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Mass spectrometric analysis of complex mixtures then and now: the impact of linking liquid chromatography and mass spectrometry

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Abstract

On-line compound isolation (using column switching) for the analysis of complex mixtures encountered in the pharmaceutical development process has been investigated. The strategy was used for the analysis of low-level compounds that responded poorly (or not at all) under standard atmospheric pressure ionization LC/MS conditions. Analytes were prepared using small secondary columns after the analytical separation. Subsequently, the retained compounds were eluted and interrogated using experimental conditions designed to maximize mass spectrometric information content; these conditions included optimized solvent systems, optimized flow rates, chemical manipulation of the sample, extended acquisition time, and other appropriate mass spectral techniques. The challenges of obtaining comprehensive qualitative information about a mixture component under the restrictive conditions of validated regulatory HPLC methods are discussed in the context of the historical framework of direct mixture analysis using mass spectrometric approaches. (Int J Mass Spectrom 212 (2001) 111–133) © 2001 Elsevier Science B.V.

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1. Introduction

In a 1976 publication [1], it was stated that the development of mass spectrometry "has, to a notable extent, been impelled by the problem of mixture analysis to which it has, in turn, made important contributions." The subsequent twenty-five years have borne witness to the ongoing validation of that observation. Many of the advancements in analytical chemistry have been made possible by new types of instruments and their novel utilization for solving important problems in chemical analysis. The direct analysis of complex mixtures is one area of mass spectrometry that has benefited substantially from the evolution of instrumentation. Beginning with the initial report describing a reverse-geometry mass-ana-

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Dedicated to Professor Graham Cooks with appreciation and admiration on the occasion of his 60th birthday and in recognition of his pioneering and diverse contributions to the field of mass spectrometry.

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lyzed ion kinetic energy (MIKE) spectrometer [2], Cooks and coworkers published numerous accounts describing novel instrumental configurations [3–6], new analytical schemes [7–16], and the characterization of samples having both biological [17–39] and chemical importance [40–48].

The two seminal themes in much of that work, which were carried through into many future applications, were the ideas that mass spectrometry could provide more than a single dimension of information and that analyses could be conducted with little or no preparation of the sample. In the case of the original work using the MIKE spectrometer, the two analyzers of a double-focusing instrument were decoupled and operated independently (in a low-resolution mode) to yield information about mass-selected ion populations. This use of consecutive stages of analysis (i.e., tandem mass spectrometry or MS/MS) [49–68] was shown to yield information that was complimentary to other dual-stage methodologies (e.g., GC/MS and, later, LC/MS) in the interrogation of chemically complex samples. In a fashion analogous to GC/MS [69], tandem mass spectrometry probed chemically complex mixtures by combining a separation technique with subsequent structural characterization. The notable difference between the two methodologies was that tandem mass spectrometry performed the separation after the ionization process rather than before it. Therefore, the separation depended not on chemical processes (occurring on a chromatography column) but, rather, on physical properties of charge and mass. This reversal in the traditional sequence of sample ionization and separation was responsible for one of the most important attributes of the direct mixture analysis approach—speed. In comparison with techniques that required extensive sample preparation and employed chromatographic separations (where retention time was rate limiting), very short cycle times were possible. A variety of compounds could be characterized in a brief period of time, as samples were introduced in their original form and analytes were accessed randomly rather than sequentially as they eluted from a chromatographic medium. The extreme versatility and ruggedness of the technique was largely due to the use of chemical ionization (CI) [70] with sample introduction via a heated direct insertion probe. This interface was successful because it was not prone to some of the restrictions encountered in GC/MS analyses (e.g., thermal decomposition occurring in the GC injection port) and required little, if any, sample preparation or pretreatment such as extraction or derivatization. The direct insertion probe facilitated introduction of incredibly complex and intractable sample matrices into the source of a mass spectrometer, which could not be introduced by other means (chromatography, etc.). Raw biological fluids, various types of intact plant or animal tissue, and solvent-refined coal liquids are but some of the complex materials that were analyzed directly and successfully using MS/MS with no sample pretreatment. Chemical ionization provided analytical selectivity and flexibility but was not without drawbacks. Ion–molecule reactions sometimes resulted in differential sensitivity and formation of undesirable products. Both of these events could complicate the appearance of mass spectra. Thermolysis of polar compounds also was unavoidable. A growing emphasis on the analysis of high molecular weight, thermally fragile biomolecules such as proteins, oligonucleotides, and complex polysaccharides eventually rendered conventional chemical ionization (and the direct insertion probe) impractical. Conventional CI ultimately gave way to other methods of ionization such as laser desorption (LD) [71], fast-atom bombardment (FAB) [72], thermospray (TSP) ionization [73], and finally, the API techniques of electrospray ionization (ESI) [74] and atmospheric pressure chemical ionization (APCI) [75]. Interestingly, none of the successors of the direct insertion probe/CI interface have been able to match its ruggedness and dependability in coping successfully with untreated or unprepared complex sample matrices.

Another key instrumental element of the mass spectrometric approach to complex mixture analysis was the selection and sequence of mass analyzers, as well as their resolving power and independence of operation. At the time of its genesis, the MS/MS technique was practiced primarily using instruments comprised of sequential arrangements of electrostatic analyzers (E) and magnetic sectors (B) [76]. The

analyzers could be arranged in a variety of configurations, could be operated at either high or low resolving power, and were limited in number only by the requirement that some measurable signal be observed at the final detector. Over the past two decades, a significant number of new instrumental configurations for carrying out tandem mass spectrometry (and mixture analysis) experiments have been commercialized. Electrostatic analyzers and magnetic sectors largely have given way to quadrupole mass filters (Q) [77], time-of-flight (TOF) analyzers [78], quadrupole ion traps (QIT) [79], ion cyclotron resonance (ICR) spectrometers (also referred to as Fourier transform or FT mass spectrometers) [80], and various combinations and arrangements thereof. The efficiencies with which devices such as quadrupoles, TOF analyzers, and ion traps transmit or collect ions allow combinations of these analyzers to be arranged sequentially for achieving multiple stages of mass analysis [81–85]. However, today, multistage analyses (so-called MS^n experiments [86–88]) are more often performed using instruments that exploit temporally rather than spatially sequential processes (i.e., trapping devices rather than spatial arrangements of "beam" analyzers). When MS/MS experiments are carried out using FT/MS instrumentation (capable of providing both MS^n analyses and accurate mass measurements that lead to empirical formulas), additional information regarding the character or identity of structures can be obtained [89]. With the shift in emphasis away from sector instruments, the high translational energy regime of collision-induced dissociation that provided access to uniquely diagnostic fragmentation processes (e.g., charge inversion [90], charge-stripping [91], etc.) virtually has been abandoned. Among currently popular instrumental configurations, only a combination of TOF analyzers (TOF/TOF) [92,93] has the potential to provide access to these high-energy collisions and the resulting structurally informative processes.

Identification or characterization of any mixture component (represented by its surrogate ion population) requires that mass-selected precursor ions (generally $[MH]^+$ or $[M\text{-}H]^-$) undergo a change in mass (dissociation). This change is the result of an energy deposition event initiated by an interaction of the mass-selected ions with a localized region of relatively high pressure (collision-induced dissociation) [94], laser light (photo dissociation) [95], an electron beam [96], or a surface (surface-induced dissociation) [97]. Although obtaining the structural information results in some loss of sensitivity, when compared to a single stage experiment there is a considerable gain in selectivity and signal-to-noise ratio (*S*/*N*). The identity of unknown compounds can be deduced using structural analogs as interpretive "templates" combined with knowledge about the nature of functional group fragmentation behavior. The primary limitation of the direct mixture analysis approach is the inability to distinguish isomeric and isobaric compounds, particularly when the first stage of mass analysis is carried out at low resolving power, as this results in a convoluted product ion spectrum. Isomer differentiation has been demonstrated for certain classes of compounds using tandem mass spectrometry, but these classes must be analyzed individually (i.e., separated by chromatography), and kilovolt energy collisions are often required to produce the spectral features required for distinction. While most structure elucidation work historically has employed positively charged precursor and product ions, negative ions (when they can be generated for analytes of interest) can offer access to unique modes of dissociation, thus enhancing the effectiveness of structure elucidation efforts. Complex samples also can be investigated using other complimentary MS/MS scan modes (i.e., precursor ion spectra or neutral loss spectra) when tandem "in space" instruments are used for the analysis. Single- (SRM) and multiple-reaction monitoring (MRM) experiments can be used to increase the sensitivity (detectability) and reliability of quantification above and beyond that achievable by the conventional scanning protocols that are used most commonly in MS/MS analyses. These techniques are analogs of single- or multiple-ion monitoring experiments used for many years in GC/MS analyses.

The role of tandem mass spectrometry in nearly every aspect of pharmaceutical research and development has been documented extensively. Areas of application include characterization of therapeutic

targets such as enzyme receptors [98], identification of synthetic compounds generated by conventional and combinatorial synthetic techniques [99], and the qualitative or quantitative determination of pharmaceutically active compounds and their related substances (e.g., synthetic intermediates and impurities, chemical degradants, and metabolites) in complex matrices of either biological (tissues and fluids) [100] or pharmaceutical (formulations) origin [101,102]. Contrary to some early predictions regarding the widespread applicability of direct MS/MS analyses (i.e., without chromatographic separation), success in analyzing complex samples encountered regularly in the pharmaceutical arena has been limited [103]. The underlying causes include interactions of analyte molecules with each other or with matrix components and the inability to distinguish isomeric species. Tandem mass spectrometry experiments are combined frequently with some separation technique (e.g., GC, HLPC, CE, etc.) to address difficulties stemming from some of these undesirable interactions. Of course, chromatographic separations introduce unavoidable time delays (i.e., retention time) that reduce the throughput of the analyses. That being said, the technique of LC/MS/MS (or LC/MS^n) has become a de facto standard method of analysis in all facets of the pharmaceutical research and development process. The selectivity of the sequential analyzers combined with the technique's inherent sensitivity (achieved through increased *S*/*N*) has created an analytical tool that is extremely powerful.

It is certainly beyond the scope of this account to comprehensively review the application of tandem mass spectrometry to pharmaceutical analysis [104]. Instead, we will focus on a few cases relevant to pharmaceutical development activities involving the characterization of related substances originating from chemical or pharmaceutical processes. Samples of biological origin (i.e., proteins, peptides, oligonucleotides, or metabolites of therapeutic molecules) will not be considered. Specifically, situations that pose significant analytical challenges caused by non-responsive analytes (compounds that respond poorly under standard conditions because of experimental constraints) will be examined in the context of a multiple-column strategy that exploits independent optimization of the separation and characterization techniques.

2. Experimental

2.1. Peak trapping

A schematic diagram of the peak-trapping system used for this work is shown in Fig. 1. The experiment was conducted according to the following scheme: first, isolating the desired compound from an analytical HPLC column (C1) in a sample collection loop; second, loading the analyte onto a micro-HPLC column (C2); third, subsequently eluting it from C2 for trapping in a second sample collection loop; fourth, loading the compound onto a capillary HPLC column (C3); and, fifth, back flushing the analyte off of C3 and through a second capillary column (C4). A summary of the columns used in this investigation is shown in Table 1. Separations were conducted using an HP1100 HPLC system (Agilent Technologies, Palo Alto, CA) equipped with an autosampler, four solvent–reservoir binary pump, solvent degassing unit, column heater with a six-port column switching valve (V1), and diode array detector with both micro and capillary flow cells. The HP1100 HPLC system was controlled using Xcalibur version 1.2 software (Thermo Finnigan).

Eluent from the analytical column (C1) was mixed with aqueous trifluoroacetic acid (5 mM) delivered by P2 in a static mixing tee downstream from the UV flow cell (HP 1100 diode array detector) to reduce the solvent strength of the mobile phase. The flow rate (f_{a1}) delivered to C1 was 1–1.5 mL/min, while P2 delivered the aqueous acid at a rate (f_{w1}) of 3 mL/min. The combined flow ($f_{a1} + f_{w1}$) was directed through V2 to the sample loop on V3 (1.5 mL) to trap the compound of interest. The timing of V2 (manual) actuation was dictated by visual observation of the arrival of the analyte at the HP 1100 diode array detector and the calculated volume of the tubing connections. Once trapped, V2 was switched to divert flow away from the sample collection loop to waste.

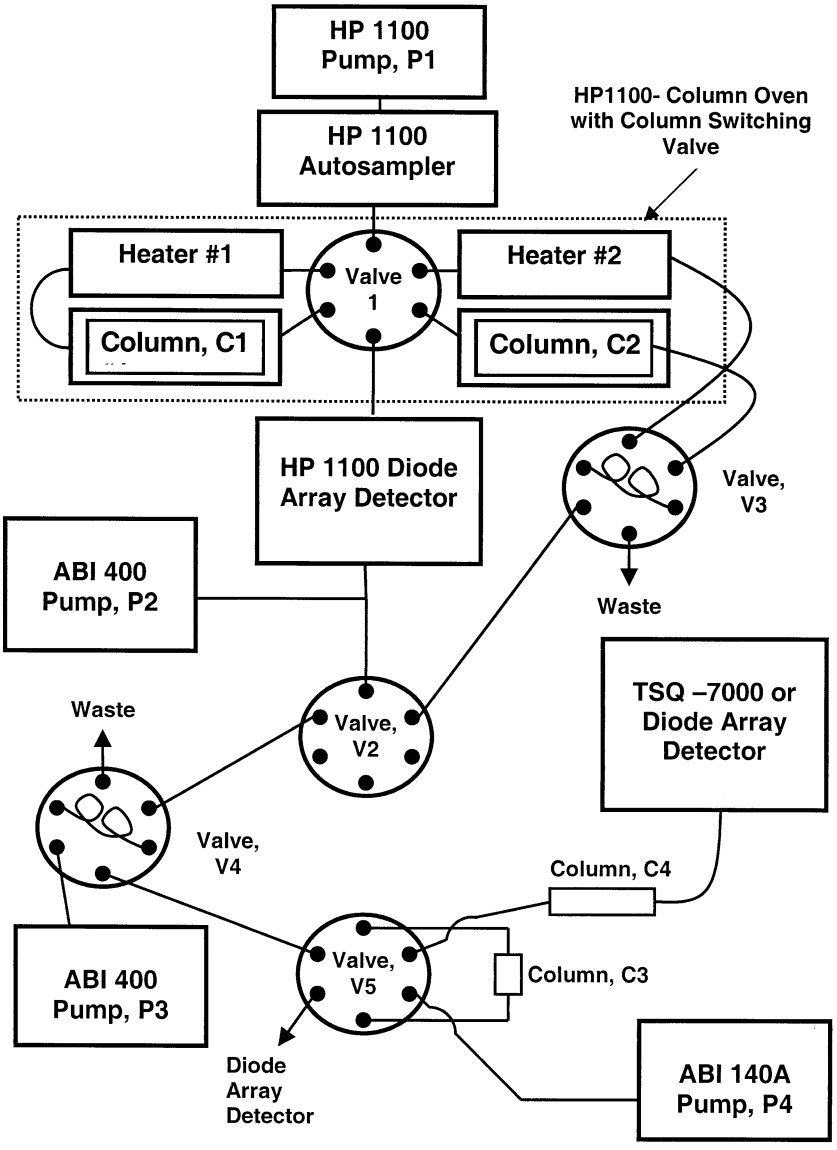

Fig. 1. Schematic diagram of the peak trapping apparatus.

The analytical method was terminated and P1 reprogrammed for solvent delivery to C2.

The contents of the V3 sample loop were loaded onto C2 at a flow rate of 0.3 mL/min (f_{l1}) with P1 delivering water for 0.5 min followed immediately by an 80/20 organic/aqueous solution. At an elapsed time of 5.5 min, the P1 flow was reduced to 0.05 mL/min (f_{a2}). Approximately 1 min later (elapsed time of 6.5 min), V3 was switched (bypassing the sample loop) to deliver the high-organic-strength solvent directly to C2. The output from P2 (now set to deliver the aqueous acid at 0.3 mL/min) was again used to reduce the solvent strength of the eluent stream, this time being combined with the output from C2. The analyte was then trapped for a second time in a sample collection loop on V4 (150 μL). Loading of the

Table 1
HPLC columns used in this study

Compound Isolated	C1	C2	C3	C4
m-chlorobenzoic acid	Symmetry C18, 4.6 × 100 mm, 3.5 μm particle size	Magic Bullet C_{30}, 3 μm particle size	Stable Bond C_{18} 0.5 × 35 mm, 5 μm particle size	Magic C_{30} 0.3 × 50 mm, 5 μm particle size
Mixture components 11 and 12	Symmetry C18, 4.6 × 100 mm, 3.5 μm particle size	Magic Bullet C_{18}, 3 μm particle size	Stable Bond C_{18} 0.5 × 35 mm, 5 μm particle size	XDB C_{18} 0.3 × 35 mm, 5 μm particle size
Tolbutamide	Luna C18(2) 2.0 × 50 mm, 3 μm particle size	Magic Bullet C_{30}, 3 μm particle size	Stable Bond C_{18} 0.5 × 35 mm, 5 μm particle size	XDB C_{18} 0.3 × 150 mm, 5 μm particle size

Note. Symmetry C18: Waters, Milford, MA; Luna C18(2): Phenomenex, Torrance, CA; Magic Bullet and Magic: Michrom Bioresources, Auburn, CA.

sample onto C3 was accomplished by switching V4, which swept the flow from P3 (water at 0.03 mL/min, f_{l2}) through the sample loop. Column 3 was then washed with water (also from P3) until the residual concentration of undesirable additives (present in the mobile phase of the original analytical separation) in the eluent was reduced to acceptable levels (monitored by diode array detection when possible).

Additional concentration of the sample was accomplished in a final elution/capture sequence utilizing C3, C4, V5, and P4. The analyte was back flushed off C3 and through C4 with either isocratic (80% acetonitrile/20% water) or gradient elution at a flow rate f_{a3} (5 μL/min for isocratic elution or 4 μL/min for gradient elution) supplied by P4 either for immediate introduction into an electrospray ionization source or for collection for later analysis.

2.2. Mass spectrometry

Electrospray ionization mass spectra were acquired using a TSQ7000 triple quadrupole mass spectrometer (Thermo Finnigan, San Jose, CA) fitted with the manufacturer's API1 interface and controlled with Xcalibur version 1.2 software. Initial LC/MS investigations utilized standard electrospray conditions (4.5 kV spray voltage, 80 psi nitrogen sheath gas, 20 psi nitrogen auxiliary gas, 250°C heated capillary) or standard APCI conditions (4 mA discharge current, 80 psi nitrogen, 20 psi nitrogen auxiliary gas, 220°C heated capillary). Eluent flow from the HPLC was reduced by a factor of two before entering the electrospray source. The second mass analyzer (Q3) was scanned across the range of *m/z* 120–600 at a rate of two scans per second.

Several modifications were made to the electrospray source for analysis of trapped compounds. A stainless steel tee (Upchurch Scientific, Oak Harbor, WA) replaced the stainless steel union at the grounding junction of the electrospray source. The standard electrospray fused silica capillary (100 μm i.d. × 190 μm o.d.) was replaced with a 30 μm i.d. × 150 μm o.d. fused silica capillary (Polymicro Technologies, Phoenix AZ). For negative-ion experiments, the spray voltage was 3.3 kV, sheath gas was delivered at 30 psi, and the heated capillary was set at 220°C. The outlet of C4 was combined with acetonitrile and delivered at 1.0 μL/min. by a syringe pump (Harvard Apparatus, South Natick, MA) to the electrospray capillary tube. Negative ion mass spectra were acquired by scanning Q3 across the range *m/z* 100–250 at a rate of two scans per second. For positive ion experiments, the spray voltage was 4.5 kV, the sheath gas was delivered at 40 psi, auxiliary gas was delivered at a rate of 20 psi, and the heated capillary was set at 220°C. Positive ion mass spectra were acquired by scanning Q3 across the range m/z 150–400 at a rate of two scans per second. Product ion MS/MS spectra acquisitions used the data-dependent scanning mode and were triggered when the total ion signal in Q1 surpassed a preset threshold. Once triggered, spectra were collected for the most abundant ion

(precursor ion) with Q3 scanning from *m/z* 15- to a value nominally above the *m/z* value of the precursor ion. All MS/MS spectra were acquired using argon as collision gas at a measured pressure of 1.6 mTorr (multiple collision conditions).

A QP-5050A GC/MS system (Shimadzu, Kyoto, Japan) was used for the analysis of small, nonpolar compounds. Samples were introduced using splitless injections (injector temperature 250°C) and eluted from a 15-m DB5-MS column (0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsum, CA) using temperature programming. The temperature program was linear, with an initial temperature of 40°C, a final temperature of 310°C, and heating rate of 40°C/min. The mass spectrometer was scanned across the range of *m/z* 50–300 at a rate of two scans per second.

Nanospray experiments were performed using a QTOF-2 mass spectrometer (Micromass, Manchester, UK) equipped with a nanospray ionization source, which was modified with a Picotip adapter assembly (Pro-ADP from New Objective, Cambridge, MA). This assembly enabled sample solutions that were collected in a capillary tube to be introduced by direct infusion at flow rates $\leq$100 nL/min. Fused silica capillary emitter tips (FS 360-50-8-D-20) were purchased from New Objective. The capillary voltage was -2.1 kV, and the cone voltage was 25 V. Nitrogen was used as the desolvation gas. Argon was used as collision gas for product ion MS/MS experiments, and the pressure in the collision cell was set to diminish the intensity of the ion beam by $\sim$40% (chamber reading of 1×10^{-4} mbar when collision gas was admitted). The collision energy was 15 eV (laboratory frame of reference, E_{lab}), and the mass resolving power was measured as 9000, full-width-at-half-maximum definition (FWHM). Spectra were acquired in profile mode with a 5-sec acquisition duration (summing of multiple scans) and a 0.1-sec interacquisition delay. The instrument was calibrated initially with a mixture of polyethylene glycols having average molecular weight distributions centered at 200, 300, 600, and 1000 D using a fifth-order polynomial, multipoint function. Spectra were obtained with the MS1 (Q_1) set to maximize beam transmission of the parent ion (LM/HM settings of 5/5) and the TOF mass analyzer set to acquire from *m/z* 50–300.

2.3. Chemicals and reagents

A pharmaceutical test mixture was prepared in-house. It consisted of an active pharmaceutical ingredient (multiply substituted aromatic compound) fortified with 17 related substances at levels of 0.2% wt/wt with respect to the parent compound. Deionized water was obtained from a Milli-Q system (Millipore, Bedford, MA). HPLC-grade acetonitrile was obtained from EM Sciences (Gibbstown, NJ). Trifluoroacetic acid and tolbutamide were obtained from Sigma Chemical (St. Louis, MO). All solvents and reagents were used as received.

3. Results and Discussion

Evaluation of product composition (indicative of both purity and stability) is an important aspect of the pharmaceutical development process. Regulatory registration of medicines requires that drug-related substances be identified at levels as low as 0.05% w/w [105]. For more than twenty years, HPLC with UV detection has played a primary role in the determination of such related substances. The task of characterizing or identifying minor components of interest in complex pharmaceutical matrices has been aided substantially by tandem mass spectrometry. However, even this highly capable technique sometimes fails to provide the desired information, particularly when used with HPLC methods primarily intended to satisfy validation criteria rather than address mass spectrometric performance issues. Because these methods are developed for eventual use in manufacturing environments, they stress optimal chromatographic performance and ruggedness. To achieve the necessary standard of performance, methods sometimes use mobile phases containing components (either additives or organic modifiers) that cause significant signal suppression in both electrospray (ESI) and atmospheric pressure chemical ionization (APCI) modes. For example, the use of acetonitrile as an

organic modifier in methods using APCI detection has been reported to reduce the sensitivity for weakly basic (nonpolar) compounds such as steroids [106]. Similarly, the presence of trifluoroacetic acid (TFA), a commonly used additive for pH adjustment of mobile phases, negatively impacts electrospray ionization results (because of ion-pair formation or other phenomena) [107]. In other cases, the API interface may not provide optimal sensitivity at the high flow rates specified by some HPLC methods, as large volumes of solvent can pose difficulties. Because method parameters cannot be modified after completion of a validation protocol, there are often few viable alternatives when standard LC/MS (or LC/MS/MS) experiments fail to yield the desired qualitative information. One possibility for overcoming such obstacles is to employ a peak-trapping strategy that effectively decouples the chromatographic separation from the mass spectrometer. While a variety of separation techniques have been coupled successfully (on-line) with mass spectrometers, an off-line approach presents a useful alternative, as the two analytical processes can be optimized independently. The ability to chemically manipulate the sample and control the time frame of the mass spectrometric experiment (leading to maximum information extraction) are considerable advantages offered by this approach. There is no disputing that this tactic sacrifices gains in efficiency and throughput associated with on-line LC/MS experiments. However, the fact remains that certain intractable analytical questions (e.g., qualitative characterization of low-level compounds that ionize poorly) require unconventional approaches. This variation of classical sample isolation techniques (i.e., collection of chromatographic fractions followed by solvent stripping and additional chromatography) provides for convenient handling of small sample quantities. Multiple-column HPLC systems have been demonstrated previously as effective means for sample clean up (before the analytical separation) in the analysis of both environmental and biomedical samples [108–112]. In contrast to those applications, this work describes the use of small, highly retentive columns of various dimensions to trap a compound of interest after (rather than before) a conventional analytical HPLC separation. Comparable strategies have been described previously, including peak trapping with UV detection [113], the direct coupling of HPLC with high-resolution GC [114] and the use of a trapping column for peak focusing with thermospray ionization [115], continuous flow fast-atom bombardment ionization [116], and particle beam ionization [117]. The peak-trapping strategy apparently has received little attention since the advent of the API techniques.

A preliminary demonstration of the current approach has been reported [118]. In that study, it was noted that several of the key factors necessary for efficient retention of analytes on trapping columns, as well as their subsequent elution for mass spectrometric detection (primarily using electrospray ionization), displayed behaviors that trended in opposite directions. For example, the efficiency with which analytes can be retained on trapping columns increases as the flow rate of eluent to the column decreases. In addition to being advantageous for sample loading of trapping columns, low flow rates are also beneficial for releasing retained compounds from those columns because they produce a concentrated analyte solution (by virtue of the elution volume being minimized). However, the need to reduce solvent strength through the addition of a substantial volume of water to the eluent stream, makes low flow rates difficult to achieve. Reduction of the eluent solvent strength is an important factor for optimal retention of compounds on trapping columns because it results in the compound being retained as a discrete band at the head of the column, minimizing diffusion into the column bed. For a typical analytical separation operating at a flow rate of 1 mL/min, a postcolumn make-up flow of water (up to 3 mL/min) was added to the eluent stream to reduce the solvent strength. Although the low organic solvent content of this composite eluent favors compound retention on the trapping column, the high flow rate (high linear velocity through the column) severely diminishes the capacity of the column to effectively retain compounds of interest. Initially, the trapping experiment used columns having the same diameter as the analytical column used for the primary separation (4.6 mm). While compati-

ble with the high flow rates resulting from the aqueous make-up flow, the relatively large elution volumes required to release the analyte from these trapping columns produced a poor mass spectrometric response because of low analyte concentration (dilution). To enhance the mass spectrometric response by reducing the elution volume, and thereby increasing the analyte concentration of the eluent being presented to the mass spectrometer, the use of smaller diameter trapping columns (2 mm) was attempted. Unfortunately, they proved to be unworkable because of prohibitively high back pressures and a failure to capture compounds from eluent streams having high linear velocities. Thus, the methodology initially was limited to 4.6-mm diameter trapping columns despite their disadvantages.

To address some of these shortcomings, the new peak-trapping approach introduced a sample collection loop between the analytical and trapping columns to serve as a transfer device. This sample loop permitted the flow rates to and from each of the columns to be optimized independently. Thus, flow rates of more than 1 mL/min (aqueous make-up flow) could be used to reduce eluent solvent strength postanalytical column, whereas sample loading onto the trapping column could be carried out at flow rate of several hundred microliters per minute. This decoupling of analytical and trapping columns also could be exploited for additional concentration of the sample through a second trapping event using a capillary HPLC column. Small-diameter trapping columns enabled analyte collection in a final peak volume of $\sim$2 μL, which represents a 50-fold increase in concentration. Gains of this magnitude can be beneficial in situations when it is necessary to elucidate the structure of low-level mixture components. Possible applications of this improved column-based compound isolation scheme include, first, structural characterization of compounds separated using a mobile phase containing high concentrations of nonvolatile additives (e.g., buffers such as borate, phosphate, or citrate salts; ion-pairing reagents such as sodium dodecyl sulfate; or chiral reagents such as cyclodextrins); second, analysis of organic acids separated using a mobile phase containing trifluoroacetic acid; third, improving the detection limits of low-level compounds through sample concentration (analogous to multiple passes of fraction collecting); and fourth, extension of data acquisition times. If one of the API techniques is most appropriate, an elution solvent can be chosen to maximize signal response (absent problematic solvents or additives). If a different ionization mode is more appropriate (e.g., electron ionization [EI] or classical chemical ionization [CI]), the sample can be retained in a small volume of solvent for transfer to another instrument. Once the compound of interest has been separated from the mixture and retained on the trapping column, the analyst has complete flexibility in choosing the appropriate conditions for optimal mass spectrometric detection irrespective of the constraints imposed by the conditions of the original analytical HPLC separation. For example, in electrospray ionization, the observed response for a compound may be enhanced in one or more ways, including, first, increasing the analyte concentration (by eluting the compound from the trapping column in a minimal volume of solvent); second, changing the composition of the eluent to reduce surface tension [119] or alter the pH; and, third, optimizing the eluent flow rate (to favor formation of optimally sized droplets). Under APCI conditions, changing the composition of the eluent can favorably alter the nature of the reagent ion plasma, thereby improving the chemical ionization response.

An understanding of the chemical stability of putative therapeutic agent depends on knowledge of its intrinsic reactivity under relevant conditions (i.e., in the presence of other components of the finished pharmaceutical formulation, when exposed to various levels of moisture, and when stored at specific temperatures). When these compounds are challenged with accelerated stress conditions (e.g., elevated temperature, elevated relative humidity, overpressure of an oxidative atmosphere, extremes of pH, photolysis, etc.) chemically complex mixtures often result. These mixtures of related substances (which can also include low-level residual synthetic by-products) often contain a variety of chemically disparate molecules because of the diversity of substructures and functional groups present in pharmacologically active mole-

Table 2
Illustration of differential detectability for complex mixture components exhibiting dissimilar chemical proterties

Test mixture component	Molecular weight (u)	Ionization mode			
		ESI+	ESI+	APCI+	APCI+
1	184	+	−	+	−
2	205	+	−	+	−
3	287	−	+	+	+
4	239	+	−	+	−
5	239	+	−	+	−
6	287	+	−	+	+
7 (active pharm. ingredient)	239	+	−	+	−
8	283	+	−	+	−
9*	173	+	−	+	−
10*	273	+	−	+	−
11	184	−	−	−	−
12	184	−	−	−	−
13	156	−	+	−	+
14	182	−	−	−	−
15	168	−	−	−	−
16	212	−	−	−	−
17	350	−	−	−	−
18	350	−	−	−	−

Note. + detected; − not detected; * coeluting isomers.

The test mixture was prepared using an active pharmaceutical ingredient and 17 known related substances. Each related substance was present in the mixture at a level of 0.2% w/w with respect to the active ingredient. The amount of text mixture injected for each of the five chromatographic analyses (UV, APCI+, APCI−, ESI+, and ESI−) was identical (5 μg). Although the UV detector exhibited a chromatographic peak for all 18 compounds, the API mass spectral responses varied widely.

cules. This diversity of chemical structure poses a challenge not only for the chromatographic methods required to separate these compounds but also for any detection schemes employed. Consequently, it is not uncommon for one or more compounds of interest to go undetected in the course of a chromatographic analysis. An illustrative example of this situation is shown in Table 2, which compares chromatographic responses generated by a typical UV photodiode array detector with those generated by a mass spectrometer equipped with an API source. While some of the related substances were detected successfully by the mass spectrometer, several compounds were missed in one ionization mode or another and others were missed entirely. Fig. 2 displays the elution profile, negative ESI mass spectrum, and product ion MS/MS spectrum for one of the compounds isolated from the test mixture using the peak-trapping apparatus. This compound produced no signal under positive ESI conditions (spectrum not shown). It is clear that this particular component of the sample mixture requires negative ion conditions for optimal detection and characterization. While not employed routinely, most commercially available instruments are capable of rapid polarity switching to achieve detection of both positive and negative ions (in alternate scans) during a given chromatographic analysis [120]. However, success in analyzing for both positive and negative ions relies not on the ability of the instrument to acquire such data but, rather, on the probability that any single set of API conditions would simultaneously favor the formation and stabilization of both types of ions. This is not to say that both positive and negative ions are not formed simultaneously under a variety of mass spectrometric conditions. However, the response surfaces for optimization of the processes underlying formation and detection of the oppositely charged species are likely to be quite different. The composition of a typical HPLC mobile phase is often more conducive to the formation of positive ions than it is to negative ions. For example, from the perspective of optimal chromatographic performance of carboxylic acids, free carboxylate anions are undesirable (prone to nonideal peak shape). Most reverse-phase HPLC separations strive to ensure that organic acids in the sample are present in the $-COOH$ rather than the $-COO^-$ form through control of mobile-phase pH (maintained well below the pK_a of common carboxylic acids). This strategy results in good chromatography but makes formation of carboxylate anions for negative ion mass spectrometric detection difficult. Another way to improve mass spectrometric detectability of a compound is to increase the concentration of analyte presented to the mass spectrometer by employing a multiple–collection cycle trapping scheme. Subsequent elution of the pooled sample produces an enhanced response. This strategy has been employed previously and was shown to yield a fivefold gain in analyte response (electrospray ionization) resulting from five successive injections onto the analytical column, followed

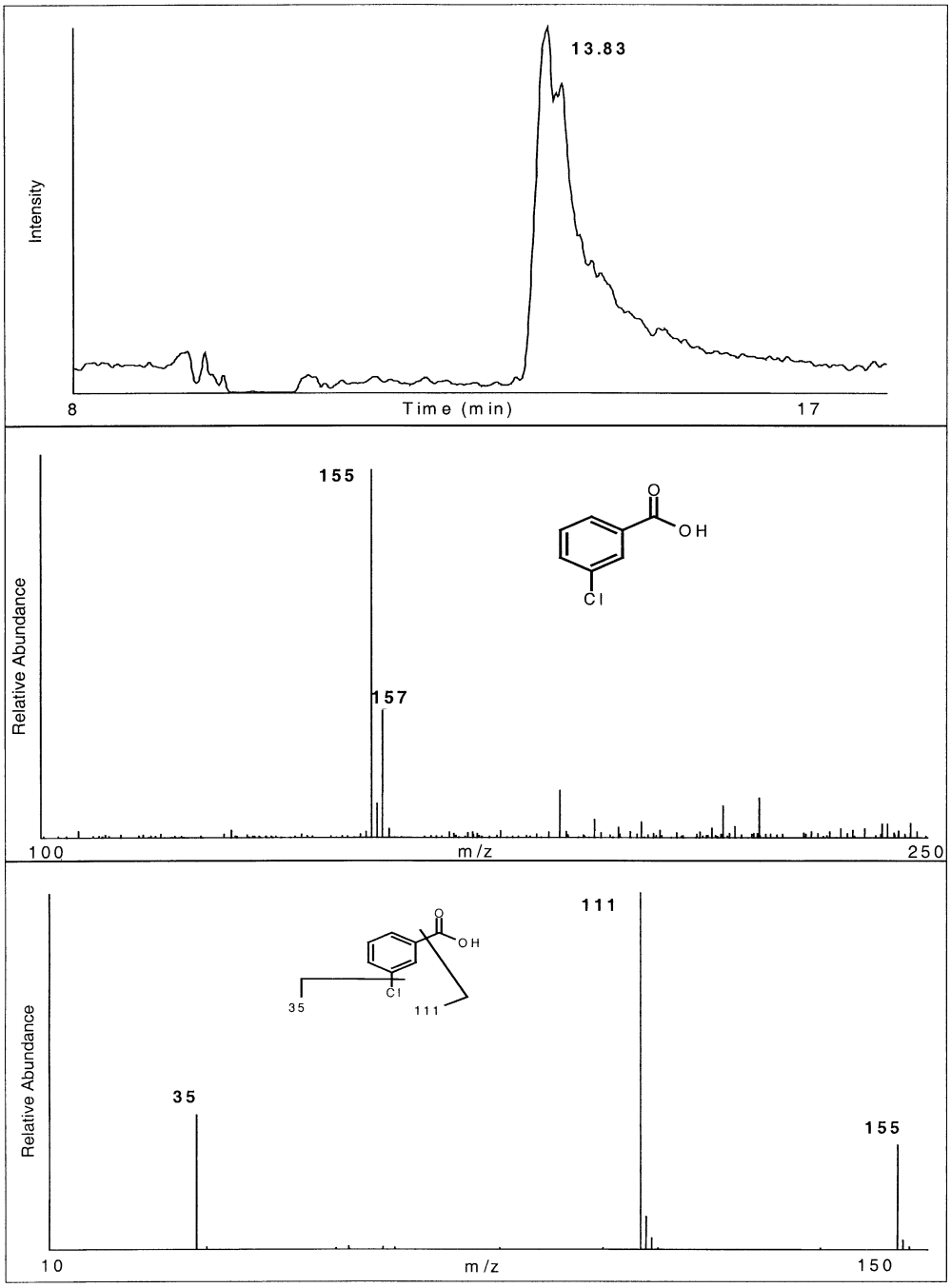

Fig. 2. Top panel: composite mass chromatogram for the $[M–H]^-$ isotopic cluster (*m/z* 155 and 157) of *m*-chlorobenzoic acid isolated and concentrated from the pharmaceutical test mixture and subsequently eluted for mass spectral characterization (under optimal conditions). Middle panel: negative electrospray ionization mass spectrum of the eluted *m*-chlorobenzoic acid. The compound was a minor component of the mixture (0.2% w/w). The total weight of mixture loaded onto the analytical column was 5 μg. Bottom panel: collision-induced dissociation spectrum (product ions) for the $[M–H]^-$ ion (m/z 155) of *m*-chlorobenzoic acid.

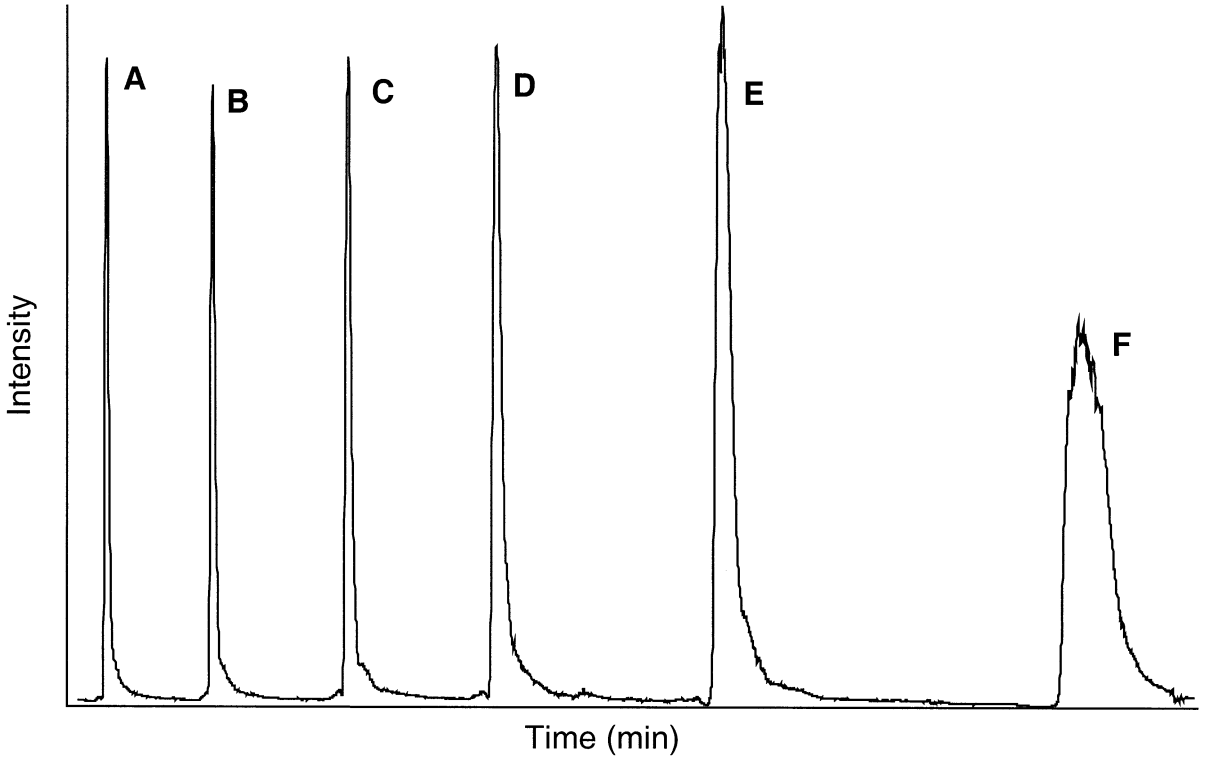

Fig. 3. Replicate injections of *m*-chlorobenzoic acid (1.3 nmoles) trapped on a Magic Bullet column and eluted using different flow rates. Injections A–F were eluted at 50, 40, 30, 20, 10, and 5 μL/min, respectively. The areas under the traces were as follows: A = 85.7e06; B = 94.6e06; C = 127.9e06; D = 193.4e06; E = 340.0e06; F = 386.9e06.

by peak trapping. The magnitude of increase in the observed mass spectral response corresponded approximately to the number of trapping cycles. The multiple-cycle trapping experiment was well tolerated by the test analyte (>50 column volumes of reduced solvent strength eluent flowed through the trapping column with negligible loss of the trapped analyte). However, this somewhat laborious process could be replaced by a single-pass collection using the new multiple-column scheme with smaller-diameter trapping columns. The use of the low flow rates associated with the smaller-dimension trapping columns also appeared to have a positive impact on the efficiency of the electrospray process in addition to boosting the ESI response by virtue of increased sample concentration. The total integrated signal obtained from a constant amount of analyte (trapped from replicate injections) increased as the eluent flow decreased. Fig. 3 displays the extracted negative ion mass chromatogram for the elution profile (replicate column loading and elution) for 200 ng of *m*-chlorobenzoic acid using both a Magic Bullet trapping column and acetonitrile as the releasing eluent at varying flow rates. An approximate fivefold increase in peak area was observed as the flow rate was reduced from 50 to 5 μL per minute. Although the elution peak volume from the trapping column was sixfold less than that from the analytical column (25 μL vs. 150 μL), the scale of the experiment may still be insufficient for isolating and analyzing low nanogram levels of compounds present in an analytical HPLC separation using only a single pass.

Despite the prevalent use of HPLC for the analysis of compounds that lack polar functional groups, mass spectrometric detection using one of the API techniques is often an inappropriate (or, at best, nonideal)

choice. Nonpolar compounds (e.g., alcohols, ethers, ketones, or aromatic and aliphatic compounds) generally do not ionize (i.e., either protonate or deprotonate) under the solution-phase conditions of the standard HPLC mobile phase and, therefore, produce little if any detectable ESI signal. APCI may produce responses for some of these nonpolar-compound classes but, perhaps, not for all. If the gas phase basicity of some of the neutral analytes is less than that of the conjugate bases of the reagent ions (e.g., $[CH_3CN]H^+$), proton transfer does not occur and no signal is observed in the positive-ion mass spectrum. Similarly, unless there is some potential site of deprotonation in an analyte molecule (forming a $[M–H]^-$ ion), there will be no response in the negative-ion mode. Changing organic modifier composition in HPLC methods (to facilitate analyte ionization) can introduce dramatic changes in the retention behavior of many compounds. So once again, we see the conflict between experimental conditions favoring optimal chromatographic behavior (e.g., higher solvent strength, lower wavelength cut-off for UV detection, lower back pressure caused by lower viscosity, etc.) and those that are ideal for mass spectrometric detection. Methanol is a more favorable choice than acetonitrile as a reagent component of the APCI plasma (producing $[CH_3OH]H^+$ ions) on the basis of its lower gas phase basicity. Thus, a wider variety of nonpolar analytes will ionize (or produce more intense signals) when this more appropriate eluent is chosen. This behavior has been illustrated previously for the on-line analysis of various steroids [106]. Acetonitrile can be used for the analytical separation (taking advantage of its positive chromatographic attributes) and then replaced (in the elution step of the peak trapping procedure) with methanol to optimize the APCI response of the trapped analyte. Alternatively, nonpolar analytes may be analyzed more appropriately using ionization modes that are better suited for the purpose, such as electron ionization or conventional chemical ionization with sample introduction via either GC or direct insertion probe, as appropriate. Maximizing analyte concentration is particularly important when samples are being prepared for GC/MS analysis because of the injection volume limitations of capillary GC columns (generally 1 μL of sample solution). For example, compounds in a typical analytical-scale HPLC separation elute in peak volumes of $\sim$100–150 μL. If such a compound were to be prepared for subsequent analysis by GC/MS, only $\sim$1% (or less) of the analyte would be introduced into the GC injector. This two-order-of-magnitude difference in sample volume could be critical to acquiring data having the necessary quality for structure elucidation of an unknown compound depending on the amount of material present in the original sample mixture. Thus, it would be desirable to increase the portion of sample available for GC/MS analysis by at least one order of magnitude, if not more. Any water that may be present in the HPLC elution volume (as a component of the original mobile phase) could also be eliminated, as its presence would be detrimental to the GC column stationary phase. Conventional sample isolation from an HPLC separation could require collection of multiple fractions, solvent/solvent extraction, and solvent stripping by rotary evaporation or other means. Losses of low-level compounds are not uncommon in such efforts because of the extensive sample manipulation (caused by surface adsorption, evaporation, etc.). The trap and elute approach described here offers significant advantages and has been used to isolate several nonpolar components observed in a HPLC chromatogram (UV detection at 226 nm) that yielded no mass spectrometric response under API conditions. Excellent information was obtained from their EI mass spectra following GC analysis (Fig. 4) once they had been separated from the original mixture (being retained on the trapping column and subsequently eluted with a small volume of organic solvent).

Successful reverse-phase HPLC separations often depend on stringent control of mobile-phase pH and ionic strength. The quality of the separation (as evaluated by peak shape and reproducibility of retention times) is highest when undesirable attractive interactions between stationary phase support and the analyte are minimized. Despite advances in HPLC column phase technology, silanol functional groups on the surface of the silica support can cause distortions in chromatographic peak shapes. One approach

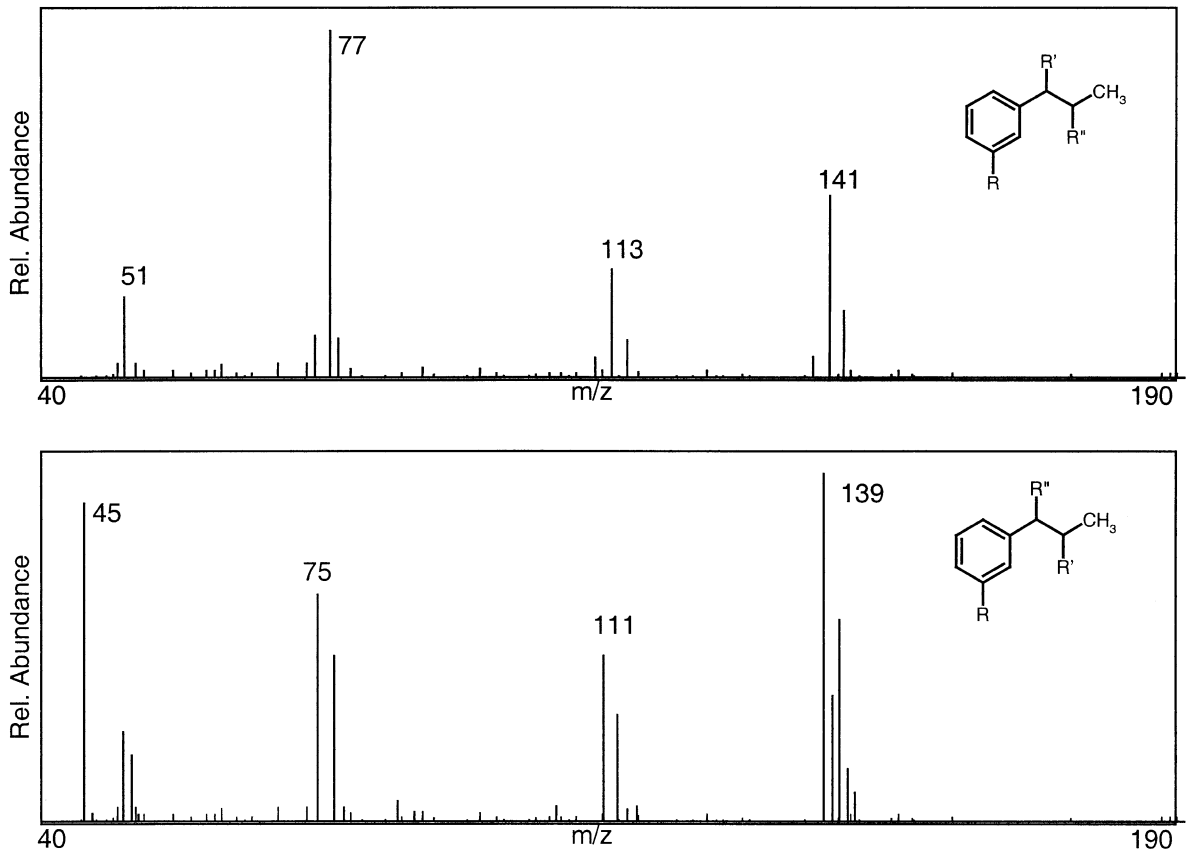

Fig. 4. Alternative detection for nonresponding compounds under API conditions. Isomeric mixture components 11 and 12 (Table 1) produced no detectable signals in any of their API mass spectra. These small, nonpolar molecules were collected (individually) as they eluted from the analytical column using the peak-trapping device. Subsequent elution from the trapping column using minimal solvent volumes produced sample solutions that were presented for GC/MS analysis. Upper trace: EI mass spectrum of component 11. Lower trace: EI mass spectrum of component 12.

for dealing with this problem is regulation of the mobile-phase pH through the use of various additives (buffers). Unfortunately, many of the buffers commonly used for this purpose (sodium or potassium salts of phosphoric, citric, or boric acid) are nonvolatile. This lack of volatility causes them to be left behind when the eluent is evaporated in the LC/MS interface, unlike organic acids such as acetic acid, formic acid, trifluoroacetic acid, or their ammonium salts. Deposition of large quantities of conductive salts on electrostatic elements of the mass spectrometer interface (or ion source) results in serious deterioration of performance or catastrophic failure because of clogging of small-diameter orifices. The use of a phosphate-buffered mobile phase at a concentration of 100 mM would result in the accumulation of $>$200 mg of salt in the mass spectrometer interface during a 20-min separation. Obviously, the buffer salt could be removed from the mobile phase to avoid the undesirable precipitation; however, peak shape and compound retention order might be compromised by such a method change. Tolbutamide (50 ng) was injected onto a standard C_{18} reverse-phase analytical column (4.6 mm $\times$ 100 mm) and eluted with a mobile phase containing phosphate buffer to illustrate the feasibility of peak trapping as a solution to this problem.

Typically, HPLC methods for such compounds are buffered with phosphate (or other suitable buffer) salts to maintain constant pH during the course of the separation. The peak of interest (tolbutamide) was retained on a trapping column, washed with water to remove the phosphate salt, concentrated, and subsequently eluted for mass spectral detection using acidified methanol (0.1% v/v or ~27 mM formic acid) to prevent the undesirable nonvolatile buffer from entering the mass spectrometer interface. Shown in Fig. 5 are the electrospray ionization mass spectra obtained for tolbutamide under standard LC/MS conditions (phosphate-buffered mobile phase with acetonitrile as organic modifier at a flow rate of 1 mL/min) and then after peak trapping. The absolute intensity of $[MH]^+$ signal at *m/z* 271 increased by a factor of 50 as a function of sample concentration and optimization of ionization conditions after being trapped. Also shown in Fig. 5 is the collision-induced dissociation spectrum (product ions) for the $[MH]^+$ ion (*m/z* 271) of tolbutamide (after peak trapping). The data demonstrate the feasibility of peak trapping for eliminating incompatible mobile phase additives to obtain high-quality spectra.

A common strategy for developing HPLC methods compatible with mass spectrometric detection is to use only volatile additives such as acetic, formic, or trifluoroacetic acid or their ammonium salts. Although most volatile mobile phase additives preclude many of the difficulties caused by nonvolatile salts (*vide supra*), they are not without their own problems. Trifluoroacetic acid (TFA) is a popular choice for adjusting mobile phase pH because of its low pK_a, its volatility, and its favorable UV absorption characteristics (producing less background interference than other organic acids such as acetic acid or formic acid). Unfortunately, TFA can form strong ion pairs with basic compounds. The survival of these ion pairs through the electrospray ionization process is recognized as a primary factor responsible for the suppression of analyte signal intensity in the positive-ion mode. Under negative electrospray ionization conditions, the presence of TFA effectively obscures the response of carboxylic acid analytes because it protonates the acids, rendering them neutral molecules rather than carboxylate anions and is present at concentrations ~20,000 times greater than any of the low-level analytes of interest. Shown in Table 3 is the effect of TFA on the negative ESI response for *m*-chlorobenzoic acid. At relatively modest concentrations (35 mM or ~0.25% v/v), trifluoroacetic acid reduced the intensity of the $[M–H]^-$ signal of the analyte by three orders of magnitude. Three ways to overcome this deleterious impact of TFA are, first, to implement postcolumn addition; second, to remove the TFA from the mobile phase; or, third, to isolate analytes of interest by peak trapping. Postcolumn addition can be a way of compensating for the nonideal mass spectrometric characteristics of chromatographic mobile phases that contain organic acids such as TFA. The addition of a suitable base (e.g., aqueous ammonium hydroxide) has proven successful in enhancing the response of carboxylic acids eluted using a mobile phase of neutral pH (i.e., containing only water and organic modifier). Raising the pH of the eluent facilitates carboxylate anion formation, which is reflected in increased ion abundance in the negative-ion mass spectrum. Unfortunately, this approach has proven to be ineffective in our hands when the mobile phase contains TFA. For positive-ion electrospray, the use of postcolumn addition (of another organic acid) has been reported to enhance the ESI response of certain compounds in the presence of TFA [107]. Removal of the TFA from the HPLC mobile phase is certainly the most straightforward remedy to this problem. The obvious drawback is the reintroduction of the deleterious factors that prompted its use in the first place (e.g., one or more peaks in the chromatogram may undergo a shift in retention time or broaden, thereby reducing resolution, specificity, and peak capacity), despite being beneficial for the mass spectrometric response of low-level acidic analytes. This alternative may be acceptable in cases of simple mixtures. However, a high degree of reproducibility must be maintained for the successful validation of regulatory methods. Thus, when the integrity of the original method must be preserved, peak trapping provides a means for optimizing the detector response for an analyte despite the presence of potentially noxious additives in the analytical mobile phase.

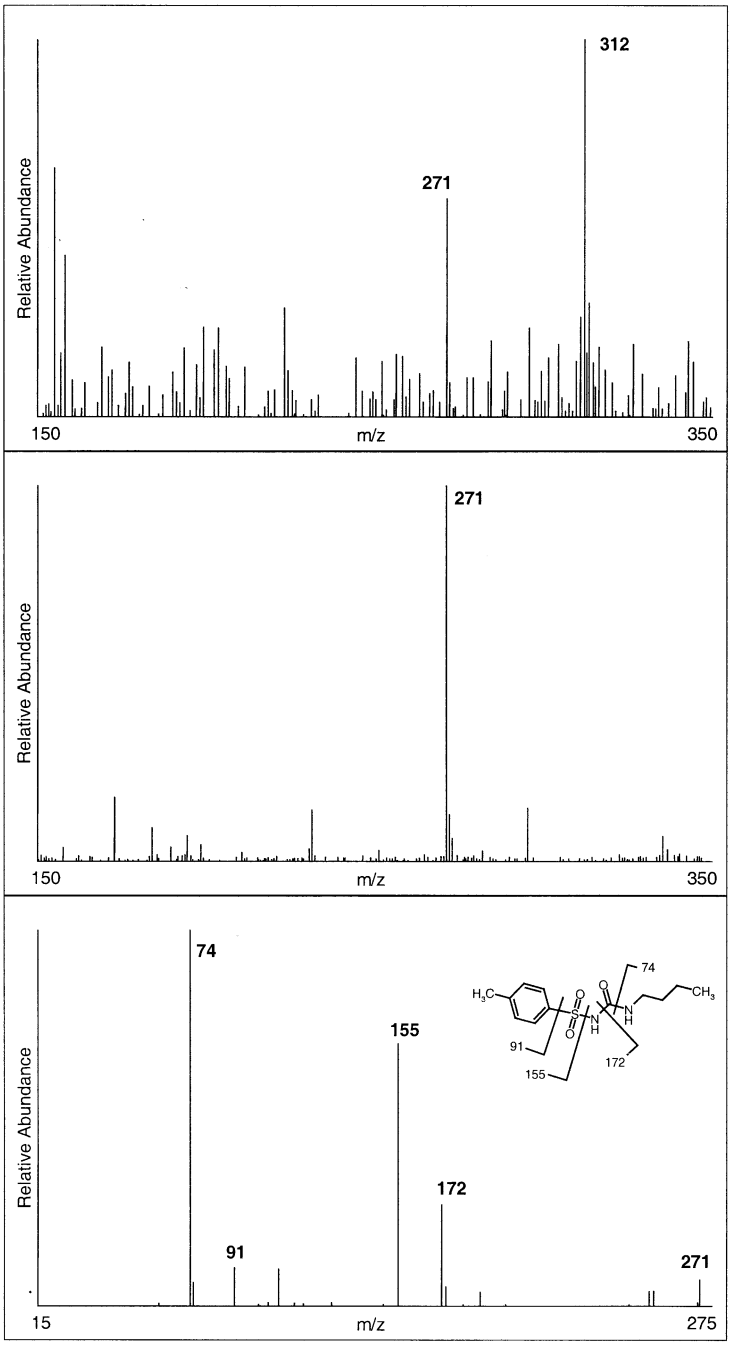

Fig. 5. Top panel: positive ESI mass spectrum of tolbutamide (~1.5 pmoles) under standard LC/MS conditions (acetonitrile used as organic modifier in a validated method). Signal-to-noise ratio (*S*/*N*) for the $[MH]^+$ (*m*/*z* 271) ion signal is very poor (~2). Middle panel: positive ESI mass spectrum of a replicate injection of tolbutamide (~1.5 pmoles) obtained after peak trapping. Absolute intensity of $[MH]^+$ signal at *m*/*z* 271 was increased by a factor of 50 because of sample concentration and optimization of ionization as a function of eluent composition (peak-trapping spectrum obtained using methanol acidified with formic acid). Bottom panel: collision-induced dissociation spectrum (product ions) for the $[MH]^+$ ion (*m*/*z* 271) of tolbutamide after peak trapping.

Table 3
Illustration of analyte anion suppression under atmospheric pressure ionization conditions (APCI and ESI) because of the presence of trifluoroacetic acid in the HPLC mobile phase. Response for the $[M-H]^-$ ion of *m*-chlorobenzoic acid as a function of TFA concentration.

TFA concentration (μM)	$[M-H]^-$ (*m/z* 155) abundance APCI (arbitrary counts)	$[M-H]^-$ (m/z 155) abundance ESI (arbitrary counts)
0	652,689	1,876,823
4	182,312	872,756
7	89,503	277,977
35	nd	nd
70	nd	nd
350	nd	nd

Note. nd = not detected above noise threshold of ~4000 counts.

In addition to using the peak-trapping apparatus to isolate and prepare analytes for mass-spectral detection, the system can also be used to conduct traditional spiking experiments for correlating chromatographic retention times. In situations where mixture components fail to yield mass spectra because of a TFA-containing mobile phase, negative-ion detection can be accomplished using a more amenable mobile phase containing either no additives or a more compatible additive such as ammonium acetate. This change in method conditions will provide access to the desired mass spectrometric information but will also likely change compound retention times. To correlate the retention times for any given compound between the two chromatographic methods, the peak-trapping device can be used to isolate the compound of interest from the ammonium acetate (or other) mobile phase and then release (co-inject) it simultaneously with a normal injection of the sample using the TFA-containing method. This variation of the classic standard spiking experiment provides retention time correlation for the analyte of interest between the two HPLC methods. Fig. 6 illustrates the spiking experiment using the peak-trapping device.

In spite of recent gains in the speed of chromatographic separations, the time scale remains relatively long with respect to the data acquisition times of most mass spectrometers. Several mass spectra routinely can be acquired in a few seconds' time. However, there are relatively few separation methods used for characterization of complex mixtures that can be performed in such a brief time interval. In fact, many of the HPLC methods included in regulatory applications for the marketing of ethical pharmaceutical products require analysis times of tens of minutes. Consequently, the mass spectrometer spends much of the time "waiting" (actually collecting redundant mass spectra of chromatographic baseline) to acquire information about relevant or interesting compounds entrained in the chromatographic eluent. For example, a standard LC/MS analysis (i.e., a regulatory method for the determination of related substances) may require ~15 min for a compound of interest to elute. If we assume a peak width of 10 s and a data acquisition rate of one mass spectrum per second (not unreasonable for a typical quadrupole mass spectrometer), we see that to acquire the 10 relevant spectra (containing information about the compound), the instrument has waited 15 min and acquired 900 spectra (with the large majority containing no useful information). Moreover, in the event that those 10 relevant spectra prove insufficient to provide the necessary information about the analyte (thereby requiring that additional data be acquired), another 900 s must be invested to yield another 10 s of potentially useable information. If instead, the chromatographic separation was conducted independently, a particular component of the sample could be captured (peak trapped) and analyzed subsequently. This rationale has been exploited by practitioners of the nanoelectrospray (or nanospray) ionization technique [121]. In nanospray ionization, the analysis of an extremely small amount of sample (typically on the order of picomoles or less of a peptide or protein isolated from a biological source and dissolved in 1–2 μL of solvent) is prolonged for several minutes. Signal averaging during this extended time period allows high-quality data to be acquired from an amount of sample that would yield little or no useable signal under conventional electrospray ionization conditions (i.e., chromatographic eluent flowing at several hundred or more microliters per minute). In a similar fashion, very small amounts of pharmaceutical related substances could be isolated and analyzed. Thus, the analysis could be performed over an ex-

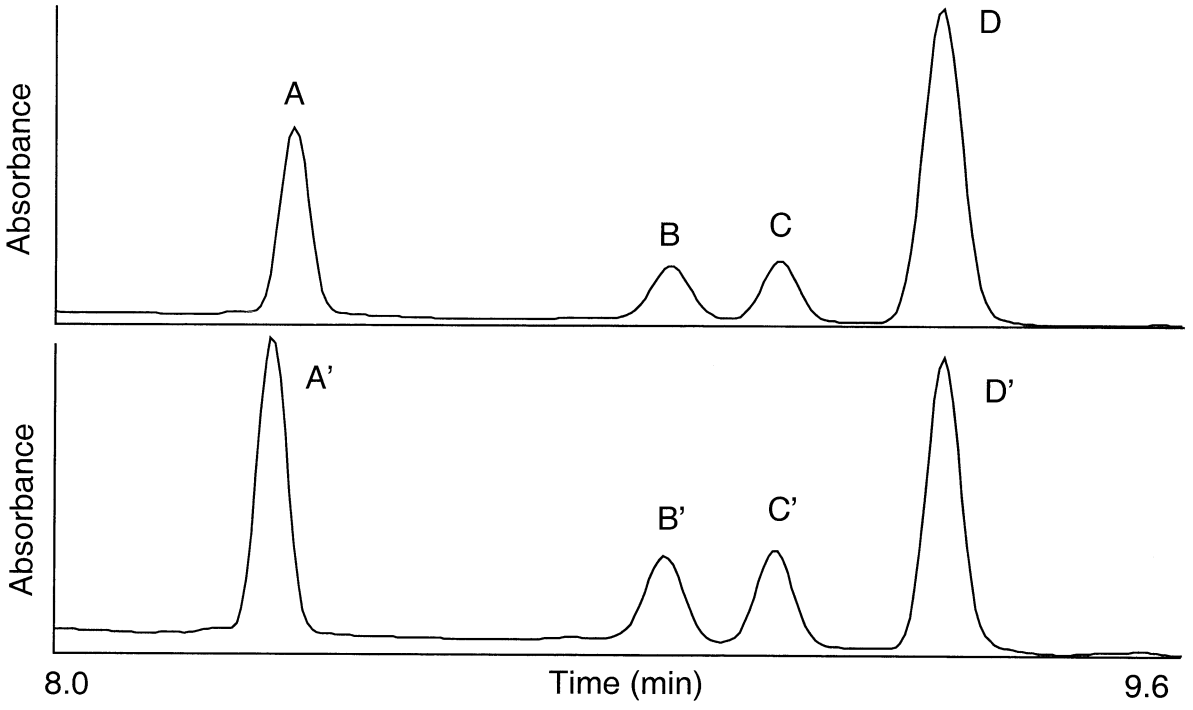

Fig. 6. Demonstration of sample spiking for retention time correlation in different HPLC methods. The peak-trapping device was used to isolate a compound of interest from a mobile phase amenable to negative-ion detection (no additives or ammonium acetate used as mobile phase buffer) and then release it simultaneously (coinject) with a second injection of the total mixture using a validated HPLC method (TFA-containing mobile phase not conducive to negative-ion detection). Lower trace: expanded region of HPLC chromatogram (UV detection at 226 nm) showing several minor components (peaks A′–D′). Peak D′ had been identified as *m*-chlorobenzoic acid using a negative-ion-compatible mobile phase. Upper trace: expanded region of HPLC chromatogram (UV detection at 226 nm) showing the same minor components (peaks A–D). The compound of interest (component 13, peak D′, which had been identified by mass spectrometry) is spiked back into the TFA-containing mobile phase using the peak-trapping device. Its coelution with peak D correlated its retention time and identity in the two complimentary HPLC methods. Peak areas: A = 47941; B = 17768; C = 18470; D = 108506; A′ = 48440; B′ = 17542; C′ = 18557; D′ = 55057.

tended period, with the duration dictated by the requirements of the mass spectrometric experiment and not the time scale of the chromatographic separation (either retention or elution time). Referring back to the example above, instead of 10 s of data acquisition time following 900 s (or multiple intervals thereof) of waiting time, an analyst could, instead, conduct the chromatographic separation once and then acquire several minutes (or more) worth of mass spectrometric data. This scenario has several obvious advantages. First, a full compliment of mass spectrometric experiments could be performed as a result of the extended acquisition time. High-quality (i.e., excellent *S*/*N*) full-scan mass spectra, dissociation spectra of different varieties (i.e., product ion, precursor ion, and neutral loss scans), positive- and negative-ion experiments, and accurate mass determinations could all be acquired as a consequence of prolonged analysis time. Although there are instruments capable of very rapid scan times and data-dependent acquisition, such a comprehensive suite of experiments would pose a significant challenge to execute on a routine or repetitive basis. In addition, chemical modifications to the sample and or the solvent/eluent such as pH changes, deuterium exchange [122], or metal ion chelation could be made before analysis. Any one or some combination of several such manipulations (maximum information extraction) could provide the

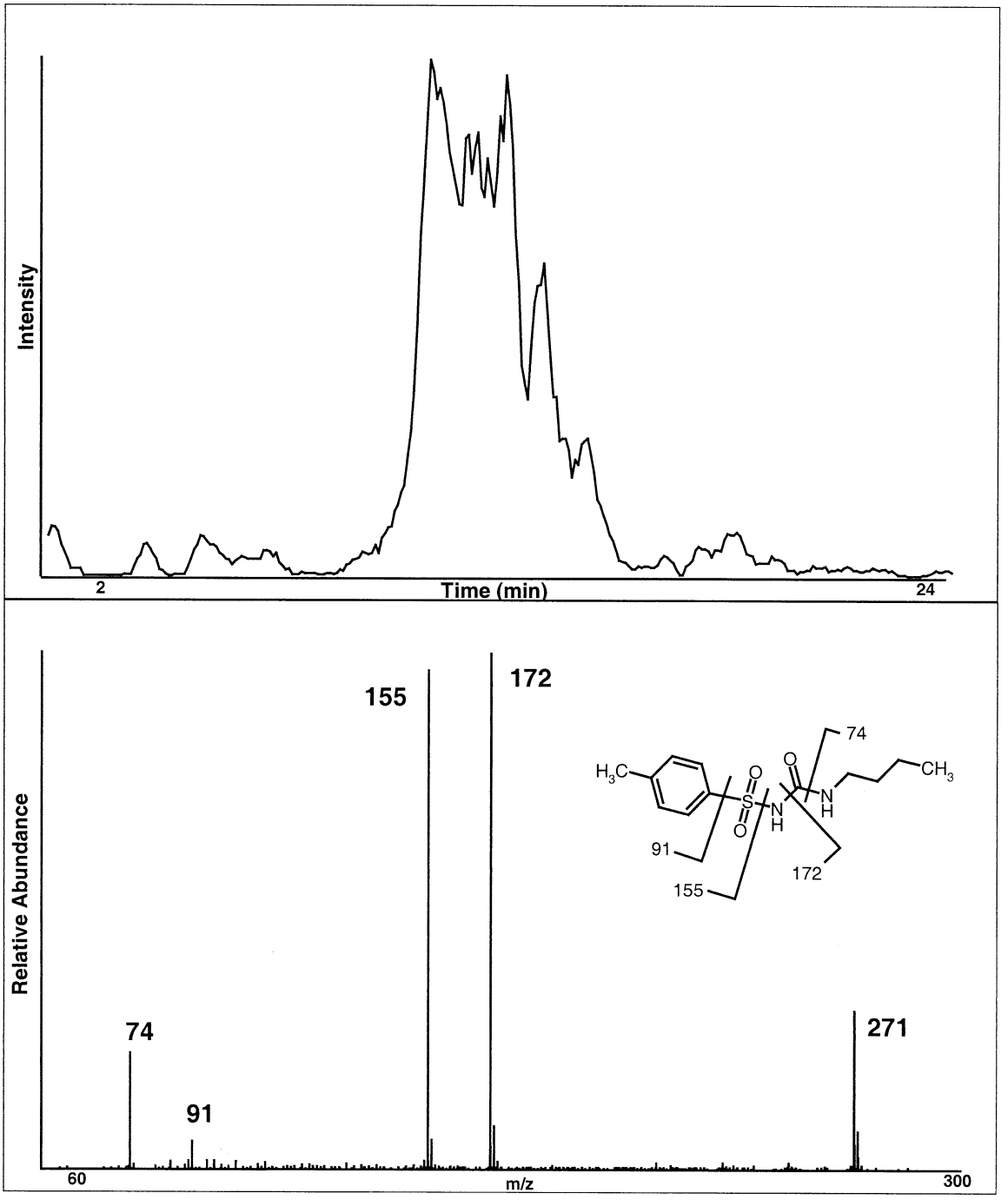

Fig. 7. Top panel: mass chromatogram for the $[MH]^+$ ion (*m/z* 271) of tolbutamide isolated and concentrated from a pharmaceutical test mixture and subsequently eluted for mass spectral characterization (under nanospray ionization conditions). Approximately 25 pmoles of the compound was trapped and eluted into the nanospray source over a period of nearly 6 min. Bottom panel: collision-induced dissociation spectrum (product ions) for the $[MH]^+$ ion (*m/z* 271) of tolbutamide.

key piece of information that enables an important analytical question to be answered. Fig. 7 shows the elution profile and MS/MS spectrum obtained for a few nanograms of the model compound tolbutamide isolated from a test mixture. This spectrum was acquired under nanospray ESI conditions from several microliters of sample solution collected from the peak-trapping apparatus. A stream of methanol from a syringe pump (flowing at ~30 nL per minute) was used to introduce the sample solution into the nanospray ion source over a 6-min timeframe.

4. Conclusions

Over the past twenty years, tandem mass spectrometry has continued to evolve and serve as an important platform for countless applications of complex mixture analysis. Many of these applications have come in areas of importance to the pharmaceutical research and development process, including those activities required for regulatory registration of new medicines. Although the application of on-line LC/MS and LC/MS/MS techniques for solving a variety of problems has become widespread, there remain situations where nonroutine solutions to problems of chemical characterization and identification are required. In many of these cases, the standard application of a prevalent analytical paradigm (i.e., the utilization of combined or hyphenated analytical techniques like GC-IR, GC-MS, LC-MS, etc.) fails to yield the desired outcome. Such failures are often caused in large part by various compromises required to achieve the dynamic linkage of two analytical techniques that possess inherently different characteristics. Conditions required for optimal reverse-phase chromatographic separations (i.e., eluent composition, pH, flow rate, ionic strength, etc.) many times are not optimal for or even compatible with efficient atmospheric pressure ionization conditions. In the past few years, there has been a shift away from elegant, if somewhat lengthy, chromatographic separations toward so-called fast separations. These rapid methods, carried out at high linear velocities on relatively short columns, increasingly depend on the specificity of detectors such as mass spectrometers for discrimination between chromatographically unresolved mixture components. In some cases, direct infusion of sample solutions is undertaken using no chromatography. Such direct mass spectrometric experiments can be executed in significantly less time than those including a chromatographic separation and result in a high degree of certainty for identity confirmation when tandem mass spectrometry is employed (except in cases where isomeric or isobaric species are possible). However, these experiments are subject to certain pitfalls because, unlike many other devices used as HPLC detectors, mass spectrometers are by their very nature chemical reactors. The environment to which analytes are exposed (e.g., high temperature, high electric field, corona discharge plasma, etc.) and the changes they undergo (desolvation or evaporation; change in mass caused by protonation, deprotonation, or some other solution or gas phase chemical reaction; excitation caused by collisional interactions [sometimes with accompanying fragmentation], etc.) during the detection process are quite different from those that occur in the flow cell of a standard UV-visible absorbance detector. And, like any chemical reactor, the conditions required for the optimization of a desired reaction, such as protonation or deprotonation (i.e., maximizing the yield of the desired product while concurrently minimizing undesirable processes and their associated by-products), can be quite specific. It, therefore, should come as no surprise that mass spectrometric detection can be compromised easily in situations where the HPLC eluent creates nonoptimal reaction conditions in the interface/ion source. As the contents of the ion source vary in composition and concentration with time, the possible outcomes can include differential sensitivity or detectability caused by differences in gas phase basicities or acidities, signal suppression caused by the presence of competing species and ion/molecule reactions that can deplete analyte ion populations (negatively affecting their characterization or detection). Thus, much of the tremendous power of the mass spectrometer as both a qualitative and quantitative detector can be negated by characteristics of the HPLC eluent that either diminish or eradicate its response for a particular analyte. In

such cases, a decoupling of the separation technique from the mass spectrometric experiment is not only advantageous but may be essential for success. Once separated from the chromatography, the mass spectrometric conditions may be optimized through modification of the eluent composition, extension of data acquisition time, or utilization of other types of instruments. Because traditional fraction collection and sample isolation techniques can be tedious, an alternative approach that employs the use of secondary trapping columns offers some advantage. Compounds eluting from the primary analytical HPLC column are retained on one or more small, highly retentive columns. Once isolated from the original mobile phase, the analyte can be washed and then released from a secondary column using an optimal organic solvent. Using this approach, not only can the composition of the eluent be optimized for maximum mass spectral response (e.g., high volatility and low surface tension for optimal droplet formation, favorable pH for formation and stabilization of either positive or negative ions, increase in analyte concentration, etc.), but the time scale of the mass spectrometric experiment can be substantially expanded to maximize data quality as in nanospray. Alternatively, more appropriate mass spectral techniques can be employed to produce the required information (e.g., EI or CI used with GC/MS). Unquestionably, these alternative approaches to the standard on-line experiment sacrifice certain desirable attributes such as efficiency and throughput. However, they can often provide insight and, ultimately, solutions to nonroutine problems in complex mixture analysis.

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