Journal of Chromatography, 626 (1992) 127–134
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 543

Enhanced chromatographic peak-purity evaluation of phenolic solutes using pH-induced spectral transformations

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 Therapeutics AB, Uppsala (Sweden)

ABSTRACT

The utility of pH-induced spectral shifts for enhanced peak-purity detection in its current form is limited. The practical option of using post-column technology to alter detection pH, rather than liquid chromatography (LC) with alkaline mobile phases, generates extra signal noise. In spite of parameter optimisation, and the use of LC-pump technology to add the post-column reagent, the increased noise is such that practical application of the post-column methodology for enhanced peak-purity determination is not realisable at levels of impurity less than 2% (w/w).

INTRODUCTION

A plethora of peak-purity/homogeneity assessment techniques exists to aid in the detection of simultaneously eluting liquid chromatography (LC) solutes. All such algorithms are limited, in part, by the spectral differences between the two or more compounds involved. This particularly applies in the analysis of pharmaceuticals. The spectral similarity of many drug molecules and their potentially related compounds, namely process impurities, degradation products and metabolites, highlights the need to maximise differences between the spectra, if low levels of related compounds are to be detected spectroscopically while eluting concurrently with the parent drug.

In compounds which contain an auxochrome, such as a phenolic group, reversible ionisation results in characteristic changes of the solute's spectroscopic properties. Several authors, including ourselves, have exploited this attribute to enhance LC solute identification using post-column reaction systems [1–3]. A variety of techniques have been used to characterise the spectral differences which arise. Hostettmann *et al.* [1] used an array of pH-shift inducing reagents, comparing the set of absorption maxima obtained for each unknown solute with data obtained from standards. Fell and co-workers [2,3] combined the spectral information obtained under both acid and alkali conditions through the use of difference spectroscopy, displaying the results graphically by employing a second-derivative transformation to highlight differences between the similar phloroglucinol derivatives examined [2]. Numerical characterisation of the pH-shifted difference spectra was also reported, based on absorbance ratios, for the characterisation of dipeptides containing a tyrosyl residue [3].

While the above techniques may be suitable for

Correspondence to: Professor A. F. Fell, Pharmaceutical Chemistry, University of Bradford, Bradford, BD7 1DP, UK.

0021-9673/92/$05.00

LC solute identification as proposed, they are not appropriate for peak-purity evaluation. The limitations of both graphical techniques and of using absorbance ratios have been previously described [4]. Recently the use of multiple-wavelength peak-area correlation has been proposed for the reliable assessment of peak-purity [5] which through the use of correlation coefficients allows multiple wavelengths to be incorporated into the calculation. The use of peak-area data gives a single figure assessment of purity which is, in principle, independent of the resolution between the overlapping components. In addition, the technique is equally applicable to isocratic and gradient elution [6].

This paper examines the use of pH-induced spectral transformations for enhancing the evaluation of peak-purity for phenolic LC solutes. The multiple peak-area correlation technique (MPACT) is used to characterise the spectral discrimination after post-column addition of alkali. The phenolic drug Olsalazine (OLZ) and several potentially related compounds are used as model pharmaceutical LC systems.

EXPERIMENTAL

Reagents

Methanol (HPLC grade, Rathburn Chemicals, UK), sodium dihydrogen phosphate monohydrate (Merck, Darmstadt, Germany) and sodium hydroxide pellets (GPR grade, BDH, Poole, Dorset, UK) were used as received. Both buffer salts were dissolved in distilled water and filtered using HVLP 0.45-μm filters (Waters, Millipore, Milford, MA, USA). All solutions were degassed prior to use. OLZ (reference material, batch 317 840) and the potentially related compounds (structures given in Fig. 1) were from Kabi Pharmacia Therapeutics AB, Uppsala, Sweden.

Apparatus

For both the LC method and the post-column reaction system the apparatus used consisted of two LKB 2150 HPLC pumps, connected using a high pressure mixer (Model No. 2152-400) and controlled using a LKB 2152 controller (all: LKB, Uppsala, Sweden). Injections were made using a Valco injection valve fitted with a 20-μl loop. The column used was stainless-steel (125 mm × 4.0 mm I.D.) packed with 5-μm Nucleosil C_{18} (Macherey-Nagel, Düren, Germany). The post-column addition of alkali was effected using a M-45 HPLC pump (Waters, Milford, MA, USA). An alkali-resistant PLRP-S 100 Å 8-μm (150 mm × 4.6 mm I.D.) column (Polymer Laboratories, Church Stretton,

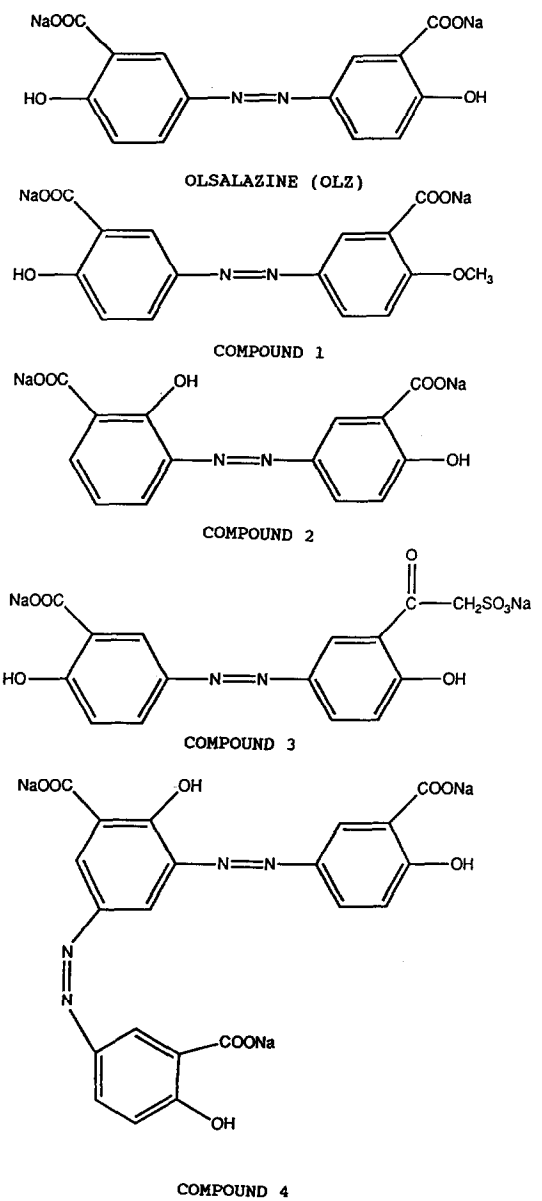

Fig. 1. Structure of Olsalazine (OLZ) and potentially related compounds.

Shropshire, UK) was placed between the pump and a zero volume tee-piece (Supelco, Saffron Walden, Essex, UK) to improve the signal-to-noise performance of the pump. Mixing of the eluent and alkali was generated by using a single-bead string-reactor (250 μm × 300 mm × 0.5 mm I.D.) (Supelco, Saffron Walden, Essex, UK) between the tee-piece and the detector. The post-column apparatus was configured as shown in Fig. 2.

Detection was effected using a HP-1040 diode-array detector (Hewlett-Packard, Waldbron, Germany). Data collection and evaluation were performed using the HP-85 computer, the HP-9000 series Workstation (with HPLC Chemstation software), the HP-7470 plotter and a 9121 dual-disc drive (all: Hewlett-Packard).

Under acidic conditions data were collected at the following wavelengths: 240, 270, 300, 330, 360, 390 and 420 nm. All signals had a bandwidth of 20 nm, and signal noise was reduced further through the use of a reference signal collected at 550 nm with a 100-nm bandwidth.

Detection of the solutes at alkali pH was performed at: 340, 370, 400, 430, 460, 490 and 520 nm. All signals had a bandwidth of 20 nm, and signal noise was reduced further through the use of a reference signal collected at 595 nm with a 10-nm bandwidth. The latter was limited by the spectral range of the detector used.

Acidic LC conditions

The mobile phase, pumped at 1.0 ml/min, consisted of methanol–10 m*M* NaH_2PO_4 buffer, adjusted to pH 5.0. Gradient elution was required to simultaneously elute OLZ and the potentially related compounds (all of which can be readily separated from OLZ using LC [7]). Using mobile phase A [comprising of methanol–buffer (10:90, v/v)] and mobile phase B [comprising of methanol–buffer (90:10, v/v)] the following gradient program was used; 100% A for 1 min, then a linear increase in mobile phase B to 100% over the next two minutes.

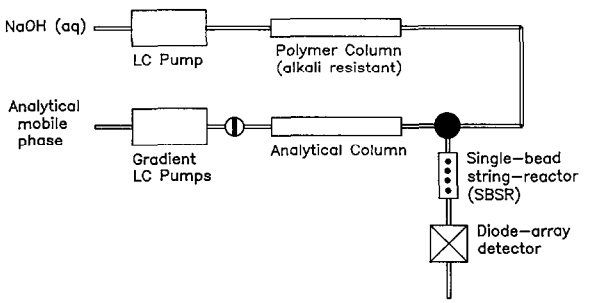

Fig. 2. Post-column continuous-flow reaction system.

Post-column noise reduction

The conditions for optimum signal-to-noise ratio, within the limits set for a suitable change in post-column pH (apparent pH of the methanolic mobile phase, pH* 12.6–12.7), were found to be: flow-rate, 0.5 ml/min; concentration of alkali added 0.7 *M* NaOH.

Computation

Correlation coefficients, *r*, were calculated using:

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

where A_{1i} and A_{2i} are the peak-areas at *i* nm for chromatograms 1 and 2, respectively [8].

It should be noted that this is the appropriate version of the correlation coefficient for use in spectral comparisons since if chromatograms 1 and 2 are of identical samples, differing only in concentration, at *i* nm: $A_{1i} = m \cdot A_{2i}$, where m = constant. Because the product-moment correlation coefficient determines deviations from the relationship, $y = mx + c$ (where c may be a non-zero value), it is not suitable for this application.

Since correlation coefficients are not normally distributed, Student's *t*-test was performed after transformation of the data to give the normalised correlation coefficient (NCC) using:

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

The values of the NCC are approximately normally distributed [9].

Since it was found that the sample standard deviations varied significantly and were dependent, in part, on the absolute value of the NCC, it was considered that the population standard deviations could not be assumed to be equal. Thus the appropriate equation used to calculate the *t* values was [10]:

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

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

Triplicate injections of each sample were made, these injections being bracketed between two sets of reference chromatograms (also in triplicate). This gave rise to nine possible correlations between each sample and each of the two reference sets, albeit with reduced degrees of freedom (DF). These sets of nine correlation coefficients were normalised and compared with the set of nine NCCs generated by correlating the two sets of reference injections using Student's *t*-test. A 95% one-way confidence limit ($t_{TAB} = 1.94$, $DF = 6$) was found to be a suitable discriminator between the reference data and significantly dissimilar sample data (*i.e.*, impure chromatographic peaks).

RESULTS AND DISCUSSION

Comparison of spectral data

It can be shown, using a spectrophotometer, that differences between the spectra of a phenolic drug, in this case OLZ, and certain potentially related compounds may be enhanced by the presence of an alkaline environment. This is shown in Fig. 3, using data collected from the chromatographic injections. Moreover, given the structure of a possible impurity which may be eluting simultaneously with the parent molecule, it is apparent that the benefit, or otherwise, of detection in alkaline conditions may be anticipated.

For the model systems presented it can be observed that the spectral characteristics of the potentially related compounds, compound 1 and compound 4 differ most from OLZ at elevated pH. It is not surprising that in both cases there is a different number of phenolic groups present in these molecules compared with OLZ.

Note that the pK_a values for the two phenolic auxochromes of OLZ are 11.0 and 11.9 [11], thus an environment of pH 13.9 is required to ensure total ionisation. Clearly this is not practicable for this type of experimentation. Thus, results are based upon the maximum achievable ionisation at a defined pH* *ca.* 12.6.

Compound 2 is spectrally more similar to OLZ at the alkaline pH. This may be explained by the loss of weak bonding between the hydrogen of the (ortho) phenolic group and the azo-bond nitrogen when ionised. The spectral differences between OLZ and compound 3 are not greatly affected by pH. This can be explained by considering the two factors involved when there are no differences involving the auxochrome. Firstly, the bathochromic shift induced by the alkali results in a loss of spectral discrimination through band broadening. Counterbalancing this is the hyperchromic shift which is also induced. This improves the signal/noise quality of the data. The application of these observations to aid in the determination of chromatographic peak-purity, and the limitations arising, are examined below.

Chromatographic peak-purity assessment

The results of applying MPACT to the LC systems containing overlapping peaks of OLZ and one of the potentially related compounds, are given in Tables I to IV and summarised in Table V as discussed below.

Acidic LC. From the results it may be observed that, in all cases, the limit of detection for the simultaneously eluting impurity is greater than 1% w/w. This highlights the limits of LC–diode-array detection often found for method development and validation.

In general, the results obtained from the present series of experiments typify those reported for most pharmaceuticals and their potentially related molecules, and arise primarily as a consequence of the strong spectral similarity between these compounds [12–16].

Comparison of the mean correlations between the sets of reference data, bracketing the samples, show both day-to-day and within-day variations. This illustrates the importance of bracketing the samples between standards when estimating the extent of signal variation due to noise.

It is possible to exploit the enhanced spectral differences between OLZ and compound 1 or compound 4 using alkali LC conditions [17]. However, a significant limitation in raising the pH, using for example polymer-based columns, is that this imposes a severe constraint on the range of pH available for optimising the separation of the known impurities. Moreover, not all impurities will be

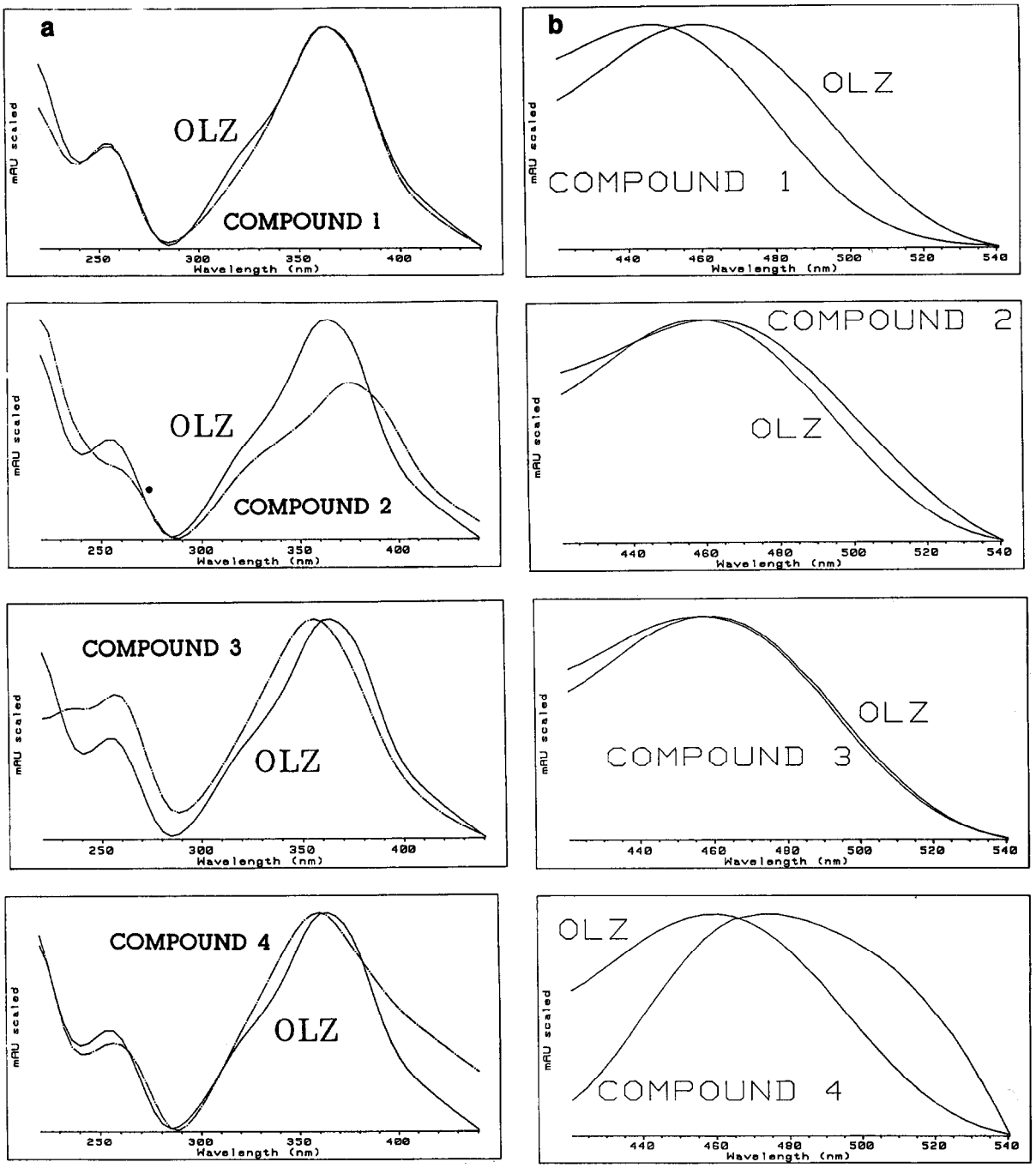

Fig. 3. (a) Spectra of OLZ and the potentially related compounds under acidic conditions (pH 5.5). Compound 1: $r = 0.999$, NCC = 3.81; compound 2: $r = 0.979$, NCC = 2.28; compound 3: $r = 0.987$, NCC = 2.51; compound 4: $r = 0.987$, NCC = 2.53. (b) Spectra of OLZ and the potentially related compounds under alkali conditions (pH* 12.6). Compound 1: $r = 0.966$, NCC = 2.03; compound 2: $r = 0.994$, NCC = 2.88; compound 3: $r = 0.992$, NCC = 2.78; compound 4: $r = 0.935$, NCC = 1.70.

TABLE I

THE LIMIT OF DETECTION FOR THE SIMULTANEOUSLY ELUTING POTENTIALLY RELATED PRODUCT (COMPOUND 1) ADDED TO OLZ

Added compound 1 (% w/w)	Reference			
	A[a]		*B*[b]	
	Mean NCC	*t*[d]	Mean NCC	*t*[d]
Acidic LC				
Ref.[c]	7.19	–	–	–
0.5	7.61	−4.60	7.64	−3.32
1.0	7.31	−1.53	7.44	−1.72
2.0	7.06	1.88	7.24	−0.32
4.0	6.93	4.17*[e]	7.34	−1.33
Ref.[c]	7.61	–	–	–
6.0	6.46	5.23*	6.49	5.03*
8.0	6.45	9.20*	6.53	8.52*
10	6.29	11.3*	6.40	9.87*
20	5.74	16.0*	5.81	15.2*
Compound 1 only	3.81	33.2*	3.82	33.1*
Post-column addition of alkali				
Ref.[c]	5.71	–	–	–
0.5	5.66	0.26	5.71	0.02
1.0	5.63	0.51	5.69	0.15
2.0	5.42	1.65	5.52	1.28
4.0	5.49	1.40	5.56	0.84
Ref.[c]	5.50	–	–	–
6.0	5.38	1.14	5.50	0.00
8.0	5.06	3.50*	5.07	3.32*
10	4.71	7.72*	4.80	6.30*
20	4.13	13.4*	4.13	12.9*
Compound 1 only	2.03	35.6*	2.03	35.6*

[a] Correlation between samples and the preceeding reference injections.
[b] Correlation between samples and the successive reference injections.
[c] Correlation between the two pairs of bracketing reference injections.
[d] $t_{TAB} = 1.94$, $p = 0.05$.
[e] * Denotes a significant difference using the *t*-test (*i.e.*, detection of the co-eluting species).

TABLE II

THE LIMIT OF DETECTION FOR THE SIMULTANEOUSLY ELUTING POTENTIALLY RELATED PRODUCT (COMPOUND 2) ADDED TO OLZ

For key to superscripts see Table I.

Added compound 2 (% w/w)	Reference			
	A[a]		*B*[b]	
	Mean NCC	*t*[d]	Mean NCC	*t*[d]
Acidic LC				
Ref.[c]	6.74	–	–	–
0.5	7.29	−2.04	7.12	−1.37
1.0	6.67	0.31	6.75	−6.20
2.0	6.13	3.10*[e]	6.24	2.56*
4.0	5.64	5.84*	5.71	5.51*
Ref.[c]	6.96	–	–	–
6.0	5.33	13.9*	5.46	12.8*
8.0	5.10	15.8*	5.21	15.0*
10	4.83	18.3*	4.90	17.7*
20	4.21	23.6*	4.24	23.3*
Compound 2 only	2.28	40.3*	2.28	40.2*
Post-column addition of alkali				
Ref.[c]	5.27	–	–	–
0.5	4.84	4.38*	5.38	−0.92
1.0	5.48	−1.35	5.39	−0.69
2.0	5.35	−1.12	5.81	−4.79
4.0	5.14	0.90	5.55	−2.59
Ref.[c]	5.21	–	–	–
6.0	6.01	−5.62	5.04	1.91
8.0	5.62	−4.28	5.16	0.52
10	5.04	2.13*	5.27	−0.61
20	4.57	8.83*	4.80	4.70*
Compound 2 only	2.88	33.2*	2.91	32.7*

preferentially discriminated in alkali conditions.

The better strategy should be post-column addition of the pH-shifting reagent. This allows the optimum separation of all known compounds by regular means through the selection of an appropriate column and mobile phase. Furthermore, detection can be effected at any pH (whether acid or alkaline) to ensure optimum peak-purity detection.

Post-column addition of alkali. Post-column continuous-flow analysis has previously been applied to shifting eluent pH [1–3], and the problem of increased signal-to-noise ratio has been described [18]. Differences in the spectral characteristics of the mobile phase and the alkali, together with the slight fluctuations in pressure from both pumps which will affect the post-column eluent constitution, are the

TABLE III

THE LIMIT OF DETECTION FOR THE SIMULTANEOUSLY ELUTING POTENTIALLY RELATED PRODUCT (COMPOUND 3) ADDED TO OLZ

For key to superscripts see Table I.

Added compound 3 (% w/w)	Reference			
	A[a]		*B*[b]	
	Mean NCC	*t*[d]	Mean NCC	*t*[d]
Acidic LC				
Ref.[c]	6.85	–	–	–
0.5	6.84	0.02	7.18	−1.04
1.0	6.60	0.97	7.01	−0.60
2.0	6.15	3.10*[e]	6.39	2.06*
4.0	5.74	5.21*	5.93	4.41*
Ref.[c]	7.46	–	–	–
6.0	5.56	7.60*	5.63	7.34*
8.0	5.29	8.70*	5.34	8.50*
10	5.03	9.76*	5.07	9.60*
20	4.41	12.3*	4.43	12.2*
Compound 3 only	2.51	20.0*	2.51	20.0*
Post-column addition of alkali				
Ref.[c]	5.55	–	–	–
0.5	6.17	−3.99	5.50	0.27
1.0	5.84	−1.73	5.32	1.21
2.0	5.49	0.24	5.49	0.33
4.0	5.26	1.86	5.84	−1.24
Ref.[c]	5.49	–	–	–
6.0	5.83	−1.49	5.27	1.86
8.0	5.80	−1.21	5.21	2.13*
10	5.30	1.30	5.04	4.06*
20	5.01	2.61*	4.72	6.21*
Compound 3 only	2.78	29.2*	2.74	29.7*

TABLE IV

THE LIMIT OF DETECTION FOR THE SIMULTANEOUSLY ELUTING POTENTIALLY RELATED PRODUCT (COMPOUND 4) ADDED TO OLZ

For key to superscripts see Table I.

Added compound 4 (% w/w)	Reference			
	A[a]		*B*[b]	
	Mean NCC	*t*[d]	Mean NCC	*t*[d]
Acidic LC				
Ref.[c]	6.74	–	–	–
0.5	7.20	−2.72	6.81	−0.34
1.0	6.99	−1.92	6.58	0.90
2.0	6.48	2.04*[e]	6.22	3.58*
4.0	5.90	6.59*	5.78	7.36*
Ref.[c]	6.60	–	–	–
6.0	5.48	10.1*	5.59	9.28*
8.0	5.22	12.6*	5.29	12.1*
10	5.05	14.2*	5.09	13.9*
20	4.46	19.7*	4.48	19.6*
Compound 4 only	2.53	37.6*	2.53	37.6*
Post-column addition of alkali				
Ref.[c]	5.66	–	–	–
0.5	5.94	−1.24	5.74	−0.33
1.0	5.66	−0.01	5.82	−0.80
2.0	5.49	0.83	5.67	−0.06
4.0	5.58	0.41	5.35	1.56
Ref.[c]	5.53	–	–	–
6.0	5.37	0.95	5.27	1.53
8.0	5.03	3.29*	5.04	2.96*
10	4.84	4.51*	4.88	4.21*
20	4.35	7.90*	4.38	7.66*
Compound 4 only	1.70	25.9*	1.71	25.8*

main factors involved in reducing the signal/noise quality of the analytical data.

An LC pump was found to be more suited to the application of adding post-column eluent than the previously used peristaltic pump. Noise was further reduced by raising back-pressure of the post-column eluent. This was achieved by placing an alkali-resistant LC polymer column before the mixing tee, allowing the post-column pump to work at its designated back-pressure specification. Alternatively a back-pressure regulator and/or pulse dampener could have been used. The signal-to-noise (S/N) ratio was optimised using a simplex method [19],

TABLE V

SUMMARY TABLE: LIMIT OF DETECTION FOR THE SIMULTANEOUSLY ELUTING POTENTIALLY RELATED COMPOUNDS

Compound added	Conditions	
	Acidic LC	Post-column addition of alkali
1	4–6%	8%
2	2%	10–20%
3	2%	8–20%
4	2%	8%

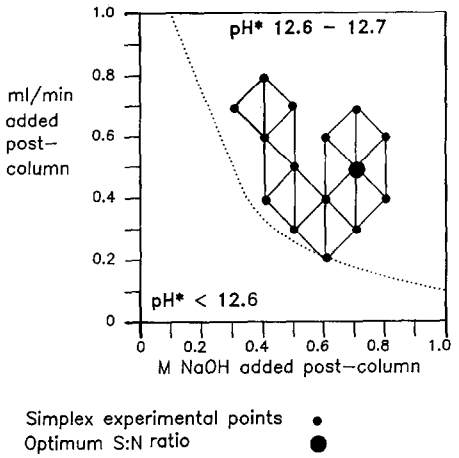

Fig. 4. Simplex optimisation of the post-column signal-to-noise ratio.

with the alkali concentration and post-column reagent flow-rate being varied within the constraint that permitted the highest achievable pH to be obtained (pH* 12.6–12.7) (Fig. 4).

Despite this, Tables I–V show that increased noise (*i.e.*, lower correlation between reference standards) results from the incorporation of the post-column system. The consequence of this is that, in all the model systems presented, a higher limit of detection for the simultaneously eluting impurity was recorded.

Further work is continuing in the Authors' laboratory to develop simpler and more effective methods of post-column pH modification. The use of alternate auxochromes with lower pK_a values, facilitating total ionisation and thus reducing noise, is also being investigated.

ACKNOWLEDGEMENTS

One of the authors (JBC) would like to thank Kabi Pharmacia Therapeutics AB for kindly providing the studentship for this research.

REFERENCES

1 K. Hostettmann, B. Domon, D. Schaufelberger and M. Hostettmann, *J. Chromatogr.*, 297 (1984) 137–147.
2 A. F. Fell, T. Z. Woldemariam, P. A. Linley, Ge Jian, M. D. Luque De Castro and M. Valcarcel, *Anal. Chim. Acta*, 234 (1990) 89–95.
3 A. F. Fell, J. B. Castledine, B. Sellberg, R. Modin and R. Weinberger, *J. Chromatogr.*, 535 (1990) 33–39.
4 J. B. Castledine, A. F. Fell, R. Modin and B. Sellberg, *J. Pharm. Biomed. Anal.*, 9 (1991) 619–624.
5 J. B. Castledine, A. F. Fell, R. Modin and B. Sellberg, *J. Chromatogr.*, 592 (1992) 27–36.
6 J. B. Castledine, A. F. Fell, R. Modin and B. Sellberg, *J. Pharm. Pharmacol.*, (1992) in press.
7 Data on file, *Kabi Pharmacia Therapeutics*, Uppsala, Sweden.
8 J. C. Reid and E. C. Wong, *Applied Spectroscopy*, 20 (1966) 320–325.
9 G. M. Clark and D. Cooke, *A Basic Course in Statistics*, Arnold, London, 2nd ed., 1983, pp. 333–337.
10 J. C. Miller and J. N. Miller, *Statistics for Analytical Chemistry*, Ellis Horwood, Chichester, 2nd ed., 1988, p. 57.
11 Data on file, *Kabi Pharmacia Therapeutics*, Uppsala, Sweden.
12 A. F. Fell, H. P. Scott, R. Gill and A. C. Moffat, *J. Chromatogr.*, 282 (1983) 123–140.
13 P. C. White, *Analyst*, 113 (1988) 1625–1629.
14 T. D. Wilson, W. F. Trompeter and H. F. Gartelman, *J. Liq. Chromatogr.*, 12(7) (1989) 1231–1251.
15 J. G. D. Marr, G. G. R. Seaton, B J. Clark and A. F. Fell, *J. Chromatogr.*, 506 (1990) 289–301.
16 H. K. Chan and G. P. Carr, *J. Pharm. Biomed. Anal.*, 8 (1990) 271–277.
17 J. B. Castledine, *PH.D. Thesis*, University of Bradford, Bradford, 1992.
18 J. B. Castledine, A. F. Fell, B. Sellberg, R. Modin, M. D. Luque De Castro and M. Valcarcel, *J. Pharm. Biomed. Anal.*, 8 (1990) 1079–1082.
19 J. C. Berridge, *Techniques for the Automated Optimisation of HPLC Separations*, Wiley, London, 1985, p. 126.