Microcolumn Liquid Chromatography: A Tool of Potential Significance in Biomedical Research

Millos Novotny

Recent progress in the area of microcolumn HPLC is reviewed with an emphasis on biomedically important directions. The potential of high efficiency separations, additional advantages of solvent economy, and the development of new detection and ancillary techniques of high-performance liquid chromatography are also discussed.

Additional Keyphrases: metabolic profiling • capillary gas chromatography

It is well recognized that mixtures of biological compounds, such as tissue extracts or physiological fluids, are extremely complex. Hundreds or even thousands of different molecular species may be present in various concentrations in a sample of interest, yet only a few compounds are typically monitored in cases pertaining to different conditions of sickness and health. Modern chromatographic methodology appears best suitable to perform multicomponent analyses of biological mixtures, but many practical considerations of a clinical laboratory (such as procedural simplicity and time of analysis) handicap this general analytical approach. The utilization of chromatographic methods in routine clinical chemistry has been gradually increasing, but is not currently widespread. Specificity of the determinations of single compounds or compound classes that are well established criteria of health is frequently secured by other analytical principles (specific reactions, enzymes, antigen-antibody interactions, etc.); simple chromatographies are occasionally used to improve further the specificity of a given determination.

Entirely different considerations are valid in studies of the molecular basis of human and animal diseases, etiology of various disorders, biochemical/pharmacological investigations, etc. Here, the forefront separation and identification methodologies are among the best hopes for our improved understanding of such conditions. If most or all of the constituents of a complex and biomedically interesting specimen are unknown, both qualitatively and quantitatively, the best available methodology should be applied. Thus, comparative multi-component chromatographic analyses, known as "metabolic profiles" (1, 2), constitute one of the possible powerful approaches to the general problem. Certain successes of this direction can be seen in the representative applications to human genetic disorders (3–6), molecular abnormalities of human and experimental diabetes (7–9, and manuscript in preparation), evidence for the polyol pathway in multiple sclerosis (10), and others. In most of these applications, high-resolution gas chromatography (GC) has played a significant role.

The use of high-resolution (capillary) GC in metabolic profiling has both advantages and drawbacks. The numbers of theoretical plates available with today's efficient glass or fused-silica capillary columns are in the range of $10^4$ to $10^6$, most frequently between 50 000 and 200 000. The columns are capable of separating several hundred components in a single run, within 1 to 2 h of analysis time. Typical complexity of a physiological fluid sample is shown in Figure 1, obtained in the author's laboratory as previously described (11). Although this separation concerns relatively volatile urinary components, other classes of biologically important compounds (steroids, polyols, amino acids, urinary acid metabolites, etc.) can be successfully gas-chromatographed after conversion to more stable and volatile derivatives. Such techniques are quite common today. On the other hand, GC is not the best alternative for numerous organic compounds that are either too large or unstable. High-performance liquid chromatography (HPLC), developed during the last 10 to 15 years, is clearly a better alternative for such cases.

The scientific literature of the last decade clearly attests to the growing importance of HPLC in biochemical and biomedical research. Potential of the method for metabolic profiling was already indicated in the earlier works by Hamilton (12) and Scott (13, 14). With recent advances in column and detection technology and shorter analysis time, HPLC appears even more attractive today. However, typical column efficiencies seldom exceed a few thousand theoretical plates, whereas the lack of chromatographic resolution is frequently overcome by the use of selective detectors. This latter is a convenient solution, provided that one knows or suspects that certain components are in a given sample; it is a less valuable approach in general metabolic profiling.

The recently introduced techniques of microcolumn HPLC (15–19) may help overcome the complexity of nonvolatile biological mixtures and improved detection methods. Here I will review the investigations in this area that are pertinent to biomedical separation problems and point out new directions associated with these developments. Properties of the individual microcolumn types and certain detection aspects will also be discussed.

Chromatographic Resolution and Speed of Analysis: Basic Considerations

There is a certain lack of resolution of very complex biological samples even with the best capillary GC methodology available today. Just how bad is the situation in HPLC, where larger, less-volatile molecules often have to be handled and the number of possible isomers increases quite dramatically with increasing molecular mass? The importance of this question is difficult to assess for the actual biomedical samples, but the incentive of the recent development of high-resolution electrophoresis (20, 21) of very large biological molecules is likely to have tremendous impact on future biochemical research. In the less developed area of separations.
(the range of, say, 500–10 000 daltons), we do not know what the impact of HPLC columns with $10^6$ plates combined with a universal detector might be.

A fair question to ask is whether the separation potential of HPLC has been utilized or not. Many years ago (22), Giddings assessed the comparative potential of gas and liquid chromatography and concluded the latter technique was superior. However, the time-of-analysis factor concerning such separations was not sufficiently emphasized, and the more recent theoretical treatments by Guiochon (23) and Knox and Gilbert (24) are more appropriate. Such theoretical analyses are quite favorable to the future of "ultrahigh-efficiency."

Resolution of components in chromatography is given by the following equation, an extension of the well-known treatment by Purnell (25)

$$R = (\sqrt{N}/4) \times (\alpha - 1/\alpha) \times [k/(1 + k)]$$

where $R$ = resolution between adjacent peaks, defined as $R = \Delta t/w$; $\Delta t$ = difference in retention times, and $w$ = average retention width at the peak base; $N$ = number of theoretical plates; $\alpha$ = relative retention of two adjacent peaks with reference to the peak of non-retained solute; and $k$ = capacity ratio (related to the partition coefficient as well as to the amounts of both phases in the column).

Although column selectivity is recognized as a powerful approach to resolution of certain substances, as based on very specific molecular interactions, it is of limited use with very complex mixtures, in which new compound overlaps are created when the previous ones have been removed, by merely changing retention. Thus, according to equation 1, for very similar substances (approaching unity), increasing the "brute force" (i.e., the number of theoretical plates) is likely to offer the best approach to resolution. The optimum range of the capacity ratios, $k$, should be 0–6.4 (23).

The peak capacity, $n$, or number of resolved peaks in a complex chromatogram, is given (26) by:

$$n = (\sqrt{N}/4) \ln (1 - k)$$

It has been calculated (23) that for a packed HPLC column some 500 fully resolved components can be obtained with $10^6$ theoretical plates in about a one-day run. For biomedical research problems, this analysis time may be quite acceptable. Note, however, that another price to be paid for the above achievement is the high inlet pressure (22, 23), in our case about 1300 atm (131.7 MPa). Although this pressure value is of some concern in ordinary HPLC, it is substantially less of a technological problem in microcolumn LC. An increase of column temperature can decrease the time of analysis up to fivefold (23), certainly not a negligible circumstance.

How can one achieve $10^5$–$10^6$ theoretical plates in practice? Substantial gains in HPLC column efficiency have been realized by reduction of particle sizes to 5–10 $\mu$m; however, common experience shows that a simple serial connection of prepacked short columns does not yield a situation where the overall efficiency is a sum of equivalent, additive column segments. Poor engineering could partly be blamed for this situation, but nonuniform permeability of conventional (4 mm i.d.) columns and the heat-of-friction problems (16, 27) are believed to be the primary causes of the undesirable situation. On the other hand, microore packed columns (18, 17) do not seem to suffer from the problem.

The potential of capillary (open tubular) HPLC has also been briefly assessed more recently (24, 28–31). Similar advantages of high column permeability, favorable time of analysis, and high column efficiency (as experienced in capillary GC) are applicable here. However, major technological advances in column and instrument design are needed to take advantage of this approach. A recent theoretical analysis by Knox and Gilbert (24) indicates that capillaries in HPLC should be faster than packed columns already for $N > 30 000$ (not an unusually high efficiency), whereas time of analysis could be decreased by 27-fold for a 10-$\mu$m column diameter and $N = 10^6$ plates. The potential of higher column efficiencies in biomedical field is beyond dispute; however, only a few illustrations of the resolution of complex mixtures exist at this time. Recently developed packed HPLC microcapillaries (17–19) appear to be a suitable compromise between the packed microore columns and "true" capillaries.

Microcolumn Types

There are three different approaches to higher efficiencies in HPLC:

1. Long microore packed columns (typically, 1 mm i.d.) are prepared by connecting 1-m segments to a desired length (15, 16); the columns are slurypacked under very high pressure.

2. Thick-walled open microtubular columns (i.d. 60 $\mu$m or less) are drawn to a desired length (28–31) by the procedure conventionally used for GC glass columns, their inner surface is provided with a thin liquid film, a chemically bonded layer, or a thin layer of in situ-formed adsorbent.

3. Packed microcapillaries (17, 18), with i.d. 70 $\mu$m or less and the lengths somewhat comparable to the open microtubular column variety, are made from glass tubes that are first filled with an appropriate packing material, then drawn down to a desired diameter; the prepared microcolumns can be di-
rectly used in an adsorption mode, or provided (18) with additional chemically bonded stationary phase.

Conceivably, all three approaches will eventually lead to methodologies having advantages of their own. The present limitation is primarily insufficient instrumental design, although further developments in column technology are also desirable. Equipment miniaturization is essential for all of these column types, although the demands are much less for the microbore columns than the "true" capillaries. Thus, the former column types are currently considered to be "more practical" than the latter, although technological advances may change this situation. The essential trend in equipment miniaturization initiated by Iahii et al. (32) will undoubtedly continue, with the ultimate aim of matching the potential of already developed column approaches.

Very high efficiencies have already been demonstrated with microbore columns by Scott and Kucera (15, 16). Alternatively, efficiency can be exchanged for the speed of analysis (33). If long analysis times are tolerated, such columns can produce separations at half a million plates or so. A molecular exclusion chromatogram was obtained with 250 000 theoretical plates on a 10-m column (15). Certain complex mixtures were also analyzed by Scott and Kucera (15, 16). Packing of microbore columns with various chromatographic materials appears feasible, but the procedures involved appear less reliable (23) than the current conventional HPLC column technology.

Investigations with open microtubular columns have thus far concentrated more on fundamentals than on the separation of real mixtures. In the straightforward correspondence of column efficiency to the Golay equation (34), open microtubular columns will start "paying off" if the column diameter is less than about 30 μm (24). The available sampling and detection technology is not yet compatible with this figure. However, the published work on 50-60 μm columns indicates that this approach deserves further attention. Technology for coating (29) and bonding (30) stationary phases inside such columns has been under development, as have the necessary surface treatments; much can be learned from the knowledge obtained in glass capillary GLC (35, 37). In a recent example (31), the inner surface of a glass capillary column was provided with a thin adsorptive layer by alkaline surface corrosion. A chromatogram of a model mixture obtained with such an adsorption-type capillary is shown in Figure 2.

The packed microcapillary column introduced by our group (17-19) can be considered a "hybrid" between the columns discussed above. Figure 3 shows a photomicrograph of a packed microcapillary section, indicating its semipermeable nature; the particles are partly imbedded into the glass wall, lending the column stability against the flow shear. Irregular flow patterns are likely to occur that contribute to rather uncharacteristic plate-height vs velocity curves (17). Good column efficiencies are obtained with packed microcapillary columns at moderate pressures as evidenced by (e.g.) the separation of a model mixture shown in Figure 4. Studies on column diameter and particle optimization in this area remain to be made. Although they share some potential advantages of open microtubular columns, including the extremely low

---

**Fig. 2.** Liquid chromatographic separation of diocetyl, dibutyl, dioctyl, and dimethyl phthalates on a 5.75 m X 43 μm, i.d., glass capillary column prepared by the surface-etching technique of Ishii et al. (37)

Reproduced from ref. 31, with permission; copyright, Elsevier Scientific Publishing Co.

**Fig. 3.** Photomicrograph of a section of alumina-packed microcapillary column; i.d. 75 μm; average particle size, 30 μm

Reproduced from ref. 17, with permission; copyright, American Chemical Society

**Fig. 4.** Chromatogram of a standard mixture on a 29 m X 75 μm (i.d.) (30 μm particle size) alumina-packed microcapillary column

Mobile phase: n-hexane with 0.5 mL of methanol per liter; flow linear velocity, 0.65 cm/s. Solute: benzene, methyl benzene, and quinoline (in order of elution); number of theoretical plates for quinoline, 85 000; inlet pressure, 1500 psi; column permeability 6.1 × 10⁻⁸. Arrows at left indicate that splitter is open for sample injection, then closed soon afterwards. (Reproduced from ref. 17 with permission; copyright, American Chemical Society)
flow-rates, packed microcapillaries have a greater sample capacity. A variety of selective packings can be prepared by in situ bonding of various silane compounds (18).

The separations of “real” mixtures with such columns mostly include polycyclic aromatic hydrocarbons with high molecular masses (19, and manuscript in preparation). Figure 5 shows an example in which the aromatic fraction of coal tar is displayed as detected with a miniaturized spectrofluorometric detector; the separation took 23 h.

Although the high resolving power of these new column types can have numerous implications for various analytical problems, some additional advantages of the microcolumns should not be overlooked. Primarily, the flow-rates (on the order of microcolumns per minute) make this type of chromatography extremely economical and environmentally desirable. Secondly, such low flow-rates may significantly affect development of novel detection and ancillary techniques, including the widely discussed liquid chromatography/mass spectroscopy combination. Certain detection aspects will be discussed below.

Development of New Detection Techniques

Very low flow-rates typical of microcolumn HPLC introduce certain advantages concerning detection capabilities of this method. Typical flow-rates for open microtubular or packed microcapillary columns are around 1 μL/min, and about 50-fold greater for microbore columns. Both are substantially less than typical flow-rates for common HPLC (1.0 mL/min).

For use with microcolumn HPLC, conventionally used detectors (such as ultraviolet/visible, spectrofluorometric, or electrochemical monitors) had to be miniaturized. Scott and Kucera (16) observed the benefits of decreasing an ultraviolet-detector cell volume for microbore columns to 1 μL; Ishii et al. (32) reported a similar detector type with a volume of 0.1 μL, which they used in work with open microtubular columns (29–31) and packed microcapillaries (18). Cells with 0.1-μL volume were also developed by Hirata et al. (19, 38) for spectrofluorometric and electrochemical detection. A chromatogram of certain electrochemically active solutes detected with a miniaturized detector (38) is shown in Figure 6.

Miniaturization of the detectors is essential to record accurately the relatively narrow bands emerging from a microcolumn and to avoid band dispersion related to dead volume. Further optimization of such detectors is still needed. However, even at this stage of development, some benefits of the microcolumns for trace analysis are becoming clear; a recent paper by Scott and Kucera (39) points out the advantages of reduced detector noise and increased mass sensitivity.

The second group of detectors of potential importance includes devices that can greatly benefit from, or would not be feasible without, the extremely low flow-rates typical for the microcolumns. Considering both the potential importance and

![Chromatogram of the aromatic fraction of coal tar with use of a stepwise gradient](Image)

**Fig. 5.** Chromatogram of the aromatic fraction of coal tar with use of a stepwise gradient

(A) Column: 55 m × 70 μm, i.d., packed with basic alumina (30 μm), treated with octadecytrichlorosilane. Mobile phase: 1, methanol/water (80/20 by vol); 2, methanol/water (90/10 by vol); 3, methanol; 4, 10 mL of methylene chloride per liter of methanol; 5, 30 mL of methylene chloride per liter of methanol. Components: a, benzene; b, naphthalene; c, biphenyl; d, fluorene, e, phenanthrene; f, anthracene; g, fluoranthene; h, pyrene; i, triphenylene; j, benz[a]anthracene; k, chrysene; l, benzo[a]pyrene; m, perylene; n, benzo[a]pyrene; o, dibenzo[ghi]perylene; and p, coronene. (B) After 20 h, the conditions were changed as follows, inlet pressure, 200 atm; 60 °C; detector sensitivity setting increased fivefold. (Reproduced with permission from ref. 19; copyright, Elsevier Scientific Publishing Co.)
the current agones of liquid chromatography/mass spectroscopy combination (40), microcolumns clearly could solve at least part of the many technological problems of this ancillary technique. Similar considerations are applicable to various transport/detector devices (41), for which only a very small portion of the total column effluent can be deposited on a wire, belt, strip, disc, etc., with conventional HPLC. Some implications of the advantages of microcolumns in this direction have already been given (42), but much technological improvement is still needed.

Spectroscopic devices based on flames or plasma-related phenomena present yet another potential direction of the microcolumn HPLC detection. Recently, a microflame photometric detector, with total effluent consumption, has been developed in our laboratory (manuscript in preparation). The detector has been so far characterized only in a phosphorus-emission mode, but other flame-related phenomena (thermionic, luminescent, etc.) are under investigation. The present sensitivity of the phosphorus-sensitive device is 75 pg of phosphorus per second. Detection of phosphinylated biological compounds (43) by microcolumn HPLC presents some interesting possibilities.

The solution of numerous biomedical problems associated with complex mixture analyses could greatly benefit from the techniques of microcolumn HPLC currently under development. Advances are being made toward reliable instrumentation in this area. Low solvent consumption and potential availability of new detection and ancillary techniques are additional valuable attributes of microcolumn HPLC.

This work was supported by the research grant No. GM 24349 from the National Institute of General Medical Sciences, U.S. Public Health Service, National Institutes of Health.

References


