High-Performance Liquid Chromatography/Mass Spectrometry: State of the Art for the Drug Analysis Laboratory

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The combination of HPLC and mass spectrometry has great promise for the toxicology laboratory. In the past five years, significant progress has been made toward producing a reliable interface for these techniques. Thermospray, liquid ion evaporation, ion-spray, and the particle beam separator are all viable "second generation" approaches with significant advantages and disadvantages. I review the operation of these interfaces with an orientation to their use in the drug analysis laboratory. Thermospray, ion-spray, and liquid ion evaporation primarily supply information about molecular mass. The benefits and limitations of using tandem mass spectrometry to obtain additional structural information will also be discussed.

The marriage of "high-performance" liquid chromatography (HPLC) and mass spectrometry (MS) has been a sought-after union for 15 years. The first practical HPLC/MS interface was reported by Baldwin and McLafferty in 1973 (1). A number of investigators attempted to improve this interface, which basically directed the liquid flow into the mass spectrometer. The direct liquid introduction interface was never widely accepted because of the modifications required to the vacuum system, the production of only a few ions from each compound, and the microliter per minute flow rates required at a time when most HPLC pumps were not capable of delivering them reliably. The moving belt interface was developed to produce electronic ionization spectra from HPLC effluent. The effluent was deposited on a moving belt or wire, which entered the vacuum system (2). Solvent was evaporated in a heated zone, and the desolvated analyte of interest carried into the mass spectrometer and vaporized from the belt. A conventional MS source was then used to produce ions. Problems with the numerous moving parts, adsorption of compounds onto the belt, and other mechanical difficulties frustrated many users.

In the intervening years, the state-of-the-art in both HPLC and mass spectrometry has advanced tremendously. The development of pumps capable of reliably delivering solvent at flow rates of microliters per minute and the availability of 4.6- and 2-mm (diameter) columns with high efficiency have been major contributors to these advances. In mass spectrometry, discovery of surface ionization mechanisms, such as liquid secondary ion mass spectrometry, and continued improvements in hardware, such as quadrupoles capable of resolving ions at 2000 atomic mass units and tandem mass spectrometers, have increased interest in this technique. Several excellent reviews of HPLC/MS have appeared (3, 4). Table 1 summarizes a variety of interfaces that have been reported. In the last three years, several of these interfaces have become sufficiently reliable to make them commercially viable.

All HPLC/MS interfaces have several elements in common. First, there must be a method to remove the liquid chromatographic eluent from the compound of interest. Except for the moving-belt interface, this process consists of nebulizing the liquid into a fine mist of particles, after which a combination of heat and reduced pressure is generally used to remove the solvent. A wide variety of nebulizing techniques have been used, either singly or in combination, including Raleigh disruption, pneumatic, thermal, and ultrasonic disruption. Second, the compounds must be efficiently transported to the mass spectrometer. Finally, provision for ionization must be made. Ionization occurs in a medium-pressure region, producing mainly protonated molecular ions and molecular ion-solvent adducts. If ionization occurs in a region of high vacuum in the presence of an electron beam, ionization with significant fragmentation is most likely. Interestingly, in some approaches to an HPLC/MS interface, all of these processes are occurring simultaneously. Nevertheless, it is useful to recognize that distinct processes are occurring, each with its attendant limitations.

HPLC/MS holds great promise for measuring drug concentrations in body fluids, particularly because many drugs are either large or labile molecules that do not lend themselves to analysis by gas chromatography/MS without laborious derivatization or modification. As with gas chromatography/MS, HPLC/MS can be envisioned in two distinct areas of application in drug analysis: qualitative identification of a drug or drug metabolite or quantification of a drug. Both applications, to be useful, require adequate limits of detection. In the former application, structural information from predictable fragmentation processes must also be available. The use of HPLC/MS in the analysis of drugs through 1983 has been reviewed (12). I will primarily review the advances since 1983, with particular emphasis on the advantages and disadvantages of each technique.

Thermospray (Figure 1)

The discovery of this phenomenon arose from the serendipitous observation that ions were being produced in a heated stream of liquid despite the fact that the filament in the mass spectrometer source had burned out. Blakely and Vestel (6) reported on the important effects of the operation of the thermospray interface. Basically, the liquid effluent is heated in a narrow-bore stainless-steel tube such that a plume of fine droplets is ejected from the tube. Development of a stable plume is a function of the inner diameter of the tube, the temperature of the probe tip, and the flow rate and composition of the mobile phase. Ions are sampled from the plume by a skimmer cone at the entrance of the mass spectrometer. Because the interface is capable of handling flow rates of 1-2 mL/min, it was the first popular HPLC/MS interface. Blakely and Vestel (6), using single ion monitoring, were able to demonstrate a limit of detection of about 10 pg for theophylline and caffeine.
which we have duplicated in our laboratory. As might be expected, the technique is very sensitive to flow fluctuations (pulses) in the HPLC pump.

The initial work on thermospray was performed in primarily aqueous solution with 0.1 mol/L ammonium acetate buffer. It was postulated that the ions were produced from statistically determined charge retention on the droplets which, on evaporation, gave rise to large surface potentials. The mechanism of thermospray is still not clearly delineated; however, some investigators have presented evidence for chemical ionization in the gas phase from interactions with the gaseous \( \text{NH}_4^+ \) ions. In either case, ionization is not very energetic (i.e., it is "soft") and therefore only limited fragmentation is observed. We and others (13) have observed that the relative number of ions formed is a function of the longitudinal position of the probe tip from the skimmer cone. This appears to be related to the droplet size and to the presence of "competing" ions in the droplets. The practical conclusion of these observations is that careful physical optimization of the interface is required to achieve maximum sensitivity.

The amount of heat available in the source block is also important in determining the limit of detection. For example, adding a heating element to the outside of the block of an original Vestec (Houston, TX) interface decreased the limits of detection for bile acid conjugates by 50-fold. Newer versions of the thermospray interface have incorporated additional heating elements. This serves to illustrate the dynamic nature of research in HPLC/MS.

The thermospray ionization mechanism functions well only in mobile phases containing a large volume fraction of water. In other mobile phases, addition of either a filament or a discharge electrode is required for ionization. As shown in Figure 2, thermospray ionization is most effective with greater than 60% water in the mobile phase, whereas discharge mode is required with decreasing volume fractions of water. In the Vestec interface, little or no advantage was found with the "filament on" ionization mode. The success of the filament vs. the discharge approach depends on source design. In the discharge mode, as in the thermospray mode, relatively little fragmentation is observed, as can be seen from the mass spectrum of cortisol and two related steroids (Figure 3). Cortisol, a neutral compound, also serves to demonstrate one of the limitations of thermospray, in that the limit of detection in an HPLC/MS assay was on the order of 5 ng, in contrast to the limits observed for theophylline. Unfortunately, in many circumstances, this compound-specific ionization efficiency is impossible to control or influence. Some commercial thermospray interfaces have incorporated an electrode opposite the sampling cone to direct ions into the mass spectrometer, with some degree of success.

In summary, thermospray is a reliable HPLC/MS inter-

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Table 1. Commercially Available HPLC/MS Interfaces

<table>
<thead>
<tr>
<th>Interface design</th>
<th>Nebulization/principle of operation</th>
<th>Volume reduction</th>
<th>Ionization modes</th>
<th>Reference no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moving belt</td>
<td>Deposition on belt</td>
<td>Evaporation of liquid from belt in oven; flow rates 0.2–0.5 mL/min for reversed phase</td>
<td>Electronic ionization (EI) or chemical ionization (CI) available</td>
<td>2</td>
</tr>
<tr>
<td>Direct liquid introduction</td>
<td>Liquid jet expansion through 0.5-μm orifice</td>
<td>Evaporation in MS source; flow rates 5–50 μL/min</td>
<td>CI only (solvent-mediated ionization)</td>
<td>1, 5</td>
</tr>
<tr>
<td>Thermospray</td>
<td>Supersonic jet expansion via thermal evaporation</td>
<td>Solvent plume is pumped; only small region of plume is sampled; flow rates 0.5–1.5 mL/min</td>
<td>CI only (&quot;thermospray&quot;; discharge assisted; filament assisted)</td>
<td>6</td>
</tr>
<tr>
<td>Liquid ion evaporation</td>
<td>Pneumatic nebulization; charge induction by high voltage (2–3 kV) field</td>
<td>API technique; flow rates 0.5–1.5 μL/min</td>
<td>Molecular ion</td>
<td>7</td>
</tr>
<tr>
<td>Electrospray</td>
<td>Charge induced on droplet by high voltage (2–3 kV) between capillary and source</td>
<td>API technique; flow rates 1–10 μL/min</td>
<td>&quot;Field desorption&quot;? (molecular ion)</td>
<td>8</td>
</tr>
<tr>
<td>Ion spray</td>
<td>Pneumatically assisted nebulization from charged capillary</td>
<td>API technique; flow rates 1–100 μL/min (up to 0.8 mL/min with thermal assist)</td>
<td>&quot;Field desorption&quot;? (molecular ion)</td>
<td>9</td>
</tr>
<tr>
<td>Particle beam separator</td>
<td>Axial pneumatic nebulization (HP); thermally assisted nebulization (Extrel); desolvation chamber to remove solvent</td>
<td>Differentially pumped beam separator; flow rates 0.5–1 mL/min</td>
<td>&quot;EI-like&quot; or CI</td>
<td>10, 11</td>
</tr>
</tbody>
</table>

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Fig. 1. Schematic diagram of the thermospray HPLC/MS interface

The vapor plume is formed at the tip of the vaporizer probe. The mass spectrometer would be above the ion sampling cone, at the upper left corner where the ions are shown exiting the interface. Excess gas vapor is pumped away with a mechanical pump shown at bottom. (Reprinted from the Vestec HPLC/MS interface manual, with permission)
Fig. 2. The relative amount of ionization produced for cortisol (m/z 301) in the thermospray, discharge, and filament on" modes of ionization with a Vistaec Thermospray interface. Studies were carried out in a continuous introduction mode by using a Hewlett-Packard 1090 HPLC and 5965A mass spectrometer.

Fig. 3. Mass spectra obtained for cortisol (top), prednisolone (middle), and prednisone (bottom) in the thermospray discharge mode.

Other conditions as described in Fig. 2.

Fig. 4. Atmospheric pressure ionization source design as implemented by Sciex Corp. and the apparatus necessary for liquid ion evaporation. The quadrupoles can be seen at left, within the cryoshell. (Courtesy of Dr. Tom Covell, Sciex Corp., Thornhill, Ontario, Canada. Reprinted with permission.)

face, but is not the ultimate answer to the interface problem. The spectra obtained are reproducible, but fragmentation is not predictable a priori and no libraries are available for computerized searching. The sensitivity of the technique is compound dependent and variable. Because of the dependence of ion production on solvent composition, it is not straightforward to use the interface with gradient elution. On the positive side, it has been very helpful in solving a large number of specific research and clinical problems, as evidenced by a large volume of literature on the technique. Almost all mass spectrometer manufacturers have available some version of a thermospray HPLC/MS interface.

Liquid Ion Evaporation/Electrospray/Ion Spray

All of these approaches have the common features of using an electrical field to induce ionization of the compounds of interest and using an atmospheric pressure ionization source. The analytical utility of the atmospheric pressure ionization source, first described by Horning et al. (14), is notable for the high sensitivity observed and for the ease of use and cleaning. The atmospheric pressure ionization source has been further developed by Sciex Corp. (Thornhill, Ontario, Canada) and is commercially available (Figure 4). The key feature of the source is the curtain of N₂ gas, which prevents un-ionized interferents (such as solvent vapors) from entering the mass spectrometer and also disrupts clusters of solvent and analyte. Ionic species produced in the area in front of the orifice are attracted into the mass spectrometer by potential difference.

Liquid ion evaporation HPLC/MS was described by Thomson et al. (7). Pneumatic nebulization of the liquid effluent is used to produce a fine mist of solvent droplets, which pass in close proximity to a needle held at a potential of 2000–3000 V. The corona discharge produces charged droplets, which evaporate and emit ions from the surface. Theoretically this is the result of field desorption processes. These ions enter the orifice and are analyzed in the mass spectrometer. All pre-ionized species appear as protonated (M+H)⁺ or de-protonated (M–H)⁻ molecular ions, with essentially no fragmentation. Many neutral compounds, such as the anabolic steroid dianabol (15, 16), also appear as protonated molecular ions. This interface is capable of handling liquid flow rates of 1 mL/min. The sensitivity of the atmospheric pressure ionization approach is reflected by the fact that a single dose of dianabol could be detected as the epi-dianabol metabolite for 16 days.

Whitehouse et al. (8) have described the use of a high potential (4.5 kV), applied between the capillary and a cylindrical electrode, to produce ionization. The liquid is extracted or pumped from the capillary at flow rates of 5–20 µL/min, and the droplets are dispersed by charge repulsion. Once again, evaporation of the solvent results in desorption of charged species, which can then be analyzed in the mass spectrometer. Bruins et al. (9) combined the electrostatic charging atomization of the electrospray interface and pneumatic nebulization to produce the ion-spray interface. In this approach, the capillary itself is charged to several thousand volts, and co-axial N₂ gas flow is used to assist atomization. This allows the use of flow rates as great as 100 µL/min, which makes the technique compatible with microbore HPLC. More recently, thermally-assisted pneumatic nebulization has increased acceptable flow rates even.
higher. Sciex will be marketing a version of the ion-spray interface. The absence of heat in these interfaces makes the detection of labile species, such as sulfate conjugates of drugs, possible. For example, the ion-spray interface has been used to detect picogram quantities of the labile anabolic steroid metabolite boldenone sulfate. This allowed detection in urine of the metabolites from a single dose of boldenone in a horse 45 days after administration (17).

Particle Beam Separator

Browner et al. (10, 11) have developed an HPLC interface that will provide electronic ionization spectra that can be compared with spectra in the library of the National Bureau of Standards. The initial intent of the interface was to provide a monodisperse stream of solvent particles, which could be desolvated and passed through differentially pumped regions to the mass spectrometer. Because of the monodisperse aerosol concept, the initial interface was given the moniker MAGIC (Monodisperse Aerosol Generation Interface for Chromatography). Operationally the interface makes use of pneumatic nebulization into a desolvation chamber to produce small particles enriched in solute (Figure 5). The particles are then further enriched by passage through a momentum separator, which is repeated in a second stage. The desolvated particle beam then passes into a standard mass spectrometer source, which is maintained in the usual low pressure region (1.33 mPa; 10⁻⁵ Torr). The mechanism of ion production is not known at this time, but appears to require collision of the particles with a surface within the source. Presumably, the gas phase atoms produced can undergo either electronic ionization or chemical ionization to yield spectra identical to gas chromatography/MS or probe spectra. Because the particle beam separation seems more important than the nebulization technique, the approach is now referred to as the particle beam separator HPLC/MS interface. Two commercial interfaces have been developed based on the particle beam separator approach: ThermoBeam (Extrac, Pittsburgh, PA) and Particle Beam Separator (Hewlett-Packard, Palo Alto, CA). The main difference between the two is in the nebulization region; in Extrac a thermally assisted nebulization process is used, whereas in the Hewlett-Packard instrument the pneumatic approach is used.

Because the ionization process occurs in a low-pressure region, classic electronic ionization spectra can be obtained. The Particle Beam Separator (PBS) and NBS library electronic ionization spectra for testosterone glucuronide are shown in Figure 6. Note that in the process of nebulization, transfer, and ionization, the glucuronide moiety was lost and the spectrum is that of testosterone. Not all compounds lend themselves to electronic ionization, however. Digoxin, for example, does not produce significant ions in the electronic ionization mode, whereas with NH₄⁺ chemical ionization, very strong molecular ions are observed along with ions from the sequential loss of carbohydrate moieties. The PBS interface is capable of handling HPLC flow rates of up to 1 mL/min, which is primarily determined by the nebulization technique and the gas load capabilities of the mass spectrometer. Limits of detection for the PBS interface are somewhat compound-dependent, but are in general in the 10–100 ng range. Because the interface is in the early stages of its development, one could hope for increased sensitivity in the future.

Tandem Mass Spectrometry

With the exception of the PBS approach, all of the HPLC/MS interfaces discussed above give rise to predominantly molecular mass information, which, although valuable, does not help one identify the structure of a compound. One potential solution to this problem is the use of tandem mass spectrometry (18, 19). In this technique, ions produced in the source region by any technique can be separated on the basis of their mass/charge ratio in a first mass spectrometer. The selected ion is given a certain amount of momentum by acceleration through a potential field, then fragmented by collision with a neutral gas target atom or molecule. The collisionally activated dissociation (CAD) is a function of the energy provided to the ion, the mass of the target atom and the ion, and the target gas thickness (18). In the "low energy" collision region explored by quadrupole mass spectrometers, nitrogen, argon, or xenon gas is generally used as the target gas. The collision chamber frequently contains an RF-only quadrupole to minimize the scatter of ions produced in the collision. The fragment (daughter) ions are then mass analyzed in a final mass spectrometer. When magnetic sector mass spectrometers are used, higher energy collisions with He are used, which results in some differences in the ions produced, but the instrumental arrangement is similar (18).

Two potential applications of HPLC/MS/MS in drug analysis are structural identification of compounds through inspection of the daughter ion spectra and selected monitoring of parent/daughter ion combinations to provide low

![Image](image_url)
The limits of detection [selected reaction monitoring (SRM)]. The electron ionization spectrum of dianabol and daughter ion spectrum of the (M + H)^+ ion of dianabol are shown in Figure 7. Inspection of the spectra indicates that there are significant differences. This is to be expected, because in the former case the mechanism of fragmentation is from the "odd electron" ion produced in the electronic ionization process, whereas the latter arises from the fragmentation of a protonated molecular ion from the "soft" ionization and mass selection in the HPLC/MS portion of the instrument. The decreased fragmentation and differences in the mass of fragments precludes the use of searches of daughter-ion spectra with standard MS libraries. Whether standard MS/MS libraries can be generated will require further study, because collision cell geometry and target energy can have a significant effect on the daughter spectra. Nevertheless, it is interesting to note that similar fragmentation may occur. For example, the base peak at m/z 122 in the electronic ionization spectrum is the result of cleavage between the A and B rings of the steroid structure. From preliminary studies with deuterated compound, the m/z 121 peak in the CAD spectrum also arises from this region of the molecule.

Selected reaction monitoring in MS/MS is analogous to selected ion monitoring in standard MS experiments. A protonated molecular ion is selected in the first mass analyzer, fragmented by collision with a target molecule, and one or more daughter ions are monitored. The net result is an improvement in selectivity and a reduction in the background noise, which improves detection limits. The latter feature is moderated somewhat by the fact that ion transmission through the instrument is not 100%, so some diminution of signal will also occur. As an example, the HPLC/MS chromatogram obtained by monitoring the protonated molecular ion of dianabol (m/z 301) contained several peaks and was clearly not specific for the compound of interest. By selecting the m/z 301 ion from the first mass analyzer, colliding the molecule with Ar, and monitoring the m/z 121 and 149 ions, selectivity is enhanced, because very few if any other molecules of mass 300 that are eluted from the HPLC column at the same time would produce the same daughter ions. If the ratio of m/z 121 and 149 ions is correct, we have further assurance that our results are accurate. Finally, by using a deuterated internal standard to follow the recovery, we improve the analysis still further.

Conclusions

In the last three years the possibility of interfacing an HPLC and a mass spectrometer has become a reality. The thermospray, liquid ion evaporation, and particle beam separator interfaces are all commercially available. Although the mechanisms of operation are not totally understood, each of the interfaces is easy to operate and can produce mass spectra reliably. These interfaces are not as simple to optimize as gas chromatography/MS has become with the advent of the mass selective detector and the ion trap. For example, with thermospray it may take a few hours to optimize conditions with a particular probe and a particular set of chromatographic conditions. In some cases, it may be necessary to change established chromatographic conditions to be compatible with the interface (e.g., the presence of 0.1 mol/L ammonium acetate in thermospray). Except for the PBS, HPLC/MS interfaces produce predominantly information on molecular mass. Prediction of the fragmentation that does occur is not simple, but can usually be explained a posteriori by using well-established rules. We have used thermospray to identify unknown drug metabolites and have confirmed the structure with synthetic compounds based on the HPLC/MS spectra. The use of tandem mass spectrometry to obtain structural information is in its infancy and is, at present, an expensive approach. No libraries exist for comparison of MS/MS spectra.

The minimum detectable quantity in most HPLC/MS interfaces is compound related. For systems using traditional MS sources, detection limits are generally in the 5–100 ng range. For specific compounds, such as theophylline or caffeine, it can be significantly better. The atmospheric pressure ionization-based interfaces are two to three orders of magnitude better.

The PBS interface appears to be an important addition to the field. Both classical electron ionization and chemical ionization spectra can be generated. It is too early in its evolution to predict the role of this interface in drug analysis, but with a modest improvement in sensitivity, it could be used directly for confirming drugs present in urine screens for drugs of abuse. With the explosive growth of HPLC/MS interface technology in the past three years, one can look forward to routine HPLC/MS in the near future.

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References