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Note

Limitation of assessing high-performance liquid chromatographic peak purity with photodiode array detectors

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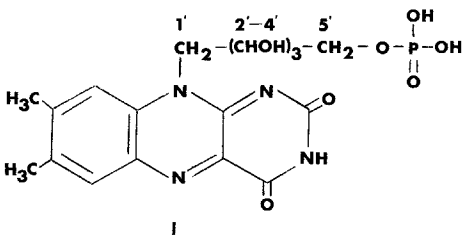
Many publications have now appeared in the literature describing the use of photodiode array detection (PDAD) in liquid chromatography for determining peak purity from on-the-fly, UV–VIS spectra[1]. Typically, techniques employing absorbance ratios[2,3], spectral suppression[4,5], and comparison of normalized up-slope, apex, and down-slope spectra[6] are used.

One criterion for determination of peak purity by these techniques is an impurity spectrum that is reasonably different from the analyte spectrum. The greater the difference between the spectra, the lower is the detection limit that is obtainable for a coeluting impurity. However, it is well known that UV–VIS spectra characteristically lack the fine structure necessary for elucidating subtle differences between similar compounds, especially when the chromophore is well insulated from the structure difference. Since isomeric impurities and analyte degradants are, in many cases, similar in structure to the analyte, failure of the PDAD techniques to detect some coeluting impurities might not be an uncommon occurrence. This paper describes three systems in which known, chromatographically resolved or nearly resolved impurities have spectra that are so similar to that of the analyte that the impurity peaks would not be readily detected in a hypothetically unresolved system.

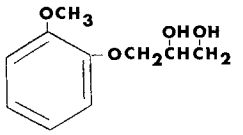
EXPERIMENTAL

Reagents

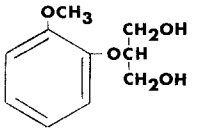
Riboflavin-5′-phosphate (I) (Sigma, St. Louis, MO, U.S.A.) and hydrocortisone (IV) (Roussel, New York, NY, U.S.A.) were used as received. Guaifenesin (II) and 2-(2-methoxyphenoxy)-1,3-propanediol (III) described previously[7] were used. All other reagents were reagent grade.



I



II



III

Apparatus

A ALC/GPC 204 high-performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with a Model 7125 fixed-loop injector (Rheodyne, Berkeley, CA, U.S.A.), a Model 6000A reciprocating pump (Waters Assoc.), and a HP 1040A photodiode array detector (Hewlett Packard, Palo Alto, CA, U.S.A.), was used.

Columns and eluents

The eluent used for I was acetonitrile–0.1 *M* sodium acetate (10:90) adjusted to pH 5.7 with 1 *M* acetic acid (prior to adding acetonitrile). The reversed-phase column used for I was a 25 cm × 4.6 mm I.D. Zorbax® C_{18} column with 5-μm packing (Dupont, Wilmington, DE, U.S.A.). For II, III and IV, the eluent was methanol–water (55:45). The column used for IV was a 25 cm × 4.6 mm I.D. μBondapak® C_{18} column with 10-μm packing (Waters Assoc.). A similar column, but one that had lost efficiency through long usage, was purposely selected for II and III. The flow-rate was 1.5 ml/min in all cases.

Sample preparation

Compound I was dissolved in eluent at a concentration of 300 μg/ml and 50 μl was injected into the chromatograph. Solutions containing compounds II and III, both together and individually, were prepared in eluent at a concentration of 600 μg/ml for II and 120 μg/ml for III. Aliquots of 10 μl were injected. Compound IV was dissolved in methanol–0.1 *M* sodium hydroxide (10:90) at a concentration of 100 μg/ml and allowed to stand for 7 h. A 20-μl aliquot was injected.

RESULTS AND DISCUSSION

A striking example of spectra similarity is the high-performance liquid chromatographic (HPLC) examination of commercial lots of riboflavin-5′-phosphate (I). As shown in Fig. 1, seven major peaks are eluted corresponding to I, 3′- and 4′-phosphates, 4′,5′-; 3′,5′-; and 3′,4′-diphosphates, and riboflavin (Ia)[8]. However, the normalized UV–VIS spectra of four of the peaks are coincident, as shown (the remaining three peaks also have spectra similar to I). The chromophore is totally insulated from the presence or absence of phosphate functional groups on the side chain, although the hydrophobicity of the molecule is greatly affected. The presence of any of these compounds coeluting with I would not be detected at any level. In fact, the presence of isomers actually coeluting with I cannot be discounted.

Other examples of impurities or degradants with spectra similar to that of the analyte have been found. Guaifenesin (II) contains an isomeric impurity (III) at levels ranging from 0.2–2% of the II concentration and is easily resolved from III[7]. How-

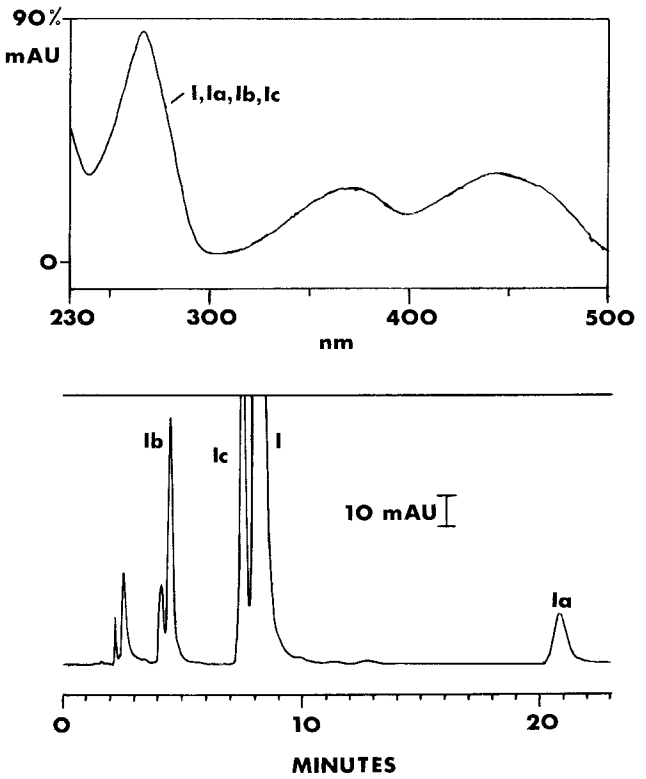


Fig. 1. HPLC and UV–VIS spectra of 300 μg/ml of riboflavin-5′-phosphate (I), riboflavin (Ia), and riboflavinphosphate analogues. A volume of 50 μl was injected. Detection was at 266 nm. For other parameters, see text.

ever, for this work, a deteriorated column was used with an eluent sufficiently strong to cause poor resolution between II and III, and the concentration of III was increased to 20% of the concentration of II. The unresolved peak shown in Fig. 2 was deconvoluted by injecting II and III individually at the same concentrations as in the mixture. Although the severe tailing results in some III being present throughout the width of the unresolved peak, the ratio of absorbance (and approximate concentration) of II:III ranges from about 3.5:1 at point A to about 14:1 at point D. Nevertheless, the four normalized spectra shown in Fig. 2 are virtually coincident, except for a slight difference in the 210–220 nm region, a region which is frequently susceptible to interference from solvent impurities. Absorbance ratios at 210/280 nm and 230/280 nm were also constant between points A and D.

Like the previous example, the resolved hydrocortisone (IV) degradants possess nearly the same normalized spectra as the parent compound (Fig. 3). The broadband steroid chromophore is not greatly affected by the structure changes associated with the degradation. Had one of these degradants coeluted with IV, unambiguous detection with PDAD techniques could be expected only at a relatively high concentration, if at all.

In summary, the similarities in the normalized spectra of each of these systems

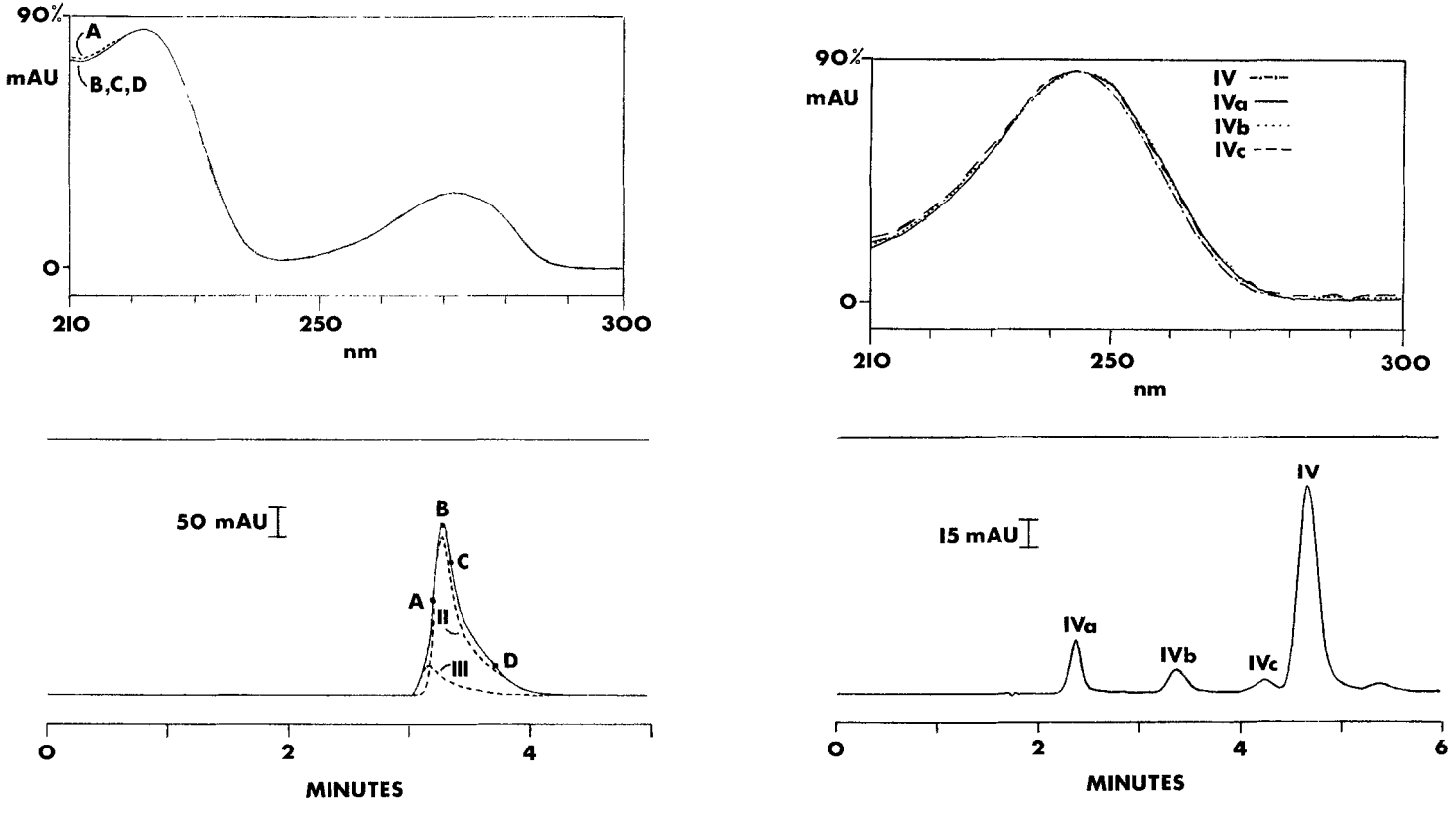


Fig. 2. HPLC and UV spectra of 600 μg/ml guaifenesin (II) and 120 μg/ml isomeric impurity (III). A volume of 10 μl was injected. Detection was at 280 nm. For other parameters, see text.

Fig. 3. HPLC and UV spectra of 100 μg/ml hydrocortisone (IV) and degradants. A volume of 20 μl was injected. Detection was at 254 nm. For other parameters, see text.

representative of analyte degradants and isomeric impurities make it apparent that purity determination using PDAD techniques may frequently lead to a false indication of peak purity. In any event, the appearance of a constant absorbance ratio, complete analyte suppression, or coincident normalized spectra obtained throughout the peak width should be considered an indication of peak purity, not a proof. For HPLC methods in which impurities are likely to be similar in structure to the analyte (*e.g.*, assays of aged pharmaceutical samples), it remains that peak purity can be assessed with the highest degree of certainty only by peak collection followed by analysis with a different chromatographic system or with a spectroscopic system such as infrared or mass spectroscopy that is sensitive to minor structure differences.

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REFERENCES

1 R. E. Majors, H. G. Barth and C. H. Lochmuler, *Anal. Chem.*, 56 (1984) 300R–349R.
2 P. A. Webb, D. Ball and T. Thornton, *J. Chromatogr. Sci.*, 21 (1983) 447–452.
3 A. C. J. H. Drouen, H. A. H. Billiet and L. de Galan, *Anal. Chem.*, 56 (1984) 971–978.
4 G. T. Carter, R. E. Schiesswohl, H. Burke and R. Yang, *J. Pharm. Sci.*, 71 (1982) 317–321.
5 A. F. Fell, H. P. Scott, R. Gill and A. C. Moffat, *J. Chromatogr.*, 282 (1983) 123–140.
6 J. C. Miller, S. A. George and B. G. Willis, *Science*, 218 (1982) 241–246.
7 G. W. Schieffer, W. O. Smith, G. S. Lubey and D. G. Newby, *J. Pharm. Sci.*, in press.
8 P. Nielsen, P. Rauschenbach and A. Bacher, *Anal. Biochem.*, 130 (1983) 359–368.