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CONSTRUCTION AND EVALUATION OF A PROGRAMMABLE GRADIENT LIQUID CHROMATOGRAPH

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SUMMARY

A programmable gradient synthesizer has been constructed which is capable of forming flow-programs and gradients (linear and non-linear) of variable duration. The synthesizer is constructed from simple digital integrated circuits and is interfaced to an inexpensive two-channel high-pressure reciprocating pump. Other components necessary to complete the chromatograph are specified to provide high performance and flexibility at moderate cost.

The application of the system to the analytical and preparative separation of some azodye reaction mixtures is presented. A new procedure for reproducible sample application in preparative liquid chromatography, employing flow-programming, is introduced.

INTRODUCTION

Recently[1,2] the popularity of gradient elution in high-pressure liquid chromatography (HPLC) has grown rapidly due, no doubt, to the power of the technique and the availability of commercial instruments with built-in gradient forming capabilities. Though desirable, commercial instrumentation may be too expensive to justify in some laboratories.

A number of publications have appeared describing gradient formers that can be constructed inexpensively[3–8]. The majority of these instruments rely upon gradient formation prior to pressurization with a high-pressure pump. An alternative method described here employs two high-pressure pumps[9] with suitable electronic flow control providing variable eluent composition at a fixed flow-rate.

The merits and limitations of single- and dual-pump gradient formers have recently been brought to light by Billiet *et al.*[10]. The performance of a single-pump

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instrument of their own design was compared with several commercial instruments which employed both approaches to gradient formation.

This publication deals primarily with the design and construction of a typical dual-pump instrument and provides several practical examples of its use in the authors' laboratory.

The instrument performs many of the functions of more expensive commercial instruments but at a lower cost (approx. US$ 300 for electronic gradient control and US$ 1300 for a two-channel pump). Several important features incorporated into the design include:

(1) A programmable memory which allows linear, non-linear, step-gradient and flow-programs to be stored and run. No programming experience is required to operate the instrument.

(2) The flexibility of the design allows considerable operator intervention in the event any unforeseen difficulties arise during a separation. The operating program can be terminated at any time, returning total manual control of eluent composition and flow-rate to the operator. (On the other hand, because the design has been kept simple, more operator interaction is required to run the instrument than would be needed for a commercial instrument.)

(3) The familiarity with the "hardware" gained by constructing such an instrument allows the user to perform maintenance or modification more quickly and inexpensively than with comparable commercial instrumentation.

MECHANICAL ASPECTS OF THE DESIGN

Using a two-channel pump (or two individual pumps) it is possible to vary the composition of the flow stream while maintaining a fixed flow-rate by reducing the flow-rate of one channel and increasing the flow-rate of the second channel. A simple way to accomplish this is to attach gears to the flow-rate controls of the two pump channels and drive them with a single, central motor-driven gear. A fourth "inverting gear" provides an opposite sense of rotation to each of the flow-rate controls. This arrangement is schematically depicted in Fig. 1. The inverting gear also serves to disengage channel 2 from the motor drive, providing one channel that can be manually controlled. (In the current design, channel 1 remains engaged to the motor at all times and must be electrically driven.)

In practice, the mechanical layout will be depicted by the actual pump used (a Milton Roy two-channel instrument minipump, in this case), and the gears that are

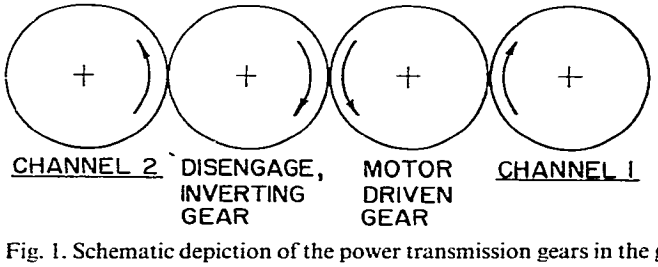

Fig. 1. Schematic depiction of the power transmission gears in the gradient attachment. The arrows refer to the direction of rotation of the gears.

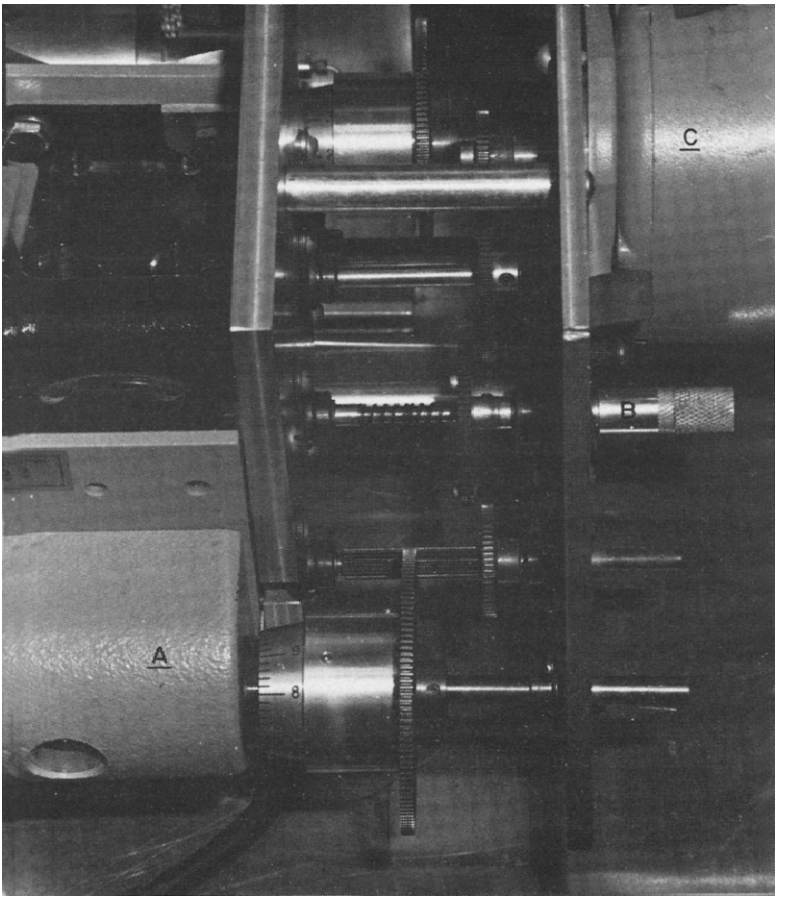

Fig. 2. The actual arrangement of the gearing in the present instrument. A = Instrument minipump and flow control with attached drive gear; B = disengageable gear; C = stepping motor.

available. The unit described here (Fig. 2) was constructed from gears acquired from inoperative equipment. Many other arrangements are possible depending upon what is available to the builder. The motor (200 steps per revolution, 60 oz.-in. torque) was obtained as surplus from American Design Components (New York, NY, U.S.A.).

ELECTRONIC ASPECTS OF THE DESIGN

To construct a linear gradient at a given flow-rate, the rate of change of the flow stream composition and the required duration of this rate of change must be specified. These functions are performed by a programmable timer which determines the length of time that the output of the programmable oscillator is connected to the stepping motor drive unit.

Low-power complementary metal oxide semiconductor (CMOS) digital integrated circuits were used throughout the instrument. Besides providing some logic

elements not available in the more familiar transistor–transistor–logic (TTL) family of circuits, they also consume much less power in comparison, simplifying power supply design. A Motorola MC14522 programmable divide-by-N 4-bit binary coded decimal (BCD) counter[11] was used for timing purposes and frequency division. A BCD number between 0000 (0_{10}) and 1001 (9_{10}) applied to the program inputs will cause the circuit to count down to zero from that preset number before resetting. Using the appropriate "cascade" outputs of the circuit, multi-digit numbers can be represented.

Three decades were used for the programmable timer allowing linear gradient programs from 0 to 999 sec to be run when a 1-Hz clock frequency is used. Longer programs could be run by lowering the clock frequency or increasing the number of decades available for counting. The circuit also has BCD output lines which allows digital display of the timing process.

With the inputs appropriately arranged, frequency division by a preset BCD number is possible. In this case three circuits are cascaded together allowing division of the input frequency by as much as 999. A 100-Hz square-wave signal applied to the stepping motor will produce a flow-rate change of about 1 ml/min/min as the system is presently structured. This maximum rate of flow-rate change has proven adequate for all experimental work.

In an instrument designed only to produce linear gradients, the program input states can be connected directly to BCD thumbwheel switches. The instrument described here has five memory locations to store duration and rate of change information. Non-linear gradients of many shapes can be constructed from several linear segments. The memory is wired from a Motorola MC14076 4-bit register, each register representing a single BCD number. A block diagram (Fig. 3) shows the arrangements of timing, frequency division and memory components. Detailed schematics and operating instructions can be obtained from the authors.

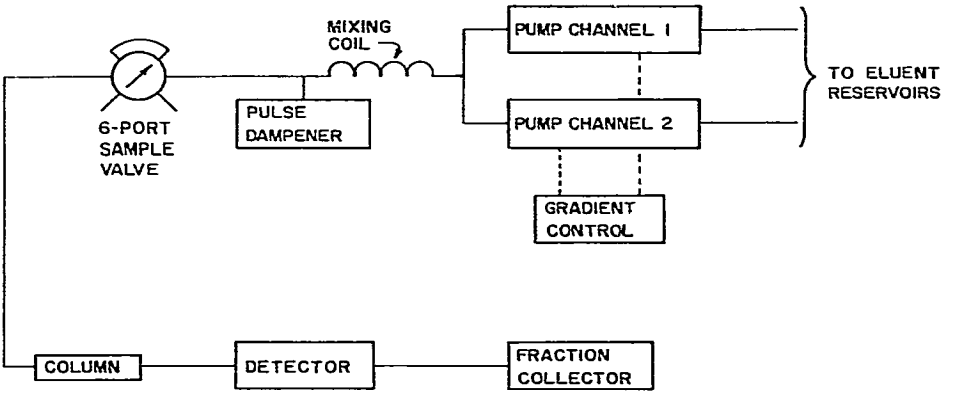

Fig. 3. Block diagram of the electronics of the programmable gradient maker.

EXPERIMENTAL

Reagents

All reagents used in this study were at least of reagent grade. The solvent

system was composed of isopropanol and a 2 *M* triethylammonium bicarbonate buffer pH 9–9.5 (abbreviated IS–TEA) and was used for all the chromatographic separations. Its preparation and use for the analytical and preparative thin-layer chromatographic (TLC) separations of cobalt(III) transition metal complexes, azodye ligands, amino acids and peptides has been described elsewhere[12].

Efficient degassing and filtration of the solvent system or its components was accomplished by attaching a Millipore Swinex inline filtration unit, fitted with a 0.45-μm membrane (Millipore No. HAWP02500), to a 4-l vacuum filtration flask with polyethylene tubing (0.062 in. I.D., 0.082 in. O.D. Intramedic PE-205 No. 7446 or equivalent) and a 10–12 in. length of 0.040 in. I.D., 0.062 in. O.D. stainless-steel tubing inserted in the rubber stopper of the flask. Upon completion of the filtration step, a solid rubber stopper was substituted and stirring continued at room temperature for approximately 30 min under house vacuum. The solvent system was degassed each day before use.

The liquid chromatograph

Aside from the electronic gradient control interfaced to the Milton Roy instrument minipump, which has already been described, the remainder of the system (Fig. 4) consisted of a Laboratory Data Control Model 709 pulse dampener, a Valco P-20 sample injection valve, and an ISCO UA-5 UV-VIS monitor equipped with a 19-μl low-dead-volume flow cell. After using this system for several years, a number of suggestions to the potential builder can be made:

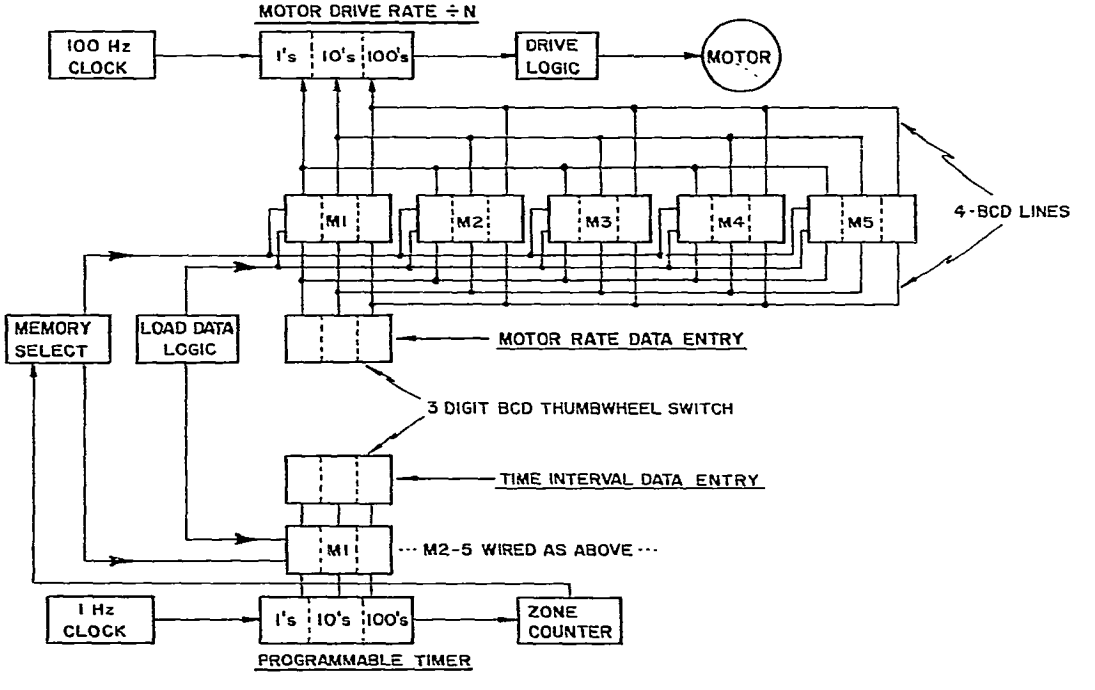

Fig. 4. Configuration of the remainder of the instrument.

(1) It has been found that the pulse dampener does remove pressure fluctuations from the solvent delivery system, however its inclusion in other systems may not be warranted. None of the instabilities commonly associated with pulsed solvent flows have been noted in the baselines generated by the UV–VIS monitor at the 0.1 absorbance scale (or larger scales) at flow-rates ranging from 1–10 ml/min with the pulse dampener switched off. Also the 1200 p.s.i. maximum pressure rating, coupled with the excessive remixing of gradients that occurs in the internal bellows when using gradient elution, limit this unit's utility.

(2) In order to utilize the UV–VIS monitor most efficiently, an instrument was chosen that could be used on the liquid chromatograph with low-dead-volume flow cells capable of operation at high back pressures (for bubble supression), as well as with conventional gravity flow chromatography columns using larger volume flow cells. Since the optical unit is separate from the electronics section, very short runs of tubing were used to connect it to the column outlet, minimizing extra column peak broadening. The remainder of the detector, a chart recorder and a fraction collector, were kept on a cart allowing the detector/collection system to be moved to other locations in the laboratory when the liquid chromatograph was not being used.

(3) PTFE tubing (0.020 in. I.D., 0.062 in. O.D.) and miniature tubing fittings (both Unimetrics and Altex brands have been used) were employed on the low-pressure side of all columns for connection to the detector. When using glass chromatography columns (see below) this tubing was installed from the sample valve to the column and also for the sample loops. When operating with metal columns the system was readily converted to stainless-steel fittings and tubing (0.020 in. I.D., 0.062 in. O.D.).

Columns and packing materials

A variety of column bodies and packing materials have been evaluated on the chromatograph including 4 and 10 mm I.D. stainless-steel columns, 7, 9 and 25 mm I.D. glass columns packed with 10–20-μm ion-exchange resins, C_{18} reversed-phase materials and conventional silica. Of all possible combinations of the above listed columns and stationary phases, 10–20-μm silica (Whatman LP-1) used with 9- and 25-mm glass columns has been most successful in terms of resolution, maintenance and cost for the separation of coordination complexes and azodye compounds. Since most of the compounds separated in this study were intensely colored, the glass columns permitted continual visual evaluation of the progress of the elution which was helpful in optimizing separations. The rather low operating pressures of these columns was occasionally an inconvenience, especially when trying to remove a strongly retained component, however their efficiencies were comparable to the metal-bodied columns. The ease of maintenance and visibility of the column bed made up for any loss in speed.

Columns of comparable efficiency were obtained by dry packing or slurry packing the stationary phase. The dry packing method should be carried out in a hood due to the fine silica dust liberated during the procedure. To dry pack a column the outlet flow adapter was attached to a vacuum pump and a powder funnel was placed on top of the column. A suitable amount of silica was then placed in an erlenmeyer flask (about three times the volume of the silica to be used) and shaken vigorously for several minutes. The air–silica suspension was rapidly poured into the

column body with the vacuum pump turned on. The column was repeatedly tapped along its length during the initial settling process. Thereafter the column was allowed to sit undisturbed with pumping for an additional 30 min. The column was then connected to the liquid chromatograph and equilibrated with the desired solvent system [usually IS–TEA (70:30, v/v)] at normal flow-rate, *e.g.*, 1–2 ml/min for the 9-mm column, 5–10 ml/min for the 25-mm column.

To slurry pack a column, several volumes of isopropanol were combined with the desired volume of silica. The mixture was then placed in a filter flask, sealed and evacuated until gas evolution ceased when the mixture was agitated. Swirling the flask at hourly intervals was sufficient. When the mixture was degassed, the vacuum line was removed and any silica on the walls of the flask was washed down with isopropanol. The mixture was then allowed to settle overnight undisturbed. After settling, the excess isopropanol was decanted off without disturbing the packing material. Fresh isopropanol was then added, a few milliliters at a time, with gentle swirling, until a thick, pourable slurry was formed. The slurry was kept sufficiently thin so that air bubbles were not entrained when the mixture was agitated. Once the desired composition was reached (this took some practice) the slurry was kept in motion until poured into the column. The upper flow adapter was installed and the column was pumped as rapidly as possible with isopropanol. After approximately 30 min, the flow-rate was reduced to a normal level (see above) and the column was equilibrated to the desired starting solvent with a linear gradient, 1 h in length.

The efficiency of a column was determined by injecting a 20-μl sample of a napthalene solution (50 mg/100 ml eluent) and eluting with IS–TEA (70:30). The peak detected at 280 nm was symmetrical if the column was packed correctly. The number of theoretical plates was determined at various flow-rates and evaluated by using the following relationship[13],

$$N = 5.54\left(\frac{V}{W_{1/2}}\right)^2$$

where N is the number of theoretical plates, $W_{1/2}$ is the peak width at half height and V the retention volume measured in the same units. Comparison of plate numbers, usually ranging from 3000–6000 per meter, provided a simple method of evaluating packing techniques and column-to-column variability. Initial separations with "real" mixtures were run at flow-rates approaching maximum efficiency. Often it was found that slower rates provided better overall resolution which was not degraded by additional diffusion broadening due to the decrease in flow-rate.

Sample preparation

Whenever possible, the sample solution was made up in the solvent system being used for the elution. This posed no problem for analytical samples, but for preparative samples stronger solvents (a higher proportion of the buffer in the IS–TEA or water alone) though undesirable, were often necessary to obtain reasonable concentrations of solute, 100–200 mg/ml. (The application of flow-programs to apply these preparative samples to columns is discussed in the Results and discussion section.) In order to protect the sample injection valve and columns, samples were filtered to 0.45 μm before introduction to the chromatograph.

RESULTS AND DISCUSSION

Gradient formation

To illustrate the shapes of the gradients that could be formed, an aqueous solution containing the dye *p*-(2,4-dihydroxyphenylate)-benzene sulfonic acid was pumped by one channel and pure water by the other. The solvent delivery system was connected directly from the outlet of the sample injection valve to the inlet of the UV monitor. Several linear gradients ranging from 2 to 20 min in duration and a non-linear gradient, made up of five linear segments, are depicted in Fig. 5.

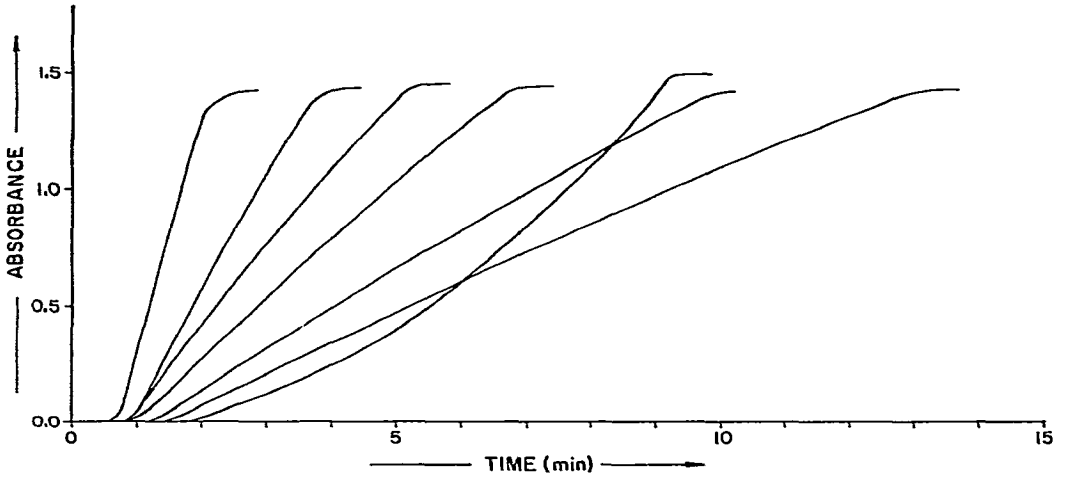

Fig. 5. Gradient profiles generated by the instrument.

Due to the flexibility of the electronic controller, a gradient program once entered can be run in an ascending or descending manner by reversing the direction of rotation of the stepping motor. (This can be accomplished by a single switch in the drive logic section of the programmer.)

During the performance evaluation of the programmer, each of the linear gradients in Fig. 5 were run several times in both directions to demonstrate the reproducibility of the system. Though only representative ascending traces are shown, the gradients generated by a given program, run in the same direction, were superimposable upon one another. Ascending and descending profiles for a particular program sequence were always superimposable mirror images. This reproducibility is inherent in the stepping motor drive system, provided that the machining of the drive system is performed accurately, and the pumps micrometer flow controls are of good quality.

Upon close inspection the gradients produced by the instrument are slightly convex, the maximal deviation from linearity being approximately 1–2% referred to the total length of the gradient. This is presumably due to some mismatch between the flow-rates of the two pump channels at any given setting[10]. This difference could be "tweaked" out by further calibration of the flow-rate controls of the pump.

Separations

In our efforts to diazotize tyrosine and histidine residues in proteins, enzymes and hormones[14] to form "chelating sites" where Co(III) could be subsequently incorporated[15,16], a considerable number of model reactions were run on the free amino acids to establish suitable conditions of pH, ionic strength and time.

To evaluate these conditions the products were separated utilizing the IS–TEA solvent system and the 9-mm analytical column previously described. Chromatographic conditions were established that allowed reaction mixtures to be evaluated rapidly. The chromatograms for a typical diazotized arsanilic acid–histidine reaction mixture are shown in Fig. 6.

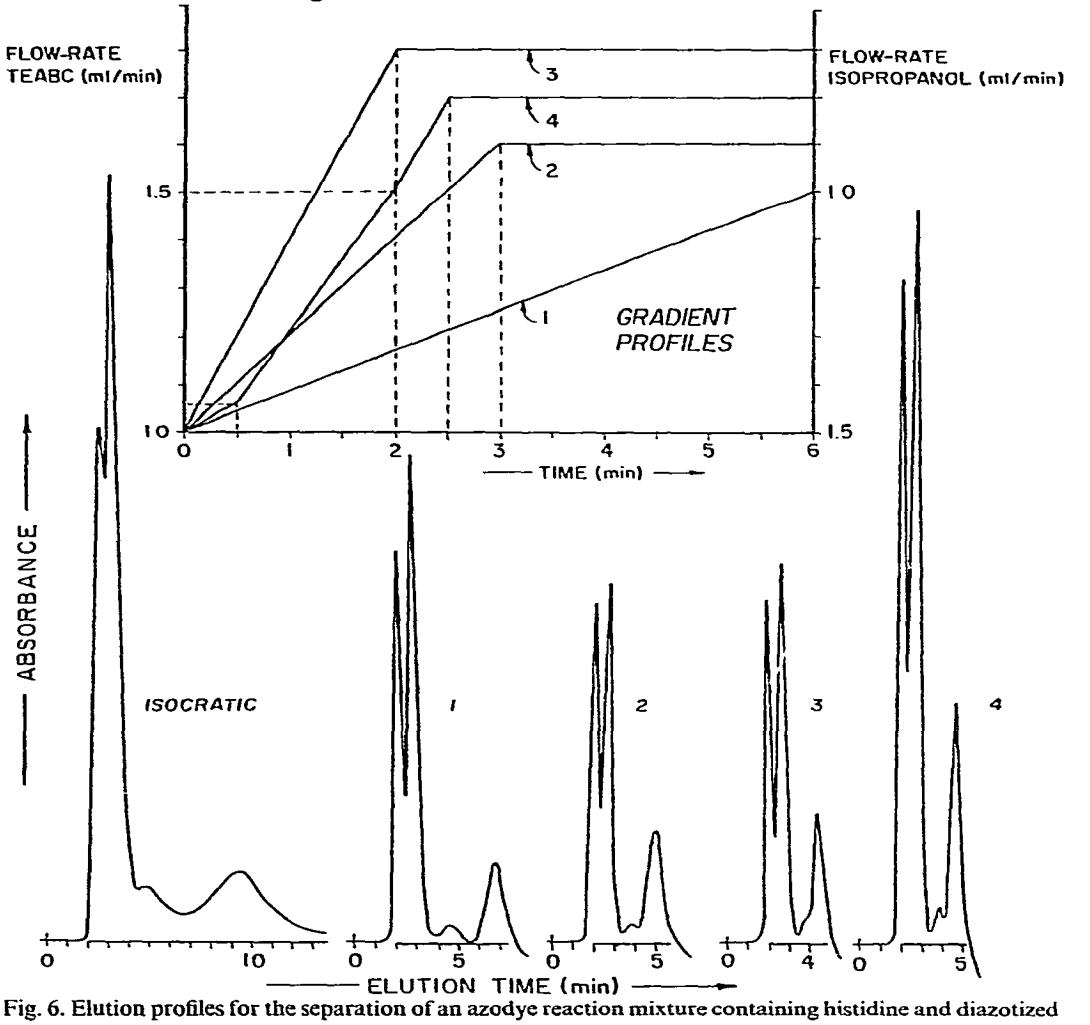

Fig. 6. Elution profiles for the separation of an azodye reaction mixture containing histidine and diazotized arsanilic acid. Gradient separations 1, 2, and 3 were mechanically compressed to fit under the insert. The isocratic elution was performed with IS–TEA (60:40) at a 2.5 ml/min flow-rate (1.5 ml/min isopropanol, 1.0 ml/min TEABC buffer). Samples ranged from 20–50 μl of 1 mM histidine diazotization reaction mixtures. The eluent was monitored at 280 nm, 1 absorbance unit full scale. The column consisted of a 250 × 9 mm glass body packed with 10–20-μm Whatman LP-1 silica.

The isocratic elution performed at 2.5 ml/min with IS–TEA (60:40) resolves four peaks corresponding to the self-coupling product of diazotized arsanilic acid: 2-(4-arsanilazo)-phenol; the desired product, arsanilazohistidine; unreacted histidine; and bis(arsanilazo)histidine.

All gradients were started at IS–TEA (60:40), 2.5 ml/min, but finish at increasingly stronger compositions; gradient 1, IS–TEA (40:60); gradient 2, IS–TEA (36:64); gradient 3, IS–TEA (28:72).

Gradients 1 and 2 improved the resolution between the components and shortened the separation time, but a point was reached where the solutes were eluted so rapidly, gradient 3, that overlapping of the last two components occurred. A nonlinear gradient, composed of three linear sections, designed to combine shallow gradients for resolution, with a steep gradient for speed, resulted in a separation very similar to number 2.

These increases in resolution were not secured without cost, since it required additional time to reequilibrate the column with the weaker solvent after the separation. This was accomplished by rerunning the gradient program with the stepping motor driven in the direction opposite to that used for the elution. The weaker solvent was then pumped across the column until the pH of the effluent was the same as the original solvent system. Running the gradient backwards required less solvent and time to reequilibrate the column than merely switching from the strong eluent composition at the end of a separation to the weaker starting eluent.

In addition to analytical chromatography of the azodye reaction mixtures, it was necessary to isolate larger quantities of the products for conventional chemical characterization techniques. Preparative TLC was not always able to provide sufficient resolution to obtain a pure product.

Initial attempts at loading 1–3 ml of 50-mg/ml samples onto a 30 × 2.5 cm column by simply switching the loaded sample loop into the flow stream resulted in either precipitation of the sample at the origin of the column or bleeding across the column without any components being resolved.

After considerable experimentation, it was found that placing the loaded sample loop in the mobile phase stream with the flow control set at zero, and slowly increasing the flow-rate to its normal value, with the gradient programmer running only one-pump channel, resulted in reproducible sample application. The success of flow-programming for sample injection can be rationalized in the following way. Initially the flow-rate is kept very low to allow the sample solution (which is more viscous and perhaps stronger than the mobile phase) to equilibrate with the column rather than precipitate or channel. If the sample was applied at a constant low flow-rate, the back end of the band was found to diffuse extensively. This was in part due to diffusion in the sample loop and associated plumbing over the time required to load the sample and the beginning of elution of the sample across the column. Increasing the flow-rate slowly during loading resulted in very narrow, well defined bands being introduced to the column.

The results of two separations on a reaction mixture containing N-acetyltyrosine and diazotized arsanilic acid are shown in Figs. 7 and 8. The first elution indicates the type of problem that can occur during optimization procedures. The monoazo derivative and two other components elute rapidly, completely resolved from one another, and are not shown. Another desired product, bis(arsanilazo)-N-

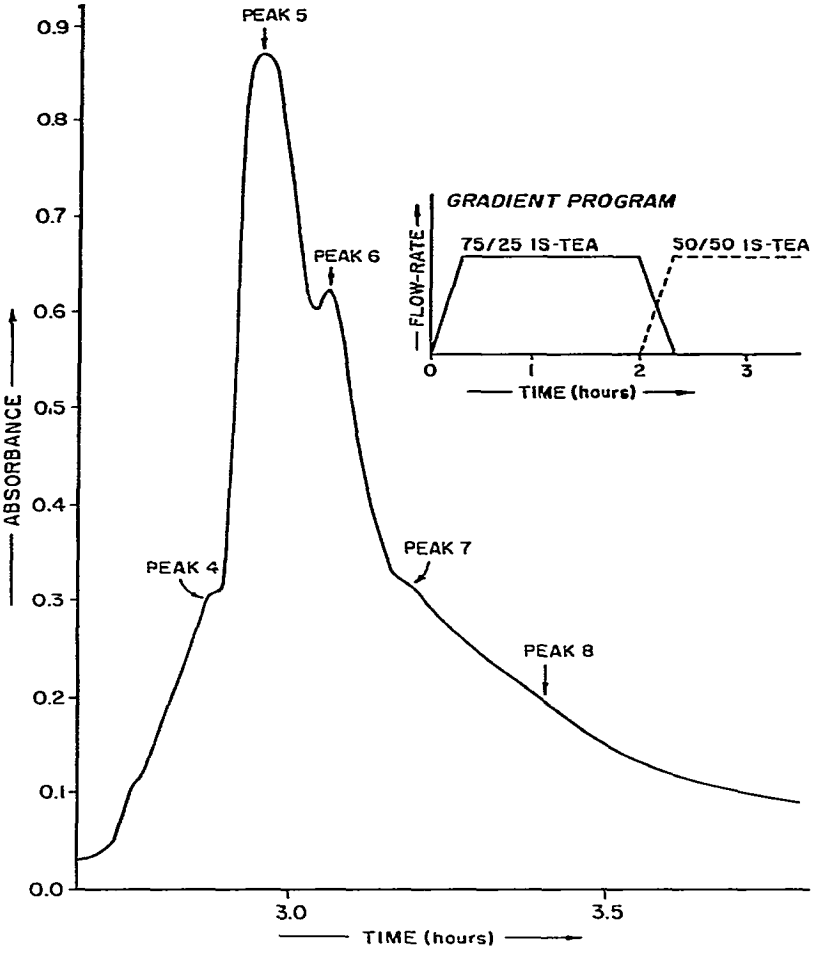

Fig. 7. Elution profiles for the unsuccessful separation of an N-acetyltyrosine diazotization reaction mixture. Inserts refer to the flow and gradient programs used to produce the separation. The eluent was monitored at 510 nm, 2 absorbance units full scale. The flow program was 20 min in length, ending at 5 ml/min flow-rate. Sample size ranged from 50–100 mg of crude reaction/ml water.

acetyltyrosine (peak 5) was not resolvable from components in peaks 4 and 6 by preparative TLC. When a sample of the reaction mixture (approx. 50 mg) was applied to the column, the components in the region of peak 5 were clearly resolved from each other (due to their characteristic colors visual identification is possible) early in the elution. A linear gradient was then applied in order to rapidly remove the resolved components from the column. Unfortunately, the components remixed before they could be completely eluted.

In contrast to the analytical separations where the two components of the solvent system were pumped separately, the preparative separations were performed with two premixed IS–TEA solutions of differing strength. This allowed small changes in the eluent strength to be defined by relatively large changes in the flow-rate of

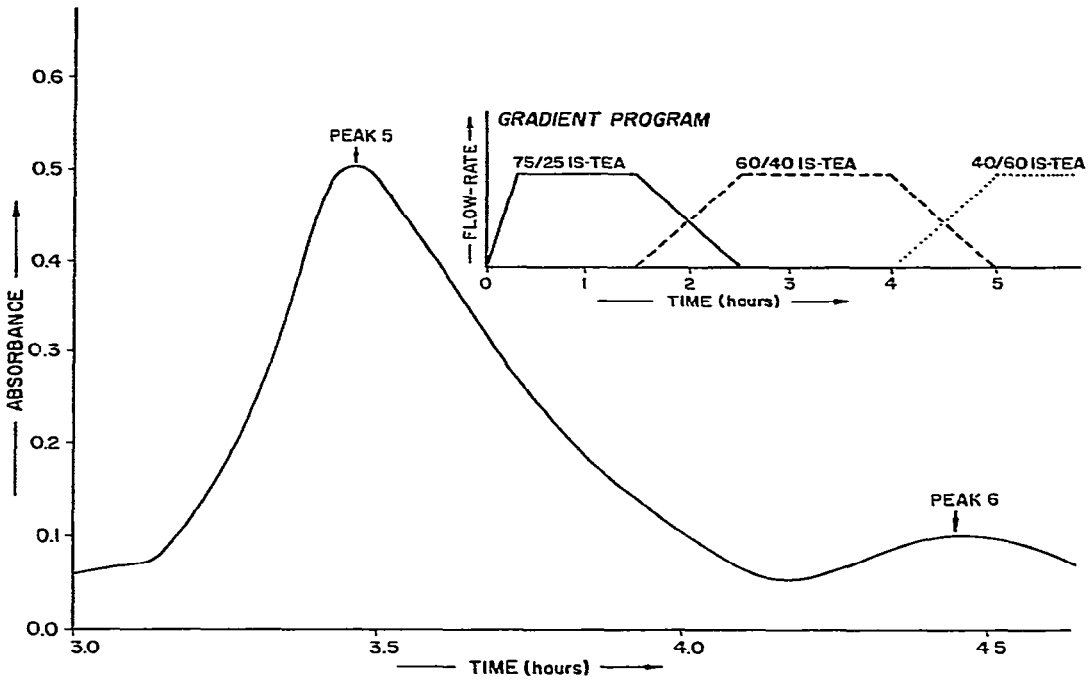

Fig. 8. The successful separation of peak 5 from the diazotization reaction mixture utilizing a somewhat longer gradient (insert) to a solvent of intermediate composition. Other conditions are the same as in Fig. 7.

either pump. Proper choice of gradient shape and solvent strength resulted in the complete resolution of peak 5 (Fig. 8). The third solvent shown on the insert in Fig. 8 was used to remove other strongly retained components in a reasonable period of time.

CONCLUSIONS

The liquid chromatograph described here, in combination with a suitable solvent system and columns, has been used to perform separations that previously were confined to TLC or were not possible at all. Gradient elution and flow-programming played a crucial role in the ability to perform preparative separations quickly and reproducibly. Additionally, the low cost of this system is an advantage where the high cost of a commercial unit cannot be justified.

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