CHROM. 4785

A SYSTEM FOR SIMULTANEOUS 25-COLUMN, CAPILLARY COLUMN LIQUID–SOLID CHROMATOGRAPHY

PER VESTERGAARD AND ERIK JACOBSEN
Research Center, Rockland State Hospital, Orangeburg, N.Y. 10962 (U.S.A.)
(Received April 23rd, 1970)

SUMMARY

A system for simultaneous 25-column, capillary column liquid–solid chromatography is described. It uses compressed nitrogen to drive the eluant through the capillary columns. A comparison with a system using chromatographic pumps shows that the nitrogen system costs 80% less per channel, has better resolution and higher reproducibility and there are fewer maintenance problems. The problem of the more variable flow rate has been overcome by the addition of an internal standard.

INTRODUCTION

We first described a system for multi-column liquid chromatography in 1960[1,2]. This system has been gradually expanded in capacity and improved in the decade since then. The first multi-column system used gravity feed to drive the eluant through the columns and the colorimetry was done manually. An improved system[3] pumped the liquid through the columns with chromatographic pumps and at the same time an automated read-out system with computer calculation of data was introduced. The system gained much in practicality and overall performance characteristics when capillary columns, first glass[4] then teflon[5], supplanted the wider-diameter thermostatted glass columns previously used. A technique was described for the machine filling of the capillary columns[6] and an improved multi-column fraction collector using magazines[7] was constructed. This new type of collector formed the nucleus for a high-capacity multi-channel computerized read-out system[8] with a capacity of 100 chromatograms per day expandable to 400 chromatograms per day. Practical work with this system has made it clear that we would have to simplify the chromatographic technique to fully exploit the increased capacity made possible by the new read-out system.

The main obstacle to very high capacity multi-column systems are the chromatographic pumps used to pump the eluant through the capillary teflon columns[3]. These pumps are expensive, costing several hundred dollars per channel, and this for most laboratories would make it prohibitively expensive to consider systems incorporating for example a hundred or more of these pumps.

We have, consequently, explored the possibility of building a system using compressed nitrogen to drive the eluant through the capillary columns thereby greatly reducing the cost per channel in multi-column chromatography.

GENERAL DESCRIPTION OF SYSTEM

A general view of the system is given in Fig. 1. The capillary teflon columns used in the chromatography[5,6] are in the waterbath. Sample transfer is through the wider-diameter cartridges of teflon tubing shown above the waterbath. The elution solvent for gradient elution is mixed in the stainless steel cylinder shown to the left

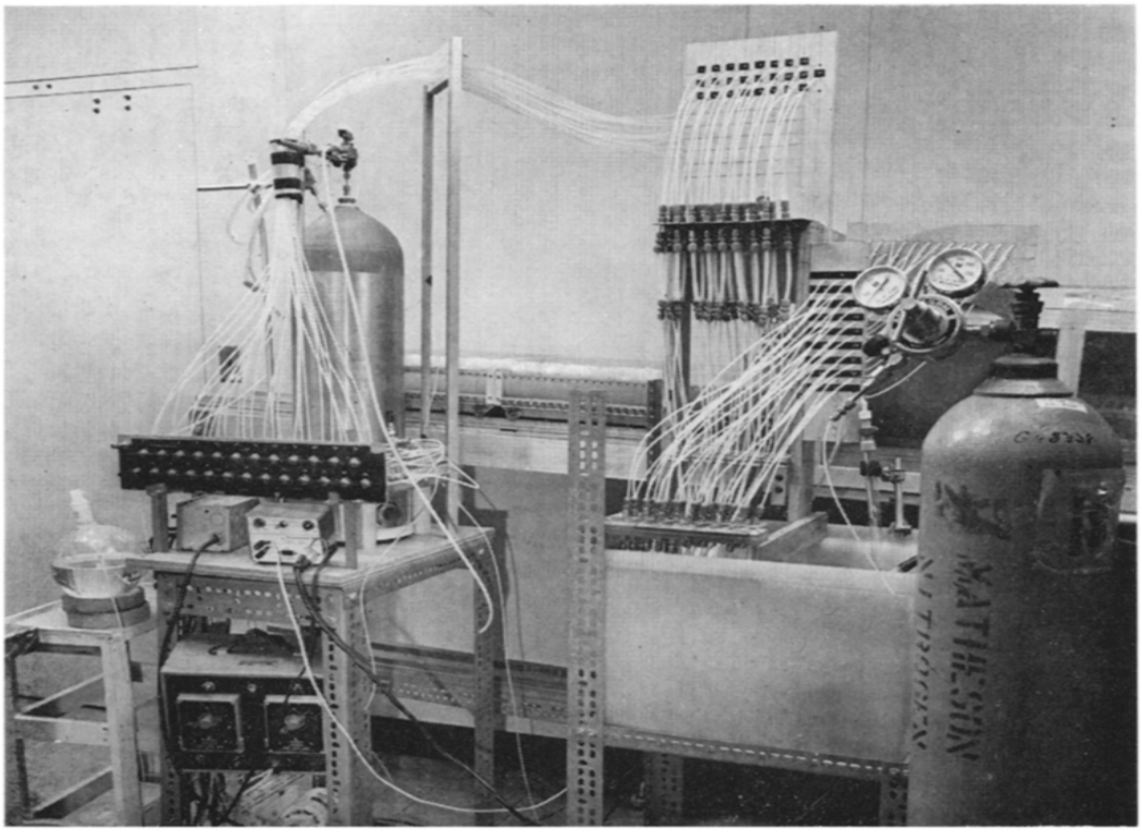

Fig. 1. Mixing and distributing cylinder for the formation of the gradient used for elution is to the left. The capillary columns are in the center with the filling cartridges on top. The nitrogen tank with its regulator is to the right and the multicollector is seen in the background.

and is then sent through the panel of needle valves located in front of the cylinder to the chromatographic columns. The pressure to drive the solvent through the capillary columns is supplied by the nitrogen tank with regulator positioned to the right in the figure. The effluent is collected in the 25-column multi-collector shown in the background. The gradient is formed by pumping a more polar solvent into a less polar solvent in the stainless steel pressure cylinder using a chromatographic pump positioned on the floor.

DETAILS OF CONSTRUCTION

Mixing and distributing cylinder

The mixing and distributing cylinder (Fig. 2) has been made from a stainless steel sampling cylinder (Hoke, Cresskill, N. J., No. 8LS5-G) of five gallons' capacity. The round bottom of this cylinder was cut off and a flat piece of 1-in.-thick stainless steel was welded on as a bottom. The stainless steel used has been of the non-magnetic type to make magnetic stirring of the solvent inside possible.

In the periphery of the bottom of the cylinder 25 L-shaped holes have been drilled and swagelock fixtures (Hoke No. 2CM2) have been fitted to each of these holes. Teflon capillary tubing leading the solvent to the needle valves have been placed inside the swagelock fittings. The teflon tubing from the outlets at the bottom of the cylinder are connected to the 25-valve needle valve (Hoke No. 1325GY)

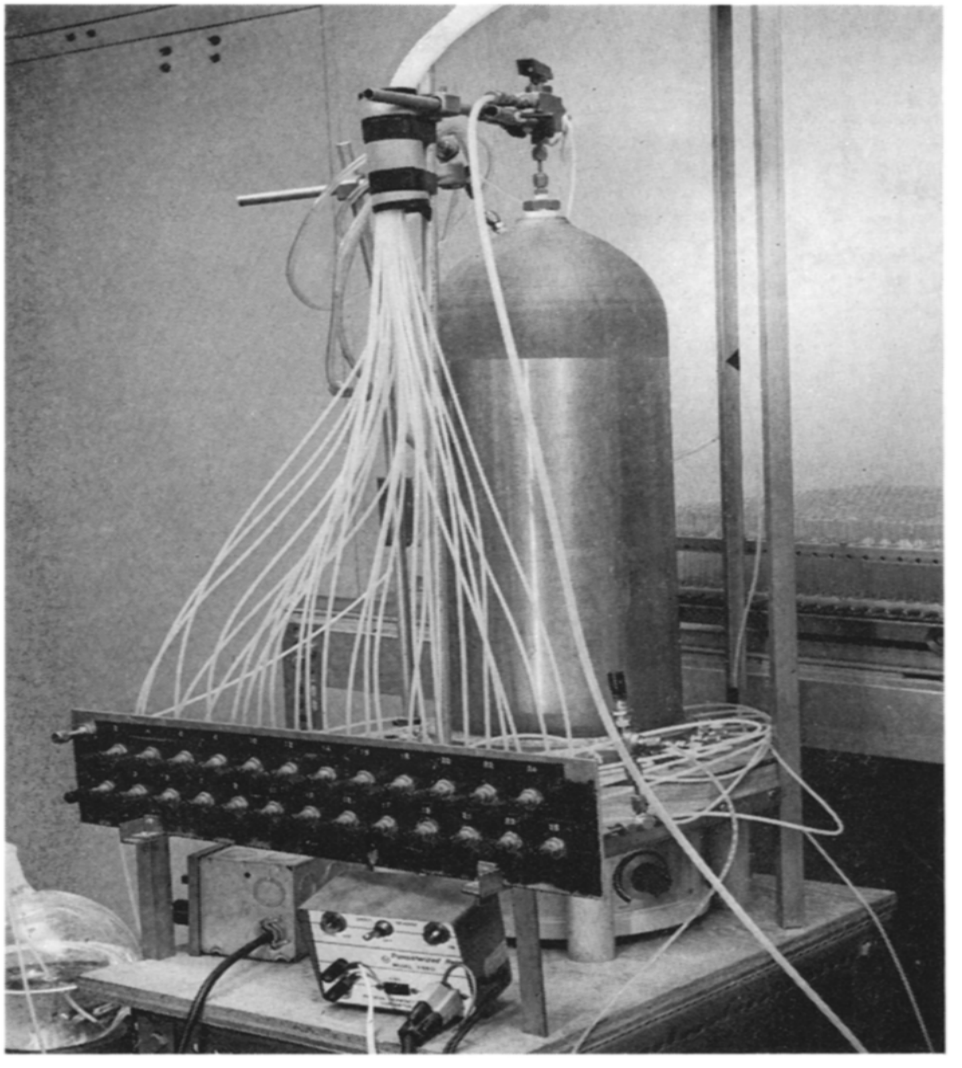

Fig. 2. The mixing and distributing cylinder. Teflon tubing goes from the outlets at the bottom of the cylinder to the needle valves in front to pass from here to the chromatographic columns. Two ball valves on top for solvent introduction and the application of nitrogen pressure to the system. A magnetic stirrer is placed under the cylinder for mixing of the gradient in the cylinder.

panel shown in the foreground of Fig. 2. From these valves solvent is passed through capillary teflon tubing to the chromatographic columns located in the waterbath.

Two ball valves (Hoke No. 7165 G25) lead to the inside on the top of the cylinder. One serves to enter the main solvent used in the chromatography into the cylinder before the run is started and to introduce the gradient forming solvent during a run. The other ball valve connects to the nitrogen tank through a reduction valve and regulator that allows the selection of the proper pressure for the chromatography.

The cylinder is placed on top of a magnetic stirrer and during operation a teflon-covered magnet on the bottom of the cylinder provides the stirring action needed to mix less polar and more polar solvents during the chromatographic run.

Chromatographic column arrangement

We have previously[7] described a very concentrated arrangement of capillary teflon columns spirally wound in a small space in a small waterbath that could be placed close to the multi-collector used in the fraction collection. This system had

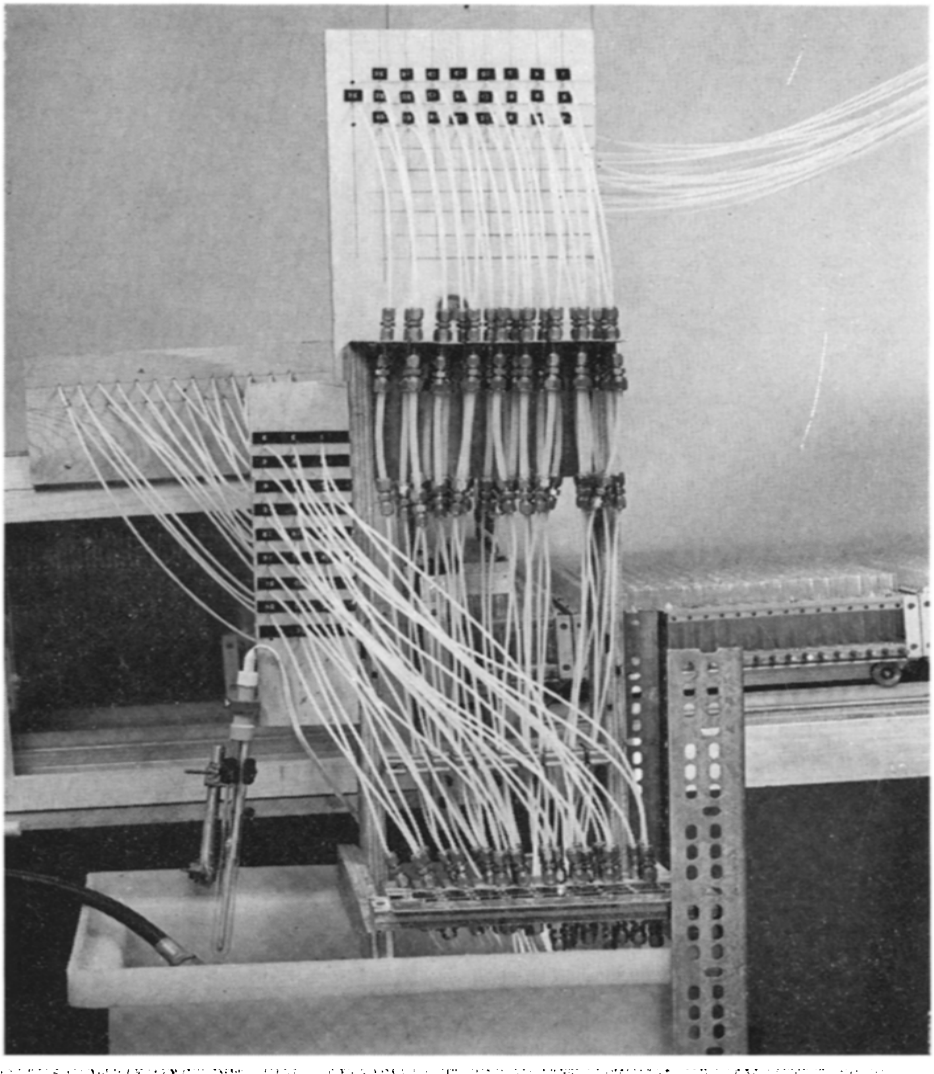

Fig. 3. The column arrangement. The capillary columns are in large loops in the waterbath. The tubing over the water level serves for transfer of the sample to the top of the capillary columns.

the undeniable advantage of giving very little mixing of the eluent as it leaves the columns. It was, however, found quite time-consuming to use the system in daily routine runs and we have therefore changed over to the system shown in Fig. 3. The sample is transferred in the proper solvent to the top portion of the capillary column and the wide-diameter teflon cartridges shown in the centre of the figure. The total transfer volume is about 3 ml, which allows one 1-ml transfer and four washes with 0.5 ml solvent to assure complete transfer to the column of a dry extract. The capillary column itself is loosely wound into a couple of circular loops held together with two pieces of plastic-covered wire as used in an ordinary household for the closing of plastic bags for freezer or refrigerator. The column is then placed in the waterbath.

The 25-column multi-collector

This (Fig. 4) is similar to the original design[1] only enlarged in capacity to accomodate 25 rows of test tubes. The reason we prefer the older desing over the magazine-type collector for high-capacity multi-column chromatography[7] is that this type of collector can be stacked one on top of each other so that for example four 25-column collectors can conveniently be combined into one 100-column collector.

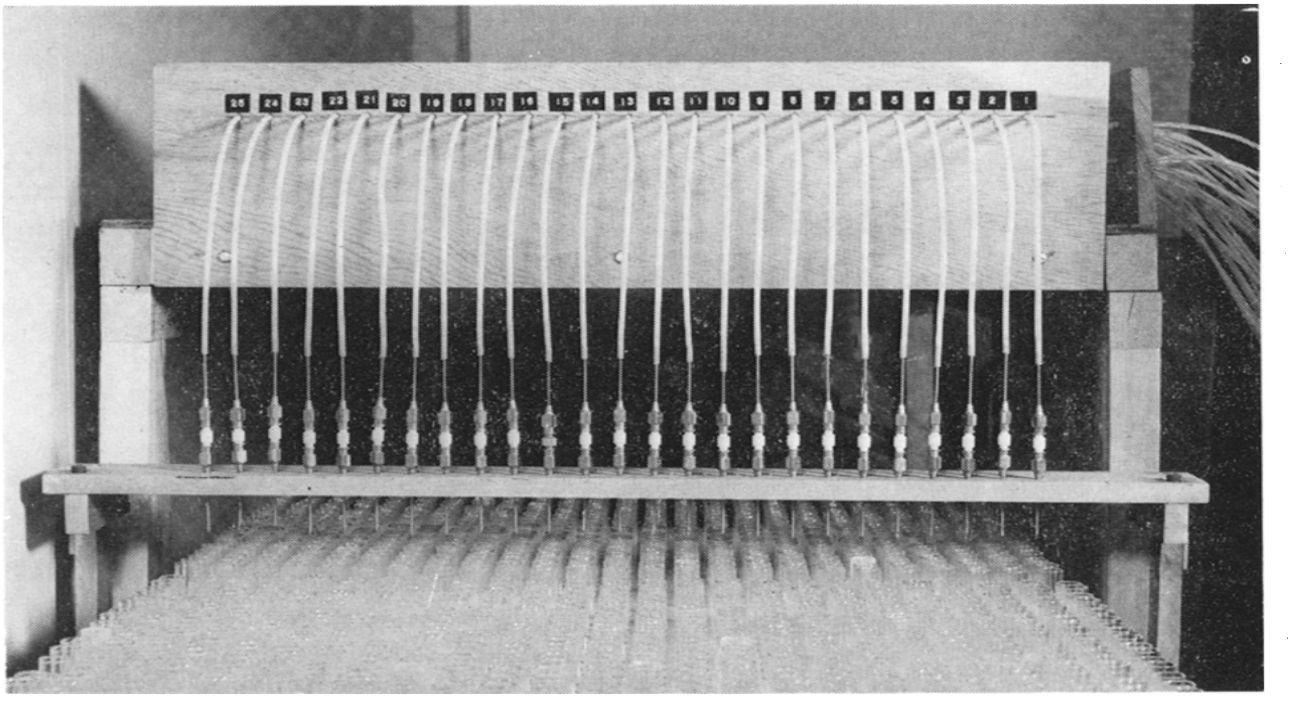

Fig. 4. This figure shows the 25-column multicollector and the way the effluents from the chromatographic columns are transferred to the collector tubes.

OPERATION OF THE SYSTEM

Preparing the columns for chromatography

The capillary teflon columns are dry-filled by machine in a one-batch operation as previously described[6]. They are loosely bound into large loops as described above

and arranged in the waterbath where they are connected to the wider-diameter teflon tubing on the cylinder side and the tubing leading to the fraction collector on the other side.

Non-polar solvent is now introduced into the stainless steel cylinder through the T-valve at the top of the cylinder with the needle valves closed. Pressure is applied to the cylinder through the valve connected to the nitrogen after closure of the other ball valve and the needle valves are kept open until all air has been flushed out of the tubing and the chromatographic columns. The valves are then closed and the solvent remaining in the cylinder is let out of the cylinder through a separate needle valve attached to one of the outlets.

Application of the sample to the columns

The swagelock fitting on top of the wider teflon tubing is opened up. The solvent down to the top layer of the chromatographic column is aspirated with a syringe to which has been attached a thin needle equipped with a piece of thin capillary teflon tubing at the end. The sample is introduced using the same small syringe with teflon tubing attached .Because of the wider teflon tubing attached to the capillary tubing a transfer of an extract from a test tube can be done with an initial 1-ml transfer of sample in solvent followed by four ½-ml washes to ensure complete transfer of the extract. The system is connected up again after the transfer of the sample and the columns are now ready for chromatography.

Filling the cylinder

The appropriate amount of non-polar solvent is introduced through one of the ball valves located at the top of the cylinder. This is done with the needle valves closed. The solvent feed valve, a T-valve, is turned towards the gradient pump that pumps the more polar solvent into the mixing cylinder. The system is now started up by turning the magnetic stirrer on, starting the gradient pump pumping, opening the needle valves to the chromatographic columns and opening the valve leading to the nitrogen tank. The nitrogen pressure is set to an appropriate value and the fraction collector is started up.

PERFORMANCE

Resolution

We were somewhat surprised to find in initial runs with the system that the resolution in the nitrogen pressure system seemed to be better than in a system using pumps to get the solvents through the capillary columns. However, this was repeatedly found to be so and was proven by the following experiment.

Two sets of twelve batches of standards each containing the following seven common urinary 17-ketosteroids, *viz.* dehydroepiandrosterone (DHEA), androsterone (A), etiocholanolone (E), 11-ketoandrosterone (OA), 11-ketoetiocholanolone (OE), 11-hydroxyandrosterone (OHA) and 11-hydroxyetiocholanolone (OHE), were run in a 12-column system using the new nitrogen pressure system.

Identical conditions were maintained throughout. The packing of the columns were done in the same manner, the gradient was the same, the temperature was kept the same and so on. Approximately 50-μg amounts of each of the standards were applied to each column.

The conditions for the chromatography were: 6-ft.-long capillary columns with an I.D. of 1.5 mm were used. Neutral alumina for chromatography, Merck, deactivated with 6 % water was the substrate. The gradient was ethanol in benzene. The pressure was approximately 8 atm. in the nitrogen system. Total run time was 20 h.

Theoretical plate numbers were calculated after the standard formula[9]: $N = 16(R'/w)^2$, where R' is the corrected retention volume and w is the width of the peak defined as that part of the peak that is within two standard deviations on either side of the peak maximum assuming that the peak follows a Gaussian curve. Since we do not have a continuous curve in our system we have based the width calculation on the fact that 95.44 % of the area of a normal curve is within two standard deviations on each side of the curve maximum and we have consequently included in the calculation of peak width enough fractions to give 95.44 % or more of the total area of a given peak.

A comparison of the resolutions found in the system using chromatographic pumps as compared with the nitrogen pressure system is given in Table I. It can be seen from this table that the theoretical plate numbers are consistently higher, on an average 50 % higher, in the system using compressed nitrogen.

TABLE I

AVERAGES FOR THEORETICAL PLATE NUMBER CALCULATIONS FOR TWELVE SETS OF SEVEN 17-KETOSTEROIDS RUN IN A NITROGEN PRESSURE SYSTEM AS COMPARED WITH A CHROMATOGRAPHIC PUMP SYSTEM

For abbreviations used for 17-ketosteroids, see text.

	DHEA	*A*	*E*	*OA*	*OE*	*OHA*	*OHE*	*Average for all seven 17-ketosteroids*
Nitrogen system	3730	5133	5737	6893	7700	7503	7923	6374
Pump system	2664	2951	3637	4739	5077	4692	5324	4155

The difference in the performance is shown in Fig. 5 that compares the best of the chromatograms from the pump and the nitrogen runs. It can be seen that there is considerable less tailing in the nitrogen system.

We believe that the difference in resolution between the two systems is due to the segmentation of the eluant stream produced by the release of dissolved nitrogen as it passes from the column as shown in Fig. 6. The deliberate introduction of gas bubbles to obtain sample separation in continuous stream analysis is one of the important features of the widely used Technicon Autoanalyzer system and it seems probable that the higher resolution in the pressurized system is due to the nitrogen bubbles serving as separating segments between each stretch of eluant solvent.

Reproducibility

The reproducibility of the two systems was compared by calculating coefficients of variation for the two 12-column runs for the seven 17-ketosteroids estimated. As can be seen from Table II somewhat better reproducibility is obtained overall in the nitrogen system.

Application of the nitrogen system to routine urinary steroid analyses

Use of an internal standard and R_F values relative to this standard. One of the drawbacks the nitrogen system has relative to the pump system is that whereas in the pump system elution volume per hour of elution time is quite constant (within 1 %) from column to column because of the high reproducibility of the chromato-

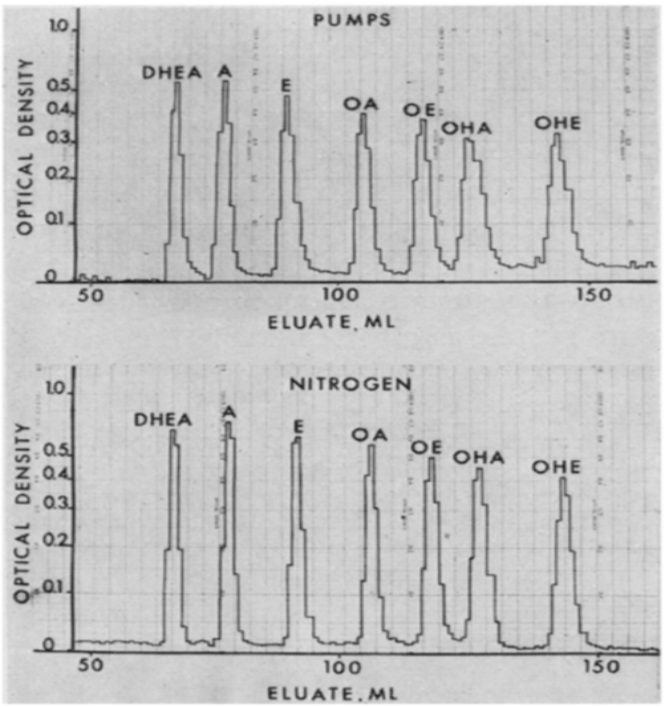

Fig. 5. Two chromatograms of seven 17-ketosteroids from the 12-batch runs mentioned in the text. On top the best of the chromatograms form the pump run, at the bottom the best of the chromatograms from the nitrogen system. For abbreviations used for 17-ketosteroids, see text.

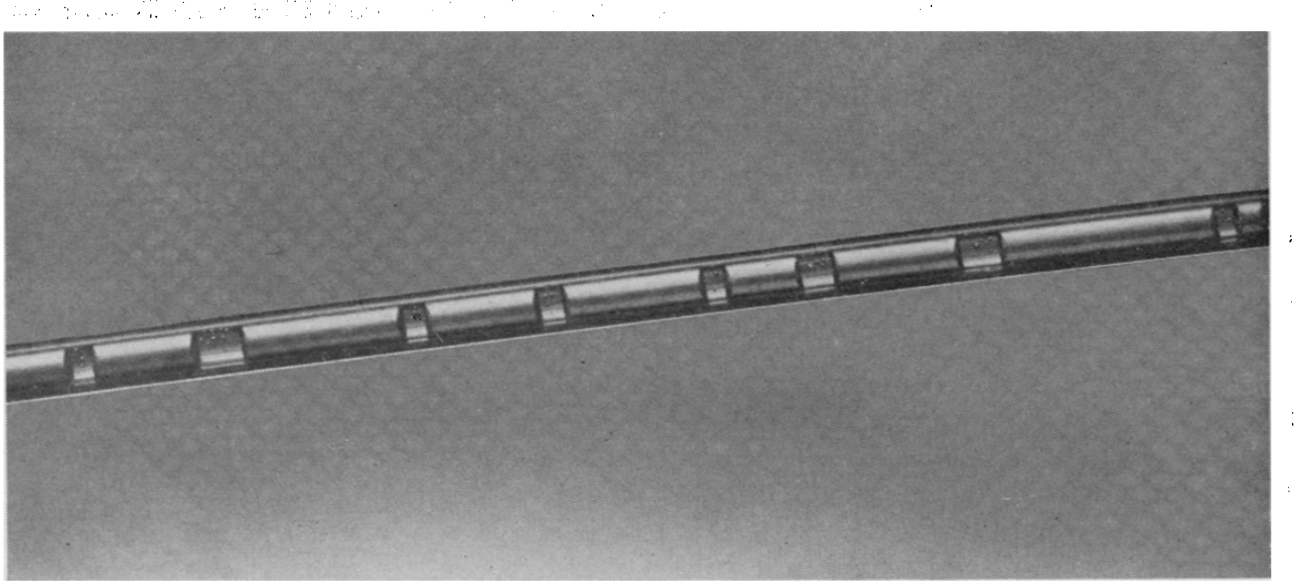

Fig. 6. A photograph of the effluent stream in the teflon tubing leading from the chromatographic column. The segmentation of the stream by nitrogen bubbles is clearly visible.

TABLE II

AVERAGES WITH COEFFICIENTS OF VARIATION FOR TWO 12-COLUMNS RUNS OF BATCHES OF SEVEN 17-KETOSTEROIDS RUN USING THE NITROGEN SYSTEM AND THE CHROMATOGRAPHIC PUMP SYSTEM

Approximately 50-μg amounts were used. Values refer to a DHEA standard and are uncorrected for differences in chromogenicity. Coefficient of variation is the standard deviation in per cent of the mean. For abbreviations used for 17-ketosteroids, see text.

	DHEA	*A*	*E*	*OA*	*OE*	*OHA*	*OHE*	*Average*
Nitrogen system								
Average (μg)	48.5	50.2	47.7	44.9	46.2	47.2	43.4	
Coefficient of variation	1.6%	2.4%	2.1%	2.5%	2.8%	2.6%	2.3%	2.3%
Pump system								
Average (μg)	47.1	49.8	47.5	44.7	46.1	48.9	45.9	
Coefficient of variation	3.7%	1.5%	2.0%	2.8%	4.4%	3.8%	5.2%	3.8%

graphic pumps this is not the case in the nitrogen pressure system. Differences in elution volume from column to column generally may vary as much as 10% around a mean if no attempt is made to adjust the flow through the needle valves. This of course is possible, but generally much too time consuming to be practical in a routine run.

To get around this problem in our routine analysis for urinary 17-ketosteroids we have added an internal standard to each urinary extract before transfer to the chromatographic column. We have for this particular analysis chosen to add 11-ketoandrosterone (OA). It is the center peak in the chromatogram and is of little physiological and biological interest and besides it is usually the smallest peak in a chromatogram of urinary 17-ketosteroids. The addition of such an internal standard makes it possible to calculate relative R_F values for the other main 17-ketosteroids when compared with the internal standard. One prerequisite for this is that so much of this standard is added that it can easily and unequivocally be identified from the chromatogram.

As can be seen from Fig. 7, showing the chromatogram of a urinary extract with OA added compared with a standard chromatogram, such a strategy is quite feasible. The important question is now how reproducible relative retention times for the different 17-ketosteroids as compared to OA are in daily routine use.

Constancy of R_{OA} values. To get retention values for the various 17-ketosteroids relative to OA twelve sets of standards each containing the seven 17-ketosteroids commonly present in urine were chromatographed and average values for the retention time relative to OA (R_{OA}) were determined for all the steroids together with the range. R_{OA} intervals for each compound were calculated by making the limits between intervals equal to the mean of the two adjoining R_{OA} values.

The values are given in Table III. Many subsequent routine determinations of urinary 17-ketosteroids have proven that R_{OA} values for a given steroid always have fallen within the R_{OA} intervals given in this table. It should be emphasized that we always in multi-column work include a set of standards with any given run. This will protect against misinterpretations should major errors in the chromatographic technique occur, for example errors in the deactivation of the alumina used or in the gradient formation. Such errors have not been made in the chromato-

graphic runs during the last half year when the method has been in routine use. However, the inclusion of a set of standards with any given run protects against misinterpretations should errors occur.

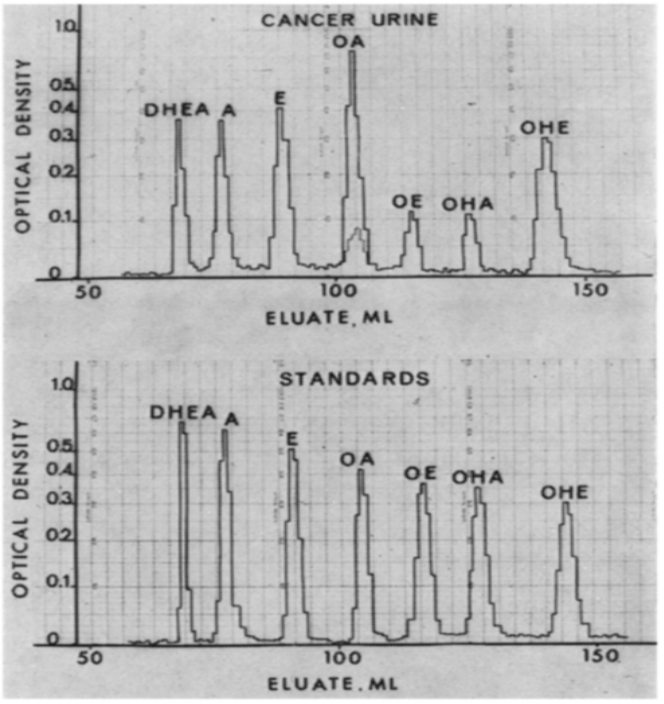

Fig. 7. From a routine run of urinary steroids. A chromatogram of an extract from a cancer patient is on top. OA has been added to this urine as an internal standard. The amount of OA present in the same urine without addition of internal standard is shown with dotted lines. At the bottom a chromatogram of standards as run in the same multi-column run. For abbreviations used for 17-ketosteroids, see text.

TABLE III

RETENTION VALUES OF DIFFERENT 17-KETOSTEROIDS RELATIVE TO THE INTERNAL STANDARD OA (R_{OA} VALUES)

For abbreviations used for 17-ketosteroids, see text.

	DHEA	*A*	*E*	*OA*	*OE*	*OHA*	*OHE*
Average	0.61	0.73	0.86	1.00	1.12	1.21	1.38
Range	0.60–0.64	0.72–0.74	0.85–0.86		1.10–1.13	1.20–1.23	1.35–1.46
Interval	0.55–0.67	0.67–0.79	0.79–0.93		1.06–1.16	1.16–1.29	1.29–1.50

Value of the R_{OA} values for computer programming. Very high capacity work with the multi-column system is not possible without computer calculation of the chromatographic data as previously discussed[8] since, if manual calculations are used, a point is reached as the capacity increases where more personnel is needed for the calculation of data than for performance of the analytical work. The use of an internal standard in the chromatographic analysis has proven of great value for the computer calculations. Relating to this internal standard in the computer

programming by using R_{OA} intervals makes the identification and labeling of individual steroids in a urinary extract more reliable than just comparing with a set of standards run simultaneously in a multi-column run although this in a system using pumps generally is quite adequate. The internal standard becomes a necessity, however, in a system using compressed nitrogen to drive the solvent mixture through the chromatographic columns.

DISCUSSION

The main reason for building the multi-column system using nitrogen to move the solvent through the capillary columns has been to cut the cost of high-capacity multi-column chromatography. A considerable saving has been achieved in this respect. The total cost of cylinder and needle valves used in the nitrogen system is approximately $ 1000 for a 25-column system, whereas the cost of pumps and mixing cylinder in a 25-column pump system is approximately $ 5000. The cost per channel has therefore been cut by 80 %.

This saving per chromatographic channel has not been obtained at the cost of reduced performance. Actually because of the better resolution in the nitrogen system performance is significantly improved in the nitrogen system as compared with the pump system both as regards resolution and reproducibility.

The use of an internal standard has been found to be a necessity in the nitrogen system where the flow rate varies considerably more from column to column than in a pump system even though columns are made of the same diameter tubing of the same length and other parameters are kept as similar as possible.

Extra amounts of an already present compound added to the chromatogram as an internal standard as in the chromatographic system for urinary 17-ketosteroids described here can be subtracted from the total value of the peak and an estimate of the amount of this compound present before addition of internal standard can thus be arrived at. This estimate will clearly be less accurate than for directly estimated compounds but will probably be adequate in most cases. Such an addition of extra amounts of an already present compound in an insensitive area of the chromatogram would appear to be a possibility for the addition of an internal standard in many situations. In other cases a compound with a retention time different from that of the compounds being determined can be used or it may be possible to use a colored substance with a proper retention time as an internal standard.

The addition of an internal standard has been found of considerable value for the computer calculation of compounds in extracts of unknown composition since retention times relative to such an internal standard make it possible to get compound identification and labeling performed by the computer with greater certainty than if no internal standard is used.

The compressed nitrogen system has been used for the routine assay of urinary steroids for the last half year. It has been found convenient and practical in use. A definite advantage this system has when compared with a system using chromatographic pumps it that the maintenance problem that is considerable when a battery of 25 pumps has to be used has been eliminated almost entirely.

This system overall evaluated and compared with the previously used pump

system is much cheaper per chromatographic channel, has higher resolution and better reproducibility and very little maintenance is needed to keep the system going. The disadvantage of a more variable flowrate has been overcome by the addition of an internal standard to the extracts to be chromatographed. This internal standard has proven of considerable value in the computer calculation of the chromatographic data.

It is our conviction that multi-column capillary column chromatography has much to offer the analytical chemist. It combines high capacity with high chromatographic resolution. With the current rapid developments in fast liquid chromatography it can also be expected over the coming few years to become an increasingly fast technique in many applications.

We hope in future developments of the system to improve the techniques further. A greatly simplified read-out system with computer calculation of data is currently under development. It will use a small on-line laboratory computer for direct calculation of data and should cut the cost of multi-column chromatography further.

ACKNOWLEDGEMENTS

This work was supported by grants GM 11940 and AM 04262, contract PH42-67-1465 and institution grants MH 07292 and FR 05651.

REFERENCES

1 P. VESTERGAARD, *J. Chromatog.*, 3 (1960) 554.
2 P. VESTERGAARD, *J. Chromatog.*, 3 (1960) 560.
3 P. VESTERGAARD AND S. VEDSØ, *J. Chromatog.*, 19 (1965) 512.
4 P. VESTERGAARD, J. F. SAYEGH AND C. S. WITHERELL, *J. Chromatog.*, 24 (1966) 417.
5 P. VESTERGAARD AND J. F. SAYEGH, *J. Chromatog.*, 24 (1966) 422.
6 J. F. SAYEGH AND P. VESTERGAARD, *J. Chromatog.*, 31 (1967) 213.
7 P. VESTERGAARD, C. WITHERELL AND T. PITI, *J. Chromatog.*, 31 (1967) 337.
8 P. VESTERGAARD, L. HEMMINGSEN AND P. W. HANSEN, *J. Chromatog.*, 40 (1969) 16.
9 L. R. SNYDER, *Principles of Adsorption Chromatography*, M. Dekker, New York, 1968, p. 17.