TECHNIQUE FOR SIMULTANEOUS MULTIPLE-COLUMN GRADIENT ELUTION CHROMATOGRAPHY

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Several methods for gradient elution chromatography have been proposed since ALM *et al.*[1] published the first detailed study of this technique. They have all been worked out for use with a single column.

The technique presented in this article has been primarily developed as a method for running multiple simultaneous chromatograms using the gradient elution method. It does, however, also offer a system for single column gradient elution chromatography that combines relative simplicity with flexibility. The fact that the gradient can be changed at will in a predetermined way at any time during the run by simply turning a screw control should prove especially useful.

The first problem we ran into in developing the technique was the unavailability of an accurate device that could deliver small volumes of eluant to a developer and at the same time could be varied continuously and easily in a calculated way.

The dropping funnel described by LAKSHMANAN AND LIEBERMAN[2] in their gradient elution method was tried but not found accurate enough for very small volumes, and the adjustment to a given volume was a long and cumbersome procedure.

A variable speed syringe drive was developed to solve these problems.

VARIABLE SPEED SYRINGE DRIVE

This device consists (Fig. 1) of a Krogh-Keyes syringe pipette arranged so that it can slide freely on two metal bars, a continuously variable speed changer with a screw control, and a synchronous motor, all mounted on a 1/4-in. thick aluminum plate.

A Krogh-Keyes syringe pipette (MacAlaster Bicknell No. 34197) of appropriate size is fitted with four 2-in. long 1/8 in. × 3/8 in. aluminum bars. They are placed so that they are parallel with the part of the Krogh-Keyes syringe that is mounted around the collar of the syringe barrel and are attached to the four corners of the metal frame of the syringe pipette. The grooves in these vertical aluminum bars slide over two horizontal stainless steel rods mounted on two small aluminum posts screwed into the 1/4 in. aluminum base. With this arrangement the syringe can move freely back and forth in a horizontal position. A circular piece of stainless steel plate 1/32 in. thick is glued to the end of the syringe piston to ensure a flat surface.

The threaded rod in the Krogh-Keyes syringe is connected to the variable speed

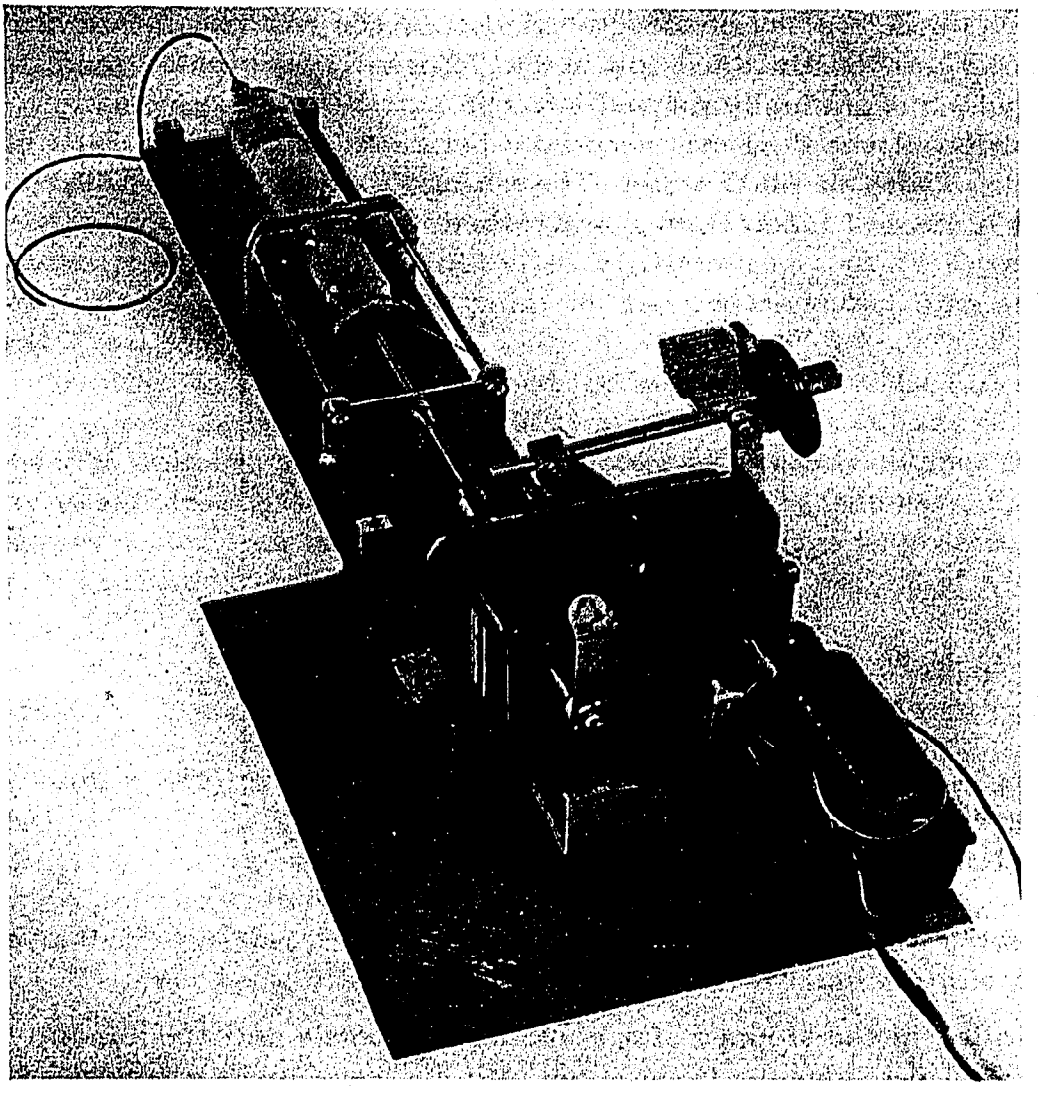

Fig. 1. The variable speed syringe drive with Krogh-Keyes syringe pipette, variable speed changer with screw control and counter and synchronous motor.

changer (Revco, Minneapolis, Model 14R) with a small cylindrical brass connector.

A counter is mounted on the screw control of the variable speed changer. A 1-in. diameter 48 pitch brass spur gear is fastened on the axle of the counter and a similar gear is put on the threaded rod which drives the screw control. One turn of the screw control registers as one turn on the counter and the screw control has a range of either 0 to 50 or 0 to 500 if subdivisions are used. This corresponds to a continuous variable speed reduction of the input speed from the motor of 1/4 of motor speed to zero.

The variable speed changer is connected to a synchronous motor. The speed selected for this motor is 1 r.p.m. for our application. If delivery greater than approximately 10 ml/h is necessary, a faster motor must be used.

The device operates in such a way that the rotating movement from the synchronous motor shaft is transferred to the output shaft of the variable speed drive

with whatever speed reduction is set on the screw control. The output shaft from the speed drive drives the threaded rod in the Krogh-Keyes pipette against the end of the piston which is gradually pushed into the barrel of the syringe.

Calibration of the device is necessary before it can be used. This is most conveniently done with distilled water. The syringe is filled with distilled water and a needle with a piece of teflon tubing is attached. A hole slightly larger than the teflon

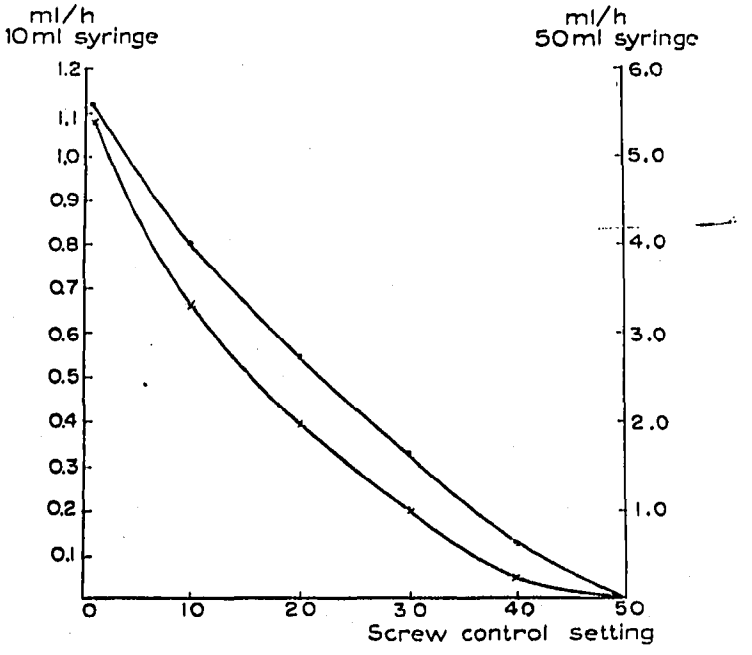

Fig. 2. Calibration chart for 50 ml (×—×—×) and 10 ml (o—o—o) syringes in the variable speed syringe drive.

tubing is drilled in the stopper of a polyethylene bottle and the amount of distilled water delivered per hour (or larger time unit for very small amounts) is measured. Fig. 2 shows such a calibration chart for a 50 ml and a 10 ml syringe. Syringes with interchangeable pistons like the multifit type are preferable, since one calibration can then be used for a number of syringes with the same capacity.

The accuracy and reproducibility of the syringe drive was tested by running 10 calibrations of both a 50 ml and a 10 ml syringe at a slow setting of the screw control (40.0) and again at a fast delivery rate (screw control set at 10.0). The means and

TABLE I

REPRODUCIBILITY OF SYRINGE DRIVE

Output per hour given as mean value with standard deviation for 10 determinations at a slow and fast delivery rate.

	Screw control set at 10.0		*Screw control set at 40.0*	
	Mean ml/h	*Standard deviation*	*Mean ml/h*	*Standard deviation*
50 ml syringe	3.327	0.027	0.219	0.0035
10 ml syringe	0.807	0.0075	0.132	0.0025

standard deviations have been calculated for both these settings. Table I shows the degree of accuracy obtained, with a standard deviation around 1 % for the faster delivery rate and close to 2 % for the slower delivery rate.

Many chromatographic columns have been run in single column runs with a 10 ml syringe set for delivery of 0.110 ml/h, and a high degree of reproducibility of chromatographic patterns with the same substances peaking within the same number of tubes from the start of the column was obtained. This confirms that the reproducibility of the syringe drive, from a practical point of view, is sufficient also at the slow delivery rates.

The syringe drive is specifically designed for low flow rates. The largest syringe available is of 100 ml capacity. One can modify the device and use a bank of 3 syringes; however, this gets unhandy and one would be better off using for higher flow rates a commercially available precision metering pump (Kruger Instruments, San Gabriel, Calif.). This instrument has sufficient accuracy for the higher flow rates, and thus supplements the syringe drive to give coverage up to rates of 600 ml of added eluant per hour.

TECHNIQUE FOR GRADIENT ELUTION CHROMATOGRAPHY ON A SINGLE COLUMN

The column used is of a special design (Fig. 3). It consists of a piece of glass tubing approximately 18 in. long and with an internal diameter of 6 mm. It is surrounded with a water jacket for thermostatting. At the bottom end a perforated glass plate is melted in to give support for the column and a standard tapered 10/30 joint is fused on at the top. A teflon stopcock with a built-in needle valve (Fischer & Porter Co., Patboro, Pa., No. 80 × 2440 B) is melted to the chromatographic tube below the perforated glass plate.

To pack the column one first connects the water jacket to a constant temperature circulator and the column is then filled three quarters of its length with solvent from the bottom by applying light vacuum to the top. Two circular pieces of filter paper are pushed down with a long glass capillary so that they cover the perforated glass plate. The column is now filled from the top with solvent and an enlarging type of glass adapter with a 10/30 joint at the bottom and a 24/40 joint at the top is put on top of the column. The adapter is filled with solvent and a 250 ml filling funnel with a bottom 24/20 joint is pressed down into the adapter. The tip must be cut off the filling funnel so that it will fit. It is very important at this point that any air bubbles trapped in the adapter be removed. This can be done by introducing a piece of thin teflon tubing through the hole in the stopcock. Trapped air will escape if a few pumping movements are performed.

Approximately 100 ml solvent is now introduced into the filling funnel and the adsorbent is added. It will gradually pass from the filling funnel to the adapter, where it will be whirled around before it slowly settles in the column. The column is tapped gently for about a minute after it has settled and then it is pressed under light pressure. Columns prepared this way are quite uniform in composition and give consistently

good separations. The technique is particularly useful in serial analyses where a number of columns can be filled rapidly and conveniently, since the columns can be left to settle after the adsorbent has been added to the filling funnel.

To construct the mixing chamber, a piece of capillary tubing is melted to the bottom of either a Pyrex bottle or an Erlenmeyer flask. A teflon adapter is then turned on the lathe so that it will have at one end a hole that will fit tight around the glass capillary. At the other end it is tapered to fit a hypodermic needle. Another teflon adapter is made to fit the top of the column. It is tapered to fit a 10/30 joint at one end and to fit a hypodermic needle at the other. A collar is left in the middle during the turning of the adapter. This serves as a support for a small stainless steel ring with two hooks. Rubber bands or metal springs secure the adapter to glass hooks on the column.

A piece of teflon spaghetti tubing (Allied Plastics, Elizabeth, N.J.) size AWG 24

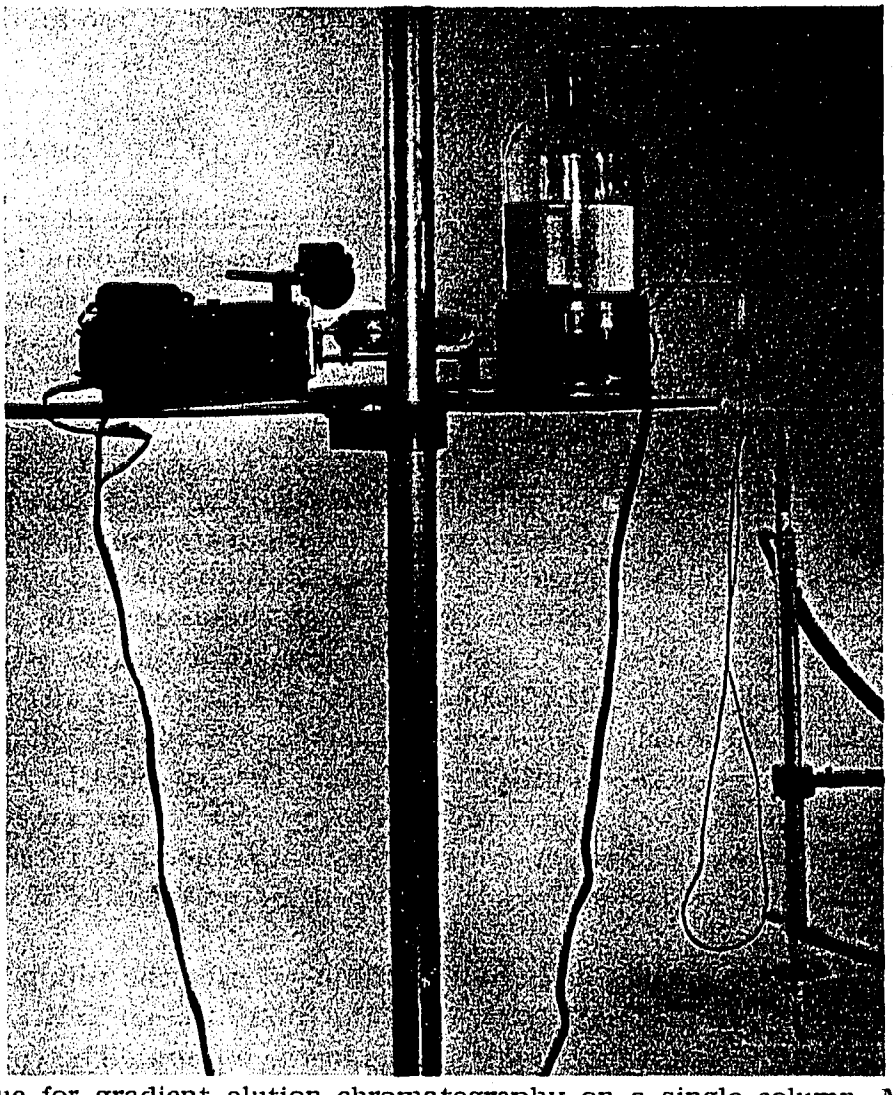

Fig. 3. Technique for gradient elution chromatography on a single column. Mixing chamber, magnetic stirrer and variable speed drive are on a platform sustained by a "polecat". The needle valve stopcock for regulation of flow rate is at the bottom of the column.

of proper length is used to connect two size 20 hypodermic needles. Care must be used to avoid perforation of the teflon tubing with the needle tip when it is attached. The needles are then put on the teflon adapters. The needle size and the diameter of the teflon tubing can be changed if faster flow is needed.

The syringe drive is connected to the mixing chamber through a needle and a piece of teflon tubing. The tubing is carried down into the bottle through a polyethylene stopper in which two small holes have been drilled, one to admit the tubing, the other to serve as a vent.

The mixing chamber is then placed on a magnetic stirrer together with the syringe drive and the whole assembly is placed on a "polecat" platform that can move freely up and down. (Polecat, Inc., Old Saybrook, Conn. manufactures this adjustable aluminum column that is spring-held between ceiling and the floor or tabletop.)

To run a chromatogram on a single column the following steps are taken. The extract to be chromatographed is transferred with a suitable amount of solvent to the column or is transferred to a filterpaper disc which is then put on top of the column. Some solvent is pressed through the column which is then filled. Teflon adapters, needles and teflon tubing are connected together and then connected to the mixing bottle. The teflon adapter fitting the column is lifted to the neck of the bottle and a calculated amount of solvent is added to the bottle. The bottom adapter is now lowered as far as it will reach and tubing and adapter is flushed with a few ml of solvent to remove air from the connecting system. This solvent is added to the mixing bottle to avoid loss. The adapter is then gently pushed down into the 10/30 joint on top of the column. Care must be taken to avoid air bubbles at this stage.

The column is now ready for calibration of flow rate. There is a linear relationship between distance from tip of the column to surface level in the bottle and one can therefore change the flow through the column in a precalculated manner by raising or lowering the platform on the "polecat". It is also possible to set the platform at a height where the outflow from the column is calculated to be somewhat higher than the flow rate needed and then by turning the screw on the needle valve adjust the flow to the proper rate. The first of these methods is the easier and faster one when single columns are run.

The amount of liquid used in the calibration is measured and a similar amount added to the mixing chamber.

The syringe drive is now filled with eluant and the run started by connecting syringe drive and magnetic stirrer to the line and opening the column stopcock.

TECHNIQUE FOR GRADIENT ELUTION CHROMATOGRAPHY SIMULTANEOUSLY ON SIX COLUMNS

The technique described for single columns is modified so that it can be used for 6 columns (Fig. 4) and is then combined with the technique for simultaneous fraction collection from 6 columns described elsewhere in this issue[3].

One modification necessary to run 6 columns at a time is in the columns. In order that needle valves on the columns are readily accessible for calibration it is necessary to arrange the columns in two parallel rows with approximately 4 in. between each

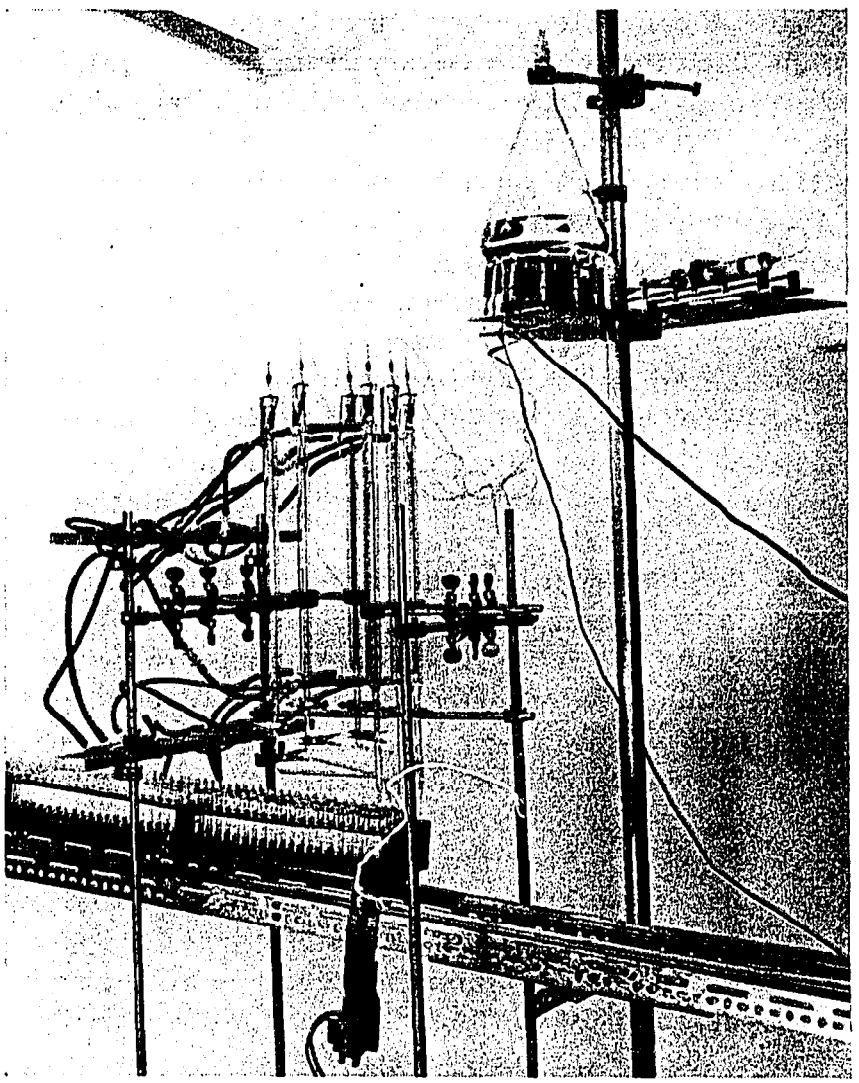

Fig. 4. Technique for gradient elution chromatography on six columns simultaneously. Six columns arranged in two rows and connected to common mixing chamber on "polecat".

row. Half of the columns are therefore modified slightly by adding a 4-in. S-shaped extension with a ball joint to the tip of the columns. The mixing chamber for the multiple run is a large Erlenmeyer flask with six 10/30 standard tapered female joints melted to the bottom. Teflon adapters similar to those used for the column portions are used to connect to the mixing bottle instead of the type used on the single column mixing bottle.

The columns are otherwise set up as described under single column runs. All six are connected to the mixing chamber and the columns are ready for calibration of the flow rate.

The optimal conditions for gradient elution chromatography on a single column are first established, as well as the optimal values for the three factors that determine the gradient: outflow from the column, initial volume in the mixing bottle and inflow from the syringe drive have first been experimentally determined. The syringe drive is set to deliver 6 times the flow rate that was found optimal on single column run. The mixing bottle is filled with 6 times the volume of solvent used in single column run, the outflow is then calibrated.

This is accomplished by moving the platform on the "polecat" to a position where the distance between liquid surface in the bottle and the tips of the columns is slightly greater than the one calculated from the calibration charts to give the needed flow through the column. Stopcocks and needle valves are now opened and the flow determined over a 10 min period by collection in measuring cylinders. At the same time measurements are made with a stopwatch to determine the time it takes, for example, for 10 drops to form. The flow per 10 min is read from the measuring cylinders

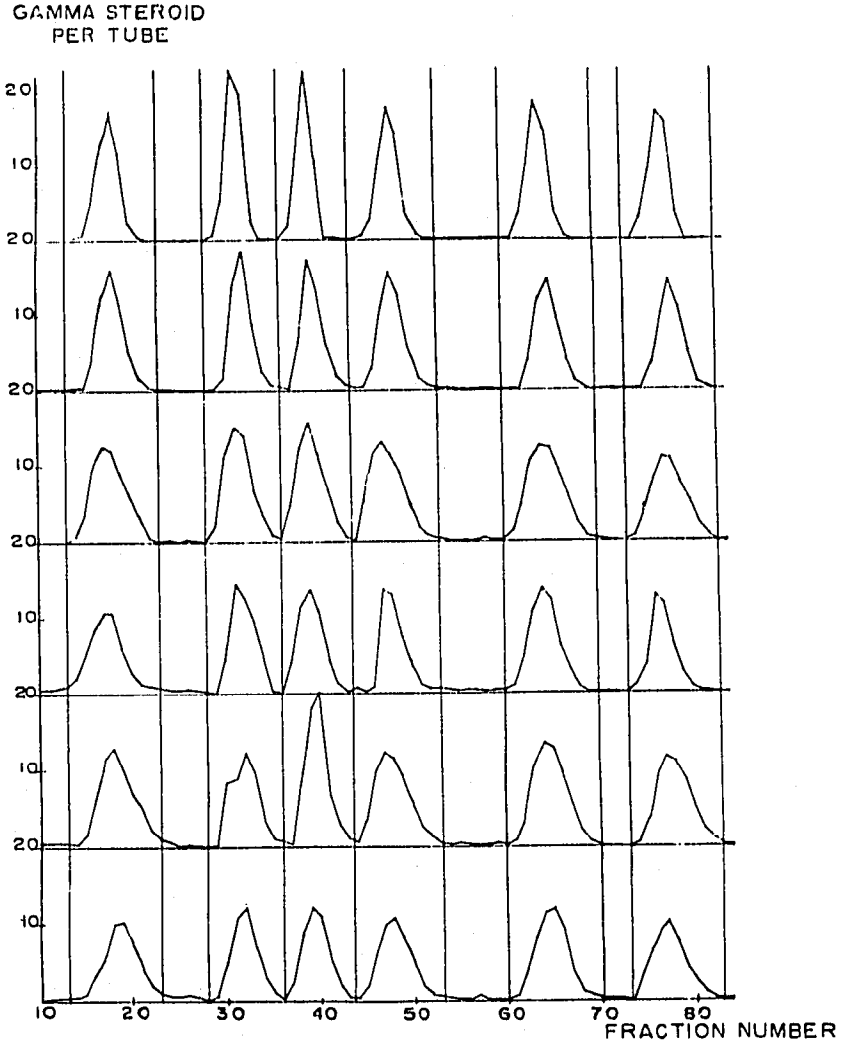

Fig. 5. Simultaneous gradient elution chromatography of 50-γ portions of six 17-ketosteroids on six chromatographic columns.

and the change in drop time that should give the needed flow per 10 min is calculated for each column. The initial maximum 10% difference between the fastest flowing and the slowest flowing column is caused by the non-uniformity of the columns. The differences are now corrected with the needle valves by adjusting the time taken for 10 drops to form to correspond to the calculated flow. The flow from the columns is fairly easily regulated within approximately 2 % difference, not including the possible error on the 10 ml measuring cylinders. From a practical point of view this error was found sufficiently low to give good agreement between runs from the different columns.

RESULTS

An example of the results obtained when running 6 columns is shown in Fig. 5. A six-column elution chromatogram was run with six 17-ketosteroids present in human urine. The conditions for the run were as follows: starting volume in mixing chamber: 3000 ml pure benzene. Syringe drive set to deliver 0.660 ml/h ethanol from a 50 ml syringe. Distance from column tip to benzene surface at start: 30 in. Flow rate at the start for each column, 5.15 ml/20 minutes $\pm$ 2 % (uncorrected for errors in the measuring cylinders). Columns 1, 3 and 5 counted from the bottom were run with transfers of the steroid standards on paper disc; in the other cases, transfers were in benzene solution. The adsorbent was neutral aluminum oxide Woelm with 6 % water added. 11.50 g were used per column. The length of the columns varied from $14^7/_8$ in. to 16 in. owing to differences in the diameter of the glass tubing used for their construction. The temperature of the cooling water was 16.2° $\pm$ 0.1°. 50-γ portions were run of androstanedione, dehydroisoandrosterone, androsterone, etiocholanolone, 11-ketoetiocholanolone and 11-hydroxyetiocholanolone given in the order of elution from the column. Mean recovery of all steroids was 99.4 % with a variability from 93 % to 106 %. 20 min intervals were used and 85 fractions collected.

As can be seen from Fig. 5, clear separations are obtained of all substances and all the steroids come out in corresponding tube areas with no overlap of areas from different columns.

DISCUSSION

The main advantage of the proposed method lies in the possibilities it gives for running many gradient elution chromatograms at one time. This should help spread the use of this technique, which for many applications will be superior to simple chromatography.

The fact that the identical gradient is used for both a set of standards and a number of mixtures of unknowns should make the determination of substances from their position on the chromatogram more reliable than determinations on chromatograms obtained through consecutive runs.

A special feature of the setup is the ease with which the gradient can be changed during a run. This means that it is possible to work not only with upwards concave and convex or straight line gradients but also with S-shapes and other shapes. It is

possible to bring about such changes in the gradient automatically by coupling a synchronous motor to the screw control and have it monitored from the necessary number of timer-activators.

The proposed technique has been used for a system of analyses for 17-ketosteroids in blood and urine and work is in progress on methods for corticosteroids in blood and urine. Details will be given elsewhere.

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SUMMARY

A technique for simultaneous gradient elution chromatography on a number of chromatographic columns is described. The same gradient is used for a set of standards and for mixtures of unknowns. The technique has great flexibility and allows easy change of gradient during a run.

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