Technical Considerations in the Use of “High-Performance” Liquid Chromatography in Therapeutic Drug Monitoring

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I review the methodology of “high-performance” liquid chromatography as applied to therapeutic drug monitoring. Aside from direct injection of sample, sample cleanup involves miscible and immiscible organic solvent extractions, solid-phase extraction, and size separations. Column considerations are bonded phases, column dimensions, particle size, guard columns, stability, pH range, and reproducibility. In a section on mobile phase, the reversed-phase mode is discussed along with temperature and degassing. Absorbance and fluorescence detectors are used most commonly. The parameters “capacity factor,” “efficiency,” and “asymmetry value” are helpful for interpreting chromatograms, as are the aspects of peak tailing, peak quantitation, “complex solutes,” and “crowded chromatograms.” Finally, automation, competing methodology, and prospects are discussed.

Additional Keyphrases: sample handling · column variables · detectors · factors affecting the chromatogram · chromatogram interpretation · future developments

Introduction

“High-performance” liquid chromatography (LC) is one of the most important analytical techniques used in therapeutic drug monitoring (TDM). Among its useful features are the ability to simultaneously analyze for and discriminate closely related drugs and metabolites such as the tricyclic antidepressants, its high precision and accuracy, and its easy adaptation for new drug assays, often with automation. Like any method, it also has shortcomings. Samples require cleanup and cannot be assayed simultaneously, in contrast to many immunoassays. The routinely used detectors are generally no match for those in gas chromatography. But current advances against these and other limitations are under way. For example, column switching conveniently provides automated sample-cleanup for LC; rapid throughput is available from “high-speed” and “box-car” LC, and scanning absorbance detection with uninterrupted flow enhances peak identification.

Here I review some practical considerations, and the current status and likely future developments in LC methodology for TDM. Only certain drug applications will be cited. For more general coverage of the applications literature, one should consult the articles and compendia in the AACC’s Therapeutic Drug Monitoring Program, and three current books (1–3). Pre-1978 literature on LC in TDM has been summarized as a part of a general review of clinical LC (4). I have chosen to focus here on the most common aspects and techniques in routine TDM by LC. The major considerations being addressed in this article are sample pretreatment, column, mobile phase, detection, chromatogram, automation, competing methodology, and future directions.

Sample Pretreatment

Before chromatographic separation, the sample may be pretreated. Four reasons for doing so are (a) to release the drug from protein binding sites, (b) to isolate the free drug, (c) to remove extraneous proteins and other potential interferents that may collect in the column and lower its performance, and (d) to concentrate the drug for more sensitive analysis. In certain cases, direct injection of the sample, with no sample pretreatment, can provide acceptable results. Otherwise, a miscible organic solvent, immiscible organic solvent, solid-phase extraction column, or size-separation technique is used for sample pretreatment.

Direct Injection

The technique of direct injection has been used mostly for uncomplicated drug analyses, such as for theophylline. This drug is therapeutically present in relatively high concentration in serum (milligrams per liter), and it possesses good characteristics of ultraviolet absorption, so that a relatively small volume of serum can be injected directly onto the LC column, thus minimizing the amount of non-eluting sample contaminant placed on the column with each injection. In this procedure, mobile phase (and perhaps also the column packing) release the drug from protein binding sites as the sample enters the column. Unfortunately, there is an increased likelihood of interferences, because there is no sample-cleanup step, and there is more rapid degradation of the column or of a “guard” column (vide infra) from accumulation of non-eluting sample components such as proteins. This limits the direct-injection approach to special cases such as theophylline. However, as the costs of LC columns continue to decrease, column washing techniques improve, and multi-wavelength detectors are used to detect interferences, this approach is likely to be used more widely. In a recent article, direct injection was used in the analysis for salicylate and naproxen in serum (5).

Water-Miscible Organic Solvent

With addition of a water-miscible organic solvent such as acetonitrile to the serum sample, there is precipitation of some protein in the serum, along with release of the drug from protein binding sites. The mixture is then centrifuged, and an aliquot of the supernate, which contains the drug, is injected. Both low and high proportions of the organic solvent have been used. When only a small proportion of organic solvent is introduced into the serum (e.g., one-tenth of the final volume is acetonitrile), only little protein precipitation occurs, although the most offending (i.e., column-contaminating) proteins perhaps are removed. In contrast, diluting serum one- to threefold with organic solvent causes more protein precipitation (e.g., approximately 90% protein
precipitation based on biuret analysis, with an equal volume of acetonitrile\(^1\), which probably prolongs column lifetime more than the other approach involving a small organic solvent volume. Nevertheless, whatever the proportions of organic solvent, the drug peak can still be accompanied and complicated by several other peaks (Figure 1). Thus, the likelihood of interfering peaks is probably about the same as with direct injection, because most if not all drugs and analogous physiological solutes will be included in the supernate in these samples. Moreover, small peaks (including late-eluting peaks from previous samples) may co-elute with the drug peak, thereby distorting peak measurements and adversely affecting precision. The potential for late-eluting peaks to degrade assay precision in TDM has been pointed out (7). Although acetonitrile is probably the most popular protein precipitant, acetone has recently been reported to be better (8).

**Water-Immiscible Organic Solvent**

Sample cleanup by extraction with a water-immiscible organic solvent such as chloroform or methylene chloride noticeably improves the overall appearance of the chromatograph (cf. Figure 2 with Figure 1). Additional selectivity for acidic or basic drugs can be achieved by adjusting the sample pH either before the initial extraction or between the steps of a double-extraction process. For drugs that are therapeutic in microgram per liter concentrations, such as the tricyclic antidepressants, additional sample cleanup (e.g., double extraction) may be necessary for better sample cleanup (see, e.g., 10). In this case, additional sample extract must be placed on the column for more sensitivity, increasing the size of interfering peaks. A water-miscible solvent such as acetonitrile can be made immiscible by adding sufficient salt to the aqueous phase, a technique useful for extraction of such very polar drugs as cimetidine (11).

**Solid-Phase Extraction Column**

Sample pretreatment by solid-phase extraction involves applying the sample to a small column filled with adsorbent material, which binds the drug before elution. Such columns fall into two groups, depending on whether they retain all the sample or only the drug and related substances. Miniscale disposable columns of both types are available commercially from several manufacturers.

In the first group of columns, those that trap the entire sample, a hydrophilic packing is used, such as particles of diatomaceous earth. The sample is completely adsorbed as a thin film onto the surfaces of these particles in the column, even including the water in the sample. An immiscible organic solvent such as methylene chloride is then poured through the column, which extracts the drug from the aqueous sample film (see, e.g., 12).

The second category is more selective, involving either hydrophobic or ion-exchange retention of the drug and related substances. For hydrophobic binding, a packing such as polystyrene, charcoal, or a C\(_{18}\) bonded phase silica (C\(_{18}\)alkyl chains covalently anchored to the surface of silica particles) adsorbs and extracts the drug hydrophobically from the sample. Once again, the drug is eluted with an organic solvent, but now either a water-miscible or water-immiscible solvent can be used, because little sample water remains on the column. An example is the extraction of anticonvulsant drugs from serum with a commercial C\(_{18}\) bonded silica column before LC analysis (13).

The second type of drug retention, by ion exchange, has been applied to highly polar, ionizable drugs such as gentamicin (e.g., 14) that are therefore not extracted by hydrophobic packings. The cleanup of gentamicin on unmodified silica particles (15) also belongs to this category, because the binding no doubt involves, or is dominated by, ionic attraction of the positively charged gentamicin to ionized silanol sites (SiO\(^-\)) on the silica. Elution—in this case with an organic solvent—was carried out after modification of the

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\(^1\) Dolan, J., and Giese, R. W., unpublished observation.
immobilized gentamicin for detection purposes with o-
phthalaldehyde, which neutralized at least some of the
positive charge of this drug and made it less polar. The
sample was then analyzed by reversed-phase LC with
fluorescence detection (15).

Separation by Size

Sample cleanup by size separation involves use of ultrafil-
tration or dialysis to isolate the free drug from the protein-
bound drug in the sample (16, 17). Free-drug measurements
will be used more commonly as improved sample pretreat-
ments are developed for this purpose and the clinical corre-
lation with free-drug concentrations is more widely
established. However, the number of assays for free drug
will probably always remain a small proportion of those for
total drug. The clinical utility of the latter assays is well-
established, and these assays are more convenient and less
expensive. Free-drug assays are more difficult when the
percentage of free drug in the sample is low, placing greater
demands on the selectivity and sensitivity of the analysis.

Column

This section focuses on the popular reversed-phase col-
umns in TDM, involving alkyl chains bonded to porous
silica packings. We will also discuss the use of related
bonded phases, and the role of column dimensions, particle
size, guard columns, column stability, and column reproduc-
ibility in TDM by LC.

Reversed-Phase Columns

There are many reasons for the much greater popula-
reversed-phase over normal-phase columns, not only for
TDM but also more generally for clinical and biochemical
LC analysis, as has been reviewed (18). Advantages of
reversed-phase LC include resistance to contamination, wa-
ter-based mobile phases, ease of equilibration, and broad
applicability. The most popular reversed-phase columns
contain porous silica particles surface-bonded with "C₈",
(C₈H₁₇) or "C₁₈" (C₁₈H₃₇) alkyl chains. (Residual silanol
groups, SiOH, are also present on these surfaces, as will be
discussed later.) The difference in chromatographic prop-
ties between these packings are subtle rather than major,
and probably most if not all applicable separations can be
achieved on either column. At higher flow rates, chromato-
graphic efficiency decreases less with C₈ than with C₁₈, but
the difference is small. Any apparent differences in selectiv-
ity (relative peak separation for a comparable retention)
between commercial C₈ and C₁₈ phases are probably over-
shadowed by selectivity differences among C₈ or C₁₈ col-
umns from different manufacturers (19). Selectivity differ-
ences are less likely between C₈ and C₁₈ than between
either of these alkyl phases and other, more polar bonded
phases such as cyano and phenyl, which can also be used in
the reversed-phase mode by incorporating adequate water
in the mobile phase. For example, the cyano-bonded phase
provides a good selectivity for separating tricyclic antide-
pressants (20).

Column Length and Particle Size

Other important factors influencing separation efficiency
are column length and the particle size of the packing. In
this regard, laboratories are increasingly using columns 15
cm long, packed with porous particles that are nominally 5
µm in diameter. A more recent advance is the development of
"very-high-speed liquid chromatography," involving even
shorter columns (e.g., 3 to 8 cm) with a conventional
internal diameter (e.g., 4.6 mm) packed with 3-µm particles
and operated at a relatively high flow rate, such as 2 to 5
mL/min (21). By using such columns along with a small-
volume flowcell (2.4 µL) and a detection system with a fast
response time, analysis times can be shortened for small
sample volumes (<6 µL) by four- to fivefold without sacrific-
ing resolution (22). Higher than usual operating backpres-
sure is required (about 3000 to 5000 psi), due primarily to
the combination of high flow rates and small particles.
Although these pressures are within the capability of cur-
rent LC pumps, there may be faster wear of the pumping
system, and the overall system is less able to tolerate
further increases in pressure that may be encountered
during routine use. Such a system was used to analyze for
theophylline in serum with a retention time of about 1 min
(23), and two of the antiepileptic drugs (along with an
internal standard) in about 1.6 min (24). (Under more
nearly ideal LC conditions for "high-speed" chromatogra-
phy with small particles, much higher analysis speeds have been
achieved, e.g., the normal-phase separation of six model
compounds in 3 s (25).) Use of a short column also usually
means less solvent consumption.

A second strategy with short columns packed with 3-µm
particles is to increase the internal diameter—e.g., to 6.2
mm—and use a flow rate that is only moderately higher,
perhaps twice the conventional (26). In this case, the result-
ing chromatogram is about the same in overall appearance
as with a more conventional column (i.e., 5-µm particles, 15
cm length, 4.6 mm i.d.), but is obtained in a third to half the
time. Analysis time is shorter with this 3-µm-particle column
because the column is substantially shorter while the linear velocity of the mobile phase is about the same.
Band volume is approximately unchanged because the
decrease in band broadening from the use of smaller parti-
cles is traded off against the extra sample dilution that
results when a wider internal column diameter is used. Not
only is the analysis time considerably shortened, but also
the extra dilution of the peaks on the column also minimizes
extra-column effects (both pre-column and post-column),
allowing conventional LC equipment to be used.

There are some potential difficulties with either of these
two strategies for LC columns packed with 3-µm particles,
or with related techniques. The first is the increased likeli-
hood, owing to the closer spacing of the particles in the
column, for more rapid contamination or plugging from
extraneous particulate matter. Such matter can consist of
aggregated proteins that may be present in the sample,
microprecipitates of buffer (arising at cooling or solvent-
mixing sites), or particles in the mobile phase originating
from valve wear (27). Secondly, plugging is more likely in
the lower-porosity filters and frits (e.g., 0.5-µm pores vs 2.0-
µm conventional) required in such systems. Much greater
care is therefore necessary in the clarification of samples
and mobile phase that are to be applied to these columns,
particularly in the case of direct injection of physiological
samples. Finally, temperature effects tend to be more im-
portant for small-particle columns (28).

Column Internal Diameter

Aside from the analytical radial compressed columns
supplied by Waters Associates (Milford, MA 01757), some of
which have an internal diameter of 8 mm, most analytical
LC columns have internal diameters in the range of 3 to 6
mm. This is because such dimensions provide good balance
among (a) ease of packing the column to give high efficiency
(including a relatively minor contribution from "wall ef-
fects"—usually the presence of looser and/or larger particles
at the walls—which tend to degrade the performance of the
column: 29, 30); (b) compatibility of the sample and peak
volumes on these columns with the injectors and detectors.
in routine use; (c) adequate sample capacity for small-scale semipreparative purposes; (d) limited elution volume of costly mobile phase; (e) limited amount of costly packing in each column; and (f) moderate pressure drop. Thus, any departure from this currently popular range of column internal diameters can enhance the usefulness of the column in some respects only at the expense of others. For example, wider-bore columns, unless they are also shorter, will require much more solvent at a given linear velocity of mobile phase.

Column Stability

Within the last couple of years there have been major advances in the stability of commercially available columns. In practical terms, these columns can withstand more mechanical stress such as being dropped onto a counter top or subjected to a reversed flow of solvent. Use of a sample-injection valve with a bypass-loop to reduce the pressure pulse during injection can also extend the useful lifetime of the column, at least for columns packed with 3- to 5-μm particles and subjected to repetitive injections with relatively high flow rates (31). Although present columns may be just as susceptible to accumulation of contamination from impure samples, with proper sample cleanup and injection techniques a column can be used for more than 2000 analyses. Column lifetime is also extended by the use of a "guard column," a short, low-cost column inserted between the sample valve and the analytical column. Its purpose is to protect the analytical column from contamination and plugging. Nevertheless, more work is needed on regenerating columns contaminated by impure samples, such as the usefulness of strong solvent injections between each sample analysis (32).

Mobile Phase pH

The pH range within which bonded-phase silica-based packings can be used is typically about 2 to 7. At lower pH, the bonded groups are hydrolyzed. At higher pH, the silica matrix itself dissolves, also leading to release of the bonded groups from the surface. Although the chemical stability of these columns at low pH has not changed significantly in recent years, the stability at higher pHs has been extended by the use of a silica-packed precolumn between the pump and the sample valve, allowing some presaturation of the mobile phase with silica before it enters the analytical column (33, 34). The use of amine vs alkali-metal buffers also improves column stability at higher pH values (35). For the non-TDM application of leukotriene analysis, reversed-phase LC at pH 10.5 (for solute stability) was successfully done by incorporating a silica precolumn, washing the analytical column when it was not in use, and periodically repacking the top of the analytical column (36). Mobile phases with a high pH can be used in normal-phase LC with silica particles—e.g., to analyze for the tricyclic antidepressants (37)—apparently because a high organic content in the mobile phase minimizes the solubility of the silica.

Column-to-Column Reproducibility

This topic often is not adequately addressed in published LC methods for TDM. Obviously, it is important that the same chromatographic conditions for a drug analysis give similar results with each new column. There is much progress, but different batches of columns from the same manufacturer still can require different compositions of the mobile phase for comparable retentions, and separations of certain drugs, especially for drugs that are susceptible to active sites in the column. The fundamental nature and difficulty of this problem has been pointed out (38). Nevertheless, with continued advances, such as the recent appreciation of the role of surface pH of silica-based packings (39), we can expect improved column reproducibility. For the time being, given the columns currently available, the LC user is challenged to develop mobile-phase conditions that not only achieve the separation required but also minimize column-to-column differences. Probably the most severe test of column-to-column reproducibility is the relative retention of acidic and basic substances analyzed simultaneously. Separations of neutral compounds are far easier to reproduce from one column to another. Although many drugs are neutral, avoiding major problems in column-to-column reproducibility when analyzed by LC, some of the potential interferences may be acidic or basic, and so their retention may vary from column to column. Thus, this more severe test of column-to-column reproducibility needs to be addressed for TDM, particularly along with sample-pretreatment protocols that do not provide for removal of all acidic or basic contaminants.

Mobile Phase

A major advantage of LC over gas chromatography is the opportunity to influence the separation of drugs by changing the composition of the mobile phase. This flexibility and control of the mobile phase provides a useful tool for the laboratory; in practice, however, this approach is underutilized. This section addresses the mobile-phase aspects of organic co-solvent (for example, methanol vs acetonitrile), temperature, and degassing. Elsewhere in this review, the role of pH and organic modifiers such as triethylamine are discussed.

Organic Co-Solvent

The two most commonly used organic co-solvents are methanol and acetonitrile, with methanol usually the first choice, other considerations aside, because of its lower cost. However, acetonitrile is a less-polar organic solvent, typically providing an equivalent solvent strength at a lower concentration, which decreases the actual difference in cost between the two solvents. Acetonitrile also yields a mobile phase with water that has a lower viscosity, which makes the column more efficient and allows a lower pumping pressure. Selectivity differences between these two solvents also can arise, largely because acetonitrile is a hydrogen bond acceptor while methanol is a hydrogen bond donor. Coworkers and I have observed, for example—as have others (40)—that the retention order of propranolol and its less-polar metabolite, N-acetylprocainamide, can be changed on reversed-phase LC simply by changing the co-solvent from methanol to acetonitrile.

A third organic solvent, which should be considered after methanol or acetonitrile, is tetrahydrofuran. Because it offers further contrasting solubility properties (less polar, weaker dipole moment than acetonitrile, nonhydroxylic relative to methanol), one might consider using this solvent when attempts to use methanol and acetonitrile are unsuccessful.

And one should not overlook the potential of a ternary mobile phase, i.e., a combination of two organic solvents plus water. Drug separation can be significantly affected by even a small proportion of a second organic solvent, especially if this solvent is a strong eluent relative to the mobile phase being modified (41). This is because such a solvent tends to concentrate in, and change the characteristics of, the bonded phase. An example of this phenomenon is the observation that a separation of the tricyclic antidepressants on a reversed-phase column is markedly improved by
adding 5 mL of methylene chloride per liter to the mobile phase.\(^2\)

A second example of a useful ternary mobile phase is the analysis of theophylline by reversed-phase LC involving a methanol/acetonitrile-buffer mobile phase at room temperature (42). Previous workers reported that a low proportion of acetonitrile was needed to adequately resolve theophylline from theobromine in a binary mobile phase of acetonitrile and buffer (43); they also used a higher column temperature to shorten the analysis time (see the following discussion on column temperature). In this ternary analysis one can calculate that the added methanol in the mobile phase allowed a low proportion of acetonitrile to be maintained for selectivity purposes, thus providing a higher solvent strength, as opposed to use of a higher column temperature, for a faster analysis.

Temperature

The temperature of the mobile phase is controlled by increasing the temperature of the column and the preceding capillary tubing. The primary effect of increasing the temperature of the mobile phase is to increase the solubility of drugs in the mobile-phase solvent, thereby diminishing their chromatographic retention. A higher mobile-phase temperature also improves column efficiency, largely because the mobile phase is less viscous, allowing more rapid molecular diffusion. A disadvantage of a higher mobile-phase temperature is decreased column stability. More details on the contribution of temperature to resolution in reversed-phase LC are available (44).

Degassing

Mobile-phase degassing serves two purposes.

The first is to avoid formation of gas bubbles in the chromatographic system. The three most likely sites for this are the gradient mixer (in gradient LC), the pump, and the flow cell. Outgassing may occur when solvents are mixed, due to the positive or negative heat of mixing and associated differences in the solubility of air when the solvents are mixed. In the pump and detector, gas bubbles tend to arise from non-degassed solvents whenever large changes in pressure are encountered by the solvent, as from the sudden low pressure impulse during the intake stroke with a reciprocating pump. The problem with these bubbles is that they can perturb the mixing of the solvents in a gradient mixer, the solvent delivery of the pump, the flow in the column, or the signal in the detector flowcell.

The second reason for solvent degassing is to overcome interference of dissolved gas, especially dissolved oxygen, with the performance of the detector. For example, dissolved oxygen (either inherently or as solute–oxygen complexes) can interfere with electrochemical or fluorescence detectors, or absorb light in an absorbance detector at a low wavelength, thus limiting the sensitivity of the analysis. Oxygen also tends to oxidize amino-bonded phase columns. More intensive degassing may be needed to solve these oxygen problems because of their propensity to occur at oxygen concentrations below the outgassing level.

In a paper comparing methods for removing oxygen from methanol, based on the residual absorbance of the methanol at a low wavelength, the order of decreasing effectiveness for oxygen removal was reflux > helium sparging > vacuum > ultrasonication (45). Nevertheless, to minimize outgassing, the LC user may find that any of these four types of methods—or even the mild degassing that can accompany vacuum filtration of a solvent—can be adequate. Further, with the advent of improved pumps that are less subject to gas bubble formation and the availability of sturdier detector flowcells that can tolerate a high pressure on the outlet side, degassing of solvents can often be completely avoided for outgassing purposes.

Detection

Several detection approaches have been developed for LC in therapeutic drug monitoring but only two are commonly used: ultraviolet and, to a lesser extent, fluorescence. Both are very convenient and sensitive for TDM purposes. The only drugs not detectable by ultraviolet are those with low absorptivities, even at low wavelengths (i.e., in the 200-nm region) or those with a very low sample concentration. A good example of a problem drug for ultraviolet detection in both respects is digoxin, which absorbs ultraviolet poorly and for which the therapeutic concentration is low—about 1 to 2 µg/L. Thus, this drug currently is not routinely monitored by LC. In contrast, sodium valproate, although a weak ultraviolet absorber, can be monitored at a low wavelength because its therapeutic concentration in the blood is relatively high (46). Although the tricyclic antidepressants are present therapeutically only in microgram per liter concentrations in serum, their absorptivities are sufficiently high that they can be adequately monitored by ultraviolet (see, e.g., 37, 47).

Ultraviolet Detection

The two general categories of ultraviolet detectors are fixed- and variable-wavelength. The traditional advantages of the fixed-wavelength detectors have been their lower cost and much higher sensitivity at the wavelengths available, owing to the greater light intensity of the light from discrete-wavelength sources. This high sensitivity tends to overcome the shortcoming of these detectors, which is that the discrete wavelength usually does not coincide with the most intense absorption wavelength for a given drug. However, aside from cost considerations, for general purposes a variable-wavelength detector is now the first choice. Not only have these detectors become extremely sensitive (e.g., 0.001 A full scale over the whole wavelength range), but their ability to select particular wavelengths, whether very low (e.g., 190–200 nm) or relatively high (e.g., >300 nm), can provide additional sensitivity and specificity over fixed-wavelength detectors. (I will comment later on multi-wavelength and rapid scanning detection.)

Fluorescence Detection

This mode of detection is not as widely used as ultraviolet in TDM because not many drugs are capable of fluorescence. Nevertheless, when it is applicable, this detection method is more sensitive than ultraviolet and generally yields a clean chromatogram because it is quite specific. This is illustrated for a low-concentration drug, propranolol, in Figure 3 (48). The higher specificity of fluorescence as compared with ultraviolet detection also facilitates analysis for this drug in plasma with only an acetonitrile pretreatment (49).

With some drugs, such as gentamicin, a pre- or post-column reaction can be used for ultraviolet or fluorescence detection, even though these drugs inherently lack these spectral characteristics. Figure 4 shows a chromatogram involving a post-column fluorescence reaction for gentamicin (14). Note the good separation and peak characteristics for the several forms of gentamicin, which differ in extent of methyl substitution, even after post-column reaction involving mixing the drug with a reagent stream of o-phthalaldehyde in a mixing tee before detection. Reaction detection in LC has been reviewed (50). Because of its specialized nature,
however, this detection technique for TDM will be considered only for those drugs that defy all other types of routine LC detectors.

Electrochemical Detection

The technique of electrochemical detection also is used for TDM, e.g., in the analysis for morphine (51, 52) or phenothi- azines (52, 53) in serum, and for acetaminophen in cerebrospinal fluid (54). This detection mode is appealing because of its high sensitivity and specificity along with a low cost. Unfortunately, relatively few drugs are electrochemically active. The main application of this detector in clinical LC analysis is for catecholamines (see, e.g., 55).

Temperature

Variation of the temperature in the detector causes changes in most if not all types of LC detection processes, which leads to baseline noise or drift. The effects are most significant at the more sensitive detector settings, for example at 0.005 A full-scale ultraviolet detection. Temperature fluctuations in the detector most often arise from such variations in the incoming mobile phase and include temperature fluctuations caused by pressure pulses from the pump. Although ambient temperature fluctuations and drift can be moderated by passing the column effluent through a heat exchanger before it goes to the detector cell, some chromatographic efficiency may be lost because of peak dispersion in the exchanger. Because temperature noise is generally manifested as slitheren effects, special detector-cell designs such as tapered cells, which overcome slitheren effects (56), can help overcome temperature disturbances.

Chromatogram

The chromatogram is the final graphic representation of the results of the chromatographic process, revealing the overall performance of this process. With some experience, the analyst can "read" the chromatogram, judging whether the chromatographic variables are optimally adjusted and recognizing early warning signs that problems are developing.

The three most simple and useful characteristics of chromatographic peaks are $k'$, $N$, and $A_2$ values; i.e., capacity factor, efficiency, and asymmetry values, respectively (30). In this section, I will discuss these variables, as well as peak tailing, quantification of peaks (peak height vs peak area), and the special problems of "complex solutes" and "crowded chromatograms."

Capacity Factor

The capacity factor is given by the following equation:

$$k' = \frac{t_r - t_b}{t_b}$$

where $t_r$ is the retention time of the peak of interest, and $t_b$ is the retention time for a nonretained peak. For routine work, it is usually acceptable for $t_b$ to be approximated by the earliest peak or baseline disturbance in the chromatogram. We should realize that $k'$ is simply the number of void volumes of mobile phase in the column, after the nonretained peak, required for elution of a given drug. Thus, $k'$ normalizes for differences in column dimensions. The practical importance of determining the $k'$ value for a drug peak is to make sure that the solute is not being exposed to too little (generally $k' < 2$) or too much (generally $k' > 10$) chromatographic retention in the column. (The LC user should appreciate that all drug peaks eluting from a given column...
have not undergone the same "amount of chromatography." This is because chromatography is a separation process based on the interaction of a given solute with the stationary phase in the column, and the faster a solute is eluted from the column in terms of \( k' \), the less chromatographic separation the solute undergoes.

A low \( k' \) value for a drug peak is undesirable because the decreased separation increases the likelihood of interferences. In practice, \( k' \) values of <1 should be avoided.

The opposite problem, an inappropriately high \( k' \) value, is that the analyst is probably paying too high a price in terms of analysis time, solvent consumption, and (or) loss of sensitivity (i.e., peak height) in order to minimize interferences in the analysis. He should either strengthen the mobile phase so as to elute the drug(s) more rapidly in terms of \( k' \), or, if this degrades resolution or introduces interfering peaks, change the selectivity of the separation by using different components in the mobile phase or the stationary phase, or both.

**Plate Number**

Efficiency, or plate number, \( N \), is a measure of the ability of the column to provide narrow rather than broad peaks. Obviously, narrow peaks are to be preferred, because they stand out more distinctly from both interfering peaks and baseline noise. This translates into faster, more reliable and sensitive analyses. One equation that is frequently used to estimate \( N \) is:

\[
N = 5.54 \left( \frac{t_r}{W_{1/2}} \right)^2
\]

where \( t_r \) is the retention time of the peak at the apex, and \( W_{1/2} \) is the peak width at one-half the peak height. Similar equations are available which allow calculation of \( N \) based on the peak width at the base, or on the peak area (30). However, there are two general pitfalls to avoid in measuring column efficiency with any of these equations.

The first potential problem is that the efficiency with which a peak is produced depends not only on the column, but also on the characteristics of all of the other instrument components in the system, such as the injection valve, the detector, and the tubing, fittings, and any filters. Broadening of a peak—particularly of narrow peaks—from these non-column sources is termed "extra-column band broadening." These effects may take place pre-column and (or) post-column. In either case, the source of the problem is usually an oversized flow region (e.g., in a tubing connector or in the detector flowcell), which causes undue mixing of sample with solvent. A rule of thumb, at least for a conventional detector flowcell, is that its volume should be less than a tenth the effluent volume of a peak of interest if noticeable broadening of this peak is to be avoided (30). A large sample volume also constitutes pre-column band broadening, and a large time constant for the detector/recorder can contribute to post-column band broadening. A rule of thumb in this latter case is that the time constant should be less than 10% of the peak width measured at half-height (57).

Two mechanisms are behind the greater influence of extra-column effects on an earlier (typically narrower) peak. In the first, which applies to pre-column band broadening, the extra-column dilution is the same for all of the sample components, because they all occupy the same volume before the column. Importantly, this type of dilution imposes essentially the same absolute increment of band broadening on both early and late peaks, causing relatively more spreading of the earlier peaks owing to their smaller, post-column volumes. The relatively small loss in chromatographic efficiency for peaks with higher \( k' \) when the injection volume is increased substantially—e.g., from 10 \( \mu \)L up to 100 \( \mu \)L—with conventional LC systems, similarly results from the tendency of the effluent volumes represented by the peak to increase by only the absolute increment by which the injection volume is greater as long as trace-enrichment effects are absent. Because earlier peaks generally are eluted in smaller volumes, they are also more susceptible to dilution in post-column mixing regions. The early-eluting peaks are more completely surrounded by solvent in these regions and so undergo relatively greater band-broadening, tailing, or other distortion than does a later, broader peak, which is eluted more gradually into these same mixing regions.

To minimize peak broadening, it is necessary for high-quality injection and detection systems (up to the performance level of the column) to be used, connected properly so that their contribution to band broadening becomes negligible relative to that occurring inherently in the column. Only under these conditions does \( N \) reveal the true ability of the column to achieve separation. In routine TDM, extra-column band broadening should not arise with properly maintained, modern equipment.

**Solute Type**

The second caution with use of the above equation (and its related forms) is that the plate number of a column can be solute dependent. Here it is useful to categorize substances arbitrarily as either "simple solutes" or "complex solutes," depending on how they behave in terms of \( N \). Generally, complex solutes tend to have a greater variety and extent of both polar and nonpolar groups, including a larger overall molecular size, than do simple solutes, although the array of the functional groups on a molecule can also play a role (58, 59). For example, we routinely observe a value for \( N \) of about 5000 theoretical plates for carbamazepine (which possesses both polar and nonpolar groups) on the same column that gives 8000 plates for toluene (a completely nonpolar substance) under the best conditions currently available in regard to \( N \) for each. Thus, one's expectations of \( N \) may need to be revised for drugs that behave as complex solutes relative to the \( N \) values provided by the column manufacturer for drugs or other compounds that behave as simple solutes.

Related to this second problem, the above equation only approximates the true \( N \) value of the peak (unless the peak is perfectly symmetrical), and this approximation worsens rapidly as the peak assumes a non-gaussian shape, including tailing that may be solute related. The most nearly correct method for calculating \( N \) involves calculation of the second central moment, a complicated calculation best done by a computer (60).

Although it has been stated in the literature that values for \( N \) of a few hundred theoretical plates are satisfactory for TDM, this is incorrect, because there are some important advantages to be obtained from higher values for \( N \), especially for a given column length, \( L \). A higher value for \( N/L \), or stated another way, a lower theoretical plate height, \( H \), where \( H = L/N \), can decrease the susceptibility to interferences, shorten analysis time, and increase sensitivity.

**Asymmetry**

The peak asymmetry value, \( A_r \), is the ratio of the back (trailing edge) to front (leading edge) lengths of the peak along a line parallel to and 10% distant from its base in terms of peak height, as shown in Figure 5. \( A_r \) is the most widely used simple parameter for measuring peak distortion, especially peak tailing. It is useful to assess tailing with \( A_r \), rather than by visual examination, not only because \( A_r \) provides a quantitative, arbitrary value for
tailing, but also because visual perception of tailing tends to fail for sharper peaks. Thus, it is not uncommon for the early eluting peaks in a chromatograph to “appear” less tailed than the later peaks, even through the $A_t$ values may be nearly the same for both. As a rule of thumb, one should use chromatographic conditions in which $A_t$ values for the peaks are less than 1.2. (Perfectly symmetrical peaks have $A_t = 1.0$.) Only a small degree of peak tailing ($A_t < 1.2$) is truly acceptable, owing to the inherent nature of the chromatographic process.

Peak Tailing

Excessive peak tailing is undesirable, no matter what the cause. Analysis time is increased because resolution is poorer; interferences are more likely to go undetected because they can blend into the tail; sensitivity is decreased because of the reduced peak height; and quantification is less precise. The reason for this last problem is that peak height is more likely to be nonlinearly related to concentration, and peak area is more difficult to evaluate with tailed peaks.

There are numerous causes of peak tailing—along with numerous remedies—and the reader is encouraged to read the chapter on this topic in Snyder and Kirkland’s recent book on LC (30). Overall, the sources of peak tailing can be divided into the two categories: “within-column” and “extra-column.” Extra-column peak tailing is basically the same problem as extra-column band broadening, because the causes are much the same. This source of tailing should be under control in any responsible laboratory doing TDM by LC. This leaves “within-column” tailing, the most common source of which is “active sites” in the column. These are molecular adsorption sites that interact strongly with the drug and are so limited in number that they delay the passage of only some of the drug molecules. The result is a tailed peak. Conditions and columns that expose the peaks to such sites should be avoided. Measures for doing so in reversed-phase LC are discussed later.

The presence of tailing due to active sites raises a concern for column-to-column reproducibility, especially with silica-based bonded-phase packings. Even though a given analysis may be satisfactorily performed on a series of several columns, sooner or later the manufacturer, because of an uncontrolled change in the silica or in the bonding conditions, is likely to supply a column with different active sites. When this happens the same separation may require new chromatographic conditions—with no guarantee that previously resolved interferences, such as related drugs, will be separated.

Tailed peaks are also a bad omen for column stability with bonded phases, because the active sites tend to be either exposed sites of the underlying silica matrix or sample contamination that has adsorbed onto the particles. Exposed silica sites provide spots for further silica to dissolve; adsorbed contamination changes the chromatographic characteristics of the column.

Tailing of Basic Drugs

On bonded reversed-phase columns, tailing from active silica sites has been a significant problem, especially for the basic drugs such as the antiarrhythmics (procainamide, lidocaine, and quinidine) and the tricyclic antidepressants. The composition and mechanistic details of these active sites are not totally understood, but the sites probably involve ionized silanol groups, which bind basic drugs electrostatically. This is consistent with the reduction in peak asymmetry seen at both high and low pH values. The improved symmetry probably arises from neutralization of the charge on the drug at high pH, and protonation of the ionized silanol groups at low pH. Unfortunately, use of these measures to improve chromatographic performance is limited by the instability of the bonded-phase packing at both very high and low pH values, as discussed before.

“End capping” with trimethylsilyl groups also has helped to reduce the tailing of basic drugs somewhat, and now is a standard procedure used by most column manufacturers. In this technique, the bonded (e.g., $C_8$H$_{17}$ alkyl) silica packing is trimethylsilylated as an “end” step to “cap” as many as possible of those silanol groups that failed to react with the sterically larger $C_6$ silylating reagent. Other such measures include the use of ion-pairing agents such as n-hexyl sulfonate or of an amine modifier such as triethylamine. These substances tend to reduce the tailing of basic drugs by competing with the drug for binding to the anionic sites on the packing. In routine laboratory practice, various combinations of these measures have been used (see, e.g., 61), the result being a reasonable control over the tailing behavior of most of the basic drugs. An example is provided by the chromatogram in Figure 6, in which procainamide and $N$-acetylprocainamide are separated on a $C_8$ column with use of a mobile phase involving both an amine modifier and an

Fig. 5. Parameters involved in the measurement of peak asymmetry ($A_t$), an expression useful for quantifying the degree of peak tailing
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Fig. 6. Chromatogram of a serum sample supplemented with procainamide (PA) and $N$-acetylprocainamide (NAPA) and treated with an equal volume of acetonitrile
After centrifugation of the sample, 50 μL of the supernate was injected onto a $C_8$ reversed-phase column and eluted with a 43/57 by vol mixture of methanol/sodium phosphate (10 mmol/L, pH 6.5) containing 1 mmol of nonylnamine amine modifier and 5 mmol of bis(2-ethylhexyl) phosphate ion-pairing agent per liter. Detection was at 280 nm. Tanaka N, Ikemura R, Karger BL, and Giese RW. Unpublished results (first peak is severely cropped)
ion-pairing agent. The drug peaks are seen to be at least moderately symmetrical.

Peak Height or Area

To quantify the peaks in a chromatogram, either peak height or peak area may be measured. Peak area has the advantage that it is less susceptible to changes in conditions, except for changes in the flow rate. It also is a better choice when tailed peaks are quantified, because peak height can be non-linearly related to concentration under these conditions. Nevertheless, in an analysis that is under control, so that peak tailing is negligible and the conditions are constant, measurement of peak height is preferred, because it is less susceptible to peak overlap from interferences or other drugs. As analyses increasingly are brought under control in this manner, quantification by peak height should be used increasingly.

Complex Solutes

All of these conditions for peaks are particularly important for "complex solutes," i.e., solutes with lower $N$ values as discussed earlier. This is because complex solutes are also more likely to undergo tailing. For example, we have been more successful in using the above-mentioned measures (end capping, pH optimization, amine modifier, and ion pair) to reduce tailing for procainamide than we have for quinidine, apparently because the latter drug tends to undergo more complex sorption interactions on the C$_8$ alkyl-bonded silica packing that we were using. A second example is the LC analysis of the immunosuppressive drug, cyclosporin A, structurally a large compound possessing numerous polar plus nonpolar groups, where major band broadening and tailing are observed (62). Fortunately, increasing the column temperature improves the peak shape considerably (62, 63).

Crowded Chromatogram

The above considerations of $k'$, $N$, and $A_n$ for peaks are especially critical for chromatograms containing numerous or more closely spaced drug peaks. Control of band separation tends to be difficult here, because various drug structures or interferences are usually involved, and their relative retentions usually respond differently to changes in mobile-phase compositions or active sites. Improving the resolution between certain peaks will simultaneously tend to decrease the overlap between others. The antiepileptic drugs provide a good example because several are used commonly, sometimes concurrently, and also have several metabolites (e.g., 8). Even single drugs that are therapeutic at low concentrations tend to give rise to crowded chromatograms because interferences are proportionately greater. Worst of all are multiple, complex, basic drugs in low concentrations, as exemplified by the tricyclic antidepressants. Thus, we can appreciate why some common tricyclics were in one case separated as two groups (Figure 7).

Automation

LC for therapeutic drug monitoring has been totally automated quite recently by Technicon's "FAST LC" system (47, 64): serum samples are loaded into a tray at one end of the system and LC peaks appear on a chart recorder at the other. In between, the samples are extracted with an immiscible organic solvent, the phases are separated, and the drug is evaporated, redissolved in mobile phase, and injected onto the LC column. Figure 8 shows chromatograms illustrating the application of this system to some common anticonvulsants. A second-generation version of

Fig. 7. Typical chromatograms from the FAST-LC (Technicon) for tricyclic antidepressant drugs in calibrators in concentrations ranging from 123 to 264 $\mu$g/L.

The internal standard (IS) is clomipramine. Reprinted, with permission, from reference 47

Fig. 8. Chromatograms provided by FAST-LC assay for anticonvulsants and metabolites.

Peaks and full-scale absorbance values as follows: 1. phenylethylmalonamide; 2. ethoxyzoline; 3. primidone; 4. phenobarbital; 5. carbamazepine epoxide (at 0.8 A full-scale); 6. phenytoin; 7. carbamazepine (at 0.2 A full-scale); IS, internal standard (hexobarbital). Reprinted, with permission, from reference 64
methods

Currently, there are powerful applications where speed, convenience, automation, and reliability of these assays are critical. Some of these advances may arise from the potential application of monoclonal antibodies to drug analysis. Certain drugs, such as digoxin and the aminoglycosides, are much easier to analyze by immunoassay than by LC, due to the inability of the routine LC detectors to measure drugs without derivatization, as discussed earlier.

Several factors promote the use of LC as opposed to immunoassay methodology for routine TDM, some of which are elaborated in the next section. First of all, because it is a powerful separation technique, LC can be used to quantitatively closely related drugs and their metabolites, such as the tricyclic antidepressants. This capability is probably beyond the specificity available from antibodies. Second, when LC is used in a single analytical step by LC, whereas separate immunoassays have been required for each drug or metabolite (aside from immunoassays measuring the sum of several drugs). Third, LC is more accurate because it is a more direct method, particularly when combined with more specific detectors such as multi-wavelength or scanning absorbance detection. The potential for inaccuracy with immunoassays is illustrated by the tendency for antibodies recognizing digoxin to cross react with digoxin metabolites. A second example is the 25% higher values for quinidine by an immunoassay as opposed to the results from an LC method, apparently owing to a lower specificity by the immunoassay (70). The final advantage of LC is that it can set up new drug assays more quickly.

Future

Although LC is a well-advanced technique, we can anticipate more sophistication and improved performance of all parts of such instruments: injection valves, pumps (e.g., further reductions in pulsations), columns (especially increased column stability and reproducibility), detectors, and ease of operating/trouble-shooting/data-handling with microprocessors. To elaborate on detectors: look for improved sensitivity, more multi-wavelength and spectral absorbance detection (with uninterrupted flow) to confirm peak identification and reveal peak overlap (71), coupling of the LC to the mass spectrometer (72), novel post-column reactions (50), and laser fluorescence detection (73, 74). For example, it is impressive that detection involving use of absorbance ratios together with retention data allows identification of 27 of 29 barbiturates by LC (75). Also, by laser fluorescence detection the antitumor drugs adriamycin and duanorubicin can be detected in picogram amounts after direct injection of a 20-μL sample of a drug-supplemented urine (Figure 9).

Mobile Phases

Analogous to the general adoption of commercial reagent mixtures in the clinical laboratory, we can expect increased use of commercially pretested column/mobile-phase combinations, particularly for difficult drug analyses. It will be cost effective to have a manufacturer fine-tune the mobile phases and select reproducible columns to maintain chromatograms that are consistent both with regard to separation of related drugs and metabolites and resolution of potential interfering drugs and endogenous metabolites. Mobile phases, along with their development and control, will also become more sophisticated. Take, for example, the use of mobile phases comprising four solvents optimized automatically for a separation by computer programing (77, 78).

![Figure 9](image)

**Figure 9.** A typical chromatogram with laser fluorescence detection of a drug-supplemented, filtered urine sample (63 pg of adriamycin and duanorubicin injected).

The sample was injected directly onto a C18 column and eluted with an equimolar mixture of acetonitrile and 10 mM/L phosphoric acid. Excitation and emission wavelengths of 488 and 590 nm, respectively, were used with a fiber optic flow cell. Reprinted, with permission, from reference 76.

Columns

Interest will increase in the use of "ultra-short" LC columns—e.g., 3 to 8 cm long, packed with 5-μm (or perhaps 3-μm) particles—to conserve both time and solvents. To be most successful for TDM, this technique will require a thorough sample cleanup, careful optimization of mobile-phase selectivity, and the use of a multi-wavelength absorbance detector, because the total plate count for the separa-
tion is smaller. This attractive technique has a potential for very high throughput, without compromising reliability, for many drug analyses.

Micro-scale LC columns, with internal diameters of 1 mm or less, are currently under development (79, 80) and have several putative advantages: major reduction in solvent cost because solvent flow is reduced about 100-fold, very small sample requirements because of less sample dilution on the column, and more compatibility with solvent-sensitive detection techniques such as flame ionization and mass spectrometry. However, the rest of the LC system must also be further miniaturized (more so than with some conventional columns packed with 3-µm particles), which is not easy, considering one is starting from the relatively small size of current components.

Column Switching

Column switching is a more certain development in TDM. This refers to special plumbing and valving arrangements that involve two or more columns to process a given sample (81). In this way, entire groups of peaks can be shuttled along different flow paths, with certain advantages. When used for sample cleanup, for example, very early-eluting or very late-eluting groups of interferences can be sent to waste while the drug peak(s) can be automatically preselected and pumped into a second column through a programmed valve. This allows more chromatography as needed before detection, and also enhanced throughput and improved precision from the cleaner baseline, which is not contaminated by late-eluting peaks. Moreover, the arrangement can be used isocratically in place of gradient elution.

Figure 10 shows an example of column switching for sample cleanup. The system, which involves two valves (the two circles), two columns, and two pumps, was applied to the analysis for methotrexate and phenothiazines (82). For methotrexate assay in plasma, the drug (from an acid-deproteinized sample) is sequentially trapped hydrophobically on a concentration column, separated by an ion-exchange process on an analytical column, and quantified by ultraviolet detection. In a related system that was partly automated, drug-containing samples of plasma, urine, or saliva were injected directly onto a hydrophobic concentration column without sample pretreatment (83). Interestingly, the performance of the concentration column was unchanged, even after 1000 injections. Similar results up to 2000 injections for a fully automated LC system are reported (65).

"Box-Car" Chromatography

The advanced concept of column switching has been taken one step further with the introduction of "box-car chromatography" (84). In this technique, the samples (i.e., middle cuts from the first of two columns) traverse the analytical column back-to-back, like box-cars in a train, rather than conventionally as single samples in the column. This mode overcomes the objection to LC that it is limited in its throughput, because analysis rates as high as 50 samples per hour can be achieved with columns of conventional length and separation times. This is illustrated for the assay of primidone, phenobarbital, and carbamazepine epoxide by the box-car chromatogram shown in Figure 11.

Parallel Columns

The throughput of LC for TDM also may be increased by use of parallel columns. For example, a single pump with appropriate valving can interface several columns and mobile phases, with the result that each column can remain dedicated to a certain mobile phase for a particular drug analysis. Overall throughput is increased, and random sampling is also made more feasible by such a system, because it can switch from one chromatographic condition to another merely by rapidly re-equilibrating the mobile phase in the pump, thus avoiding the longer time necessary for column re-equilibration. More sophisticated systems also can be constructed based on this principle, involving (e.g.) more than one pump or superimposed column switching, for additional flexibility.

Other Applications

Thus, LC is continuing to develop both instrumentally and in applications for TDM, particularly along with automation. We can therefore anticipate an expanding role for LC in the clinical laboratory, including applications other than to drugs. For example, LC seems to be unrivaled in its

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**Fig. 10. Principle of the chromatographic determination of methotrexate**

The sample is brought into the loop of the high-pressure valve I and is transported by eluent A to the concentration column. After switching valve II into the injection position (dotted line), eluent B flows in the reverse direction through the concentration column and brings the concentrated methotrexate onto the analytical column. Reprinted, with permission, from reference 82.

**Fig. 11. "Box-car" chromatography: analysis of primidone (P), phenobarbital (Pb), and carbamazepine epoxide (Cb) at 55 samples per hour**

Reprinted, with permission, from reference 84.
ability to conveniently quantify the porphyrins (85); vitamins such as vitamin A (86), 25-hydroxy-D$_3$ (87, 88), and riboflavin (89); amino acids (90, 91); and sphingolipids (92). It is also a powerful method for measuring catecholamines (55) and is the basis for a proposed Selected Method of analysis for cortisol (93), and there is interest in subjecting samples to an LC separation before hormone radioimmunoassay (94). These other applications for LC in the clinical laboratory will encourage its further development as a methodology for TDM, and vice versa.

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