CHAPTER THIRTEEN

Mass Spectrometry and Drug Discovery

RICHARD B. VAN BREEMEN
Department of Medicinal Chemistry and Pharmacognosy
University of Illinois at Chicago
Chicago, Illinois

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1 INTRODUCTION

At the beginning of the 20th century, mass spectrometers were invented to help physicists and physical chemists prove the existence of isotopes of the elements. As radioactivity and nuclear physics was explored, specialized mass spectrometers were used to characterize the fission products of radioactive elements as they were created or discovered. In addition, mass spectrometers were used for the measurement of isotopic enrichment of radioactive elements, their inorganic derivatives, and even the isotopic purification of radioactive elements as inorganic compounds. As this era of mass spectrometry reached maturity in the 1940s, some physicists announced that there would no longer be any need for mass spectrometry because virtually all of the elements had been discovered and characterized. Of course, these prognosticators were wrong because the entire field of organic mass spectrometry was about to begin.

While mass spectrometers were being used for the purification of fissionable material for atomic weapons as part of the Manhattan Project of World War II, organic mass spectrometry was being invented for the analysis and quality control of aviation fuel. In 1945, the application of mass spectrometry to organic chemistry emerged as a productive new area of research and discovery. Commercial production of organic mass spectrometers began immediately, and petroleum companies became the first customers for these new analytical instruments. Early commercial mass spectrometers used electron impact (EI) ionization (see Equations 13.1 and 13.2) to generate ions from gas–phase molecules that were separated by acceleration through an electromagnetic field provided by either a fixed magnet or an electromagnet. After separation, the ions were detected using a simple impact detector such as a Faraday cup. This basic design is still in use today for the identification and quantitative analysis of volatile organic compounds.

$$\mathrm{M} + e^{-}\ (70\ eV) \rightarrow M^{+\cdot} + 2e^{-} \quad \text{formation of positive molecular ions using EI ionization} \tag{13.1}$$

$$M + e^{-}\ (2\text{–}10\ eV) \rightarrow M^{-\cdot} \quad \text{electron capture EI ionization} \tag{13.2}$$

Toward the late 1950s, organic mass spectrometers began to be used for the analysis of a wider variety of organic molecules and eventually became a fundamental analytical tool for the characterization of synthetic organic compounds. Today, mass spectrometers are used routinely to confirm the molecular weights of organic compounds and to verify their structures based on fragmentation patterns. Fragmentation results from the cleavage of chemical bonds within an ion, resulting in the formation of a product ion of lower mass and one or more neutral products. Of course, only the fragment ions and not the neutral species are detected in a mass spectrometer because this instrument measures the mass-to-charge ratio (m/z) of ions in the gas phase. The energy for fragmentation is the result of excess energy imparted to the molecular ion or during a process known as collision-induced dissociation (CID), which will be discussed along with tandem mass spectrometry (MS-MS) below. Because the fragmentation pattern reflects the relative strengths of chemical bonds in a compound, mass spectra (a plot of ion relative abundance versus m/z) provide structurally significant fragment ions for compound identification. Rules for structure elucidation of chemical structures through the interpretation of mass spectra have been developed. (For a review of EI and ion fragmentation pathways, see McClafferty et al. 1997, Section 4).

In many cases, EI imparts so much excess energy into a molecule that only fragment ions and no molecular ions are produced. Therefore, "softer" ionization techniques were developed to enhance molecular weight information. The first of these ionization methods was chemical ionization (CI). Developed by re-

Table 13.1 Types of Mass Spectrometers and Tandem Mass Spectrometers

Instrument	Resolving Power	*m/z* Range	Tandem MS
Magnetic sector	100,000	12,000	Low resolution
Quadrupole	< 4,000	4,000	none
Triple quadrupole	< 4,000	4,000	Low resolution
Time-of-flight (TOF)	15,000	> 200,000	none
FTICR	> 200,000	< 10,000	MS^n, high resolution
Ion trap	< 4,000	< 10,000	MS, low resolution
QTOF	12,000	4,000	High resolution
TOF-TOF	15,000	> 10,000	High resolution

searchers in the petroleum industry (1), CI became another standard ionization technique for organic mass spectrometry. During CI, high energy electrons (as in EI) are used to ionize a gas called a reagent gas at a constant pressure (usually ~1 Torr) in the mass spectrometer ionization source. The reagent gas in turn ionizes the sample molecules through ion–molecule reactions that usually involve the exchange of protons. Less frequently, sample molecule ionization might involve a charge exchange. Two of the most common ionization mechanisms in CI are summarized in Equations 13.3 and 13.4.

$$M + RH^+ \rightarrow MH^+ + R \quad \text{CI through proton transfer, R = reagent gas} \qquad (13.3)$$

$$M + R^{+\cdot} \rightarrow M^{+\cdot} + R \quad \text{CI through charge exchange} \qquad (13.4)$$

During the 1960s, high resolution double-focusing magnetic sector instruments became available and are now standard tools for the determination of elemental compositions using a type of analysis called exact mass measurement. In mass spectrometry, resolution is defined as M/ΔM, where M is the *m/z* value of a singly charged ion, and AM is the difference (measured in *m/z*) between M and the next highest ion. Alternatively, AM may be defined in terms of the width of the peak. High resolution is typically regarded as a value of at least 10,000. At this resolution, the molecular ions of most drug-like molecules (that is compounds with molecular weights less than ~500) can be resolved from each other. After resolving a sample ion from others in a mass spectrum, an exact mass measurement may be carried out by accurately weighing the unknown ion and comparing its m l . value to that of a calibration standard. Since the 1960s, other types of mass spectrometers capable of high resolution exact mass measurements have become available as commercial products, including Fourier transform ion cyclotron resonance (FTICR) mass spectrometers, reflectron TOF instruments, and recently, quadrupole time-of-flight hybrid (QqTOF) mass spectrometers (see Table 13.1 for a listing of types of organic mass spectrometers and a comparison of their performance characteristics). By the early 2000s, FTICR and QqTOF instruments became more popular than magnetic sector mass spectrometers for exact mass measurements, high resolution measurements, and drug discovery applications. As will be discussed below, exact mass measurements are essential to many types of mass spectrometry-based screening and drug discovery today.

Biomedical applications of mass spectrometry began during the 1960s both at academic institutions and pharmaceutical companies. These applications depended on the volatilization (usually by heating) of pharmaceutical compounds and biochemicals before their gas-phase ionization using EI or CI. To increase the thermal stability and volatility of these compounds, a variety of derivatization methods were developed to mask polar functional groups and reduce hydrogen bonding between molecules. These methods were particularly effective for use with gas chromatography–mass spectrometry (GC-MS), which was introduced during the 1960s as a practical and powerful tool for qualitative and quantitative

analysis of compounds in mixtures. Both EI and CI were immediately useful for GC-MS, because both of these ionization methods require that the analytes be in the gas phase. When capillary GC was incorporated into GC-MS, this technique reached maturity. GC-MS may be used to select, identify, and quantify organic compounds in complex mixtures at the femtomole level. The speed of GC-MS is determined by the chromatography step, which typically requires several minutes to 1 h per analysis. By the 1970s, some organic chemists were announcing that organic mass spectrometry had reached maturity and that no new applications were possible. Like the physicists and physical chemists who had pronounced the end of mass spectrometry a generation earlier, this group would soon be proved wrong.

Although GC-MS remains important for the analysis of many organic compounds, this technique is limited to volatile and thermally stable compounds that comprise only a small fraction of all organic compounds and even fewer biomedically important molecules. Therefore, thermally unstable compounds, including many pharmaceutical compounds such as nucleic acid analogs and biomolecules such as proteins, carbohydrates, and nucleic acids, cannot be analyzed in their native forms using GC-MS. (For more details regarding GC-MS and its applications, see Watson 1997, Section 4.) Although derivatization facilitates the GC-MS analysis of many of these compounds, alternative ionization techniques were needed for the analysis of the vast majority of polar and non-volatile compounds of interest to drug discovery.

During the 1970s and early 1980s, desorption ionization techniques such as field desorption (FD), desorption EI, desorption CI (DCI), and laser desorption were developed to extend the use of mass spectrometry toward the analysis of more polar and less volatile compounds (see Watson 1997, Section 4, for more information regarding desorption ionization techniques including DCI and FD). Although these techniques helped extend the mass range of mass spectrometry beyond a traditional limit of m/z 1000 and toward ions of m/z 5000, the first breakthrough in the analysis of polar, non-volatile compounds occurred in 1982 with the invention of fast atom bombardment (FAB) (2). FAB and its counterpart, liquid secondary ion mass spectrometry (LSIMS), facilitated the formation of abundant molecular ions, protonated molecules, and deprotonated molecules of non-volatile and thermally labile compounds such as peptides, chlorophylls, and complex lipids up to approximately m/z 12,000. FAB and LSIMS use energetic particle bombardment (fast atoms or ions from 3 to 30,000 V of energy) to ionize compounds dissolved in non-volatile matrices such as glycerol or 3-nitrobenzyl alcohol and desorb them from this condensed phase into the gas phase for mass spectrometric analysis (see Fig. 13.1). Protonated or deprotonated molecules are usually abundant and fragmentation is minimal.

Introduced in the late 1980s, matrix-assisted laser desorption ionization (MALDI) has helped solve the mass limit barriers of laser desorption mass spectrometry so that singly charged ions may be obtained up to m/z 500,000 and sometimes higher (3). For most commercially available MALDI mass spectrometers, ions up to m/z 200,000 are readily obtained. Like FAB and LSIMS, MALDI samples are mixed with a matrix to form a solution that is loaded onto the sample stage for analysis. Unlike the other matrix-mediated techniques, the solvent is evaporated before MALDI analysis, leaving sample molecules trapped in crystals of solid phase matrix. The MALDI matrix is selected to absorb the pulse of laser light directed at the sample. Most MALDI mass spectrometers are equipped with a pulsed UV laser, although IR lasers are available as an option on some commercial instruments. Therefore, matrices are often substituted benzenes or benzoic acids with strong UV absorption properties. During MALDI, the energy of the short but intense UV laser pulse obliterates the matrix and in the process desorbs and ionizes the sample. Like FAB and LSIMS, MALDI typically produces abundant protonated or deprotonated molecules with little fragmentation.

By the time that GC-MS had become a standard technique in the late 1960s, LC-MS was still in the developmental stages. Producing gas-phase sample ions for analysis in a vacuum system while removing the high perfor-

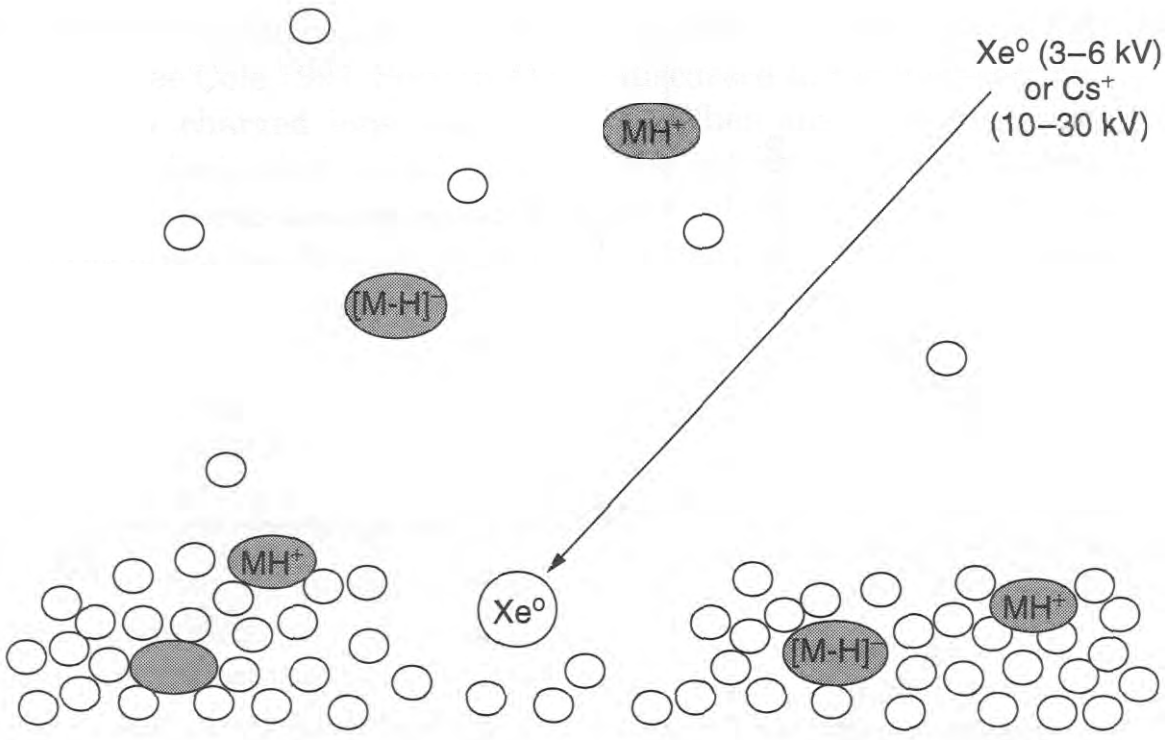


Figure 13.1. Scheme for desorption ionization using FAB or LSIMS from a liquid matrix (○).

mance liquid chromatography (HPLC) mobile phase proved to be a challenging task. Early LC-MS techniques included a moving belt interface to desolvate and transport the HPLC eluate into an CI or EI ion source or a direct inlet system in which the eluate was pumped at a low flow rate **(1–3** μL/min) into a CI source. However, neither of these systems was robust enough or suitable for a broad enough range of samples to gain widespread acceptance.

Because FAB (or LSIMS) requires that the analyte be dissolved in a liquid matrix, this ionization technique was easily adapted for infusion of solution-phase samples into the FAB ionization source in an approach known as continuous-flow FAB. Then, continuous-flow FAB was connected to microbore HPLC columns for LC-MS applications (4). Because this method is limited to microbore HPLC applications at flow rates of <10 μL/min and requires considerable operator intervention, it is not ideal for the analysis of large sample sets. Instead, more robust techniques have been developed to fulfill this requirement. However, continuous-flow FAB is still in use in some laboratories.

Like continuous-flow FAB, the popularity of particle beam interfaces is diminishing, but systems are still available from commercial sources. During particle beam LC-MS, the HPLC eluate is sprayed into a heated chamber connected to a vacuum pump. As the droplets evaporate, aggregates of analyte (particles) form and pass through a momentum separator that removes the lower molecular weight solvent molecules. Finally, the particle beam enters the mass spectrometer ion source where the aggregates strike a heated plate from which the analyte molecules evaporate and are ionized using conventional EI or CI. Particle beam LC-MS is limited to the analysis, of volatile and thermally stable compounds that are amenable to flash evaporation and EI or CI mass spectrometry. Therefore, this approach is not used for polar biochemicals such as carbohydrates, sugars, peptides, proteins, or nucleic acids.

Because thermospray became the first widely used LC-MS technique (during the late 1970s and early 1980s), this technique should be mentioned here. Thermospray facilitates the interfacing of standard analytical HPLC systems at flow rates up to 1 mL/min with mass spectrometers. Although the interface between the HPLC and mass spectrometer is inefficient and exhibits low sensitivity for most analytes, thermospray has been useful for the LC-MS analysis of many types of small molecules. During thermospray, the HPLC eluate is sprayed through a heated capillary into a heated desolvation chamber at reduced pressure. Gas phase ions remaining after desolvation of the droplets are extracted through a

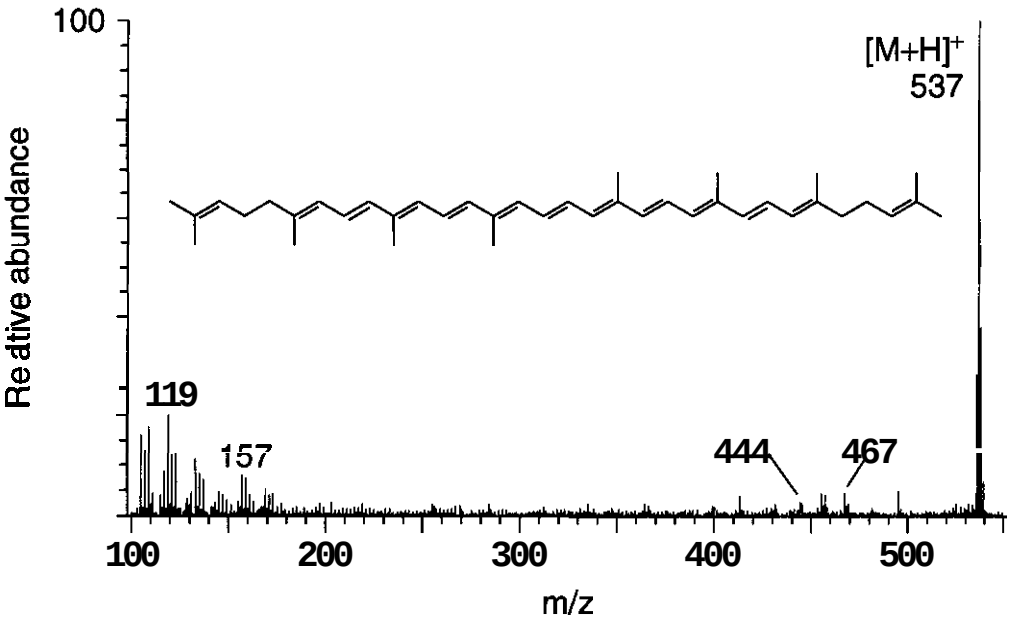


Figure 13.2. Positive ion APCI mass spectrum of the red carotenoid lycopene in a solution of methanol and tert-butyl methyl ether (1:1; v/v). In this analysis, lycopene formed a protonated molecule instead of a molecular ion, $M^{+\cdot}$.

skimmer into the mass spectrometer for analysis. The sensitivity of thermospray is poor because there is no mechanism or driving force to enhance the number of sample ions entering the gas phase from the spray during desolvation. Also, thermally labile compounds tend to decompose in the heated source. These problems were solved when thermospray was replaced by electrospray during the late 1980s.

During the 1990s, electrospray and atmospheric pressure chemical ionization (APCI) became the standard interfaces for LC-MS. Today, APCI and electrospray ionization are the most widely used ionization sources and HPLC interfaces for drug discovery using mass spectrometry. Unlike thermospray, particle beam or continuous-flow FAB, electrospray and APCI interfaces operate at atmospheric pressure and do not depend on vacuum pumps to remove solvent vapor. As a result, they are compatible with a wide range of HPLC flow rates. Also, no matrix is required. Both APCI and electrospray are compatible with a wide range of HPLC columns and solvent systems. Like all LC-MS systems, the solvent system should contain only volatile solvents, buffers or ion pair agents to reduce fouling of the mass spectrometer ion source. In general, APCI and electrospray form abundant molecular ion species. When fragment ions are formed, they are usually more abundant in APCI than electrospray mass spectra.

The APCI interface uses a heated nebulizer to form a fine spray of the HPLC eluate, which is much finer than the particle beam system but similar to that formed during thermospray. A cross-flow of heated nitrogen gas is used to facilitate the evaporation of solvent from the droplets. The resulting gas-phase sample molecules are ionized by collisions with solvents ions, which are formed by a corona discharge in the atmospheric pressure chamber. Molecular ions, $M^{+\cdot}$ or $M^{-\cdot}$, and/or protonated or deprotonated molecule; can be formed. The relative abundance of each type of ion depends on the sample itself, the HPLC solvent, and the ion source parameters. Next, ions are drawn into the mass spectrometer analyzer for measurement through a narrow opening or skimmer that helps the vacuum pumps to maintain very low pressure inside the analyzer, while the APCI source remains at atmospheric pressure. For example, the positive ion APCI mass spectrum of lycopene is shown in Fig. 13.2. The carotenoid lycopene is the red pigment of ripe tomatoes and is under clinical investigation for the prevention of prostate cancer (5).

During electrospray, the HPLC eluate is sprayed through a capillary electrode at high potential (usually 2000–7000 V) to form a fine mist of charged droplets at atmospheric pressure. As the charged droplets migrate towards the opening of the mass spectrometer because of electrostatic attraction, they encounter a cross-flow of heated nitrogen that increases solvent evaporation and prevents most of the solvent molecules from entering the mass spectrometer. Molecular ions, protonated or deprotonated molecules, and cationized species such as $[M + Na]^+$ and $[M + K]^+$ can be

formed. (For additional information on electrospray ionization, see Cole 1997, Section 4). In addition to singly charged ions, electrospray is unique as an ionization technique in that multiply charged species are common and often constitute the majority of the sample ion abundance. The relative abundance of each of these species depends on the chemistry of the analyte, the pH, the presence of proton donating or accepting species, and the levels of trace amounts of sodium or potassium salts in the mobile phase. In contrast, APCI, MALDI, EI, CI, and FAB/LSIMS usually produce singly charged species. A consequence of forming multiply charged ions is that they are detected at lower m/z values (i.e., $z > 1$) than the corresponding singly charged species. This has the benefit of allowing mass spectrometers with modest m/z ranges to detect and measure ions of molecules with very high masses. For example, electrospray has been used to measure ions with molecular weights of hundreds of thousands or even millions of Daltons on mass spectrometers with m/z ranges of only a few thousand. (For a review of LC-MS techniques, see Niessen 1999, Section 4.)

An example of the C_{18} reversed phase HPLC-negative ion electrospray mass spectrometric (LC-MS) analysis of an extract of the botanical, *Trifolium pratense* L. (red clover), is shown in Fig. 13.3. Extracts of red clover are used as dietary supplements by menopausal and postmenopausal women and are under investigation as alternatives to estrogen replacement therapy (6). The two-dimensional map illustrates the amount of information that may be acquired using hyphenated techniques such as LC-MS. In the time dimension, chromatograms are obtained, and a sample computer-reconstructed mass chromatogram is shown for the signal at m/z 269. An intense chromatographic peak was detected eluting at 12.4 min. In the m/z dimension, the negative ion electrospray mass spectrum recorded at 12.4 min shows a base peak at m/z 269. Based on comparison with authentic standards (data not shown), the ion of m/z 269 was found to correspond to the deprotonated molecule of genistein, which is an estrogenic isoflavone (6). Because almost no fragmentation of the genistein ion was observed, additional characterization would require CID and MS-MS as discussed in the next section.

When analyzing complex mixtures such as the botanical extract shown in Fig. 13.3, the use of chromatographic separation before mass spectrometric ionization and analysis is essential to distinguish between isomeric compounds. Even simple mixtures of synthetic compounds might contain isomers that would require LC-MS for adequate characterization. Another problem overcome by using a chromatography step before mass spectrometric analysis is ion suppression. No matter what ionization technique is used, the presence of multiple compounds in the ion source might enhance the ionization of one compound while suppressing the ionization of another. Usually, only some of the compounds in a complex mixture can be detected by mass spectrometry without chromatographic separation. The presence of salts and buffers in a sample can also suppress sample ionization. Therefore, LC-MS has become a powerful tool for analyzing natural products, synthetic organic compounds, and pharmaceutical agents and their metabolites.

In general, APCI facilitates the ionization of non-polar and low molecular weight species, and electrospray is more useful for the ionization of polar and high molecular weight compounds. In this sense, APCI and electrospray are often complementary ionization techniques. However, during the analysis of large or diverse combinatorial libraries, both polar and non-polar compounds are usually present. As a result, no one set of ionization conditions using APCI or electrospray is adequate to detect all the compounds contained in the library of compounds. Therefore, a UV ionization technique called atmospheric pressure photoionization (APPI) has been developed for use with combinatorial libraries and LC-MS (7). Recently, APPI became a commercially available ionization alternative to APCI and electrospray. During APPI, a liquid solution or HPLC eluate is sprayed at atmospheric pressure, as in APCI. Instead of using a corona discharge as in APCI, ionization occurs during APPI because of irradiation of the analyte molecules by an intense UV light source. Obviously, the carrier solvent must not absorb UV light at the same wavelengths, or interfer-

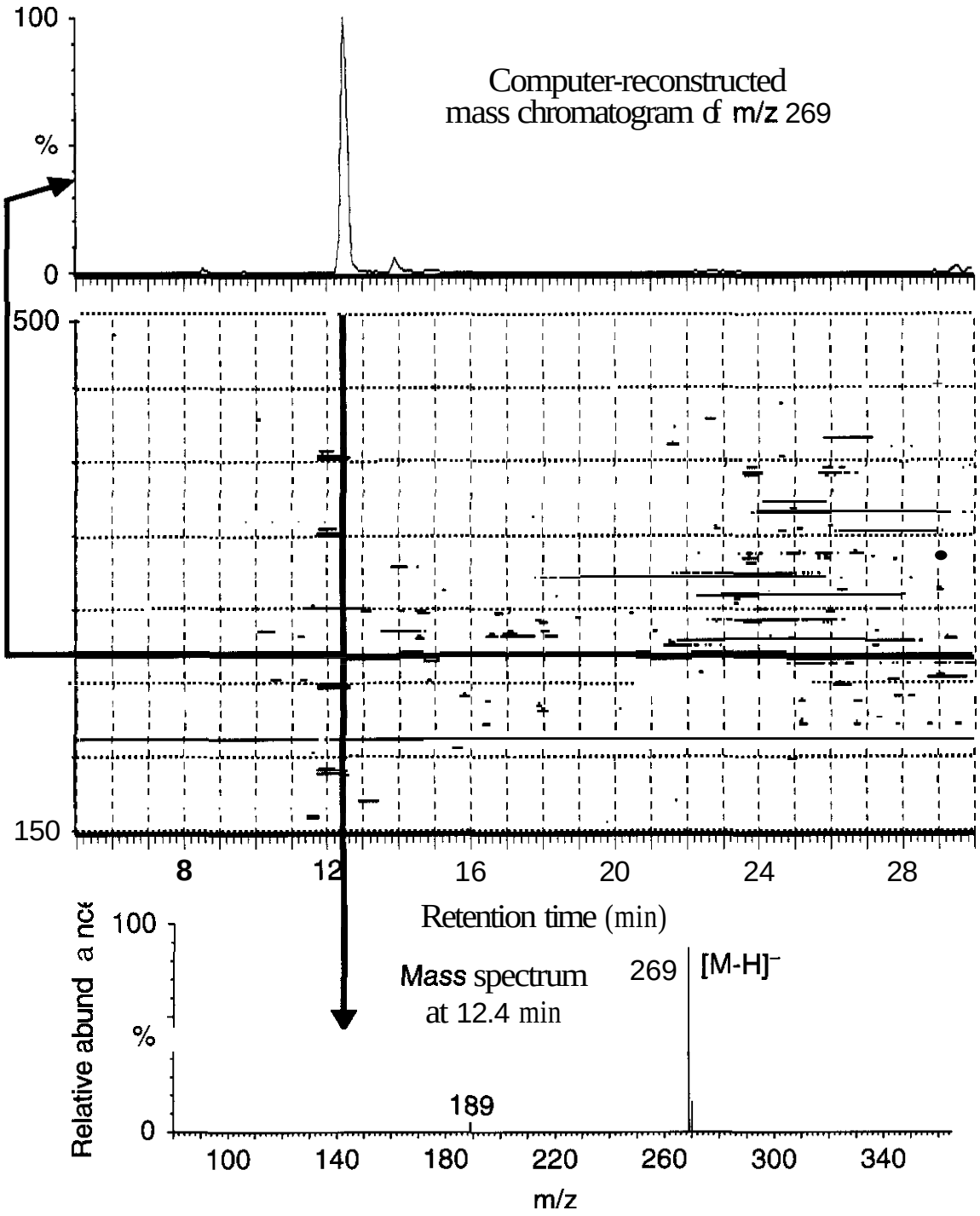


Figure 13.3. Two-dimensional map showing the LC-MS analysis of an extract of red clover under investigation for the management of menopause. Reversed phase separation was carried out using a C_{18} HPLC column in the time dimension and negative ion electrospray mass spectrometry was used for compound detection and molecular weight determination in the second dimension.

ence would prevent sample ionization and detection. The use of APPI as an alternative to APCI and electrospray for drug discovery applications is under investigation.

Desorption ionization techniques like FAB, MALDI, and electrospray facilitate the molecular weight determination of a wide range of polar, non-polar, and low, and high molecular weight compounds including drugs and drug targets such as proteins. However, the "soft" ionization character of these techniques means that most of the ion current is concentrated in molecular ions, and few structurally significant fragment ions are formed. To enhance the amount of structural information in these mass spectra, CID may be used to produce more abundant fragment ions from molecular ion precursors formed and isolated during the first stage of mass spectrometry. Then, a second mass spectrometry analysis may be used to characterize the resulting product ions. This process is called tandem mass spectrometry or MS-MS and is illustrated in Fig. **13.4.**

Another advantage of the use of tandem mass spectrometry is the ability to isolate a particular ion such as the molecular ion of the analyte of interest during the first mass spec-

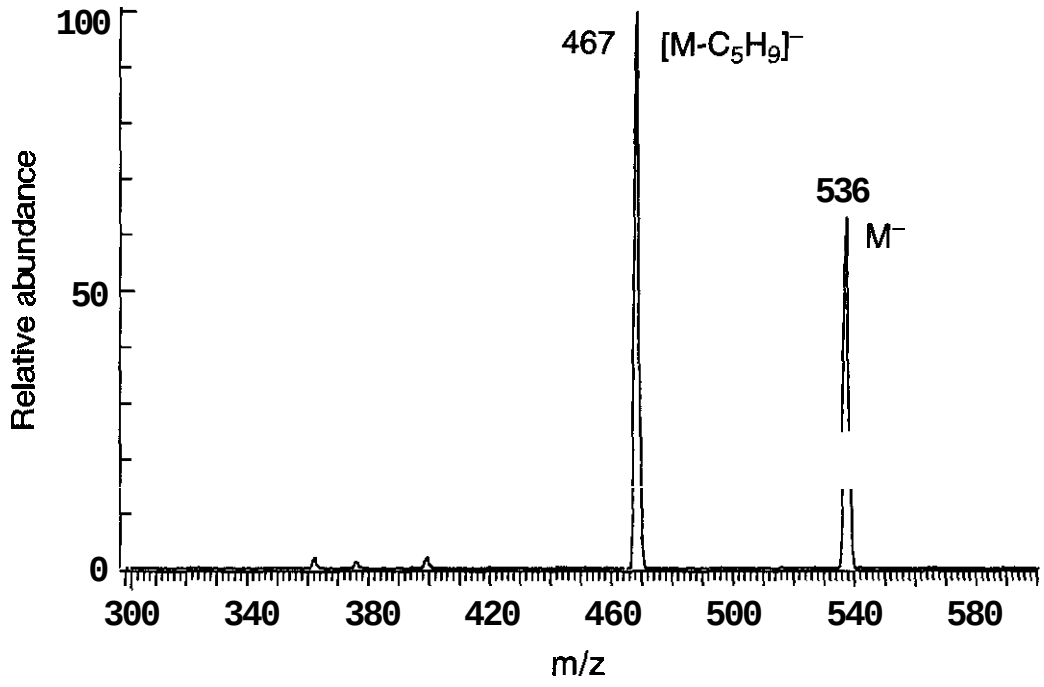


Figure 13.4. Scheme illustrating the selectivity of MS-MS and the process by which CID facilitates fragmentation of preselected ions. Negative ion electrospray tandem mass spectrum of lycopene. CID was used to induce fragmentation of the molecular ion of *m/z* **536. As** a result, the fragment ion of *m/z* **467** was formed by the loss of a terminal isoprene unit. This fragment ion may be used to distinguish lycopene from isomeric α-carotene and β-carotene, which lack terminal isoprene groups.

trometry stage. This precursor ion is essentially purified in the gas-phase and free of impurities such as solvent ions, matrix ions, or other analytes. Finally, the selected ion is fragmented using CID and analyzed using a second mass spectrometry stage. In this manner, the resulting tandem spectrum contains exclusively analyte ions without impurities that might interfere with the interpretation of the fragmentation patterns. In summary, CID may be used with LC-MS-MS or desorption ionization and MS-MS to obtain structural information such as amino acid sequences of peptides and sites of alkylation of nucleic acids, or to distinguish structural isomers such as β-carotene and lycopene. Beginning in 2001, TOF-TOF tandem mass spectrometers became available from instrument manufacturers. These instruments have the potential to deliver high resolution tandem mass spectra with high speed that should be compatible with the chip-based chromatography systems now under development.

Over the course of the last century, mass spectrometry has become an essential analytical tool for a wide variety of biomedical applications including drug discovery and development. By combining mass spectrometry with chromatography as in LC-MS or by adding another stage of mass spectrometry as in MS-MS, the selectivity of the technique increases considerably. As a result, mass spectrometry offers all of the analytical elements that are essential to modern drug discovery namely speed, sensitivity, and selectivity.

2 CURRENT TRENDS AND RECENT DEVELOPMENTS

Since the early 1990s, pharmaceutical research has focused on combinatorial chemistry (8, 9) and high-throughput screening (10) in an effort to accelerate the pace of drug discovery. The goal has been to produce, in a short time, large numbers of synthetic organic compounds representing a great diversity of chemical structures through a process called combinatorial chemistry and then quickly screen them in ***vitro*** against pharmacologically significant targets such as enzymes or receptors. The "hits" identified through these high-throughput screens may then be optimized by quickly and efficiently synthesizing and then screening large numbers of analogs called targeted or directed libraries. **As** a result, lead compounds might emerge from such combinatorial chemistry drug discovery programs in a few weeks instead of several years. Furthermore, a single organic chemist using combinatorial synthetic methods might synthesize thousands of compounds or more in a single week instead of less than five in the same time using conventional techniques, and a single medicinal chemist might identify hun-

dreds of lead compounds per month instead of just one or two in the same period of time.

Accompanying this new drug discovery paradigm, new scientific journals have been established such as ***Combinatorial Chemistry & High Throughput Screening, Journal of Combinatorial Chemistry, Journal of Biomolecular Screening,*** and ***Molecular Diversity*** (see list of journal websites in Section 4). The variety of topics published in these journals reflects the multidisciplinary nature of the current drug discovery process and ranges from organic chemistry, medicinal chemistry, molecular modeling, molecular biology, and pharmacology, to analytical chemistry. As described below, the most significant analytical component of drug discovery has become mass spectrometry. Only mass spectrometry has become an essential element at all stages of the drug discovery and development process.

Although a variety of spectroscopic and chromatographic techniques, including infrared spectroscopy, nuclear magnetic resonance spectroscopy, fluorescence spectroscopy, gas chromatography, HPLC, and mass spectrometry, are being used to support drug discovery in various capacities, some of them, such as gas chromatography and fluorescence spectroscopy, are not applicable to most new chemical entities, some are not specific enough for chemical identification (e.g., infrared spectroscopy), and other techniques suffer from low throughput (e.g., nuclear magnetic resonance spectroscopy). Unlike gas chromatography, HPLC is compatible with virtually all drug-like molecules without the need for chemical derivatization to increase thermal stability or volatility. In addition, mass spectrometry provides a universal means to characterize and distinguish drugs based on both molecular weight and structural features while at the same time providing high throughput. With the development of routine LC-MS interfaces and ionization techniques such as electrospray and APCI, mass spectrometry has also become an ideal HPLC detector for the analysis of combinatorial libraries (11), and LC-MS, MS-MS, and LC-MS-MS have become fundamental tools in the analysis of combinatorial libraries and subsequent drug development studies (12–14).

The application of combinatorial chemistry and high-throughput screening to drug discovery has altered the traditional serial process of lead identification and optimization that previously required years of human effort. Consequently, neither the synthesis of new chemical entities nor their screening is limiting the pace of drug discovery. Instead, a new bottleneck is the verification of the structure and purity of each compound in a combinatorial library or of each lead compound obtained from an uncharacterized library using high-throughput screening. Because the number of lead compounds entering the drug development process has increased, in part because compounds are entering development at earlier stages than in the past, the traditional drug development investigations concerning absorption, distribution, metabolism, and excretion (ADME) and even toxicology evaluations of new drug entities have become additional bottlenecks. As a solution to the drug development bottlenecks, high-throughput assays to assess the metabolism, bioavailability, and toxicity of lead compounds are being developed and applied earlier than ever during the drug discovery process, so that only those compounds most likely to become successful drugs enter the more expensive and slower preclinical pharmacology and toxicology studies. In support of these new combinatorial chemistry synthetic programs and new high-throughput assays, mass spectrometry has emerged as the only analytical technique with sufficient throughput, sensitivity, selectivity, and robustness to address all of these bottlenecks.

2.1 LC-MS Purification of Combinatorial Libraries

Although combinatorial libraries were originally synthesized as mixtures, today most libraries are prepared in parallel as discrete compounds and then screened individually in microtiter plates of 96-well, 384-well, or 1536-well formats. To facilitate subsequent structure-activity analyses and to assure the validity of the screening results, many laboratories verify the structure and purity of each compound before high-throughput screening. Semi-preparative HPLC has become the most

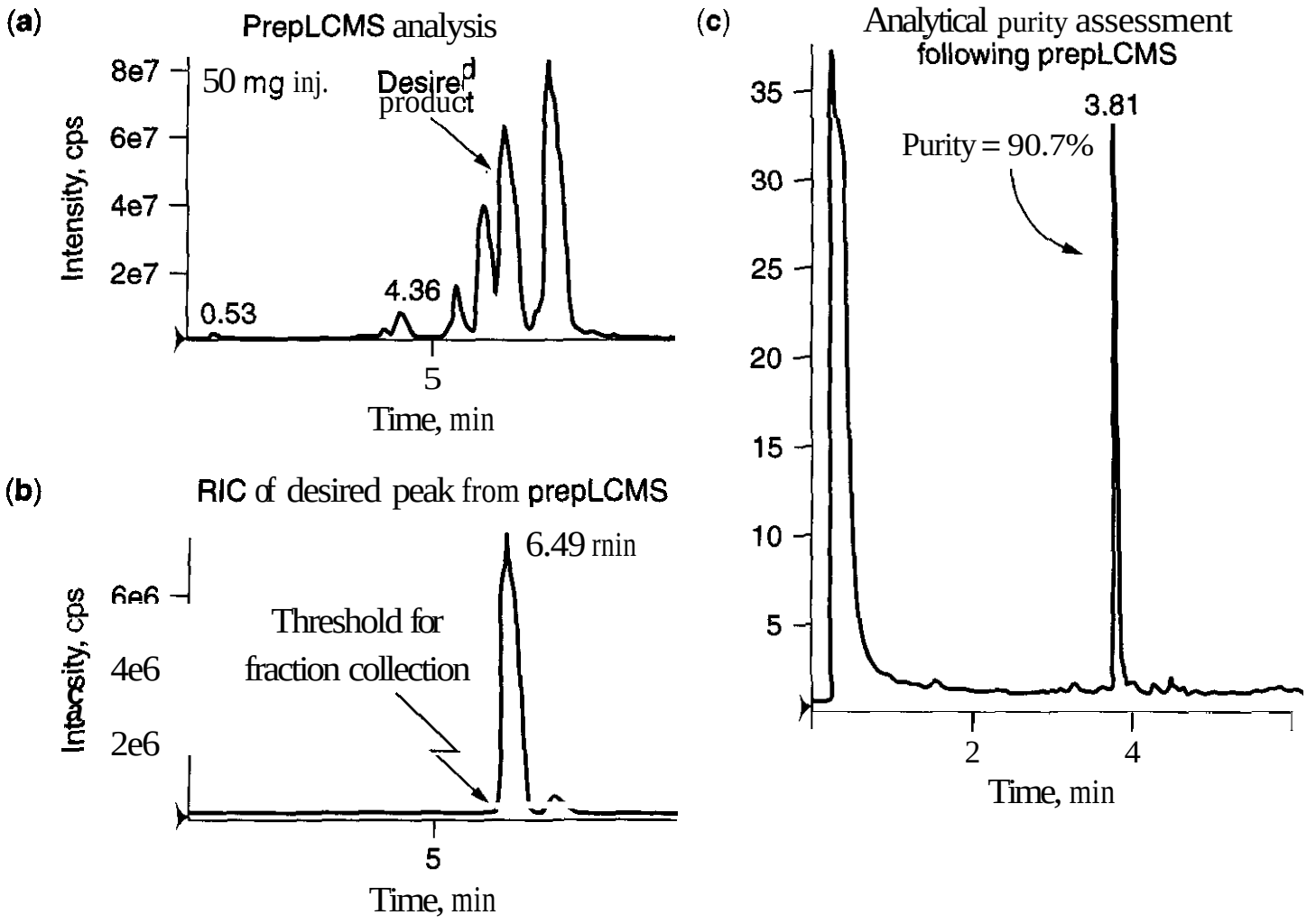


Figure 13.5. Mass-directed purification of a combinatorial library. Chromatographic separation was carried out using gradient elution of 10–90% acetonitrile in water for 7 min after an initial hold at 10% acetonitrile for 1 min. (a) Total ion chromatogram showing desired product and impurities. (b) Computer-reconstructed ion chromatogram (RIC) corresponding to the expected product. (c) Post-purification analysis of the isolated component with a purity >90%. (Reproduced from Ref. 15 by permission of Elsevier Science.)

popular technique for the purification of combinatorial libraries on the milligram scale because of high throughput and the ease of automation. Typically during semi-preparative HPLC, fraction collection is initiated whenever a UV signal is observed above a predetermined threshold. This procedure usually results in the collection of several fractions per analysis and hence creates additional issues such as the need for large fraction collector beds and the need for secondary analysis using flow-injection mass spectrometry, LC-MS, or LC-MS-MS to identify the appropriate fractions. When purification of large numbers of combinatorial libraries is required, this approach can become prohibitively time consuming and expensive.

To enhance the efficiently of this purification procedure, the steps of HPLC purification and mass spectrometric analysis may be combined into automated mass-directed fractionation (15–17). Any size HPLC column may be used, and only a small fraction of the eluant (~μL/min) is diverted to the mass spectrometer equipped for APCI or electrospray ionization. Because all of the components, including autosampler, injector, HPLC, switching valve, mass spectrometer, and fraction collector, are controlled by computer, the procedure may be fully automated. For greatest efficiency, the system may be programmed to collect only those peaks displaying the desired molecular ions, or alternatively, all peaks displaying abundant ions within a specified mass range. An example of the MS-guided purification of a compound synthesized during the parallel synthesis of a combinatorial library of discrete compounds is shown in Fig. 13.5. Although the crude yield of the reaction product was only 30% (Fig. 13.5a), the desired product was detected based on its molecular ion (Fig. 13.5b). After MS-guided fractionation, re-analysis us-

ing LC-MS showed that the desired product was >90% pure (Fig. 13.5c).

The use of MS-guided purification of combinatorial libraries provides a means for reducing the number of HPLC fractions collected per sample and eliminates the need for post-purification analysis to further characterize and identify each compound as would be necessary when using UV-based fractionation. The ionization technique (i.e., electrospray, APCI, or APPI), and ionization mode (positive or negative) must be suitable for the combinatorial compound so that molecular ion species are formed. Also, a suitable mobile phase and HPLC column must be selected. As an alternative to HPLC, supercritical fluid chromatography-mass spectrometry (SFC-MS) has been used for the high-throughput analysis of combinatorial libraries (18, 19). The advantages of SFC-MS relative to conventional LC-MS for the purification of combinatorial libraries of compounds are the lower viscosities and higher diffusivities of condensed CO, compared with HPLC mobile phases and the ease of solvent removal and disposal after analysis. However, SFC instrumentation remains more expensive and less widely available than conventional HPLC systems.

2.2 Confirmation of Structure and Purity of Combinatorial Compounds

The determination of molecular weights, elemental compositions, and structures of compounds used for high-throughput screening, whether discrete compounds or combinatorial library mixtures, is typically carried out using mass spectrometry, because traditional spectroscopic and gravimetric techniques are too slow to keep pace with combinatorial chemical synthesis. In addition, mass spectrometry may be used to assess the purity of compounds being used for high-throughput screening. The highest-throughput technique for confirming molecular weights and structures of drug candidates is flow injection analysis of sample solutions using electrospray, APCI, or APPI mass spectrometry. Typically, no sample preparation is necessary.

Although any organic mass spectrometer may be used to confirm the molecular weight of a compound, tandem mass spectrometers provide additional structural information through the use of CID to produce fragment ions. As discussed above (see also Table 13.1), tandem mass spectrometers include triple quadrupole instruments, QqTOF mass spectrometers, ion trap mass spectrometers, multiple sector magnetic sector instruments, FTICR instruments, and the new TOF-TOF mass spectrometers. In most applications, APCI or electrospray ionization is used.

In addition to molecular weight and fragmentation patterns, high precision and high resolution mass spectrometers such as QqTOF instruments, reflectron TOF mass spectrometers, double focusing magnetic sector mass spectrometers, and FTICR instruments are necessary for the measurement of exact masses of drugs and drug candidates for the determination of elemental compositions. The combination of high resolution and high precision is especially useful for determining the elemental compositions of compounds in combinatorial library mixtures without having to isolate each compound using chromatography or some other separation technique. Because FTICR instruments and the hybrid QqTOF mass spectrometers are capable of simultaneously measuring exact masses at high resolution of both molecular ions and fragment ions generated during MS-MS, these instruments are becoming extremely popular within drug discovery programs.

As an example of the exact mass measurement of a combinatorial library mixture, the FTICR negative ion electrospray mass spectra of a 36- and a 120-compound peptide library mixture are shown in Fig. 13.6. The resolution achieved in this experiment was 20,000–40,000. Although the exact masses of all components in a small combinatorial library can often be measured during a single infusion experiment, on-line HPLC separation or the analysis of discrete compounds is sometimes required to overcome ion suppression problems. However, LC-MS is a relatively slow process because of the slow chromatographic separation step. Because LC-MS is required in many instances for the analysis of mixtures and to eliminate interfering salts or buffers, two approaches have emerged to increase the throughput of this technique; parallel LC-MS and fast LC-MS. One approach to increasing

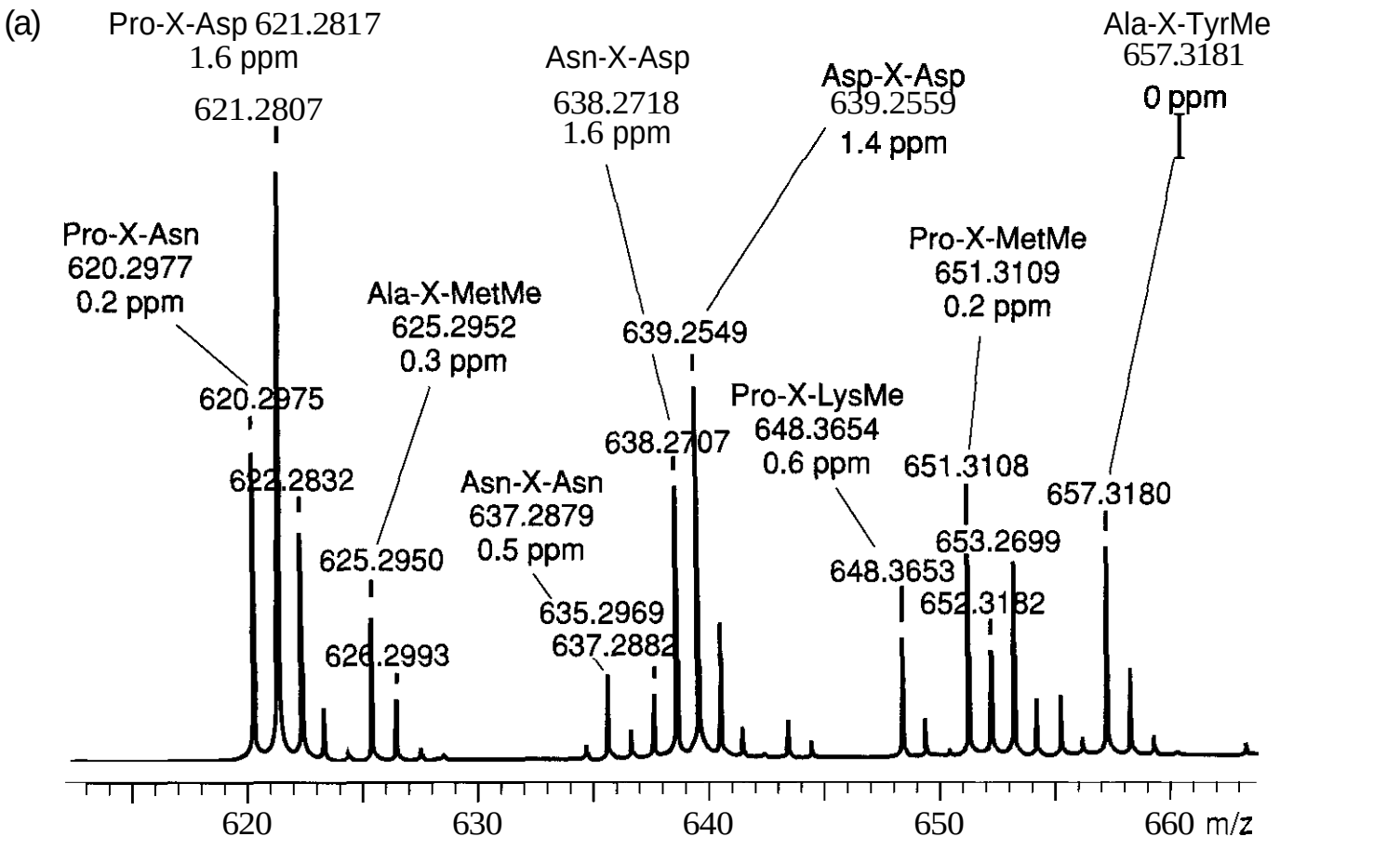


Figure 13.6. (a) Partial negative ion electrospray mass spectrum of a 36-component library mixture. Both the measured mass and the difference between the measured and theoretical values (in ppm) are shown. (b) Negative ion electrospray spectrum of the 120-component library showing the resolution of three nominally isobaric peaks. (Reproduced from Ref. 24 by permission of Bentham Science Publishers).

throughput of the rate-limiting chromatographic separation has been to simultaneously interface multiple HPLC columns to a single mass spectrometer. This approach is called parallel LC-MS. Commercial parallel electrospray interfaces and HPLC systems are now available that can accommodate up to eight HPLC columns simultaneously (20–22). Although the multiple sprays are introduced to the ion source simultaneously, these streams

may be sampled in a time-dependent manner to minimize cross contamination between channels.

Another solution to increasing the throughput of LC-MS has been to minimize the time required for HPLC separation through an approach called fast HPLC. HPLC separations are accelerated by using shorter columns and higher mobile phase flow rates. Because coelution of some species is likely to occur during fast chromatographic separations, the selectivity of the mass spectrometer is essential for the characterization and/or quantitative analysis of the target compound. However, samples of compounds prepared using combinatorial chemistry are usually simple mixtures of reagents, by-products, and products that require only partial chromatographic purification to prevent ion suppression effects during mass spectrometric analysis.

In addition to molecular weight determination using conventional MS or high exact mass measurement and structural confirmation using MS-MS, fast LC-MS is also used to assess the purity and yield of combinatorial products (15, 23). Before high-throughput screening, many researchers analyze combinatorial libraries for both purity and structural identity using mass spectrometry to assure the validity of structure-activity relationships that might be derived from the screening data. Fast LC-MS and LC-MS-MS may be carried out to satisfy this requirement using gradients (usually a step gradient with a reverse phase HPLC column) with a total cycle time of 1–3 min (24) or using an isocratic system requiring less than 1 min per analysis. A variety of HPLC columns are used for fast LC-MS that include narrow bore (2-mm) and analytical bore (4.6-mm) columns with length typically from 0.5–5 cm. The mobile phase flow rate for these fast LC-MS analyses is usually from 1.5–5 mL/min.

2.3 Encoding and Identification of Compounds in Combinatorial Libraries and Natural Product Extracts

The use of mass spectrometric identification in combinatorial chemistry is not limited to the analysis of synthetic products as a means of quality control, but also for the identification of active compounds or "hits" during high-throughput screening. Although the synthesis and screening of discrete compounds (25) enables them to be followed through the entire process by using partial encoding or bar-coding, it is sometimes advantageous to screen libraries prepared as mixtures (26) and use a technique such as mass spectrometry to rapidly identify the hit(s) in the mixture. One approach to the rapid deconvolution of combinatorial library mixtures is to prepare libraries containing compounds of unique molecular weight and then identify them using mass spectrometry. However, such libraries are necessarily small because the molecular weight of most drug-like molecules is between 150–400 Da. Because of the molecular weight degeneracy of larger combinatorial libraries, several encoding strategies have been devised to rapidly identify active compounds in these mixtures (27–29).

Because most combinatorial libraries contain compounds with degenerate molecular weights, various tagging strategies have been devised to uniquely identify library compounds bound to beads. Most of these tagging approaches are based on the synthesis of encoding molecules. For example, peptide (30) or oligonucleotide (31) labels have been synthesized on the beads in parallel to the target molecules and then sequenced for bead decoding. Alternatively, haloarene tags have been incorporated during synthesis and then identified with high sensitivity using electron-capture gas chromatography detection (32). In addition to the increased time and cost for the synthesis of a library containing tagging moieties, the tagging groups themselves might interfere with screening giving false positive or negative results.

For peptide libraries, one solution to this problem uses matrix-assisted laser desorption ionization (MALDI) mass spectrometry to directly desorb and identify peptides from beads that were screened and found to be hits (33). This technique is called the termination synthesis approach. Because the peptide library compounds are analyzed directly, products with amino acid deletions or substitutions, side-reaction products, or incomplete deprotection are readily observed. Also, because there are no extra molecules used for chemical

tagging, this source of interference is avoided. However, this approach is specific to peptide libraries and is not necessarily applicable to other types of combinatorial libraries.

Another approach that eliminates possible interference from the chemical tags, "ratio encoding," has been developed for the mass spectrometric identification of bioactive leads using stable isotopes incorporated into the library compounds (29, 34). Within the ligand itself, the code might be a single-labeled atom that is conveniently inserted whenever a common reagent transfers at least one atom to the target compound or ligand. The code consists of an isotopic mixture having one of the many predetermined ratios of stable isotopes and can be incorporated in the linker or added through a reagent used during the synthesis. The mass spectrum of the compound shows a molecular ion with a unique isotope ratio that codes for a particular library compound. For example, Wagner et al. (29) used isotope ratio encoding during the synthesis of a 1000-compound peptoid library and was able to identify uniquely all the components based on their isotopic patterns and molecular weights. Because isotope ratio codes are contained within each combinatorial compound, a chemical tag is not required. The speed of MS-based decoding outperforms most other decoding technologies, which are time consuming and decode a restricted set of active compounds.

Although combinatorial synthesis provides rapid access to large numbers of compounds for screening during drug discovery and lead optimization, these libraries are usually based on a small number of common structures or scaffolds. There is a constant need for increasing the molecular diversity of combinatorial libraries and finding new scaffolds, and natural products have always been a rich source of chemical diversity for drug discovery. The traditional approach to screening natural products for drug leads uses bioassays to test organic solvent extracts for activity. If strong activity is detected, then activity-guided fractionation of the crude extract is used to isolate the active compound(s), which is identified using mass spectrometry (including tandem mass spectrometry and exact mass measurements), IR, UV/VIS spectrometry, and NMR. Recently, a variety of mass spectrometry–based affinity screening methods have been developed to streamline the tedious process of activity-guided fractionation. These approaches are discussed in Section 2.4.

Whether lead compounds in natural product extracts are isolated using bioassay-guided fractionation or mass spectrometry–based screening, there is a high probability that the structure of the active compound(s) has already been reported in the natural product literature. In such cases, the tedious process of complete structure elucidation using a battery of spectrometric tools should be unnecessary. Instead, mass spectrometry alone may be used to quickly "dereplicate" or identify the known compounds based on molecular weight, fragmentation patterns, and elemental composition in combination with natural product database searching (35–39). Commercially available natural products databases include NAPRALERT (40), Scientific & Technical Information Network (STN) (41), and the Dictionary of Natural Products (42). Because some of these databases also contain UV/VIS absorbance data, it is also advantageous to use a photodiode array detector between the HPLC and mass spectrometer to obtain additional spectrometric data during LC-W-MS dereplication (36, 37).

2.4 Mass Spectrometry–Based Screening

The earliest approaches to combinatorial synthesis used portioning and mixing (26) and enabled the synthesis of combinatorial libraries containing hundreds of thousands to millions of compounds. Today, this approach remains the most efficient method for preparing enormous libraries of compounds. However, until the mid-1990s, efficient screening techniques did not exist to rapidly identify the "hits" within large combinatorial mixtures. Therefore, chemists were motivated to develop ways to prepare large numbers of discreet compounds using massively parallel synthesis, which could be assayed quickly for pharmacological activity using high throughput screening one compound at a time. Recently, several mass spectrometry–based screening assays have been developed that are suitable for screening combinatorial library mixtures, and some are even useful for screening natural product extracts which have always been a

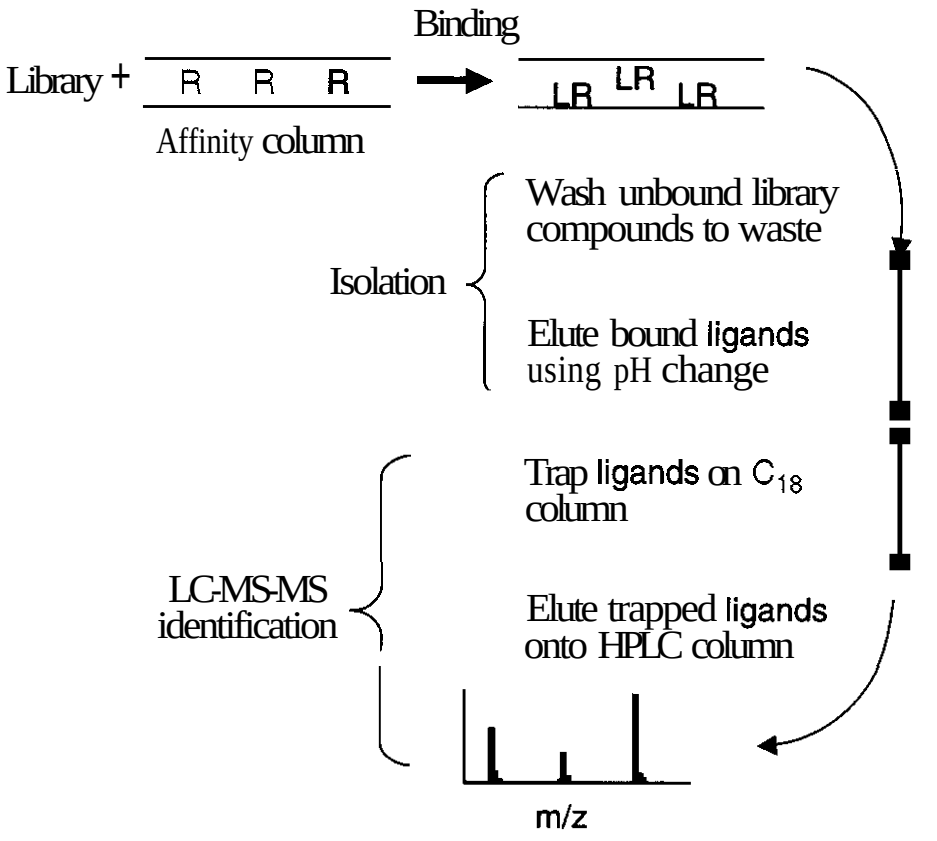


Figure 13.7. Affinity chromatography combined with **LC-MS-MS** for screening combinatorial library mixtures.

source of molecular diversity for drug discovery. All of the mass spectrometry–based screening methods use receptor binding of ligands as the basis for identification of lead compounds.

2.4.1 Affinity Chromatography–Mass Spectrometry. Since the introduction of affinity chromatography more than 30 years ago, this technique has become a standard biochemical tool for the isolation and identification of new binding partners to specific target molecules. Therefore, the coupling of affinity chromatography to mass spectrometry is a logical extension of this technique, and the application of affinity LC-MS to the screening of combinatorial libraries has been demonstrated by several groups (43, 44). During affinity LC-MS screening, a receptor molecule such as a binding protein or enzyme is immobilized on a solid support within a chromatography column. The library mixture is pumped through the affinity column in a suitable binding buffer so that any ligands in the mixture with affinity for the receptor would be able to bind. Then, unbound material is washed away. Finally, the specifically bound ligands are eluted using a destabilizing mobile phase and identified using mass spectrometry. This affinity-column LC-MS assay is summarized in Fig. 13.7.

In some applications (43), ligands are eluted from the affinity column and then trapped on a second column such as a reverse phase HPLC column. LC-MS or LC-MS-MS identification of the ligands (hits) is then carried out using the trapping column. In other systems, ligands are identified directly from the affinity column using mass spectrometry (44). For example, Kelly et al. (44) prepared an affinity column containing immobilized phosphatidylinositol-3-kinase and used it for direct LC-MS screening of a 361-component peptide library. Electrospray mass spectrometry and tandem mass spectrometry were used to identify the ligands released from the affinity column using pH gradient elution.

Advantages of affinity chromatography–mass spectrometry for screening during drug discovery include versatility and re-use of the column. Both combinatorial libraries and natural product extracts can be screened using this approach, and a wide range of binding buffers may be used. Mass spectrometry–compatible mobile phases are only required during the final LC-MS detection step. Furthermore, a single column may be used multiple times to screen different samples for ligands unless the destabilization solution irreversibly denatures, releases, or inhibits the receptor.

Despite these advantages, affinity chromatography has numerous drawbacks that have

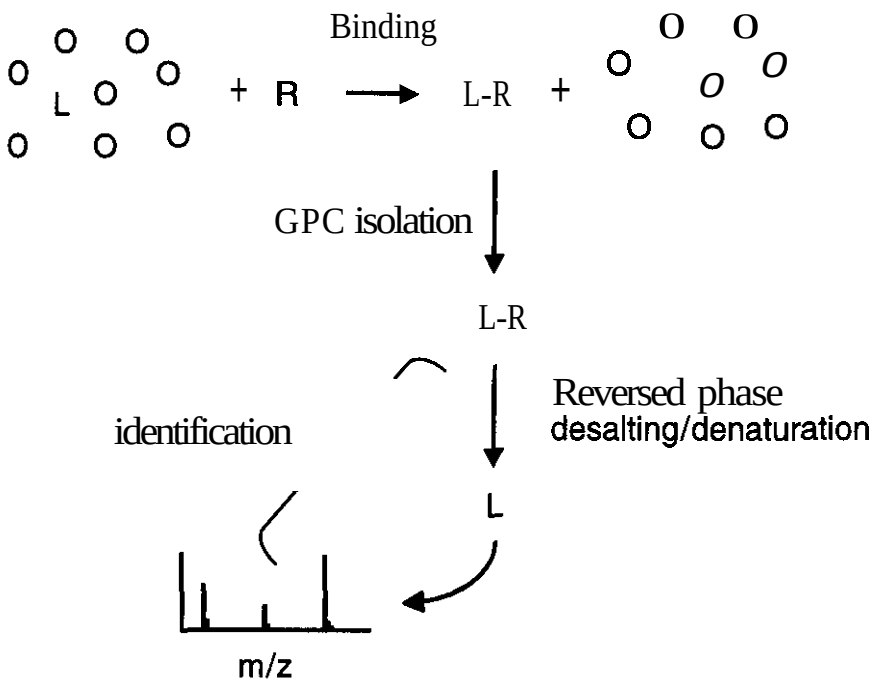


Figure 13.8. GPC followed by LC-MS-MS for screening mixtures of combinatorial libraries. After incubation of a receptor with a library of compounds, the ligand-receptor complexes (L-R) are separated from the low molecular weight unbound library compounds using GPC. Next, the L-R complexes are denatured during reversed phase HPLC to release the ligands for MS-MS identification.

prompted the development of alternative mass spectrometer screening tools. For example, immobilization of the receptor might change its affinity characteristics causing false negative or false positive hits. This is particularly problematic for receptors that are solution-phase in their native state. Also, developing and then implementing an immobilization scheme is often a slow, tedious, and even expensive process, and this process is unique for each new receptor. Finally, false positive hits are often obtained when screening large molecularly diverse libraries, because there are usually compounds in such mixtures that have affinity for the stationary phase or linker molecule instead of the receptor.

2.4.2 Gel Permeation Chromatography–Mass Spectrometry. Another type of chromatography that has been combined with mass spectrometry as a screening system for drug discovery is gel permeation chromatography (GPC) (45, 46). Also called size-exclusion chromatography, GPC separates molecules according to size as they pass through a stationary phase containing particles with a defined pore size. During GPC-based screening, a library mixture is pre-incubated with a macromolecular receptor to allow any ligands in the library to bind, and then GPC is used to separate the large receptor–ligand complexes from the unbound low molecular weight compounds in the mixture. Finally, ligands are released from the receptor during reversed phase HPLC and identified either on-line or off-line using tandem mass spectrometry. This screening method is illustrated in Fig. 13.8.

During the pre-incubation and GPC steps, any binding buffer may be used, because the binding buffer will be removed during reverse phase LC-MS analysis. However, the GPC separation step must be carried out quickly, because ligands begin to dissociate from the receptor immediately and can become lost into the size exclusion gel. Despite this disadvantage, this approach allows both receptor and ligand to be screened in solution, which avoids some of the problems associated with the use of affinity columns for screening. The GPC LC-MS-MS screening method should also be suitable for screening natural product extracts as well as combinatorial library mixtures.

2.4.3 Affinity Capillary Electrophoresis–Mass Spectrometry. Affinity capillary electrophoresis was originally used for the determination of the binding constants of small molecules to proteins (47–49). This solution-based technique is rapid and requires only small amounts of ligands. Affinity constants are measured based on the mobility change of the ligand on interaction with the receptor present in the electrophoretic buffer (50). By combining affinity capillary electrophoresis with on-line mass spectrometric detection and

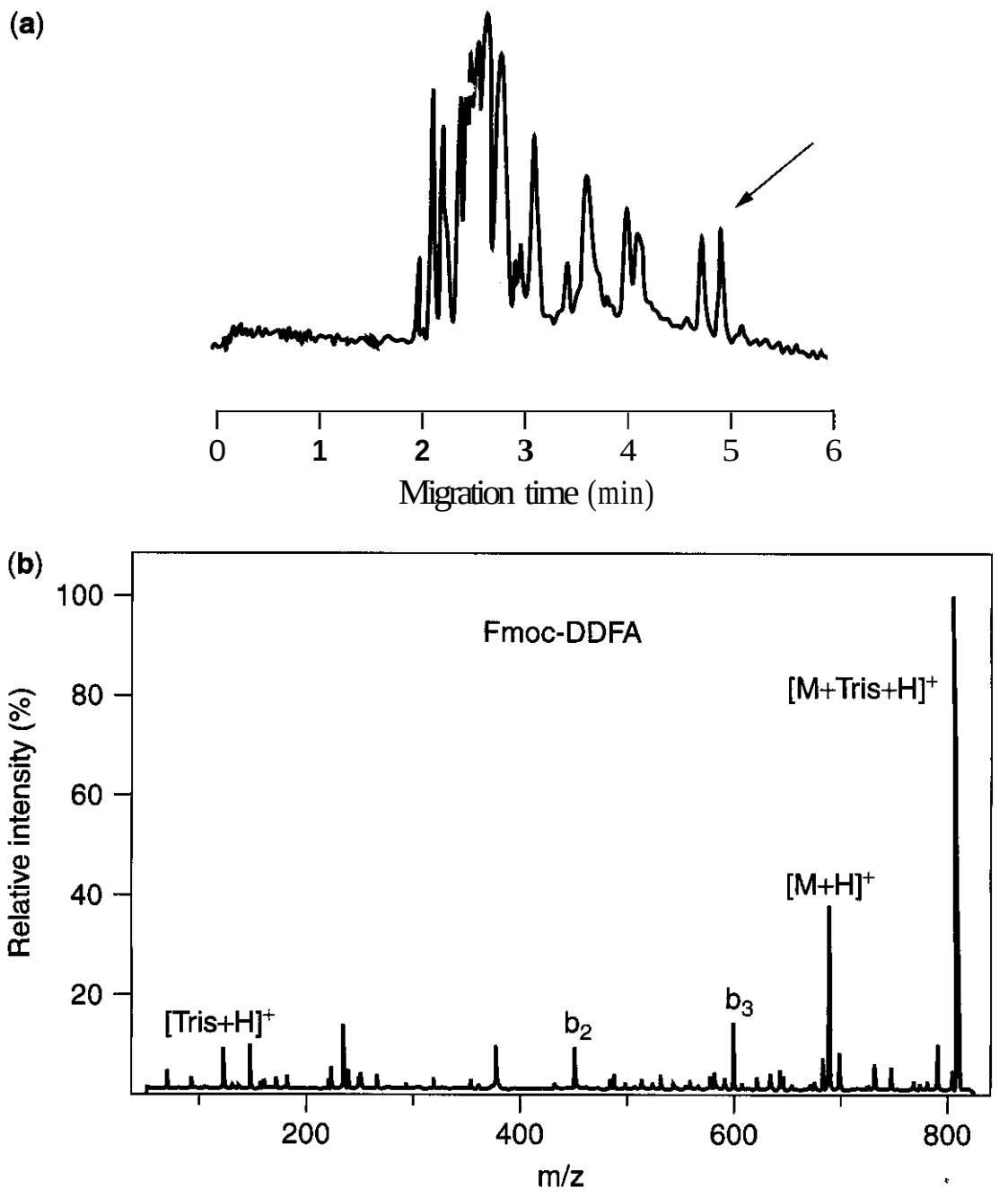


Figure 13.9. Affinity capillary electrophoresis–UV-mass spectrometry of a 100-tetrapeptide library screened for binding to vancomycin (104 μM in the electrophoresis buffer). (a) The elution of peptides was monitored with UV absorbance during capillary electrophoresis, and the elution time increased with increasing affinity for vancomycin. (b) Positive ion electrospray mass spectrum with CID of the Tris adduct of the protonated peptide detected at ~5 min in the electropherogram shown in a (Reproduced from Ref. 52 by permission of the American Chemical Society.)

identification, affinity constants for multiple compounds can be measured in a single analysis (51). Recognizing that on-line mass spectrometric detection was helpful for the identification of each ligand, Chu et al. (52) extended this approach to include the screening of combinatorial libraries as a means of drug discovery. The data in Fig. 13.9 show the results of screening a 100-tetrapeptide library for affinity to vancomycin using affinity capillary electrophoresis–mass spectrometry. Without vancomycin in the electrophoresis buffer, all the peptides eluted within 3 min. When vancomycin was present, the peptides eluted in order of affinity, with the highest affinity compounds being detected between 4.5 and 5 min. Positive ion electrospray tandem mass spectrometry was used to identify the highest affinity ligands (see Fig. 13.9b).

Note that some peptide ligands such as Fmoc-DDFA were detected as adducts with Tris, which was used in the electrophoresis buffer. Although the identification of this peptide was not prevented by the formation of this adduct, some buffers used during electrophoresis might interfere with mass spectrometric ionization and detection. Also, the types of libraries that have been screened using this approach have contained modest numbers of synthetic analogs such as peptides. Libraries exceeding 400 members required preliminary purification using affinity chromatography to reduce the number of compounds (52). As a result, this approach is probably not ideal for screening libraries containing molecularly diverse compounds or for screening natural product extracts. However, affinity capillary electrophoresis–mass spectrometry is fast; each analysis requires less than 10 min. Also, it may be used to measure affinity constants for ligand–receptor interactions.

2.4.4 Frontal Affinity Chromatography–Mass Spectrometry. Like affinity chromatography–mass spectrometric screening (see Section 2.4.1), frontal affinity chromatography uses an affinity column containing immobilized receptor molecules (53). The difference between the two screening methods is that the ligands are continuously infused into the column during frontal affinity chromatography and detected using mass spectrometry. Compounds with no affinity for the immobilized receptor elute immediately in the void volume, but the elution of the ligands is delayed. As compounds compete for binding sites on the affinity column, these sites become saturated until ligands begin to elute from the column at their infusion concentration. In this manner, frontal affinity chromatography may be used to measure affinity constants for ligands, and by using a mass spectrometer for on-line identification of ligands, this technique becomes a screening method (54, 55).

During frontal affinity chromatography–mass spectrometry, signals for all compounds eluting from the affinity column are recorded by the mass spectrometer, and the last compounds to elute at their infusion concentrations represent the highest affinity compounds or "hits." An example of the screening of six oligosaccharides with different binding affinities for an immobilized monoclonal carbohydrate-binding antibody is shown in Fig. 13.10. Compounds 1–3 eluted immediately (no affinity), whereas compounds 4–6 eluted in order of increasing affinity for the antibody. Dissociation constants were determined to be 185, 12.6, and 1.8 μM for compounds 4–6, respectively (54).

Because frontal affinity chromatography uses a conventional affinity column, this technique provides additional applications of this type of column to investigators already using affinity–mass spectrometry (See Section 2.4.1). However, the same limitations and disadvantages of using immobilized receptors still apply, such as non-specific binding to the stationary phase, the development time and cost of preparing the affinity columns, and the possibility that immobilizing the receptor might alter its binding characteristics and specificity. In addition, mass spectrometric detection creates some additional limitations. Because all library compounds must be monitored simultaneously, the compounds must be selected so that they have unique molecular weights. Also, one compound in the mixture should not suppress the ionization of another. Therefore, this approach is probably restricted to the screening of small combinatorial libraries that are similar in chemical structure and ionization efficiencies. Finally, the binding buffer used for affinity chromatography must be compatible with on-line APCI or electrospray mass spectrometry. This means that the mobile phase must be volatile and usually of low ionic strength (i.e., typically <40 mM for electrospray ionization).

2.4.5 Bioaffinity Screening using Electrospray FTICR Mass Spectrometry. Although FTICR mass spectrometry may be used to determine the exact masses of combinatorial library compounds and to confirm their structures using CID and high resolution tandem mass spectrometry (see definitions of CID and MS-MS in Section 1), electrospray FTICR mass spectrometry may be used for the direct screening of combinatorial libraries without the need for any pre-purification or chromatography. In this application, a combinatorial library is pre-incubated with a receptor in solution and then analyzed directly using electrospray to identify receptor–ligand complexes in the gas phase (56–60). Once a receptor–ligand complex is ionized and trapped in the FTICR mass spectrometer, the mass difference between the complex and the receptor alone might be measured with sufficient resolution and accuracy to determine the mass(es) and perhaps elemental composition(~) of the ligand(s). If the ligand carries a charge, then CID may be used to dissociate the ligand for subsequent analysis using tandem mass spectrometry. This elegant and simple screening approach is summarized in Fig. 13.11.

An extension of this FTICR mass spectrometry–based screening technique has been to screen a combinatorial library for ligands to two receptors simultaneously (59, 60). In this example, the two receptors consisting of RNA constructs representing the prokaryotic (16s) rRNA and eukaryotic (18s) rRNA A-site were incubated simultaneously with an aminogly-

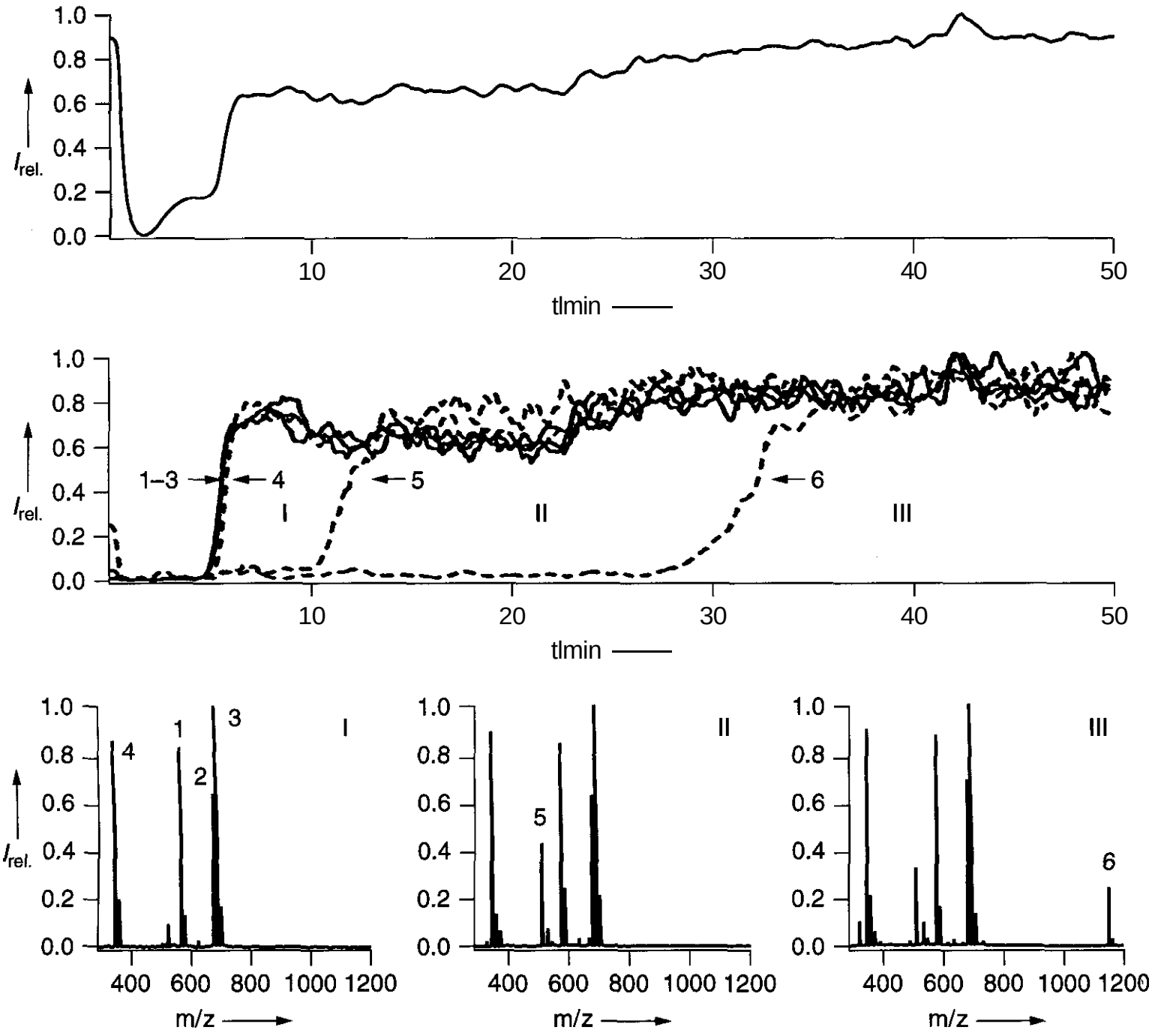


Figure **13.10.** Frontal affinity chromatography–mass spectrometry screening of a 6-oligosaccharide mixture for affinity to an immobilized carbohydrate-binding monoclonal antibody. Top: positive ion electrospray total ion chromatogram. Middle: computer-reconstructed mass chromatograms for the molecular ion species of all six compounds. Compounds **1–3** (solid line) eluted in the void volume, indicating no binding to the antibody. Break through signals for compounds 4, **5,** and 6 appear at successively later times, indicating increasing affinity for the immobilized antibody. Bottom: positive ion electrospray mass spectra recorded at times **I, II,** and III as indicated in the middle trace. The protonated molecules of compounds 1–6 **are** labeled.

coside library to identify potential ligands. By screening a target mixture against the same library, screening efficiency is enhanced and the number of analyses required is reduced.

The advantage of this screening method over other approaches is the elimination of purification steps before mass spectrometric identification. Also, the disadvantages associated with chromatographic separations are eliminated. However, the use FTICR mass spectrometric screening restricts the binding buffer and receptors that may be used. Only low ionic strength and volatile buffers are compatible with this approach (such as 10 m*M* ammonium acetate). Also, the receptor and ligand must be highly purified to avoid impurities that might interfere with ionization and detection. Therefore, this technique is probably more suitable for the screening of combinatorial libraries than complex natural product mixtures. Finally, the receptor–ligand complex must ionize efficiently during electro-

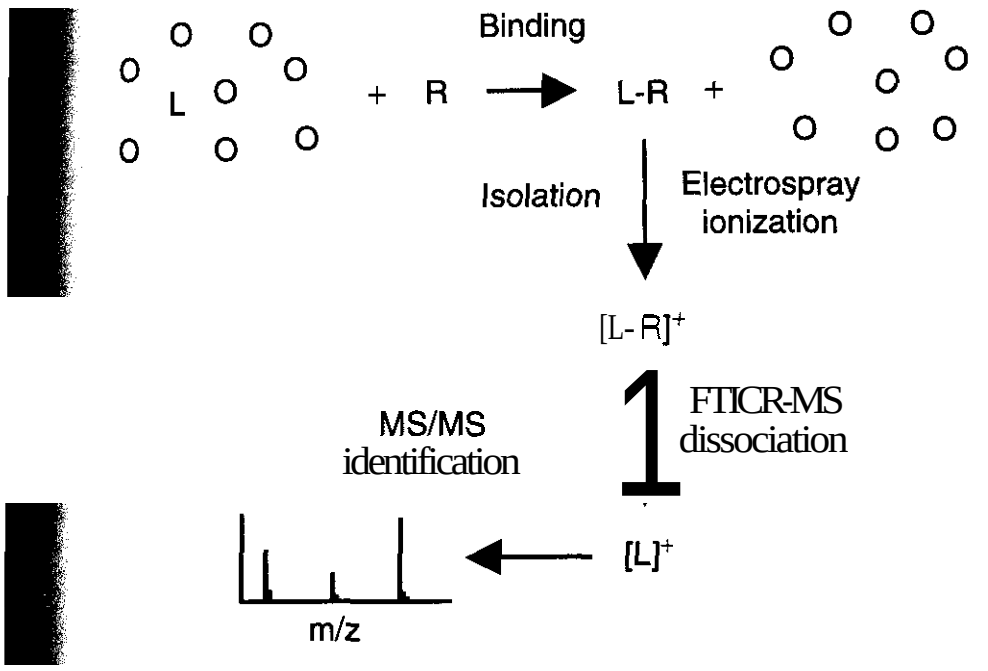


Figure 13.11. Bioaffinity electrospray FTICR mass spectrometry. The isolation and mass spectrometric identification of receptor-specific ligands are carried out entirely in the mass spectrometer without chromatography or other separation steps.

spray under solvent and ion source conditions that do not cause dissociation of the complex.

2.4.6 Pulsed Ultrafiltration–Mass Spectrometry. A versatile approach to screening solution phase combinatorial libraries and natural product extracts is pulsed ultrafiltration–mass spectrometry (61, 62), which uses a standard LC-MS system with an ultrafiltration chamber substituted for the HPLC column. The principle of pulsed ultrafiltration screening of combinatorial libraries is shown in Fig. 13.12. During pulsed ultrafiltration, ligand–receptor complexes remain in solution in the ultrafiltration chamber while unbound library compounds and buffer are washed away. After unbound compounds are removed, the hits from the library are eluted from the chamber by destabilizing the ligand–receptor complex using an organic solvent, a pH change, or a

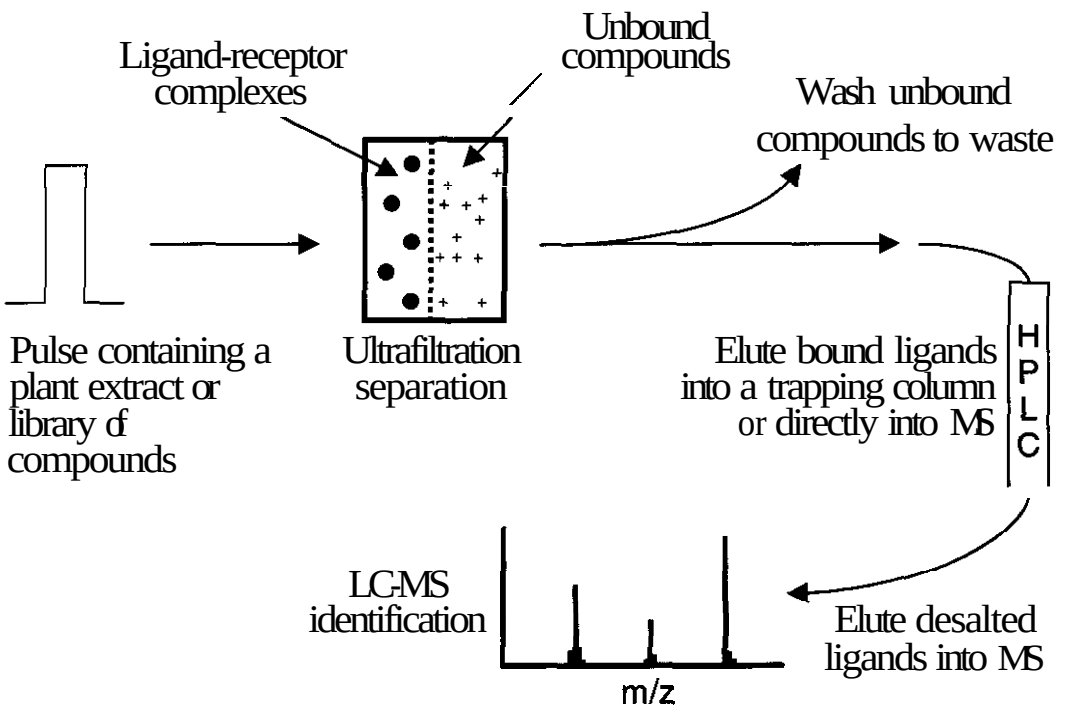


Figure 13.12. Combinatorial library screening using pulsed ultrafiltration mass spectrometry. During the loading step (left), ligands are bound to the receptor either on-line (top) using a flow-through approach or off-line (bottom two incubations). Unbound compounds and binding buffer, cofactors, etc. are washed out of the ultrafiltration chamber to waste during a separation step (middle). Bound ligands are dissociated from the receptor molecules and eluted from the chamber by introducing a destabilizing solution such as methanol, pH change, etc. Finally, released ligands are identified using mass spectrometry, tandem mass spectrometry, or LC-MS (right). (Reproduced from Ref. 64 by permission of John Wiley & Sons.)

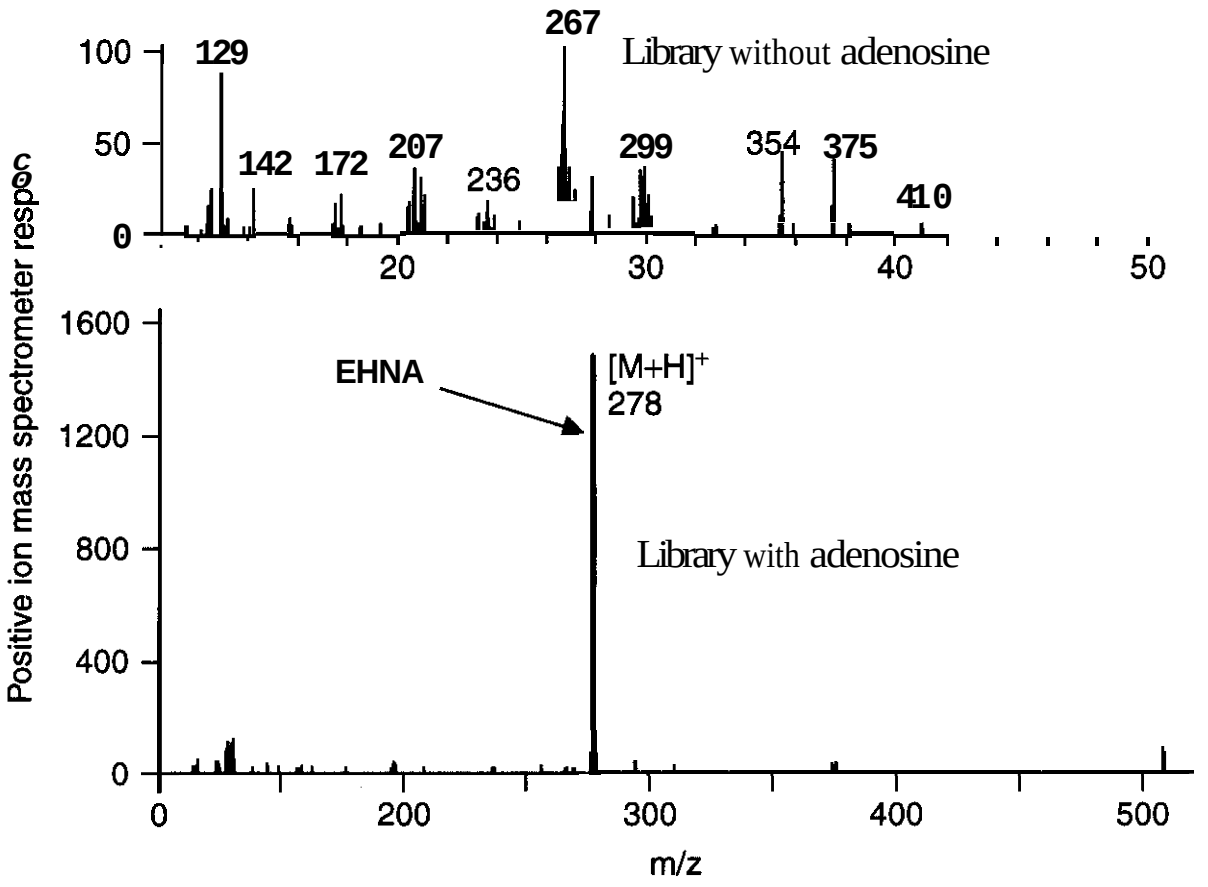


Figure 13.13. Identification of **EHNA** as the highest affinity ligand for adenosine deaminase in a combinatorial library of **20** adenosine analogs using **ultrafiltration** electrospray mass spectrometry. (Reproduced from Ref. 61 by permission of the American Chemical Society.)

combination of both. The released ligands are identified on-line using APCI or **electrospray** mass spectrometry (61) or collected and analyzed off-line using mass spectrometry, **LC-MS**, or LC-MS-MS (63).

An example of pulsed ultrafiltration mass spectrometry for the screening of a library of 20 adenosine analogs for ligands to adenosine deaminase is shown in Fig. 13.13. After a **15-min preincubation** of the library compounds (17.5 μM each except for EHNA, which was present at 1.75 μM) **with** 2.1 μM adenosine deaminase in 50 mM phosphate buffer, an aliquot **containing** 420 pmol of the receptor was injected into the ultrafiltration and washed for 8 min at 50 **μL/min** with water to remove the phosphate buffer and unbound or weakly binding library compounds. Methanol was introduced into the mobile phase to dissociate the enzyme–ligand complex and release bound ligands for identification by **electrospray** mass spectrometry. During methanol elution, only EHNA [erythro-9-(2-hydroxy-3-nonyl) adenine] was detected as the $[M+H]^+$ ion of m/z 278 (Fig. 13.13). In control experiments using the library without enzyme, no library compounds were detected during methanol elution (Fig. 13.13, Control). Despite being present at a 10-fold lower concentration than the natural substrate adenosine analogs, EHNA was easily identified because it had the highest affinity among the library compounds (K_d = 1.9 nM). This demonstrates the use of **ultrafiltration electrospray** mass spectrometry for identifying a high affinity ligand among a set of analogs that bind to a specific receptor. In a follow-up lead optimization study using pulsed ultrafiltration mass spectrometry, a **synthetic combinatorial** library of EHNA analogs was screened for binding to adenosine deaminase, and **structure-activity** relationships for EHNA binding were identified (65).

As an illustration of the versatility of pulsed ultrafiltration–mass spectrometry, binding assays for a variety of receptors have been reported including **dihydrofolate reductase (63)**, cyclooxygenase-2 (**62**), serum albumin (66, 67) and estrogen receptors (**68**). Not only is pulsed ultrafiltration useful for **identifying ligands** to different receptors, but a wide range of **combinatorial** libraries and natural product extracts in any suitable binding buffer may be screened. In addition to **combinatorial** libraries, complex natural product extracts

have been screened (68), and neither plant nor fermentation broth matrices were found to interfere with screening (62). As another example of the flexibility of this screening system, a centrifuge tube equipped with an ultrafiltration membrane (69) has been used instead of an on-line ultrafiltration chamber. Other applications of pulsed ultrafiltration–mass spectrometry include screening drugs and drug candidates for metabolic stability (70), metabolic activation to reactive metabolites (71), and the measurement of affinity constants for ligand–receptor interactions (66, 67).

Metabolism and toxicity screening applications of pulsed ultrafiltration use hepatic microsomes in the ultrafiltration chamber. For metabolic screening drugs and the cofactor nicotinamide dinucleotide phosphate (NADPH) are flow-injected through the ultrafiltration chamber (oxygen is dissolved in the mobile phase), and the metabolites formed by microsomal cytochrome P450 and any unreacted compounds flow out of the chamber for mass spectrometric identification and/or quantitative analysis (70). On-line applications require the use of volatile buffers, but LC-MS and LC-MS-MS may be used off-line to analyze the ultrafiltrate no matter what buffer had been used. Screening drugs for metabolic activation using pulsed ultrafiltration–mass spectrometry is carried out in a similar manner, except that glutathione is coinjected along with NADPH and the drug substrate (71). MS-MS may be used on-line or LC-MS-MS may be used off-line to screen for glutathione adducts as an indication that the drug was metabolized to a reactive intermediate(s) that was trapped by reaction with glutathione. Finally, pulsed ultrafiltration may be used with UV or mass spectrometric detection to measure affinity constants of individual compounds (66).

To measure affinity constants and other physico-chemical properties of binding such as the number of binding sites, two pulsed ultrafiltration measurements are carried out. First, an aliquot or pulse of a liquid is injected through the chamber, and the elution profile is recorded. Then, the chamber is loaded with a receptor, and the ligand is reinjected. If binding occurs, the elution profile will be delayed in proportion to the affinity constant. The control injection is used to control for non-specific binding to the apparatus. Because the concentration of receptor and total amount of liquid are known, and because the concentration of free ligand is measured as it elutes from the chamber over a wide range of concentrations, the affinity constant and other binding parameters may be calculated.

In most of the applications of pulsed ultrafiltration to date, serial analyses were carried out with a throughput of approximately one or two assays per hour. Because the purpose of these assays was to screen complex mixtures or to obtain metabolism data for new drug entities, the throughput of these analyses was acceptable, but was not high throughput. The rate limiting step in these analyses was the ultrafiltration separation and not the mass spectrometric detection. Two solutions have been reported to increase the throughput of pulsed ultrafiltration mass spectrometry. In the first solution, van Breemen et al. (70) used a multiplex ultrafiltration system in which up to 60 ultrafiltration chambers could be arranged in parallel and interfaced to a single mass spectrometer. This scheme is shown in Fig. 13.14. In this system, a continuous flow of the buffer or mobile phase is maintained through the ultrafiltration chambers, but the mass spectrometer samples each ultrafiltrate solution at 1-min intervals. The sampling time would be selected to correspond to the time at which a maximum concentration of metabolites would be expected to elute from the chamber. This approach was demonstrated to increase the throughput of metabolic screening using ultrafiltration mass spectrometry by 60-fold. Although used originally for metabolic screening, this approach would be applicable to toxicity screening and drug discovery screening as well.

The second solution to increasing the throughput of pulsed ultrafiltration mass spectrometry has been to miniaturize the ultrafiltration chamber volume while maintaining the flow rate and chamber pressure. Because the ultrafiltration membrane cannot withstand high pressure without rupturing, the ultrafiltration process cannot be accelerated simply by increasing the flow rate through the chamber. The approach of Beverly et al. (72) was to fabricate a 35-μL ultra-

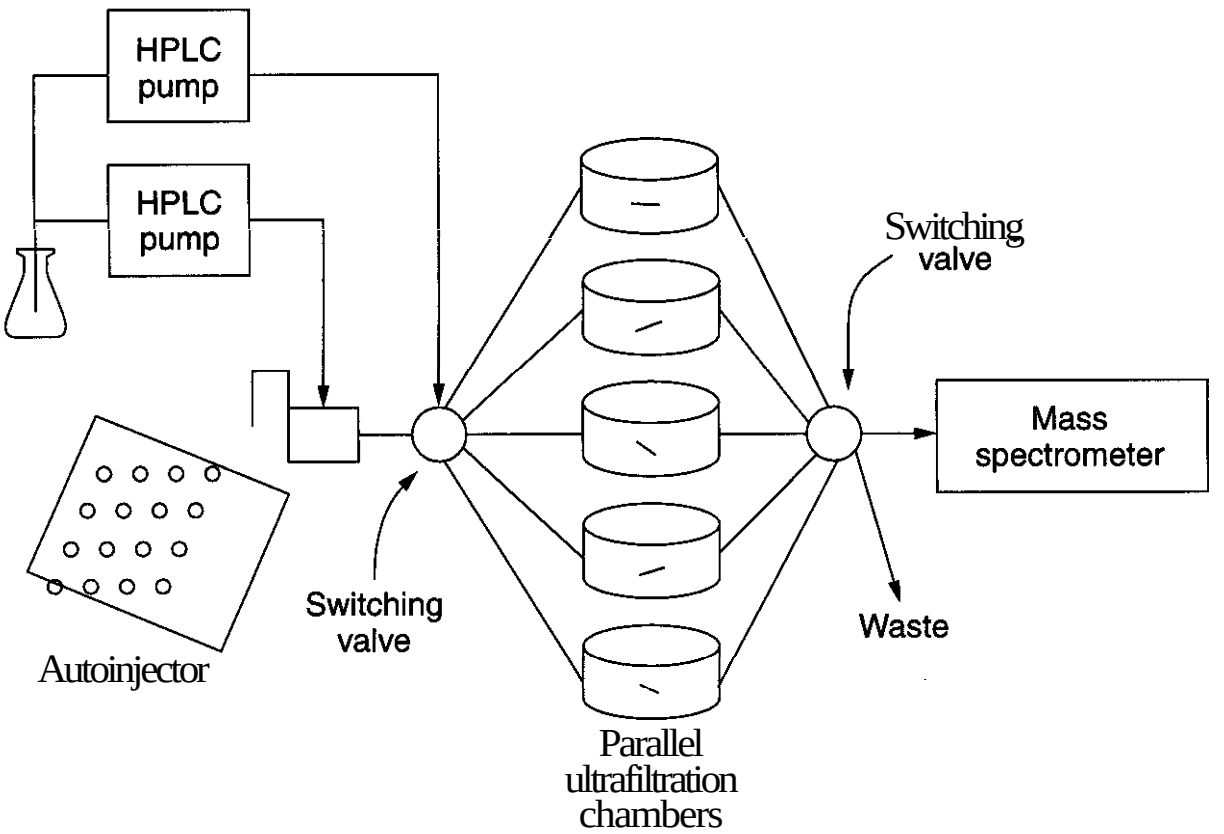


Figure 13.14. High-throughput pulsed ultrafiltration mass spectrometry system for screening drug candidates for metabolic transformation. Multiple ultrafiltration chambers are connected in parallel to a single mass spectrometer detector. After loading each chamber with liver microsomes, a different drug is injected into each chamber at intervals of 1 min (for 60 screens/h using 60 chambers). Constant flow of incubation buffer is maintained through all chambers, but only one chamber at a time is connected on-line to the mass spectrometer. Drug metabolite profiles are recorded using mass spectrometry for up to 1 min per chamber. (Reproduced from Ref. 70 by permission of the American Society for Pharmacology and Experimental Therapeutics.)

filtration chamber that was approximately threefold lower in volume than the smallest reported by van Breemen et al. (61). As a result, ultrafiltration mass spectrometric analyses could be carried out at the rate of at least three per hour, which corresponded to a threefold enhancement of throughput. This study suggests that chip-based ultrafiltration mass spectrometry would have the potential to result in a truly high-throughput system.

The advantages of pulsed ultrafiltration–mass spectrometry include the variety of different applications that may be carried out, the convenience of on-line screening, solution-phase screening, the ability to screen either combinatorial libraries or natural product extracts, the diversity of receptors that may be screened, and the freedom to use either volatile or non-volatile binding buffers. For metabolic and toxicity screening, flow injection analyses have the additional advantages that product feedback inhibition is prevented so that the metabolic profile more closely approximates the *in vivo* system *(70)*. Finally, the disadvantages of pulsed ultrafiltration screening for drug discovery include the washing step, during which dissociation and loss of weakly bound ligands might occur, and the slow speed of each experiment, which can take up to 1 h.

2.4.7 Solid Phase Mass Spectrometric Screening. Because drugs are usually in a soluble form to be transported to the active sites in cells and tissues, it is logical that most mass spectrometry–based screening methods use solution-phase analysis of these compounds, and it is no surprise that most successful mass spectrometry screening assays use electrospray ionization or APCI. However, solid phase ionization techniques such as matrix-assisted laser desorption ionization (MALDI) might be effective, provided that ligand–receptor interactions are allowed to take place in an environment similar to in *vivo* conditions and that a suitable separation step is carried out before the preparation of the MALDI sample.

To use MALDI mass spectrometry for screening, several research groups have developed immobilized receptors on MALDI targets or on solid supports that can be placed on a MALDI target for use in the affinity purification of potential drugs from test solutions. Following procedures originally developed for affinity chromatography, the preparation of affinity surfaces for MALDI mass spectrometry has been achieved quite easily. However, the use of these affinity MALDI chips for screening mixtures of small molecules during drug discovery has been unproductive. One of the problems has been the high background noise at low m/z values caused by the matrix used for MALDI. This problem may be mitigated by eliminating the matrix or using alternative sample stages such as porous silicon chips (73, 74). However, noise persists because of the affinity support and immobilized receptor molecules. Another problem that has yet to be overcome is the elimination of the high background noise caused by non-specific binding of test compounds to the affinity target. Although this problem is similar to the false positive results and non-specific binding that occurs during affinity chromatography–mass spectrometry (see Section 2.4.1), the signals for non-specific binding are magnified by the fad that the actual affinity surface is being irradiated and sampled by the MALDI laser beam. As a result, affinity-based screening coupled with MALDI mass spectrometry has not been a successful drug discovery approach.

However, progress is being made in the use of affinity probes for the capture of proteins and other macromolecules from biological solutions followed by MALDI mass spectrometric detection and identification (75–77). One affinity MALDI mass spectrometry method has been paired with the affinity probes using in surface plasmon resonance systems (78). These affinity-based MALDI mass spectrometry screening assays are promising approaches for testing blood or other biological fluids for the presence of specific proteins or other macromolecules. As a result, these have the potential to become clinical diagnostic tools or might even lead to the identification of new therapeutic targets. However, they are unlikely to become useful for screening combinatorial libraries or natural product extracts for the purpose drug discovery.

3 THINGS TO COME

Mass spectrometry has become an essential analytical tool at every stage of the drug discovery and development process. In this chapter, the various applications of mass spectrometry to combinatorial chemistry and drug discovery have been highlighted. Although the speed of mass spectrometry matches the demands of combinatorial chemistry, the slow and serial nature of chromatography in the various LC-MS applications remains a bottleneck that limits their throughput. Because mass spectrometry is highly selective, only partial chromatographic separations are needed for most measurements. In fact, the primary function of the chromatography step is usually to separate species that might otherwise interfere with the ionization process. Recognizing this limited function of chromatography during LC-MS–based screening assays, manufacturers of chromatography columns are addressing this need by developing high-throughput columns for fast chromatography for LC-MS. Improvements in this direction should continue to reduce the time required for LC-MS from a few minutes to a few seconds. Meanwhile chip-based technology is beginning to emerge for miniaturized capillary electrophoresis–mass spectrometry (CE-MS) (79). These chips are being developed to enable ultrafast and highly sensitive electrospray mass spectrometric analysis. Because of their microscopic size, CE-MS chips have the potential to hold large arrays of samples that would facilitate high-throughput analysis.

In terms of mass spectrometry instrumentation, the currently available instruments such as time-of-flight (TOF) analyzers and hybrid quadrupole-TOF analyzers are able to acquire complete mass spectra at rates compatible with fast CE separations. As CE or ultrafast chromatography replaces conventional, slow HPLC applications, TOF-based mass spectrometers will be needed to replace the less efficient scanning types of instruments such as quadrupoles and ion traps for most high-throughput applications. FTICR mass spectrometry remains unsurpassed in terms of resolution and mass accuracy for both MS and MS-MS applications. However, the throughput of FTICR mass spectrometric

analysis needs to be increased to remain useful for combinatorial chemistry applications. Advances in increasing the throughput of FTICR mass spectrometry are anticipated.

Hyphenated technologies such as LC-NMR-MS are being developed to support structure elucidation of combinatorial libraries (80). Although such technologies are still in a developmental stage, they have great potential for analyses of combinatorial libraries and for natural product drug discovery (81–83). The main impediments of applying LC-NMR-MS to combinatorial chemistry remain poor sensitivity of the NMR, the obligatory use of deuterated solvents for chromatography, and the low throughput of NMR analyses. However, efforts are in progress to improve the throughput of NMR analyses (84–86).

In conclusion, mass spectrometry provides rapid, reliable, sensitive, and selective analysis of combinatorial libraries for structure confirmation, purity analysis, and library deconvolution. In addition, mass spectrometric screening methods have been developed and are beginning to be applied to drug discovery. In the case of natural products, mass spectrometry facilitates the screening of natural product extracts and facilitates the dereplication and characterization of lead compounds. At different times during the last 100 years, first physicists and physical chemists and then organic chemists pronounced that mass spectrometry had run out of new applications and had no future. Fortunately, they were wrong. Today, medicinal chemists recognize that the potential of mass spectrometry to contribute to all facets of drug discovery has only just begun to be explored. Furthermore, applications of mass spectrometry to drug development are even less developed and are waiting to be developed. Mass spectrometry has become a fundamental analytical tool for drug discovery, and this role should continue to grow in the future.

4 WEB SITE ADDRESSES AND RECOMMENDED READING FOR FURTHER INFORMATION

- http://www.asms.org Homepage of the American Society for Mass Spectrometry. This web site contains additional information about mass spectrometry and links to a variety of reference materials regarding biomedical mass spectrometry.
- http://www.bentham.org/cchts/ *Combinatorial Chemistry & High Throughput Screening*
- http://pubs.acs.org/journals/jcchff/ Journal of Combinatorial Chemistry
- http://www.5z.com/moldiv/ Molecular Diversity
- **http://www.liebertpub.com/BSC/defaultl.asp** Journal of Biomolecular Screening
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