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Note

A novel method of generating multislope gradients for high-pressure liquid chromatography

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Carrier programming is a useful method of eluting from a column as a single injection a mixture having a wide range of distribution coefficients[1,2]. Numerous devices have been described, all of which fall into the categories of either high-pressure or low-pressure programmers. Low-pressure programmers, which generate the carrier gradient on the inlet side of the pumping system, are not usable with syringe- or gas-displacement pumps. Such carrier-supply systems require the gradient to be generated on the outlet or high-pressure side of the pump, and therefore require either a two-pump system or a time-pulsed injection[3]. Each system is costly to the user.

The exponential-dilution flask is a known method of producing a continuous precisely formed gradient, with simple low-cost equipment[4,5]. A carrier programmer using the principle has been commercially available for several years[6,7]. The principle limitation of the dilution flask is its apparent inability to generate concave, multi-slope gradients. In its simplest form it produces only an exponential gradient in which the early portion approaches a linear gradient.

In this work it is demonstrated that by the introduction of a properly balanced by-pass flow system the exponential-dilution flask can be converted into a versatile programmer, capable of delivering linear, concave or convex multislope gradients and pre-selected isocratic carrier blends.

EXPERIMENTAL

Apparatus and reagents

A Tracor Model 5100 liquid chromatograph with an ultraviolet photometer detecting at 254 nm and a pneumatic amplifier pump[8] providing output pressures up to 6000 p.s.i. was used in the work. The Tracor-Chromatec Model 6000 gradient elution accessory[7] was modified as described below to direct added flow through the by-pass thereby increasing the control range of the vernier blend valve.

Chemicals and solvents

A solution of 0.125% benzene in *n*-hexane was used as the B or final carrier. The concentration was selected to provide a full-scale recorder deflection without saturating the detector.

n-Hexane was used as the A or initial carrier. Gradient shape was determined by monitoring the increase in UV absorbance of the eluent. Spot samples of eluent were checked by gas chromatography to confirm the validity of the relationship between detector response and benzene concentration.

Description of apparatus

Although the principle of the exponential-dilution flask may be known, a brief description of a closed-vessel gradient programmer using the principle is given for reader understanding.

The modified Tracor Model 6000 gradient elution accessory illustrated schematically in Fig. 1 is a closed-vessel gradient generator employing a 10-ml mixing chamber. In normal operation, each increment of B carrier entering the mixing chamber must displace an equal volume of liquid from the chamber. The mixed effluent is a continuum in which the concentration of B increases in a gradient described by:

$$C_b = B(1 - e^{-u/v}) \tag{1}$$

where:

C_b = Instantaneous concentration of B in effluent
B = Concentration of B carrier. When expressed as unit fraction, $B = 1$ and $C_b \cdot 100 = \%$ of B in effluent.
u = Volume eluted from mixing chamber.
v = Volume of mixing chamber.

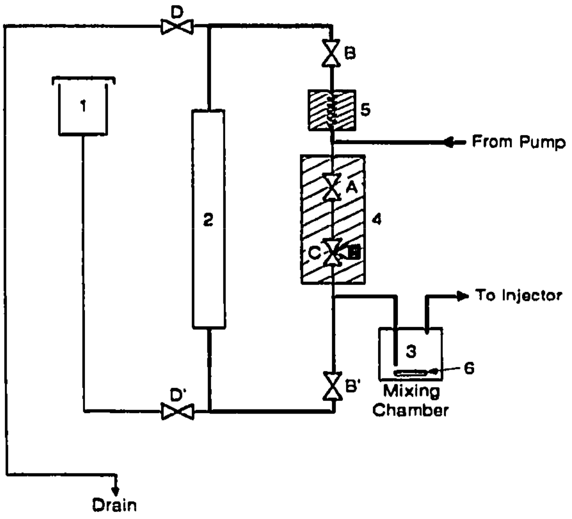

Fig. 1. Schematic diagram of Tracor Model 6000 gradient elution accessory after modification. 1 = B carrier reservoir (vol. = 2 l); 2 = B carrier hold chamber (vol. = 100 ml); 3 = 10-ml mixing chamber, 10 ml sealed and continuously stirred; 4 = A carrier by-pass; 5 = restrictor coil; shaded areas form balanced by-pass system of the A carrier. Valves: (A) A carrier shut-off; (B) and (B') B carrier shut-off; (C) blend valve, vernier knob and fine metering; (D) and (D') hold chamber, refill and drain valves.

To increase the convenience and versatility of the closed-vessel gradient accessory a flow-controlled by-pass line was added to permit A carrier to reduce the effective concentration of B carrier in the mixing chamber. Flow through the by-pass line is controlled by a "fine" metering valve (Nupro S series) with a 10-turn vernier handle. In this modification, a piece of 0.009 in. tubing was inserted in the "hold-chamber" inlet line as illustrated in Fig. 1 to force A carrier through the by-pass line such that, when both lines are fully open, 90% of the flow is forced through the by-pass line. In this configuration, blends from 10% B–100% B can be programmed either as continuous gradients or as isocratic blends by adjustment of the metering valve.

RESULTS AND DISCUSSION

In normal operation, when the blend valve is closed, the concentration curve of B carrier in the effluent is described by eqn. 1 and is illustrated by the top elution curve in Fig. 2. However, when the blend valve is open, A carrier by-passes the holding coil and dilutes the contents of the mixing chamber thus reducing the slope of the gradient. The curves shown in Fig. 2 are exact tracings of response curves superimposed on each other. The ordinate was converted to a 100% B scale by a classical geometric procedure. A 10-μl sample of 2% benzene in carrier was injected to provide a two-point alignment of the time scale. The dashed line in Fig. 2 shows the theoretical curve as defined by eqn. 1. The departure from the theoretical curve is an indication of a minor lag in the mixing chamber.

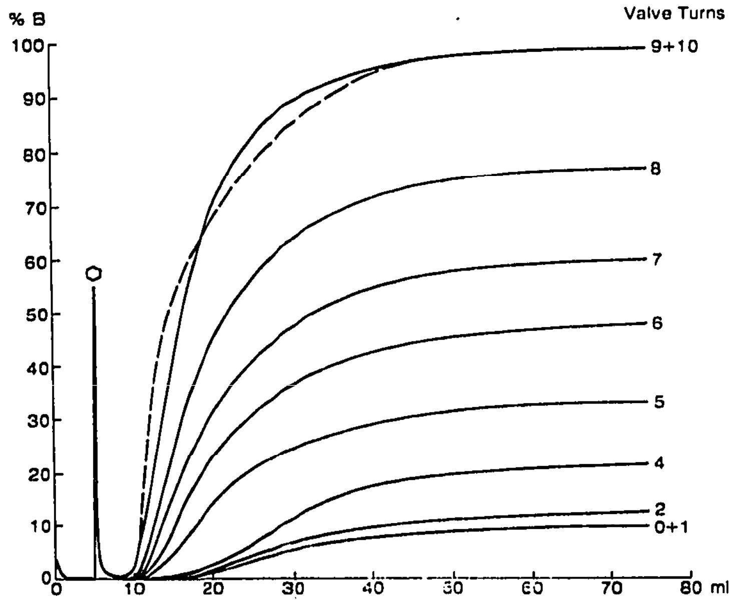

Fig. 2. Gradient curves at several blend-valve settings. A Carrier, *n*-hexane. B Carrier, *n*-hexane containing 0.125% benzene. Column, 0.46 × 25 cm Partisil-5. Flow-rate, 2 ml/min. Benzene injection, 10 μl of a 2% solution in *n*-hexane. — — —, Theoretical curve according to eqn. 1.

Assuming the B carrier obeys Beer's law, the pen deflection is a direct indication of the amount of B carrier in the effluent. The plateau of each curve indicates the maximum amount of B carrier in the effluent at each valve setting. The graph in Fig. 3 shows the relationship between vernier setting and the percentage of B carrier in the effluent for the particular restrictor used in this study. By operating on the plateau the blend valve provides a simple means of adjusting carrier composition at any level between 10 and 100% B for isocratic operation. To obtain blends of less than 10% B, either the restriction may be increased or the strength of B reduced. The plateau concentration may be achieved quickly by opening the injection port and allowing the mixing chamber to be flushed at high flow-rate. With a pneumatic pump, the mixing chamber is flushed through the injector in approximately 30 sec after which equilibrium concentration is achieved throughout the column and system in a few minutes.

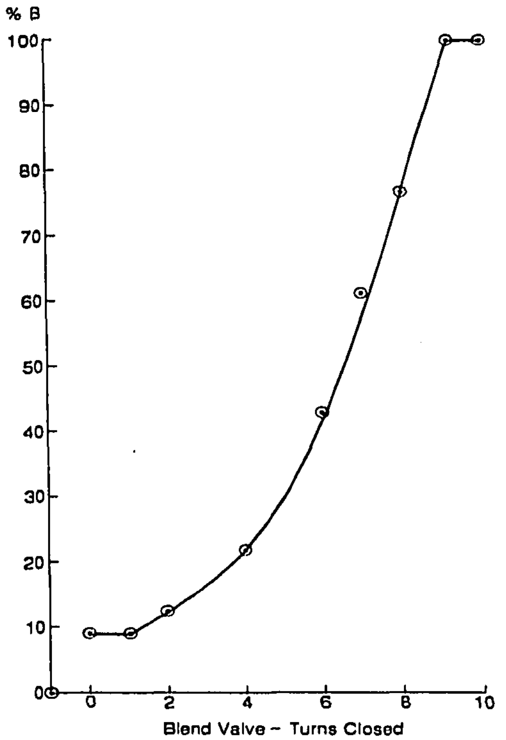

Fig. 3. Effect of blend-valve setting on effluent composition.

Concave gradients

The controlled by-pass system can be used to generate fully concave gradients as illustrated by the elution curves in Fig. 4. The program was started with the blend valve open, and then closed in small increments. However, lines 3 and 4 in Fig. 4 show that continuous concave gradients can be obtained in three increments. To maintain a continuous concave slope the elution volume between valve adjustment

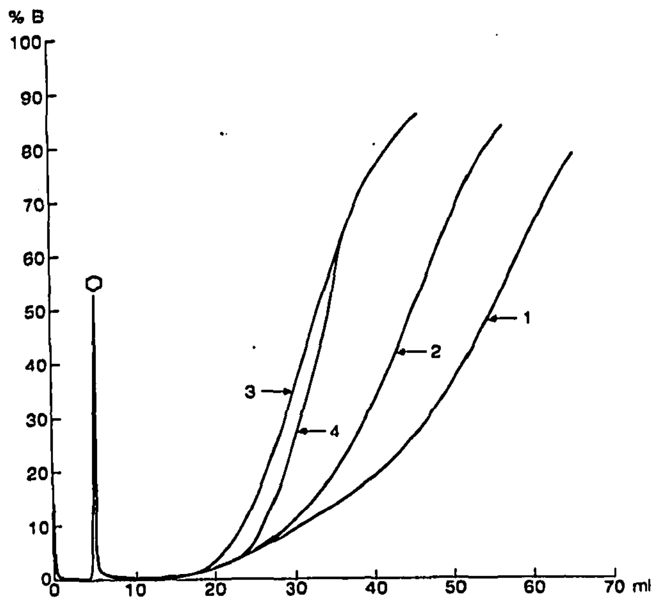

Fig. 4. Some concave gradients produced by the exponential-dilution flask with the aid of the controlled by-pass.

Curve No.	*Elution increment (ml)*	*Valve settings*
1	5	AB-1, 2, 3, 4, 5, 6, 7, 8, 9, B
2	7	2, 4, 6, B
3	7, 5	0, 5, 7.5, B
4	7	0, 2, 5, B

should be equal to or less than the volume of the mixer. Curvature is determined by the size of the respective adjustments, as illustrated further in Fig. 5. When the volume between adjustments is much greater than the volume of the mixing chamber, the gradient is converted from a concave to a multilinear gradient, which, in practice,

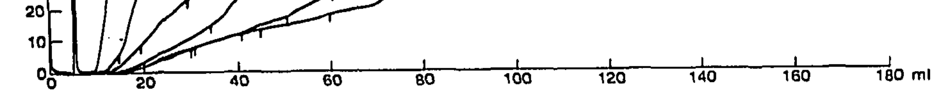

Fig. 5. Gradients produced by several valve-adjustment programs. Spikes on curves indicate valve adjustments.

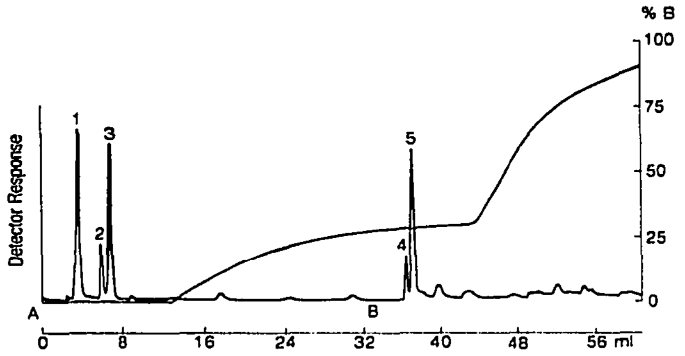

Fig. 6. Separation of fat-soluble vitamins. Column: 0.25 × 25 cm Partisil-5. Carriers: A = *n*-Hexane–chloroform (85:15); B = *n*-hexane–chloroform–50% water-saturated isopropanol (88:10:2). 1 = Retinol palmitate (vitamin A); 2 = tocophenol acetate (vitamin E); 3 = retinol acetate (vitamin A); 4 = calciferol (vitamin D_2); 5 = retinol (vitamin A).

may be as useful for many separations as a concave gradient. For example, the separation in Fig. 6 was made with a two-step exponential gradient that approaches a three-step multilinear gradient.

Operation in the isocratic or exponential modes requires no operator attention after sample injection. Operation in the convex or complex multilinear modes requires less operator attention than does peak attenuation since blend valve adjustments can be made at scheduled times.

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