Evaluation of the purity of the complete chromatographic peak involves examination of the apparent peak identity coupled with assessment of peak homogeneity. Thus the apical spectral data can be compared with reference data,

TABLE III

ASSESSMENT OF PEAK PURITY BASED ON CORRELATION OF CHROMATOGRAPHIC PEAK APEX SPECTRA WITH SULPHASALAZINE APEX SPECTRA OF SIMILAR CONCENTRATION

Wavelengths used: 300, 320, 340, 360, 380, 400 and 420 nm. Triplicate injections (10 μg on-column) of each sample were correlated with triplicate injections of pure sulphasalazine.

Retention of compound A relative to sulphasalazine	Sample	Normalized spectral correlation		
		Mean (n = 9)	t-Value	Tabulated t (P = 0.01 one-tailed)
0 min	Reference sulphasalazine	8.09		
	Sulphasalazine + 10% A	4.84	16.5[a]	2.90
	Sulphasalazine + 1% A	7.00	4.96[a]	2.65
	Sulphasalazine + 0.1% A	8.15	−0.22	2.55
+0.25 min	Reference sulphasalazine	8.19		
	Sulphasalazine + 10% A	6.91	7.43[a]	2.76
	Sulphasalazine + 1% A	7.63	3.23[a]	2.76
	Sulphasalazine + 0.1% A	8.24	−0.23	2.55
+0.45 min	Reference sulphasalazine	8.85		
	Sulphasalazine + 10% A	7.74	6.65[a]	2.55
	Sulphasalazine + 1% A	8.15	3.42[a]	2.60
	Sulphasalazine + 0.1% A	8.67	0.95	2.58

[a] Denotes reliable detection of the presence of the impurity.

and the homogeneity of the peak can be assessed by comparing spectral data through the peak with the apex spectrum.

Table III shows, as would be expected, that the ability to determine the presence of a co-eluted analyte by comparing spectral data at the apex of the chromatographic peak diminishes as the degree of resolution of an overlapping peak increases. This is shown by a general increase in the mean normalized correlation coefficient and reduction of the *t*-value obtained, for similar levels of adulteration, with increasing chromatographic resolution. Thus a sensitive peak-homogeneity assessment algorithm may also be required in cases of partial resolution between the two compounds, to detect the presence of an impurity.

A common approach to the assessment of peak homogeneity, using continuous data in the time domain, is to express the data graphically using absorbance ratio plots. Fig. 3, where a single absorbance ratio, with a well selected wavelength pair, is used for the model system with a 0.45-min difference in retention time between the two mixture components, highlights a major drawback of this method, namely the deviations from the square waveform when assessing homogeneous peaks caused by noise. This is particularly significant at the front and trailing edges of the chromatographic peak due to the variation of the signal-to-noise ratio across the peak. Moreover, as operator involvement is required to interpret the plot, this approach is not suitable for the automation required to facilitate future incorporation in an expert system.

In developing a potentially operator-independent technique to take advantage of the foregoing results, it was decided to explore the use of correlating multiple chromatographic peak areas (Fig. 4). This could overcome many of the problems such as noise, peak tailing and spectral skewing.

Previously, the peak-area approach has been applied using chromatograms at two wavelengths of detection [13], with limited success owing to shortcomings in the peak-integration software then available, together with the disadvantages that affect all forms of *AR* algorithm. As it has been shown that a reduced wavelength data set with data points spread equally over the whole spectrum of interest maintains spectral discrimination, the collection and integration of the chromatographic signal at a representative number of wavelengths is possible using the detection system described. Hence the

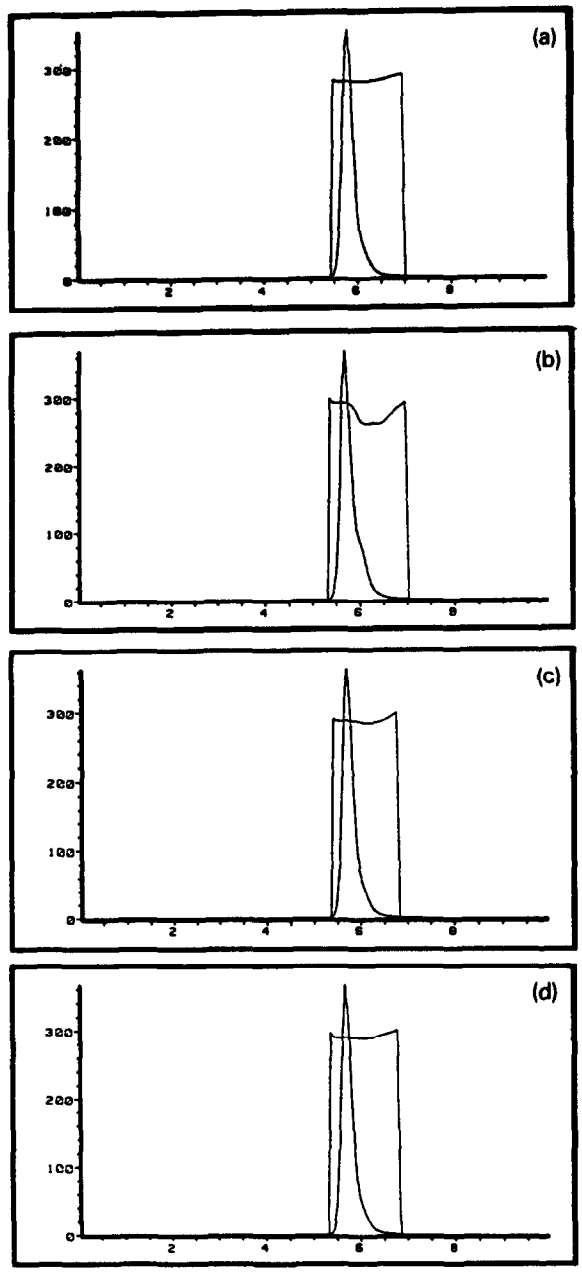

Fig. 3. Comparison of the absorbance ratio plot for (a) sulphasalazine with those obtained from partially co-eluted mixtures consisting of (b) sulphasalazine + 10% A, (c) sulphasalazine + 1% A and (d) sulphasalazine + 0.1% A. In all instances compound A elutes 0.45 min after sulphasalazine.

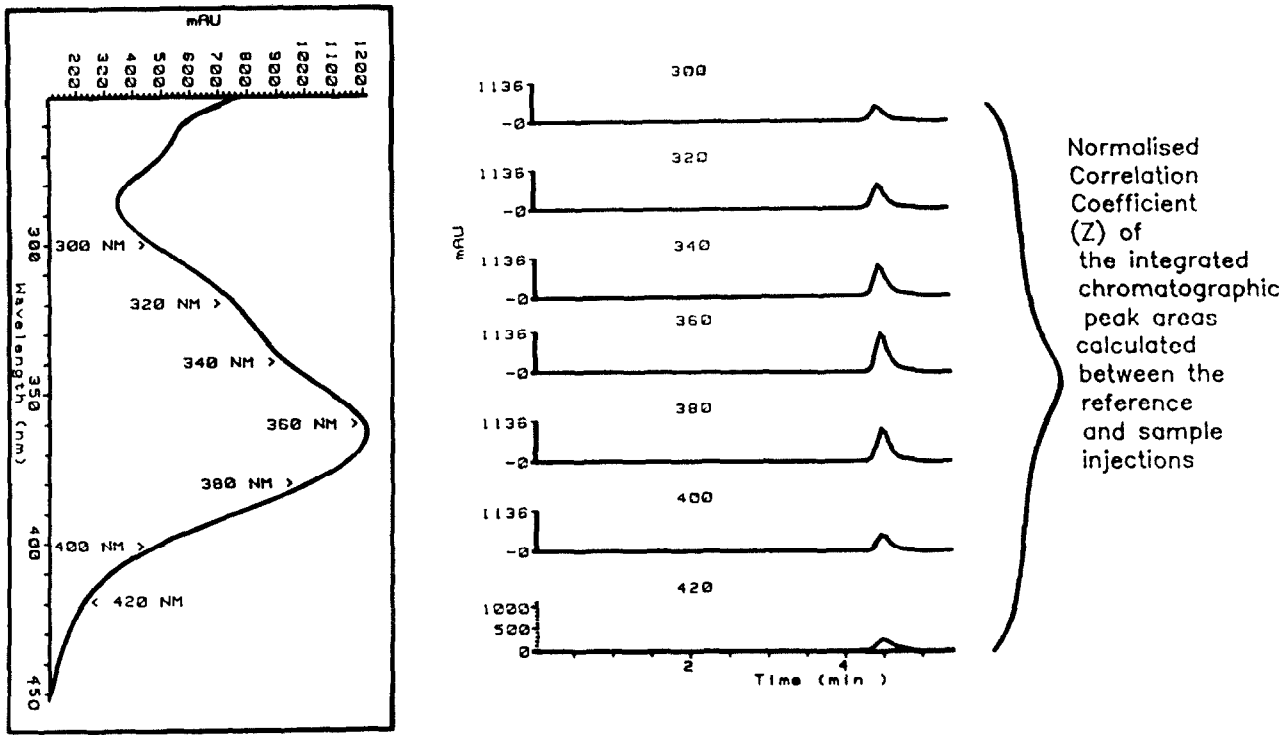

Fig. 4. The multiple peak area correlation technique.

TABLE IV

ASSESSMENT OF PEAK PURITY/HOMOGENEITY BASED ON CORRELATION OF CHROMATOGRAPHIC PEAK AREAS WITH THOSE FOR SULPHASALAZINE OF SIMILAR CONCENTRATION

Wavelengths used: 300, 320, 340, 360, 380, 400 and 420 nm. Triplicate injections (10 μg on-column) of each sample were correlated with triplicate injections of pure sulphasalazine.

Retention of compound A relative to sulphasalazine	Sample	Normalized spectral correlation		
		Mean ($n = 9$)	t-Value	Tabulated t ($P = 0.01$ one-tailed)
0 min	Reference sulphasalazine	9.45		
	Sulphasalazine + 10% A	4.77	39.9[a]	2.90
	Sulphasalazine + 1% A	7.05	22.3[a]	2.90
	Sulphasalazine + 0.1% A	9.29	1.41	2.72
+0.25 min	Reference sulphasalazine	8.26		
	Sulphasalazine + 10% A	4.74	20.7[a]	2.90
	Sulphasalazine + 1% A	6.88	8.10[a]	2.90
	Sulphasalazine + 0.1% A	8.82	−2.69	2.58
+0.45 min	Reference sulphasalazine	8.71		
	Sulphasalazine + 10% A	4.71	49.7[a]	2.82
	Sulphasalazine + 1% A	6.92	18.7[a]	2.65
	Sulphasalazine + 0.1% A	8.45	2.59[a]	2.55

[a] Denotes veliable detection of the presence of the impurity.

problems of wavelength selection can be overcome and consequently this allows operator-independent implementation. Further, high precision is now available in the integration algorithms used; indeed, a higher degree of correlation is observed between peak-area data than between apex data for many of the standards (Tables III and IV). This is analogous to the use of peak areas in analyte quantification.

It should be noted that, in the HP-1040 diode-array detector used, up to eight signal data collection channels exist, independent of spectra collection. The signal absorbance values are more accurate than spectral absorbances, as they are stored as three rather than two byte values. The use of other diode-array detectors, many of which rely on spectral data to reconstruct chromatographic plots at wavelengths other than that of the single wavelength at which the data was originally monitored, will clearly affect the implementation and the sensitivity of the peak-purity algorithm proposed.

In fact, a reliable single figure assessment of peak purity is obtained when multiple chromatographic peak area data are correlated with chromatographic data obtained from a reference sample, as shown in Table IV. Using the model system described, 1% of compound A in a mixture with sulphasalazine could be detected, regardless of the resolution between the two compounds, with quick and efficient use of the data collected, and without the need for prior knowledge of the impurity to maximize spectral discrimination. The general applicability of the *t*-test to this data is also shown in Table IV (and Tables I and III), although it is unlikely that the multiple peak-area correlation technique is truly detecting the presence of 0.1% of compound A at a 0.45-min difference in retention times. This highlights the approximate nature of the mathematical transformation and the probability-based statistical test used. In practice, such "borderline" samples could simply be re-tested. In addition, for a higher degree of confidence in the purity of a chromatographic peak, the confidence interval of the null hypothesis (*i.e.*, that the sample peak and reference peak spectral characteristics are indistinguishable) could be lowered.

CONCLUSION

The use of the multiple peak-area correlation technique minimizes the effects of variations in the signal-to-noise ratio of the data across the chromatographic peak, is not susceptible to the potential effects of spectral skewing and does not necessitate a knowledge of the spectral characteristics of possible simultaneously eluting species. Moreover, it gives a single figure purity parameter suitable for routine statistical analysis. Further work in this area is continuing.

ACKNOWLEDGEMENT

One of the authors (J.B.C.) thanks Kabi Pharmacia Therapeutics for providing a studentship for this research.

REFERENCES

1 J. Van Rompay, *J. Pharm. Biomed. Anal.*, 4 (1986) 725–732.
2 *British Pharmacopoeia 1988*, HM Stationary Office, London, 1988.
3 *The United States Pharmacopeia*, United States Pharmacopeial Convention, Rockville, MD, 21st Revision, 1984.
4 N. Ostojic, *Anal. Chem.*, 46 (1974) 1653–1659.
5 A. Bylina, D. Sybilska, Z. R. Grabowski and J. Koszewski, *J. Chromatogr.*, 83 (1973) 357–362.
6 R. N. Smith and M. Zetlein, *J. Chromatogr.*, 130 (1977) 314–317.
7 R. Yost, J. Stoveken and W. MacLean, *J. Chromatogr.*, 134 (1977) 73–82.
8 P. C. White, *J. Chromatogr.*, 200 (1980) 271.
9 A. F. Fell, H. P. Scott, R. Gill and A. C. Moffat, *Chromatographia*, 16 (1982) 69–78.
10 P. A. Webb, D. Ball and T. Thornton, *J. Chromatogr. Sci.*, 21 (1983) 447.
11 A. F. Fell, H. P. Scott, R. Gill and A. C. Moffat, *J. Chromatogr.*, 273 (1983) 3–17.
12 P. C. White and T. J. Catterick, *J. Chromatogr.*, 280 (1983) 376.
13 A. F. Fell, H. P. Scott, R. Gill and A. C. Moffat, *J. Chromatogr.*, 282 (1983) 123–140.
14 A. C. J. H. Drouen, H. A. H. Billiet and L. de Galan, *Anal. Chem.*, 56 (1984) 971–978.
15 G. B. Marcelle, M. S. Ahmed, J. M. Pezzuto, G. A. Cordell, D. P. Waller, D. D. Soejarto and H. H. S. Fong, *J. Pharm. Sci.*, 73 (1984) 396–398.
16 K. H. Law and N. P. Das, *J. Chromatogr.*, 388 (1987) 225–233.
17 P. C. White and T. J. Catterick, *J. Chromatogr.*, 402 (1987) 135.
18 P. C. White, *Analyst (London)*, 113 (1988) 1625–1629.
19 A. F. Fell, T. P. Bridge and M. H. Williams, *Anal. Chim. Acta*, 223 (1989) 175–182.
20 E. L. Inman, M. D. Lantz and M. M. Strohl, *J. Chromatogr. Sci.*, 28 (1990) 578–583.

21 A. F. Fell, J. B. Castledine, B. Sellberg, R. Modin and R. Weinberger, *J. Chromatogr.*, 535 (1990) 33–39.
22 J. G. D. Marr, G. G. R. Seaton, B. J. Clark and A. F. Fell, *J. Chromatogr.*, 506 (1990) 289–301.
23 J. Frank, Jzn, A. Braat and J. A. Duine, *Anal. Biochem.*, 162 (1987) 65–73.
24 H. J. P. Sievert, S. L. Wu, R. Chloupek and W. S. Hancock, *J. Chromatogr.*, 499 (1990) 221–234.
25 L. Huber, *Applications of Diode-Array Detection in HPLC*, Report 12-5953-2330, Hewlett-Packard, Avondale, PA, 1989.
26 J. C. Reid and E. C. Wong, *Appl. Spectrosc.*, 20 (1966) 320–325.
27 G. M. Clark and D. Cooke, *A Basic Course in Statistics*, Arnold, London, 2nd ed., 1983, pp. 333–337.
28 J. C. Miller and J. N. Miller, *Statistics for Analytical Chemistry*, Ellis Horwood, Chichester, Ellis Horwood, 1988, 57.
29 E. E. A. Askelof, N. Svartz and H. C. Willstaedt, *U.S. Pat.*, 2 396 145 (1946); *C.A.*, 40 (1946) 3472.
30 *HPLC Chemstation, Fact File*, Report 79994-90007, Hewlett-Packard, Avondale, PA, 1987.
31 A. C. Moffat (Editor), *Clarke's Isolation and Identification of Drugs*, Pharmaceutical Press, London, 2nd ed., 1986.