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MODIFIED SCANNING SPECTROPHOTOMETER AS VARIABLE WAVELENGTH DETECTOR FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple flow cell is described which enables the Beckman DB series spectrophotometer to be modified into a dual-beam liquid chromatography detector. Comparative performance data and a variable wavelength application are presented. The effect of detector response time on column efficiency and peak symmetry is demonstrated.

INTRODUCTION

Among the many new developments in high-performance liquid chromatography (HPLC) instrumentation and column technology, the use of a spectrophotometer as a liquid chromatography (LC) detector has been outstanding. However, the relatively high cost and unpredictable delivery times have put the numerous new commercial instruments out of the reach of many chromatographers. As a result, a number of self-built flow cell spectrophotometer modifications for use in LC have appeared in the literature. Michaelis *et al.*[1] have reviewed those appearing before 1974. More recently, Brinkman *et al.*[2] and Carr[3] have described spectrophotometer modifications made on comparatively expensive instruments. Davies and Mercer[4] used a flow cell assembly for a Beckman DB spectrophotometer, but the large 250 μl total volume made it unsatisfactory for modern HPLC. Thacker *et al.*[5] mentioned the use of a modified DB, but gave no design performance details.

This paper describes and evaluates simple, easily built, 4- and 8-μl flow cells that enabled the use of the common Beckman DB series spectrophotometer as a single- or dual-beam HPLC detector.

EXPERIMENTAL

Flow cell construction

The entire unit (Fig. 1) was constructed from aluminium stock (detailed drawings are available from the author). The 0.5-cm path-length cell, shown in detail in Fig. 2, had an internal volume of 4 μl. The 1-cm path-length, 8-μl flow cell was of

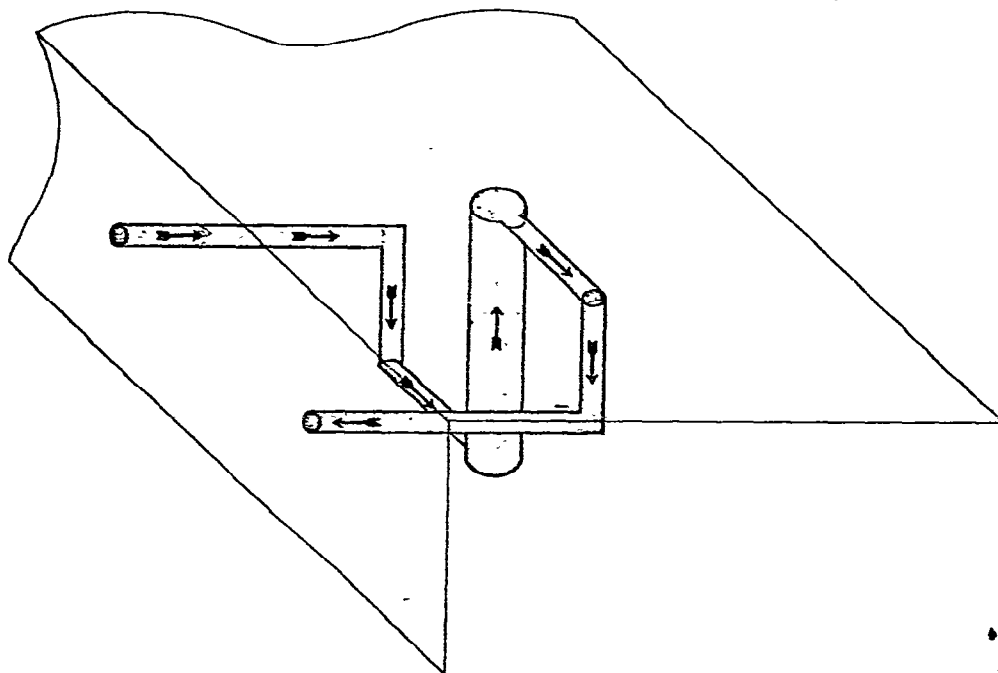Fig. 1. Dual channel, 4-μl, 0.5-cm path-length flow cell and holder assembly.

Fig. 2. Detail of 4-μl flow cell.

similar design. The 0.3 mm I.D. tubing and the fittings were PTFE components (Altex, Berkeley, Calif., U.S.A.). The 1 mm thick quartz windows (Italquartz, Milan, Italy) formed a leak-free seal with the flow cell body when 0.10 mm thick polyethylene was used as a gasket. The unit was mounted on the instrument using the four screws that originally held the sample compartment in place. The design facilitated proper alignment on each axis after the assembly had been mounted on the spectrophotometer.

Instrumentation

The chromatograph was a Hewlett-Packard Model 1010A (Grotzingen, G.F.R) modified with a high-pressure sample injection valve with a 10-μl sample injection loop (Altex, Model 905-01). The strip-chart recorder (Beckman, model 1005) had a maximum chart speed of 25.4 cm/min. The 0.25 m $\times$ 4.6 mm I.D. LiChrosorb RP8 reverse phase chromatography column was obtained commercially (Brownlee Labs, Santa Clara, Calif., U.S.A.).

The commercial detectors used in the evaluation studies were a Hewlett-Packard Model 1036A, a Schoeffel model 770 (Trappenkamp, G.F.R.), and an Altex Model 153. The Altex detector had been modified by changing two capacitors on the printed circuit board thus reducing its theoretical time constant by a factor of five. All of the above detectors were equipped with 8-μl, 1-cm flow cells.

For the performance studies, the Altex detector response time was further altered by placing a low-pass RC filter consisting of a 0–50-kΩ variable resistor and a 22-μF capacitor between the detector output and the strip-chart recorder. This enabled further incremental increases of the observed response time for the detector by a factor of *ca.* 2.

Reagents

Acetonitrile (analytical grade, J. T. Baker, Gross Gerau, G.F.R.) was used as obtained from supplier. Water was freshly distilled in glass before use. The steroid compounds were obtained commercially (Farca, Milan, Italy) or were produced by the product development group of Società Internazionale Ricerche Steroidi.

LC detector performance test conditions

Injections of 10 μl of a benzyl alcohol sample in acetonitrile were made on the RP8 column using a mobile phase of 40% acetonitrile in water pumped at a constant rate of 2.65 ml/min. The resulting column pressure drop was 77 bar. The variable wavelength detectors were adjusted to 254 nm, 1.0 O.D. In all cases, the chromatography was conducted at ambient temperature, 20–24°.

Peak symmetry and HETP calculations

The measure of symmetry, or degree of skewness as defined by Davies and Goldsmith[6], was determined according to the formula:

$$\text{Skewness} = \frac{\mu_1^0 - \text{mode}}{\sigma}$$

where the first statistical moment, μ_1^0, is the geometric mean of the peak distribution, and σ is the square root of the second statistical moment (variance), μ_2^0.

Knowledge of the variance of the peak distribution permitted use of the formula:

$$\text{HETP} = \frac{\sigma^2 L_C}{t_R^2}$$

as suggested by James and Martin[7], where t_R is the retention time as determined by the first moment and L_c is the column length.

Peak moment values were obtained by graphically measuring the peak amplitudes at regular intervals from high-speed recordings. In all cases, more than 20 measurements were made. The skewness and HETP were evaluated on a programmable calculator. The mode, or maximum peak amplitude, was determined by a parabolic curve fitting routine using the upper 20% of the peak amplitude values from which the vertex was obtained.

The HETP values obtained from this method agreed well with those resulting from the usual formula:

$$\text{HETP} = \frac{L_C\, w^2}{16\, t_R^2}$$

where ω is the peak width. The skewness values were proportional to the asymmetry factor determined at 10% peak height, as described by Asshauer and Halasz[8]. However, the approach used in this report for HETP and peak skewness determination will facilitate on-line computer application in the future.

DISCUSSION

Modification reversability

It was desirable to have the flexibility of using the instrument as a conventional scanning spectrophotometer or as a variable wavelength LC detector. The removal of the original cell holder compartment and the installation and alignment of the flow cell, and *vice versa*, required only a few minutes.

Performance

The flow cell modified Beckman spectrophotometer was evaluated by comparing its performance with that of three commercial detectors. This was done by maintaining identical experimental conditions in each case, varying only the particular detector being studied.

It has been pointed out that in many cases, the limiting factor in a chromatographic system is the detector response time[9]. A slow detector response time not only reduces column efficiency by reducing plate height but, as will be shown later, is detrimental to the peak symmetry. A response time of 0.5–3 sec is generally observed in commercial detectors. The relative response times for the four detectors considered in this study were determined by injecting acetone directly into the detectors and measuring the time necessary for an increase in absorbance from 0.1 to 0.90 O.D. No attempt was made to compensate observed response times for the contribution due to the recorder, as the same recorder was used with each detector. The observed response times are shown in Table I.

TABLE I

OBSERVED RESPONSE TIMES FOR VARIOUS DETECTORS

Detector	τ' (sec)	HETP (μm)	$Sym_{10\%}$*	Skew	r (kΩ)**
HP-1036A	0.5	42.2	1.2	0.15	—
Schoeffel	0.7	47.3	1.3	0.17	—
Modified DB-G 4 μl	1.3	53.2	1.3	0.16	—
Modified DB-G 8 μl	1.4	57.8	1.3	0.18	—
Altex 153	1.6	64.0	1.4	0.22	0
Altex + filter	1.8	65.6	1.4	0.22	10
Altex + filter	2.2	77.8	1.6	0.24	20
Altex + filter	2.6	83.0	1.7	0.25	30
Altex + filter	3.0	89.7	1.7	0.28	40

* Symmetry value at 10% peak height.
** RC filter resistance.

HETP as a function of detector response times

Benzyl alcohol had a *k'* value of 0.78 in the chromatographic conditions used in this study. The resulting 7.7 sec peak width was 15.4–2.6 times larger than the observed response times of the nine detector tests detailed in Table I. The effect of response time on the measured HETP is illustrated in Fig. 3. The least-mean-square linear regression analysis correlation coefficient, *r*, was 0.99. Extrapolation of the regression line to the zero intercept indicated that theoretically an observed response time of zero would result in a HETP value of 31.7 μm. A 2-sec change in response time was shown to vary the plate height by almost 100%. The observed ratio of response time to peak width for the two modified DB tests was *ca.* 6:1. Under these
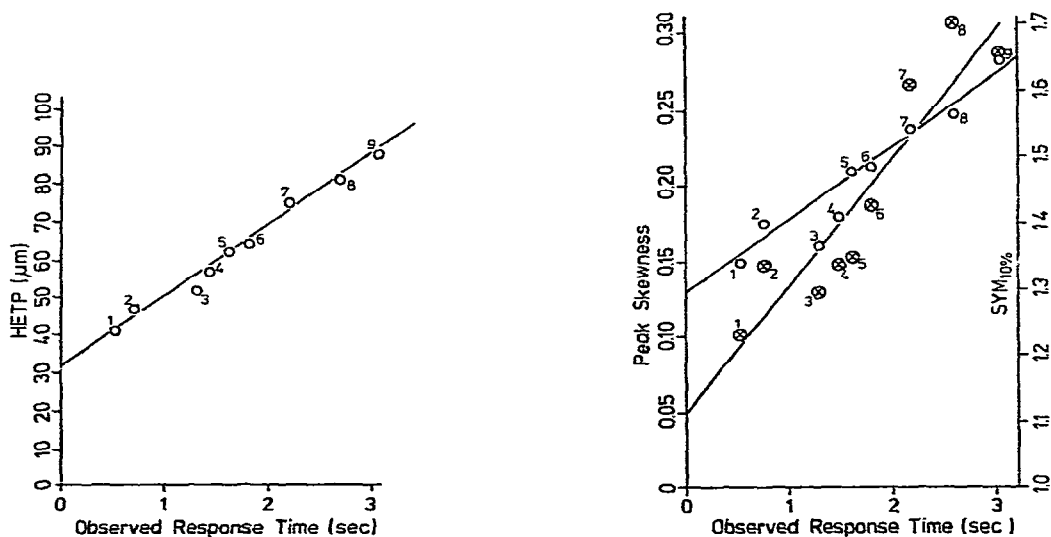

Fig. 3. Relationship between measured plate height and observed detector response time. Numbers correspond to the detector listing in Table I.

Fig. 4. Skewness (○) and symmetry at 10% peak height (⊗) relationships to observed detector response time. Linear correlation coefficients are 0.94 and 0.92, respectively. Numbers correspond to the detector listing in Table I.

conditions, the modified DB suffered a 20% loss in plate height in respect to the fast responding HP-1036A. As other compounds with larger k' values eluted from the column, the influence of the response time upon the plate height became less important.

Peak symmetry as a function of detector response time

Column efficiency in terms of plate height was not the only factor observed to be dependent upon the detector response time. Fig. 4 illustrates the linear relationship between peak symmetry and observed detector response time. The zero intercepts for the two expressions of symmetry both resulted in positive skewness values, indicating that peak symmetry is not only a function of detector response time. Although the response time was seen to have a large effect, other well-documented factors such as flow cell volume, column–detector connections, column packing technique and injection technique also contribute to peak symmetry. The performance of the modified DB was satisfactory in respect to peak symmetry. It was also observed that the performance of the 4-μl flow cell in the DB approached that of the best detector. In terms of skewness, the smaller volume compensated for the slower response time.

The flow cell modified Beckman detector was found to have a linear response and zero intercept within its 0.0–1.0 O.D. range capability. This was determined by the analysis of a series of benzyl alcohol solutions under the conditions described above.

Sensitivity

Commercial LC detectors usually have a maximum sensitivity range of at least 0.01 O.D. units equivalent to a full-scale recorder response. Without further modification, the full-scale response for the modified Beckman was 1.0 O.D. This sensitivity would not be suitable for trace analysis and samples with low solubility or weak absorption properties. However, 10 μl injections of samples having a concentration of 3–8 mg/ml and an ε value of 8000–15 000 in the UV range gave satisfactory recorder response. This made the modified spectrophotometer suitable for many types of analytical application. Generally, a much higher sample concentration can be used, further increasing the response without approaching column overload conditions.

Stop-flow scanning

Valuable qualitative data were obtained from as few as two injections by employing stop-scan analysis, as described by Carr[3]. An initial chromatogram of the related androstane derivatives was recorded (Fig. 5), so that important peaks appearing after a second injection could be anticipated. When an eluting peak of interest was near its maximum height, the chromatograph pump was stopped and a high-pressure valve, placed between the outlet of the column and detector, was closed. The UV spectrum of the trapped peak was then obtained conventionally. The procedure was repeated for the five identified peaks, as shown in Fig. 6. Thus, in a very short time, and with only a small amount of sample, the absorbance spectrum of each component of a complex mixture was obtained without having to perform a tedious and often difficult preparative chromatography operation.
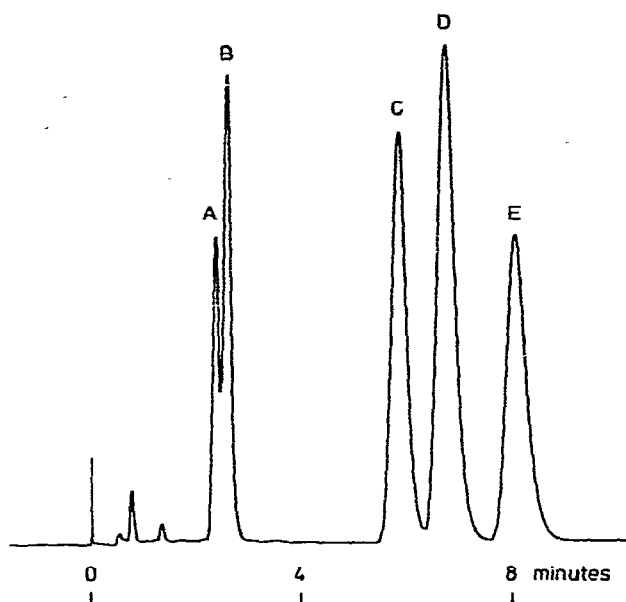
Fig. 5. HPLC separations A = 19-Nor-4,6-androstadiene-3,17-dione; B = 19-nor-4-androstene-3,17-dione; C = 17α-hydroxy-19-nor-4,6-androstadien-3-one 17-acetate; D = 17α-hydroxy-19-nor-4-androsten-3-one 17-acetate; E = 17α-hydroxy-4-androsten-3-one 17-acetate. Conditions: 0.25 m × 4.6 mm LiChrosorb RP-8 column, acetonitrile–water (1:1) mobile phase, flow-rate 2.8 ml/min. Flow cell modified Beckman DB-G detector, 260 nm.
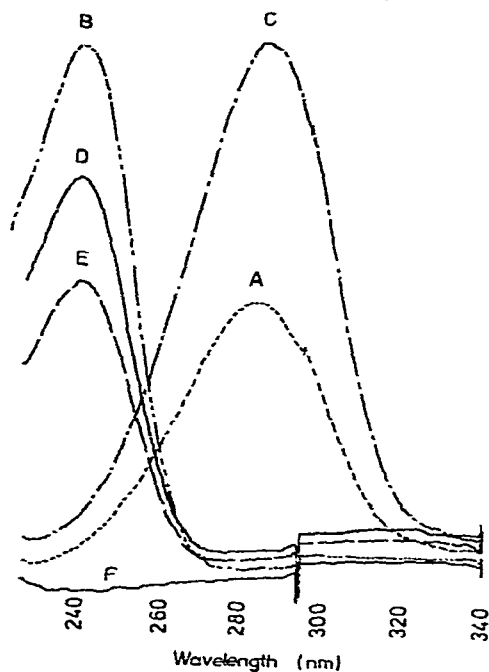

Fig. 6. Stop-scan analysis absorbance spectra of the androstane derivatives separated in Fig. 5 using the flow cell modified Beckman DB-G. A–E, as in Fig. 5; F, cell vs. cell scan.

CONCLUSIONS

The loss in column efficiency due to the detector response time decreases as the k' value of the sample increases. With ratios of peak width to observed response time of $>10:1$, the loss in performance becomes neglegible for most types of routine analysis. The modified Beckman DB is suitable for many types of LC application. In addition, it allows maximum instrument utilization since the detector also functions as a normal scanning spectrophotometer.

The widely used and rugged Beckman DB is a dependable instrument. The older models can be obtained for a modest price. The ten-year-old, tube-type DBG model used in this study has been used routinely as a LC detector for over 2 years. The only maintenance required during that period was a single replacement of the hydrogen lamp.

ACKNOWLEDGEMENTS

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