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Diode array detection of low level co-eluting species in high-performance liquid chromatography*

N.H. ANDERSON,† M.R. GRAY and C.J. HINDS

Sterling Research Group, Willowburn Avenue, Alnwick, Northumberland NE66 2JH, UK

Abstract: A simple and sensitive method for detecting low levels (0.5 and 1.0%, w/w) of co-eluting species in HPLC has been developed. This method is based on the subtraction of normalized peak up-slope and down-slope spectra from that of the apex. Visual inspection of the resultant "difference spectra" allows for a qualitative judgement regarding the integrity of the peak under consideration.

Keywords: *Chromatographic peak integrity assessment; diode array spectroscopy; HPLC; antazoline; danazol; co-eluting species.*

Introduction

The application of diode array detection to the determination of HPLC peak integrity is well established. Techniques employed in this respect range from simple spectral overlays [1] to the examination of absorbance ratio information [2] and analysis by various mathematical deconvolution routines [3, 4]. All of these are affected by several parameters including: (a) differences between the analyte and impurity spectra; (b) deviations from absolute co-elution; (c) amount of impurity present; (d) relative molar extinction coefficients of the analyte and impurity.

The relative influence of these parameters, the experimental conditions and the overall aim of the experiment will determine which technique is used. Where mere confirmation of peak integrity is all that is required, for example when validating an analytical method, the use of a rapid and sensitive test is sufficient. These requirements are satisfied by the spectral subtraction method reported here. The application of this method to two representative examples, antazoline and a co-eluting degradation product and a mixture of a non-steroidal glucocorticoid with danazol is discussed. Spectra were taken from approximately 10% of the peak height (up-slope and down-slope) and subtracted from that of the apex using a simple "macro" program (see Table 1). Prior to subtraction, each spectrum was normalized to an arbitrary value of 10,000.

Experimental

Chemicals and reagents

Antazoline hydrochloride was from R.W. Unwin. The non-steroidal glucocorticoid and danazol were from Sterling Drug Inc. Acetonitrile and methanol were Rathburn HPLC-grade. The antazoline degradation product (*N*-benzyl-*N*-phenylglycinylethylenediamine) was extracted from a refluxed basic solution of antazoline hydrochloride and recrystallized from propan-2-ol.

Equipment

A Hewlett–Packard 1090M LC system (with Revision 3.21 and Color View software) with a Hewlett–Packard 1040 diode array detector, and a Hewlett–Packard 8451A diode array spectrophotometer were used.

LC conditions

Glucocorticoid. A 10 × 0.46 cm Hypersil 5 μm ODS column was used, the mobile phase being acetonitrile with 0.5% (w/v) ammonium acetate solution at pH 4.0 (55:45, v/v). A flow rate of 2.0 ml min^{-1} and a temperature of 40°C were maintained with an injection volume of

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† Author to whom correspondence should be addressed.

Table 1

'Macro' Program

NAME PURITY	
PICK A,,START TIME,3,,	Choose
PICK B,, END TIME,3,,	section of
CHROMATOGRAM A:B	chromatogram
DRAW 3,X	
GETSCALARS	
SPECTRUM X,220:400	Take APEX
NORMALIZE	spectrum
ENTER	
PICK A,, POINT ON UP-SLOPE,3,,	
SPECTRUM A,220:400	Choose points
NORMALIZE	on UP and
SUBTRACT	DOWN-slope
PICK B,, POINT ON DOWN-SLOPE,3,,	
SPECTRUM B,220:400	
NORMALIZE	
EXCHANGE Z,Y	
SUBTRACT	
MERGE	Merge and draw
FORMAT MERGED	subtracted
SMOOTH 13	spectra
DRAW 3,X,220:400,−500:500	

20 µl. Under these conditions a resolution factor [5] from separate injections was calculated as 0.20.

Antazoline. A 30 × 0.39 cm µ-Bondapak 10 µm nitrile column was used, the mobile phase being methanol and water with an overall concentration of 1.0% (w/v) ammonium acetate at pH 6.8 (40:60, v/v). A flow rate of 2.0 ml min^{-1} under ambient conditions was used with an injection volume of 20 µl. Under these conditions a resolution factor [5] from separate injections was calculated as 0.14.

Sample preparation

Solutions of the glucocorticoid and danazol were accurately prepared at about 0.1 mg ml^{-1} in mobile phase and diluted with each other to give ratios of glucocorticoid–danazol 100:0, 200:1 and 0:100 on a % (w/w) basis.

Solutions of antazoline hydrochloride and its degradation product were accurately prepared at about 0.25 mg ml^{-1} in mobile phase and diluted with each other to give ratios of antazoline hydrochloride–degradation product of 100:0, 100:1 and 0:100 on a % (w/w) basis.

Results and Discussion

Overlaid and subtracted spectra, taken from HPLC peaks with and without co-eluting species, are presented.

Figures 1 and 2 demonstrate how spectral subtraction greatly enhances the small differences in spectra resulting from the presence of a co-eluting species. The spectral subtraction method assumes that where the co-eluting species is present at a low level, its effect on the spectrum taken from the analyte peak apex is insignificant, and that this spectrum therefore represents that of the pure analyte. Where there is no co-eluting species (Fig. 3), subtraction of normalized spectra taken at any point throughout the peak from that of the apex should result in a straight line. This was found to be the case after allowing for the noise inherent in the procedure (Fig. 4). However, where a co-eluting species is present, its effect upon a spectrum taken at a point near the peak base, say at 10% of peak height, results in a readily detectable deviation from linearity when this spectrum is subtracted from that of the apex (Fig. 2). Where the same spectra are simply overlaid an unambiguous conclusion regarding the integrity of the peak cannot be made (Fig. 1). This was also true for the examination of absorbance ratio information versus time produced using Hewlett–Packard's "Color View" software, which is not readily amenable to presentation here.

For this method to work the minor component must have a significant absorbance relative to that of the analyte at some point over the spectral range. For example, although there is apparently a large difference between the UV spectra of the glucocorticoid and

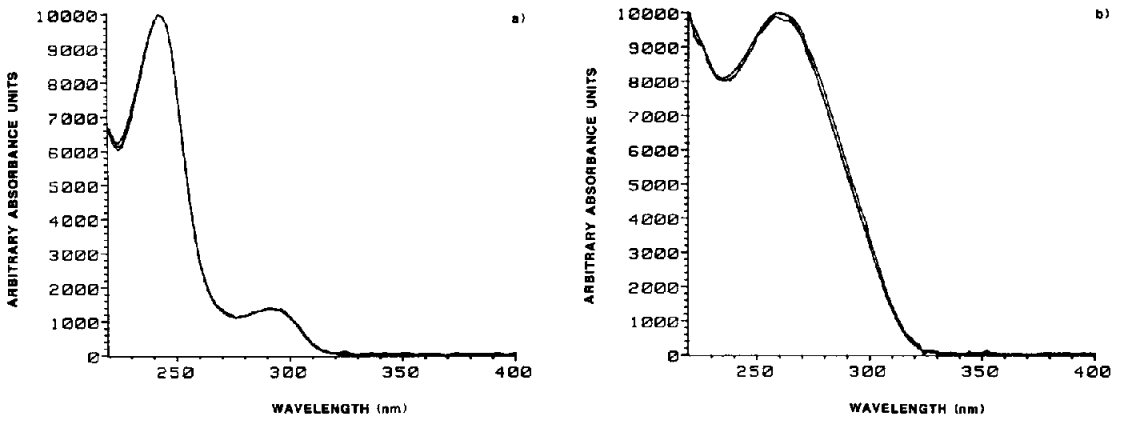

Figure 1
Overlaid spectra normalized to an arbitrary value of 10,000 taken from the apex and 10% peak height (up-slope and down-slope) of (a) an antazoline HPLC peak (152 mAu at 254 nm) containing 1% (w/w) of its degradation product, and (b) a glucocorticoid HPLC peak (238 mAu at 260 nm) containing 0.5% (w/w) danazol. The similarity of the spectra make their individual assignment impractical here.

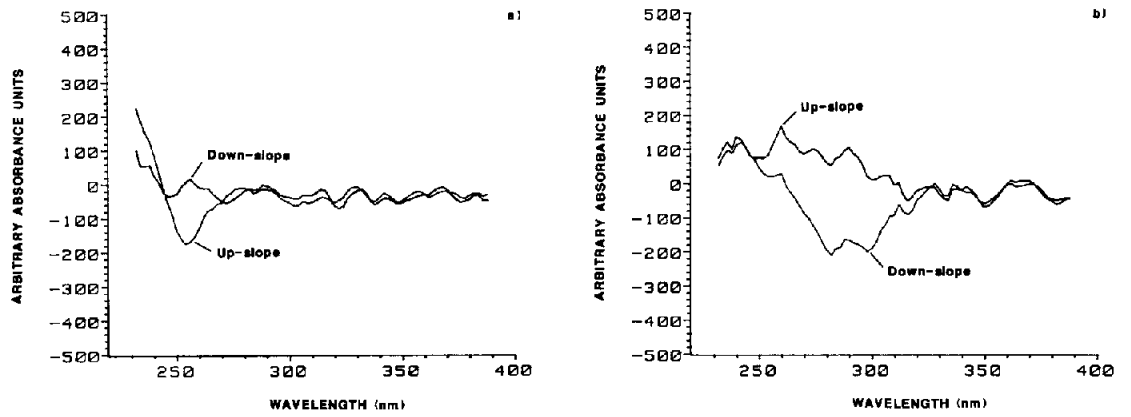

Figure 2
Up-slope and down-slope spectra presented in Fig. 1. (a) and (b) subtracted from that of the peak apex for (a) antazoline with co-eluting species, and (b) the glucocorticoid with co-eluting species. The *y*-axis is an expanded portion of the 10,000 arbitrary units to which the spectra were normalized prior to subtraction.

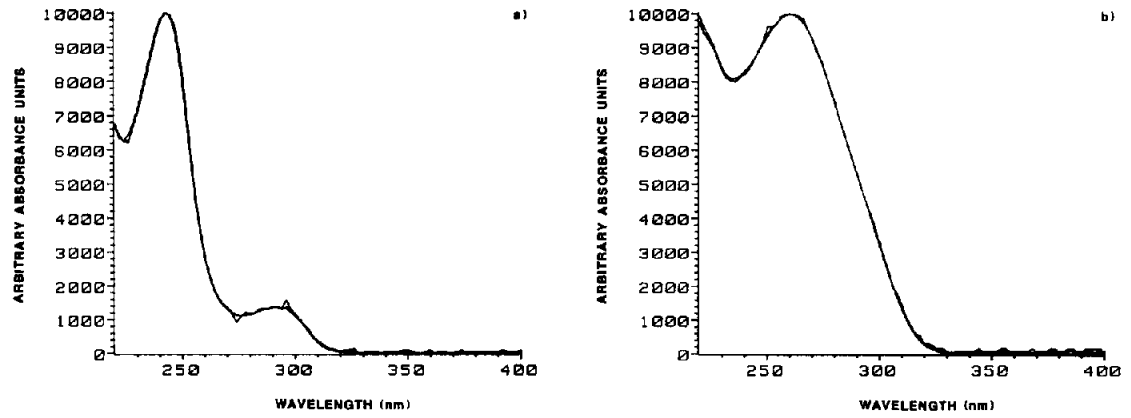

Figure 3
Overlaid spectra normalized to an arbitrary value of 10,000, taken from the apex and 10% peak height (up-slope and down-slope) of (a) an antazoline HPLC peak (152 mAu at 254 nm), and (b) a glucocorticoid HPLC peak (244 mAu at 260 nm). The similarity of the spectra make their individual assignment impractical here.

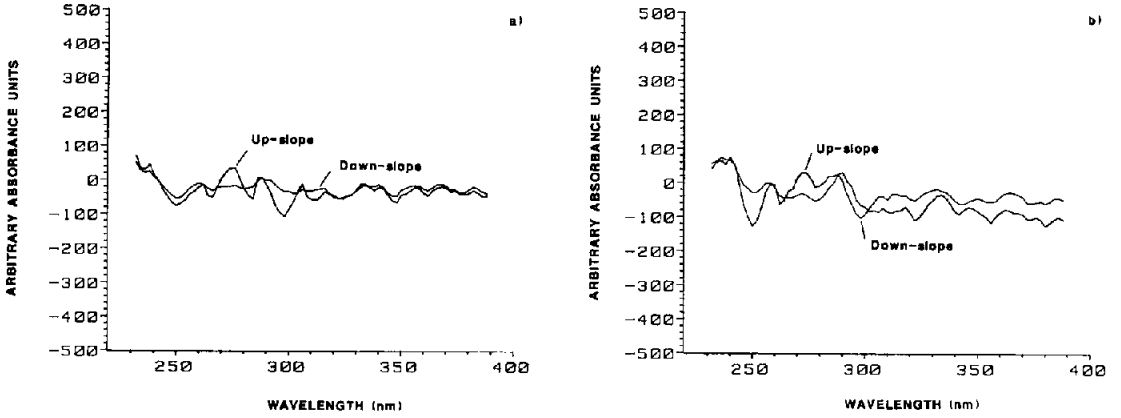

Figure 4
Up-slope and down-slope spectra presented in Fig. 3. (a) and (b) subtracted from that of the peak apex for (a) antazoline, and (b) the glucocorticoid. The *y*-axis is an expanded portion of the 10,000 arbitrary units to which the spectra were normalized prior to subtraction.

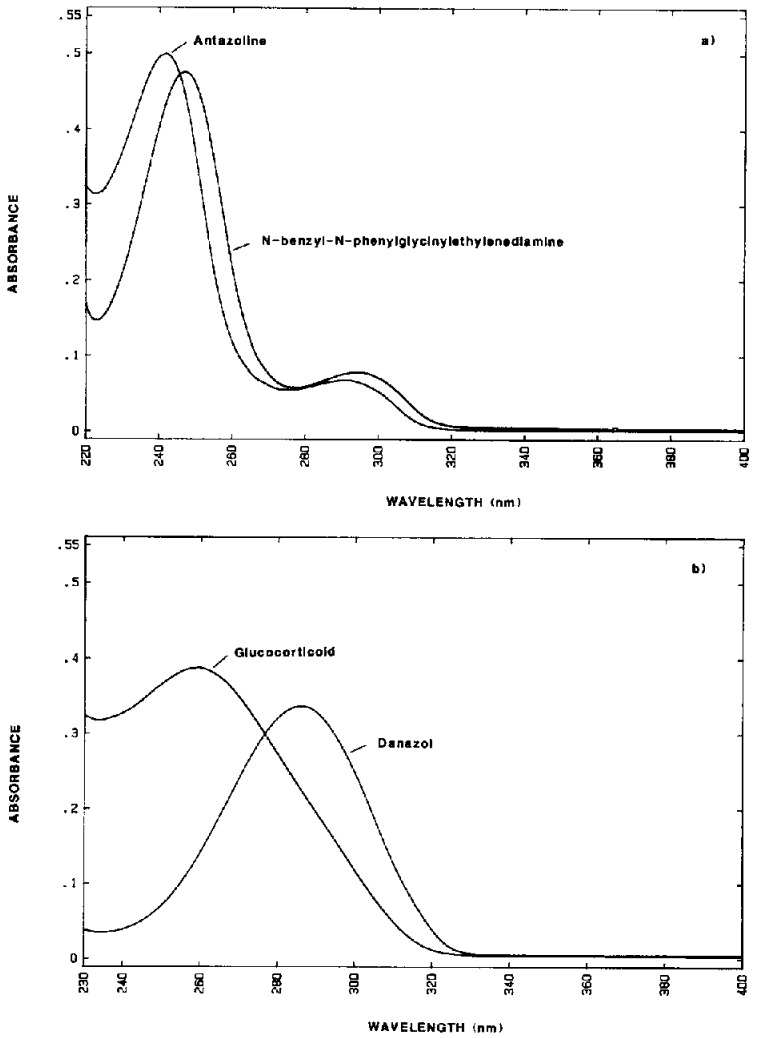

Figure 5
Overlaid spectra of (a) antazoline and its degradation product, and (b) the glucocorticoid and danazol. Molar extinction coefficients measured by spectrophotometer are, respectively, antazoline (15200 at 242 nm), *N*-benzyl-*N*-phenylglycinyl-ethylenediamine (13200 at 247 nm), glucocorticoid (18000 at 259 nm) and danazol (11400 at 286 nm) in the appropriate mobile phase.

danazol at approximately 235 nm (Fig. 5), when danazol is present at only 0.5% (w/w), its absorbance at this wavelength, and consequently the effect it produces in the respective "difference spectrum", are essentially zero. By contrast, at approximately 280 nm, the danazol makes a much larger contribution to the analyte spectrum (Fig. 5), producing an easily detectable effect on the "difference spectrum" (Fig. 2b). Naturally, the greater the difference in spectra between the analyte and co-eluting species, the more sensitive the technique becomes. It should be noted that in order to apply this method, the analyte peak height should be at least 100 mAu at the wavelength of maximum absorption. This is readily achievable by altering the amount of material injected, provided there is no loss of peak shape.

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