Simultaneous Multicolumn Liquid/Liquid and Liquid/Solid Chromatography with a Computerized Readout System

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For more than a decade, an intense and largely successful effort has been made to automate simple clinical assays. It is clear that second-generation automated methods for the clinical laboratory increasingly must put emphasis on high-resolution methods for the isolation and estimation of individual substances in complex biological mixtures. A multicolumn system has been developed as a general system for high-capacity liquid chromatography from the conviction that such systems will have an increasingly important role to play in clinical chemistry.

Additional Keyphrases AutoAnalyzer • high-capacity, high-speed analytical systems • multicolumn chromatography • steroids • advantages of liquid chromatography

Automation of assays of single biochemicals of biological importance has progressed over the last decennium to a point where, in many clinical laboratories today, the majority of the analyses performed are automated simple procedures. There is still much progress to be made in this field, but it is clear now that work must be expanded to the development of automated clinical analyses of the single components from complex mixtures of biologically active compounds.

The pioneering efforts in this field have focused on the development of automated methods for the assay of individual amino acids in biological fluids, based on the work of Spackman et al. (1). Many commercial instruments have been designed to exploit this technique, and progress has been made in optimizing the chromatographic parameters to increase the capacity of such systems from one chromatogram per 24 h to one chromatogram per hour.

Important as this development has been, it is clear, as has been pointed out by Bush (2), that there will be a need in the near future for clinical analytical tools with far higher capacity than even the most sophisticated amino acid analyzer presently possesses. Bush (2) suggests we develop systems with the potential of resolving and estimating on the order of 5000 substances per day for complex mixtures.

Multicolumn liquid chromatographic methods that use liquid/liquid and liquid/solid chromatography developed over the past decade (2-10) can potentially be developed into such high-capacity systems. Although we have never run more than 100 chromatograms in a day, separating eight substances per chromatogram, the systems have the potential to be easily expanded to four times this capacity. Admittedly, this would be costly, although not unreasonably so, compared with current prices for complex automated analytical instruments. However, improvements and simplifications in the basic system will, we are convinced, within the next years drastically expand the capacity of the multicolumn systems and at the same time bring the cost of the instrumentation down.

The need for such high-capacity, high-resolution systems has become increasingly clear in recent years. As our society puts more and more emphasis on the prevention and early detection of disease rather than on the cure of fully developed diseases, a great expansion of multiple-screening procedures can be expected. This will lead to an enormous expansion both in the use of high-capacity, automated, and simple clinical methods and in the application of more sophisticated methods for determining substances like steroids, amino acids,
column chromatography, with gradient-elution techniques. The gradient is formed in the Teflon distributing bottle by pumping a more polar solvent into a less polar solvent in the bottle and mixing with a magnetic stirrer. The eluent is then distributed through chromatographic pumps to the capillary Teflon columns, the temperature of which is controlled in a water bath, and the effluent from these columns is collected on a 12-column multicollector (in background).

To handle the large number of colorimetric readings, we first introduced a one-channel automatic readout system with punched paper tape output for calculation on a computer (9). This was later replaced with a high-capacity multichannel system with both paper tape output and direct on-line computer connection (10) (Figure 2). An appropriate, specific color reaction is performed on fractions from the multicollector after evaporation of the organic eluent, and the samples are then passed from one magazine tower in the readout machinery to the other, with an aliquot of the sample being aspirated into the colorimeter for registration on the multipen recorders. The recorded transmittance values are then sent to a data-logger and on-line computer systems for data handling.

Because of the high capacity of such multicolumn systems, it becomes necessary to use a computer for the data handling. Even a relatively moderate output of 50 chromatograms per day would require two to three full-time persons to perform the calculations; at high outputs, more people would be needed for the calculations than for the analytical work.

In our system it is important to use two independent computer systems, as shown on the right in Figure 2. A data-logger transforms the recorder values to punched paper-tape data, which is accepted by a computer. An on-line IBM 360 computer connected to the system allows transmission of calculated data back to the laboratory with a time lag of a few microseconds. The two systems working simultaneously serve two important functions. If there is breakdown in one system, the other serves as back-up and may save many man-hours of calculation time. The two systems also serve as a check on performance, since calculated values obviously must agree if both systems are working correctly.

Application

The potential of this type of system is perhaps best illustrated through its application in large-scale routine analyses. Over the past couple of years, we have run several thousand chromatograms per year with this system for the routine assay of urinary steroids.

The flow sheet for the assay of individual urinary

Fig. 1. A multicolumn chromatographic system with the gradient elution technique

The gradient is formed in the white Teflon bottle, pumped through the capillary Teflon columns in the water bath, and collected simultaneously from 12 columns in a multicollector and many others of equal biological importance.

High-capacity liquid/liquid and liquid/solid chromatography has an important role to play in this development.

System Description

Our system has been gradually developed over the past 10 years. First we used a gravity feed system for simultaneous, multicolumn chromatography on six columns, using gradient-elution techniques (3) and a specially constructed multicollector (4). The collector has recently been enlarged and redesigned to include magazine towers for the test tube carriers (5). A commercial fraction collector (the Serva Collector), similar to our original design and suitable for 10-column work, is now available in the United States (Brinkmann Instruments, Westbury, N.Y. 11590). We originally used thermostated glass columns (6) in our work and later went to capillary Teflon (E. I. du Pont de Nemours and Co.) columns (7, 8).

Figure 1 shows one of the chromatographic systems currently in routine use in our laboratory but about to be replaced by improved systems currently under final experimental evaluation. It is set up for simultaneous 12-column capillary col-
17-ketosteroids and individual corticosteroids is given in Figure 3. The urine samples are first treated with a β-glucuronidase preparation that contains some steroid sulfatase. The steroid sulfates not released by the enzyme are subsequently freed by solvolysis or continuous extraction with ether at pH 1. A preliminary fractionation into subgroups of steroids is performed on small silica-gel columns, and multicolumn chromatography is then used to estimate the individual steroids.

Figure 4 (top) shows chromatograms of the urinary steroids from a patient with breast cancer compared with sets of steroid standards (bottom). The 17-ketosteroids are assayed after column chromatography on aluminum oxide and reaction with the Zimmermann reagent in pyridine solution (9). The corticosteroids less polar than cortolones are determined after partition chromatography on silica-gel columns and reaction of the steroids with blue tetrazolium (11). The cortols and cortolones are determined indirectly after periodate oxidation of the polar fraction from the small silica-gel columns. This transforms the cortols and cortolones into the corresponding 17-ketosteroids. These are then estimated after aluminum oxide column chromatography and automated colorimetry with the Zimmermann reaction (11).

The urinary 17-ketosteroids are: dehydroepiandrosterone (DHEA), androsterone (A), etiocholanolone (E), 11-ketoandrostosterone (OA), 11-ketoetiocholanolone (OE), 11-hydroxycorticosterone (OHA), and 11-hydroxyetiocholanolone (OHE). The corticosteroids determined are allo-tetrahydrocortisone (A-THE), tetrahydrocortisone (THE), allo-tetrahydrocortisol (A-THF), and tetrahydrocortisol (THE). Cortol and cortolone, after oxidation with periodate, are transformed to 11-ketoetiocholanolone (OE) and 11-hydroxyetiocholanolone (11 OHE), respectively. The corresponding allo-cortolone and allo-cortol are transformed to 11-ketoandrostosterone (OA), and 11-hydroxyandrostosterone (OHA), respectively.

Performance

The system has been in use for about three years and a total of several thousand complex chromatographic analyses have been performed per year. It has operated well with no major breakdowns. The readout system was fabricated from standard Technicon components built for continuous use. Service problems were similar to those found with the colorimeters and recorders in the standard AutoAnalyzer system. The readout system ordinarily works with four active channels. If a colorimeter or recorder has to be serviced, the other three channels can be used and, in this way, we have never had a complete shutdown of the system. We apply preventive maintenance to the chromatographic pumps by renewing at fixed intervals parts, such as gaskets, that tend to get worn. We have always had, at any given time, three 12-column units available in the laboratory so that, if one is being serviced, we still have the
other two available to handle the routine load. This type of redundancy in the system is, in our experience, necessary if one wants to assure continuous day-by-day operation.

With a few days of indoctrination, the system can be used by the usual laboratory technicians since no special skills are necessary to handle the instrumentation. The overall reproducibility of the system, checked through double-blind duplicate analyses, has been somewhat better for most steroids than for the much simpler group assays performed manually.

Potential

Because of our interest in steroids, we have applied the multicolumn techniques initially to the analysis of these compounds in biological fluids. The same techniques obviously can be applied to any compound for which a liquid chromatographic system has been or can be developed.

With amino acid analysis, it has been possible, through the diligent work of many groups during the past 12 years, to increase the capacity of single-column instruments from one chromatogram per 24 h to one per hour. It is clear, however, that any further progress in productivity following the same approach of shortening the chromatographic run is going to be increasingly harder to obtain, as more and more extreme conditions will be required.

With the same multicolumn technique described above, amino acid analyzers could achieve a significant increase in productivity. For example, by modifying available methods for fast amino acid analyses to multicolumn techniques, a 25-column system could be set up to run 25 analyses instead of one analysis per hour. This method will become increasingly attractive as new compressed nitrogen systems (11) are developed. We are currently evaluating systems for simultaneous, 25-column chromatography which do not use chromatographic pumps and, thus, will be much cheaper per channel than the current systems using pumps.

Adding greatly to the potential of liquid chromatographic methods in high-capacity work are a number of recent developments that promise strong advances in this field in the coming years. Most interesting are the improvements in speed. Liquid chromatography, which had been by far the slowest of the chromatographic techniques, has been radically changed.

Hamilton (12, 15) was the first to explore systematically, experimentally, and theoretically the changes in chromatographic parameters necessary to increase speed in liquid chromatography. More recently, Snyder (14, 15) has taken the problem of maximum resolution per unit time in liquid chromatography under experimental investigation and theoretical consideration, and has evolved equations and charts for maximum bed efficiencies as a function of column pressure, column length, separation time, and particle size. He points out that a maximum number of 60,000 theoretical plates can conveniently be obtained, with the proper manipulation of the chromatographic parameters, while staying well within the frames of current technology. Such high-resolution systems may need long run-times; however, much better separations can be obtained than with paper chromatography or thin-layer chromatography.

Recently, rapid analyses with liquid chromatography have been achieved. For example, Snyder (14, 15) demonstrated separations of a mixture of hydrocarbons in 40 min. Horvath et al. (16), using pellicular column material (1.83-m columns and 75 atm of pressure), succeeded in separating a complex mixture of nucleotides in 75 to 90 min and they concluded that such a liquid chromatographic technique compares with gas chromatography in speed, resolution, and quantitative range.

Clearly, these examples illustrate that liquid chromatography has come a long way in combining speed with resolution and no longer can be considered a slow chromatographic method. We are only at the beginning of exciting new developments in this area. The "brush" materials developed by
Halasz and his group (17) and now introduced commercially in the United States (Waters Associates, Inc., Framingham, Mass. 01701) hold great promise for further advances in fast liquid chromatography. The work by Piel (18) with high-pressure, small-particle liquid chromatography has never been properly followed up experimentally. This technique, if further developed, might give high resolution in a few minutes for complex separations.

Advantages of Liquid Chromatography

There are many factors that make liquid chromatography attractive for the clinical chemist, as compared with other chromatographic methods. The high resolution is probably the most important advantage vis-a-vis thin-layer and paper chromatographic methods.

The fact that liquid chromatography can separate thermolabile and nonvolatile substances non-destructively without the need for time-consuming procedures for derivative formation is one factor that makes it attractive compared with gas chromatography. Also, in liquid chromatography, specific reactions (such as color and fluorescence reactions) can be applied to enhance the specificity of the assay. In gas chromatography, commonly used detectors are nonspecific and are influenced by extraneous material, so that very considerable prepurification may be necessary before a gas chromatographic assay can meaningfully be applied to a complex biological mixture.

Other advantages of liquid chromatography are the wide range of available detectors. If stream splitting is used, a number of detectors can be applied to different portions of the same chromatographic fraction—e.g., colorimetry, fluorometry, and liquid scintillation counting. The flame-ionization detector (19) and very sensitive ultraviolet detectors (20) are also available for liquid chromatography. It is clearly possible to use the paper chromatographic preparator and scanner as developed by Bush (21, 22) as a detector in column chromatography. Further advantages of liquid chromatography systems are the higher loads possible; the ease with which they can be adapted to sequential, automated chromatography for very high, combined resolution; and the relative ease with which fully automated systems can be developed.

With the many advantages liquid chromatog-
raphy has to offer, this methodology can expect a considerable renaissance and expansion in the coming years. I feel confident that multicolumn technology will play a part in this development.

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References