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PHOTOMETRIC DETECTION AT 185 nm FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH EITHER ISOCRATIC OR GRADIENT ELUTION

ASSAY OF MIXTURES OF POLYETHYLENE GLYCOL OLIGOMERS

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SUMMARY

The use of acetonitrile–water mobile phases for reversed-phase high-performance liquid chromatography allows photometric detection at wavelengths as low as 185 nm in both the isocratic and gradient elution modes. At 185 nm almost all compound types exhibit usable detection sensitivity, making the UV spectrophotometer a "universal" detector. Application of detection at 185 nm for the gradient elution separation of various polyethylene glycols allows the routine analysis of mixtures of these compounds, either in unesterified samples or in samples containing both the unesterified and esterified derivatives.

INTRODUCTION

Berry[1,2] has demonstrated the advantage of UV detection in high-performance liquid chromatography (HPLC) at wavelengths as low as 190 nm. Thus, compounds with low or minimal absorptivity at higher wavelengths, such as alcohols, ethers and ketones, can be detected at 190 nm with sensitivities in the mid-nanogram to low-microgram range. When various experimental problems had been solved, Berry[2] also showed that gradient elution can be carried out with 0–100% water–acetonitrile as mobile phase plus detection at 210 nm. This allows the routine exploratory separation of unknown samples, and leads to estimates of the best conditions for corresponding isocratic separations[3,4]. Berry[2] further found that gradient elution at lower wavelengths (190–200 nm) is not feasible with full-range (0–100%) gradients. Along similar lines, Binder[5] reported the use of detection at 188 nm for various sugars.

While attempting the reversed-phase HPLC analysis of various mixtures containing oligomeric polyethylene glycols (PEGs), we have discovered that gradient elution with limited-range water–acetonitrile gradients (5–25%) is useful for these

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samples. It is possible to detect the separated species with adequate sensitivity at 185 nm, but not at higher wavelengths. Other compound types (alcohols, ethers, ketones, alkyl chlorides) also show usable sensitivity at this wavelength, but not at wavelengths of 200 nm or higher. Some of the problems reported by Berry[2], using full-range gradient elution at 210 nm, were not encountered in this study. In any case, we consider the approach used here to be routinely applicable for many otherwise difficult samples.

This study also allowed us to compare the optimization of the separation conditions in gradient elution with recently developed theory[3]. PEGs of higher molecular weight show a systematic increase in the steepness of isocratic plots of log capacity factor (k') *vs.* the volume fraction (φ_B) of mobile phases composed of water (A) and acetonitrile (B), with C_8 reversed-phase columns. This behavior differs from that found for other sample compounds in similar reversed-phase HPLC systems[6], leading to the recommendation here that convex (rather than linear) gradients be used for optimal resolution of individual PEG oligomers.

EXPERIMENTAL

Equipment

The liquid chromatograph consisted of a Varian 5000 ternary-gradient system (Varian, Palo Alto, CA, U.S.A.) plus an LDC Spectromonitor-III variable-wavelength detector (Laboratory Data Control, Riviera Beach, FL, U.S.A.) and an SP4100 data system (Spectra Physics, Santa Clara, CA, U.S.A.). The Technicon Tricyclics column (Technicon, Tarrytown, NY, U.S.A.) on which all separations were performed is a 7-μm C_8 reversed-phase column. Other small-particle C_8 columns should perform similarly. Ambient temperature was used.

The UV spectrum of nitropropane, used as a reference to measure the relative extinction coefficients of several compounds by HPLC, was obtained with a Cary 219 spectrophotometer (Varian).

Reagents

Various polyethylene glycols were purchased from Fluka (Buchs, Switzerland). Nitric acid (A200S) and phosphoric acid (A242) were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). The acetonitrile had a UV cutoff at 188–190 nm and was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) or Fisher Scientific (product A-998). All water was purified initially by means of a Milli-Q system (Millipore, Bedford, MA, U.S.A.).

RESULTS AND DISCUSSION

Detector performance

Linearity. The linearity characteristics of the detector operated with 25% acetonitrile–buffer are illustrated in Figs. 1 and 2, for nitropropane as solute. Nitropropane was chosen for these studies because of its roughly constant absorptivity over the region 180–200 nm (Fig. 3) and its high absorptivity, which makes column overloading unlikely under these experimental conditions. The maximum solute absorbance for a linear detector response is seen in Fig. 1 to decrease with decreasing

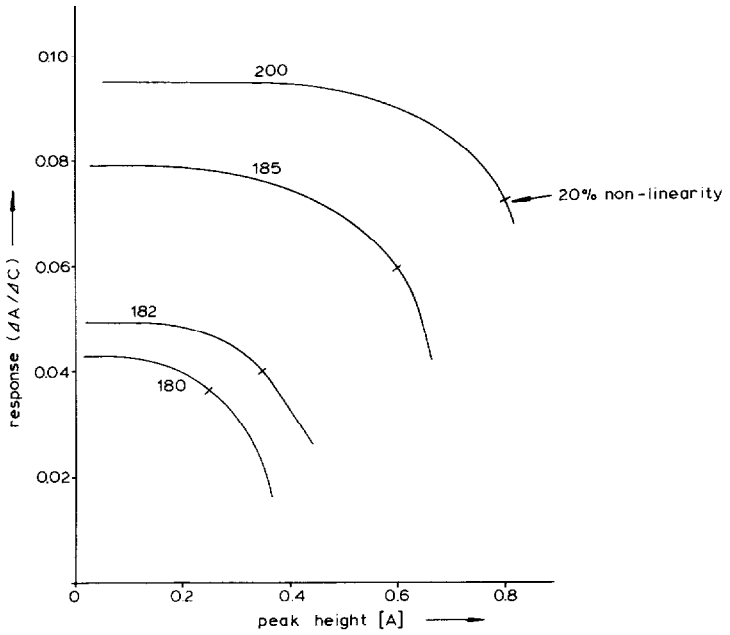

Fig. 1. Detector linearity *versus* wavelength for nitropropane as solute. Mobile phase, 75% 1 m*M* phosphoric acid plus 5 ppm nitric acid–acetonitrile; flow-rate, 6.0 ml/min. Other conditions as in Experimental.

wavelength, as expected. The absorbance for a 20% non-linear response is indicated for each curve of Fig. 1. Absorbances for 2% non-linearity are about half of these latter values, which means that at 185 nm the detector is linear within about 2% for solute net absorbance values below 0.3 (corrected for baseline absorbance).

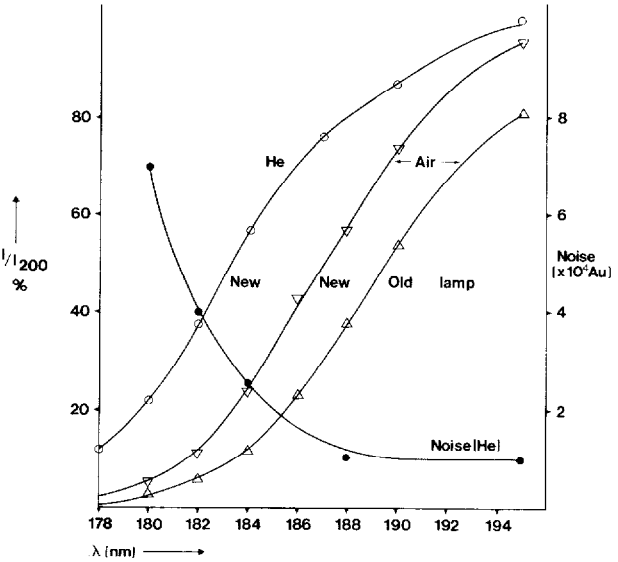

Fig. 2. Photocell response and noise *versus* wavelength. Conditions as in Fig. 1.

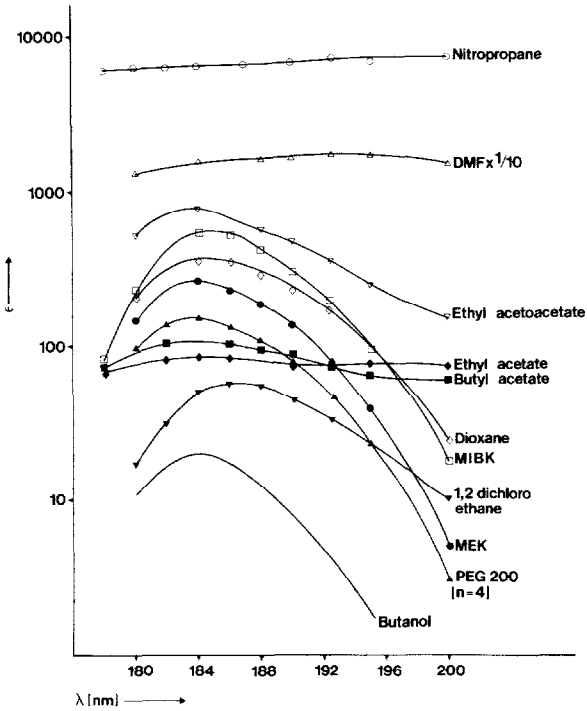

Fig. 3. Molar absorptivities *versus* wavelength for various compounds. Values determined chromatographically, using conditions as in Fig. 1. DMF = Dimethylformamide; MIBK = methyl isobutyl ketone; MEK = methyl ethyl ketone.

The lamp output intensity as measured by the reference photodiode of the detector decreases as the lamp ages and a "haze" builds up on the lamp window. This is illustrated by the "new" *vs.* "old" solid curves at the bottom of Fig. 2 (detector flushed with "air"). A "new" lamp is defined as one with less than 100 h of service, and the data in Figs. 1 and 2 are with "new" lamps. A further cause of a decrease in lamp intensity is the presence of oxygen in the detector. Although the present detector does not provide for helium or nitrogen flushing of the optical bench, it was possible to encase the entire detector in a transparent plastic bag and flush the system with helium. As indicated by the left-land curve at the bottom of Fig. 2, this had a major effect on the apparent lamp intensity at the photo-detector. It can be assumed that detector linearity (and noise, see below) will degrade as the lamp ages, but the data in Fig. 2 suggest that this could be more than compensated for by provision for helium flushing of the optical bench.

Signal-to-noise ratio. The detector sensitivity depends on both solute absorptivity and baseline noise levels. These two components of sensitivity are illustrated in Figs. 2 and 3 for various compounds. At 185 nm, there is a good compromise between increasing baseline noise and maximum absorptivity for several compound classes. For a minimum detection limit of 3.5 signal-to-noise ratio (see ref. 7), this corresponds to the detection of concentrations ranging from 35 μM for butanol to 100 nM for nitropropane. In other terms, the minimum detection sensitivity (for alcohols)

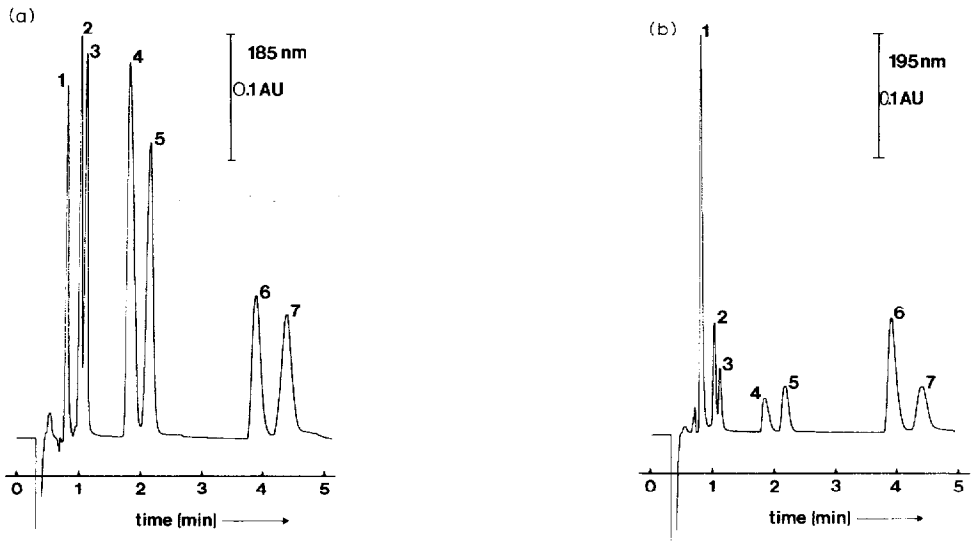

Fig. 4. Varying solute sensitivity at (a) 185 nm and (b) 195 nm. Conditions as in Fig. 1, except mobile phase contains 7.5% acetonitrile. Sample: 100 μl of 0.05 mM dimethylformamide (1), 2 mM dioxane (2), 4 mM tetraethylene glycol (3), 84 mM butanol (4), 4 mM methyl ethyl ketone (5), 9 mM ethyl acetate (6) and 1.5 mM ethyl acetoacetate (7).

at 185 nm would be about 0.3 μg of solute. Thus, in the worst case (alcohols), detection at 185 nm is at least as sensitive as with a refractometric detector[8]. For other compound types shown in Fig. 3, the photometric detection sensitivity is considerably greater than for the refractometer.

Some examples of the use of detection at 185 nm

The preceding discussion suggests that detection at 185 nm can be useful for

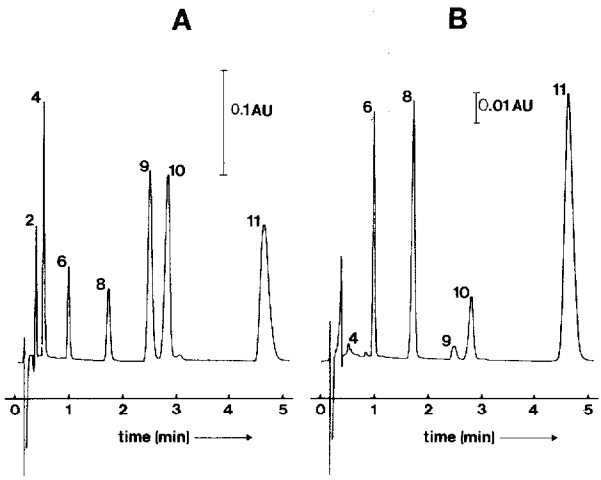

Fig. 5. Varying solute sensitivity at (a) 185 nm and (b) 200 nm. Conditions as in Fig. 1. Sample: 100 μl of 2 mM dioxane (2), 84 mM butanol (4), 9 mM ethyl acetate (6), 0.1 mM nitropropane (8), 3 mM methyl isobutyl ketone (9), 5 mM 1,2-dichloroethane (10) and 30 mM butyl acetate (11).

the more sensitive detection of certain compound types in HPLC separations. In fact, for detection wavelengths less than 200 nm the photometric detector becomes essentially a "universal" detector for most compounds. Apart from a few compound types such as saturated hydrocarbons, nitriles and amines, every compound in the sample will be detectable at concentrations above the trace level. However, it is clear from Fig. 3 that even in the low-UV wavelength region, there are substantial differences in compound sensitivity as the wavelength is varied over the region 185–200 nm. This suggests that the use of HPLC runs with detection at two different wavelengths in this region can provide additional qualitative information on the nature of sample components ("wavelength ratioing", ref. 8, p. 592). Variation of detector wavelength within the region 185–200 nm can also provide substantial selectivity in detecting certain compound types. These possibilities are illustrated by the repetitive separation of different samples under the same conditions (except for detection wavelength) in Figs. 4 and 5. For the various compound types represented in these separations, good detection sensitivity is observed for every compound at some wavelength in the region 185–200 nm. Relative response at the wavelength pairs 185/195 or 185/200 nm is seen to vary widely, however, implying substantial detector selectivity when it is desired to emphasize the detection of certain compounds while suppressing the detection of others.

An interesting practical example of the application of low-UV detection is shown in Fig. 6. An aged laboratory sample of *n*-hexanol was extracted with water and the extract was analysed by reversed-phase HPLC using the conditions given in Fig. 6, and with detection at (a) 184 nm and (b) 200 nm. No useful information resulted from the separation in (b) with detection at 200 nm. However, the separation

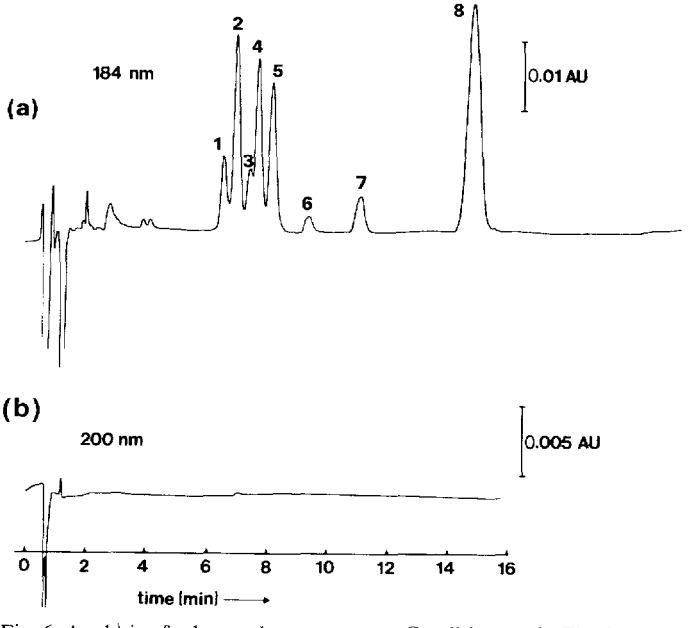

Fig. 6. Analysis of *n*-hexanol water extract. Conditions as in Fig. 1, except the flow-rate is 2.0 ml/min. (a) Detection at 184 nm; (b) detection at 200 nm. Sample size, 100 μl of extract.

in (a) at 184 nm shows the presence of hexanol (peak 8) plus seven other compounds which represent degradation products of hexanol and/or impurities in the original sample. Presumably these compounds (1–7) are mainly oxidation products which are more water soluble than the starting *n*-hexanol.

Gradient elution and the analysis of mixtures of PEGs

Figs. 7 and 8 show the HPLC separation of two PEG samples which contain different proportions of the individual oligomers. These separations were monitored at 185 nm, which is seen to yield adequately sensitive detection. PEG samples can be assayed by gas chromatography (GC) (*e.g.*, ref. 9), but the procedure requires sample derivatization before analysis and in our experience is generally more complicated and potentially less precise than assay by HPLC with low-wavelength detection. When the PEGs occur as minor products in esterified PEG surfactants (*e.g.*, lauryl esters), then GC is inapplicable, whereas HPLC analysis is still practicable.

Melander *et al.*[10] have described the HPLC separation of PEG samples under conditions similar to those described here, but with detection at 200 nm. It is clear from the data for PEG 200 in Fig. 3 that the sensitivity of detection at 200 nm is about 5% of that at 185 nm, suggesting that the latter wavelength is more suitable for the routine analysis of PEG samples by HPLC. The PEGs can be readily eluted using a 5–25% acetonitrile–water gradient, which is fortunate because of our finding of the same "baseline hump" described by Berry[2] for monitoring at 200 nm, when the gradient was extended to 40% acetonitrile. We were not able to eliminate these baseline peaks at 40% acetonitrile using the alumina purification scheme reported by Berry[2]. Therefore, until more highly purified acetonitrile becomes available, acetoni-

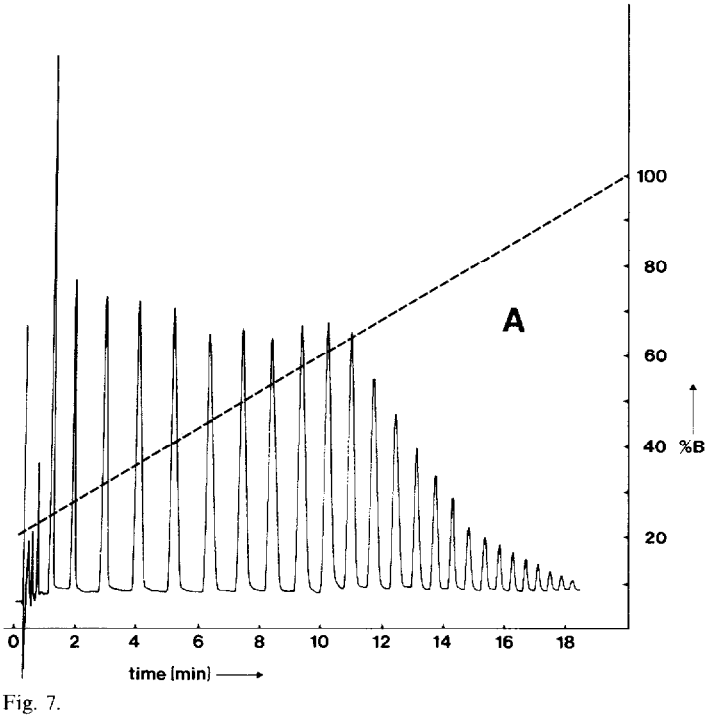

Fig. 7.

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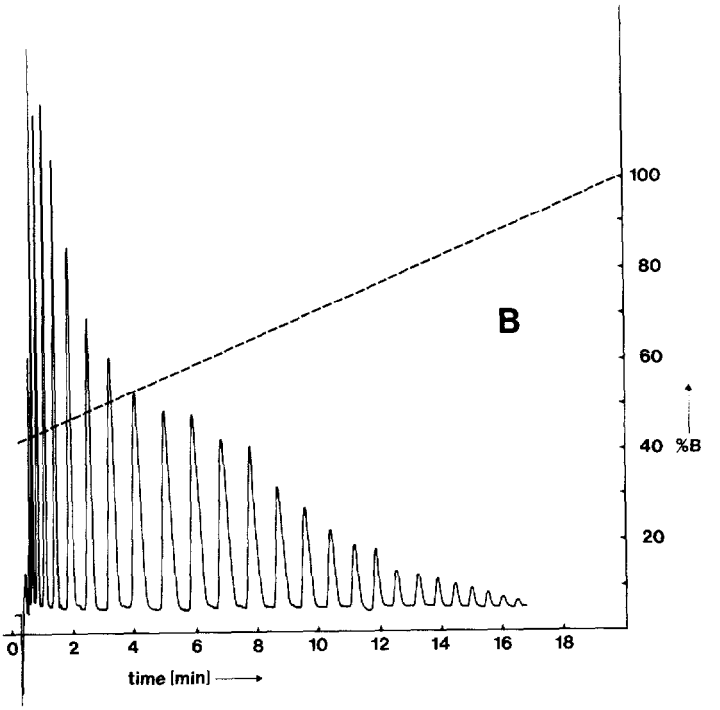
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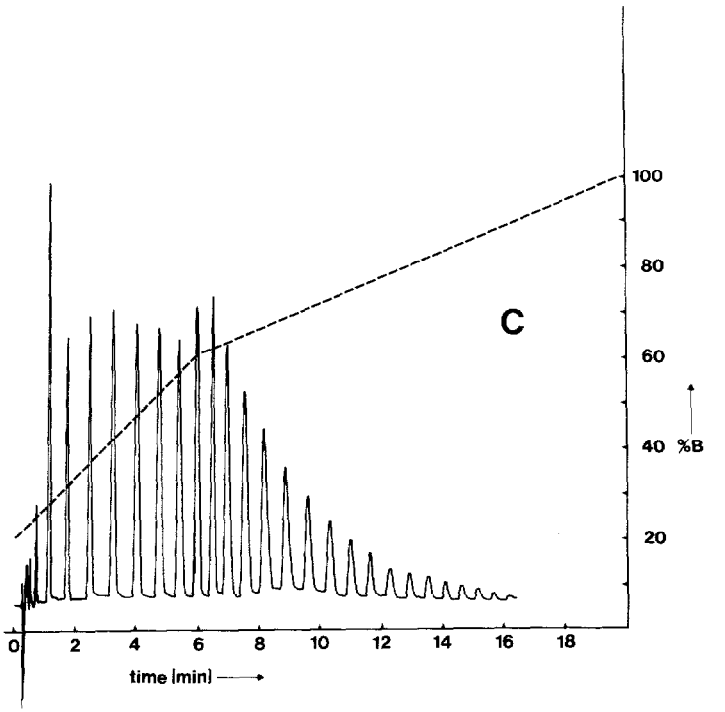
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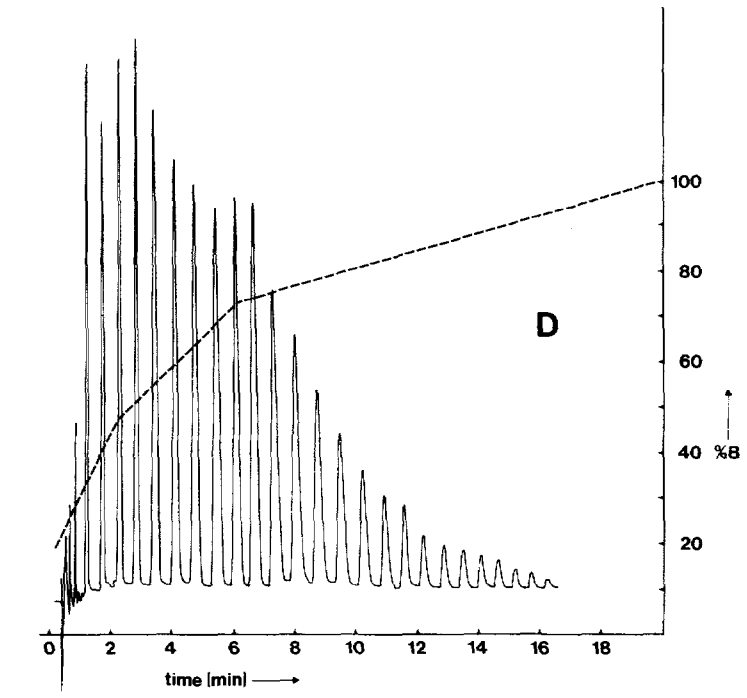
Fig. 7.



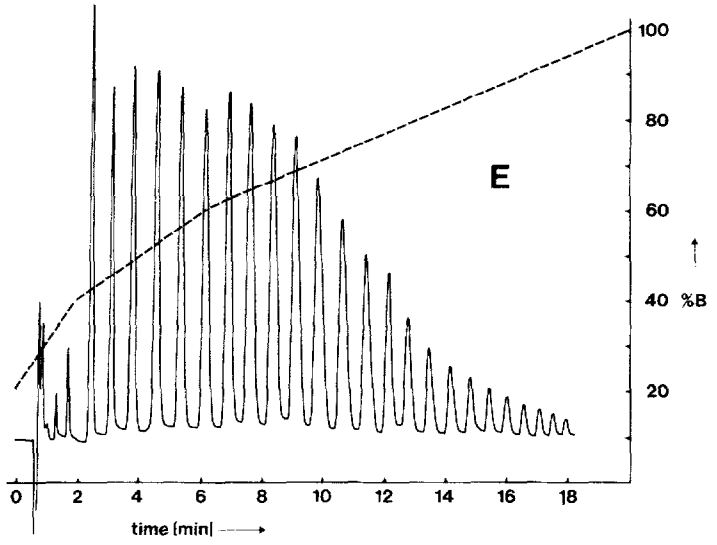


Fig. 7. Gradient elution separation of PEG oligomers from 4-mer to 27-mer. Conditions as in Experimental. Linear gradient from (A) 1 m*M* phosphoric acid, 5 ppm nitric acid, in water to (B) 25% acetonitrile, 75% A. Flow-rate: A–D, 6.0 ml/min; E, 2.0 ml/min.

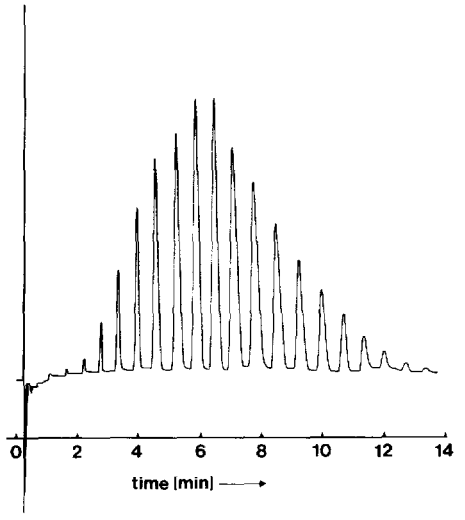


Fig. 8. Separation of PEG 600. Conditions as in Fig. 7D. Sample: 100 μl of 1 % aqueous PEG 600.

trile–water gradients should not be run past about 35 % when detection is attempted at 185 nm.

Melander *et al.*[10] reported that the separation factors (α) for adjacent PEG oligomers in polyoxyethyleneoctylphenol decrease with increasing mobile phase strength (*i.e.*, percentage of acetonitrile) in reversed-phase HPLC separations. This is equivalent to larger values of S for higher molecular weight oligomers in the relationship

$$\log k' = \log k_0 - S\,\varphi_B \tag{1}$$

Eqn. 1 has been verified for other compounds in reversed-phase HPLC systems by several workers (*e.g.*, Discussion in ref. 3), where for a given solute k_0 and S are found to be constant in a given reversed-phase system. Schoenmakers *et al.*[6] found that with acetonitrile–water as mobile phase, values of S do not increase as k_0 increases (S does increase with k_0 for methanol or tetrahydrofuran as solvents). For the PEG derivatives studied by Melander *et al.*[10], this is not the case (conformational changes complicate the situation). We have confirmed this observation. For acetonitrile–water as mobile phase, higher molecular weight PEGs give larger value of both k_0 and S, which means that S increases regularly with increase in k_0. This suggests that no generalization for all compound types can be offered for acetonitrile–water systems, in terms of the dependence of S on k_0.

From a practical standpoint, it is predicted[3] that convex gradients will be favored for the separation of PEGs in the present HPLC system. This is in fact observed, as can be seen in Fig. 7A–D. Here, the gradient is made increasingly more convex, starting out with a linear gradient in Fig. 7A. The band spacing in Fig. 7D is more nearly constant (and optimum) than in Fig. 7A. The similar separation of a sample of PEG 600 in Fig. 8 uses the optimized convex gradient of Fig. 7D, and again excellent band spacing and resolution of adjacent bands is observed.

In gradient elution with a fixed gradient time, the resolution should decrease as

the flow-rate is decreased, owing to a corresponding increase in the average value of the gradient steepness for each band during its separation. This is shown in Fig. 7E *versus* the similar separation in Fig. 7D; only the mobile phase flow-rate differs for these two separations: 6.0 ml/min in Fig. 7D *versus* 2.0 ml/min in Fig. 7E. The expected poorer resolution for the lower flow-rate in Fig. 7E is seen to be confirmed.

In order to achieve constant baseline absorbance throughout a gradient elution run with detection at 185 nm and acetonitrile–water as the mobile phase, it was necessary to add nitric acid (5 ppm) to the water (solvent A), so as to make its absorbance equal to that of the acetonitrile (solvent B). Berry[2] described a similar procedure for detection at 210 nm, using azide in place of nitric acid. In both instances, remarkably stable baselines are obtained with no drift during the gradient run. This is expected, as long as the added absorbing component is not retained by the stationary phase and is also added to sample solutions when large-volume injections of sample are made.

Practical limitations on detection at 185 nm

We have seen that acetonitrile–water gradients are limited to the range 0–35% with detection at 185 nm, owing to the presence of impurities in the acetonitrile. It may prove possible with further work, as described by Berry[2] for detection at 210 nm, to obtain adequately pure acetonitrile to obviate this problem. Similarly, we have found that various amine modifiers which are widely used as "silanol suppressors" in reversed-phase HPLC are too absorbant for inclusion in the mobile phase when detection at 185 nm is to be used. Again, this may reflect impurities in these amine modifiers, or it may mean that very much lower concentrations of these modifiers will be required than are usually used. At this point we do not know whether other spectrophotometric detectors will provide similar performance for monitoring at 185 nm.

As the choice of mobile phases is limited, stationary phase retention and selectivity are important variables. Columns packed with methyl- and cyanopropyl-silica were used to extend the range of analytes to those that were too strongly retained on C_8 silica. It can be predicted that fluorocarbon-modified silica columns would also be useful, *e.g.*, for the separation of lower alcohols that give detection problems in gas chromatography.

CONCLUSIONS

It is possible to carry out reversed-phase HPLC separations in both the isocratic and gradient modes with detection at 185 nm. The range of mobile phase compositions is at present limited to 0–35% acetonitrile–water for these separations. Compounds such as alcohols, ethers, alkyl chlorides and ketones are detected with much higher sensitivities at 185 *versus* 200 nm or higher wavelengths. Specifically, the analysis of commercial PEG samples for individual oligomers is now possible by means of HPLC with detection at 185 nm, and this appears to be the preferred routine procedure for samples containing either PEGs alone or in a mixture with various esters used as surfactants.

REFERENCES

1 V. V. Berry, *J. Chromatogr.*, 199 (1980) 219.
2 V. V. Berry, *J. Chromatogr.*, 236 (1982) 279.
3 L. R. Snyder, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography. Advances and Perspectives*, Vol. 1, Academic Press, New York, 1980, p. 208.
4 P. J. Schoenmakers, H. A. H. Billiet and L. de Galan, *J. Chromatogr.*, 205 (1981) 13.
5 H. Binder, *J. Chromatogr.*, 189 (1980) 414.
6 P. J. Schoenmakers, H. A. H. Billiet and L. de Galan, *J. Chromatogr.*, 185 (1979) 179.
7 L. R. Snyder, in S. Ahuja (Editor), *Ultratrace Analysis of Pharmaceuticals and Other Compounds of Interest*, Marcel Dekker, New York, 1983, Ch. 4.
8 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley-Interscience, New York, 2nd ed., 1979, Ch. 4.
9 B. Stancher and L. Favretto, *J. Chromatogr.*, 150 (1978) 447.
10 W. R. Melander, A. Nahum and Cs. Horváth, *J. Chromatogr.*, 185 (1979) 129.