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Isolation of the components of a complex mixture by means of column switching for their enhanced detection by mass spectrometry

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Abstract

Mass spectral characterization of low-level impurities in drug substances and formulations may be challenging when using a validated HPLC method developed for optimal chromatographic performance. In many cases, either the mobile phase contains non-volatile additives that are deleterious to the operation of the mass spectrometer, or some of the related substances fail to ionize effectively under electrospray ionization or atmospheric pressure chemical ionization conditions. This paper describes a way to capture these low-level compounds from an analytical HPLC column using a small trapping column. Mixture components are retained on the trapping column by means of reducing the solvent strength of the eluent. Subsequent elution of trapped compounds using mobile phases more amenable to mass spectral analysis yields improved detection and characterization of low-level compounds of interest. Possible applications of peak trapping and elution include: (1) analysis of compounds separated using a mobile phase containing high concentrations of non-volatile additives, (2) analysis of organic acids separated using a low-pH mobile phase (containing trifluoroacetic acid), and (3) improving the detection limit of a low-level compound of interest through multiple collections. The peak trapping apparatus and optimization experiments are described.

Keywords: Column switching; Mass spectrometry; Pharmaceutical analysis; Peak trapping; Tolbutamide; Nortriptyline; Indomethacin

1. Introduction

The development of pharmaceutical products requires due diligence in the monitoring and determination of related substances arising from synthesis, formulation, or storage. Registration requirements set forth by the International Committee on Harmonization state that such related substances must be identified and qualified at levels as low as 0.05% [1]. Most often, high-performance liquid chromatography (HPLC) with UV detection is used for this purpose. Once optimized and validated, these analytical methods are suitable for the determination of known compounds at appropriate levels. However, new compounds may arise in the formulations when changing the synthetic scheme, during the manufacturing process, or upon storage. The qualification of these substances requires that they be identified unambiguously. In this regard, HPLC with mass spectrometric detection provides valuable qualitative information for new or unexpected related substances that arise during the development process.

The development and refinement of the electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) HPLC–mass spec-

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trometry (MS) interfaces has facilitated and improved the detection of these minor components over the past few years. Still, the ionization source may be inadequate for the identification and structural elucidation of related substances when using the validated HPLC method in which a new impurity may be detected. The ionization efficiencies of the mixture components being presented to the mass spectrometer by the HPLC system may be severely diminished by the mobile phase composition and pH. For example, trifluoroacetic acid (TFA), a modifier used commonly in HPLC methods, will suppress negative electrospray ionization of carboxylic acids. TFA may also suppress positive ionization of amine bases due to ion-pair formation. In other cases, the MS interface may not provide optimal sensitivity at the 1–2 ml/min flow-rate commonly used in HPLC methods.

Additionally, any complex mixture composed of constituents having disparate chemical properties (i.e., functional groups responsible for the solution- and gas-phase acidity or basicity of the molecule) will exhibit varied mass spectrometric response for the various components. In extreme cases, the characterization of important mixture components will be precluded. In situations when standard HPLC–MS techniques prove insufficient for comprehensive analysis of these components, some isolation and pretreatment of specific compounds is needed. Because conventional fraction isolation techniques are generally tedious and time consuming (involving repetitive collections, pooling of fractions, extraction, and removal of excess solvent), we have adapted a column-switching HPLC approach for isolating and characterizing low-level impurities for reversed-phase liquid chromatography (RPLC).

Although column-switching HPLC systems are an established technology used to improve selectivity and detectability, this technique is commonly used as an on-line sample clean-up step prior to the analytical separation. A short column is generally used in the first dimension to separate the analyte from potential interferences in the sample matrix. The analyte is then eluted onto an analytical column, separated from other remaining compounds, and detected. This technique is widely used in environmental and biomedical analyses and has been reviewed recently [2–5].

In contrast, this work describes the isolation (trapping) of low-level compounds from an analytical HPLC column (4.6 mm I.D., flow-rate ~1 ml/min) using a short column with the addition of a weak solvent. Subsequent elution of trapped materials using solvents more amenable for mass spectral analysis yields improved detection for the compounds of interest.

This paper describes the experimental apparatus for peak trapping in RPLC, discuss optimization experiments, and present several applications. The intent of peak trapping in this work is to improve mass spectrometric response and qualitative characterization of trace mixture components.

2. Experimental

2.1. Chemicals and reagents

Tolbutamide and indomethcin were selected as test compounds to characterize and optimize the performance in the peak trapping experiments. Although the technique may be applied to unknown eluents, these compounds were considered representative in structure, size and functionality to pharmaceutical compounds commonly analyzed.

Tolbutamide and indomethacin were obtained from Sigma (St. Louis, MO, USA). Nortriptyline was obtained as USP reference standard (US Pharmacopeial Convention, Rockville, MD, USA). Acetonitrile and methanol were obtained from Burdick & Jackson (Muskegon, MI, USA). Formic acid and ammonium formate were from Aldrich (Milwaukee, WI, USA). Acetic acid was obtained from EM Science (Gibbstown, NJ, USA). TFA, phosphoric acid and dibasic sodium phosphate heptahydrate were from J.T. Baker (Phillipsburg, NJ, USA). Purified water was from a Picosystem Plus with Picotech system (Hydro, Research Triangle Park, NC, USA). All reagents and solvents were used as received with no additional purification.

Test solutions of tolbutamide, nortriptyline, and indomethacin (0.01 mg/ml) were prepared in water. Methanol was used to aid dissolution.

2.2. Chromatography

Chromatography was performed using an HP1100

HPLC system (Agilent Technologies, Palo Alto, CA, USA). The analytical column was a Symmetry C_{18}, 3.5 μm, 100×4.6 mm I.D. (Waters, Milford, MA, USA). Separations were performed at a flow-rate of 1.0 ml/min using a mobile phase of 100 m*M* phosphate buffer, pH 2.0–acetonitrile (70:30, phosphate MP) or a mobile phase of water–acetonitrile–TFA (60:40:0.05, TFA MP). The injection volume was 5 μl or as noted. The UV detector was equipped with a high-pressure micro flow cell (Agilent). The detection wavelength was 235 nm.

2.3. Peak trapping

The peak trapping apparatus consisted of a six-port valve (Valco, Houston, TX, USA), a polyether ether ketone (PEEK) static mixing tee (Upchurch Scientific, Oak Harbor WA, USA), and two high-pressure pumps (Beckman 110B, Beckman Instruments, Fullerton, CA, USA; or ABI 400, Applied Biosystems, Foster City, CA, USA). The high-pressure pumps delivered the weak solvent and elution solvent, which were controlled independently. Columns used for trapping were Symmetry C_{18}, 5 μm, 20×3.8 mm I.D., or Symmetry C_{18}, 3.5 μm, 10×2.1 mm I.D. (Waters). A schematic diagram is shown in Fig. 1.

At the beginning of a peak trapping experiment the trapping column was flushed with a strong solvent and then equilibrated with a weak solvent.

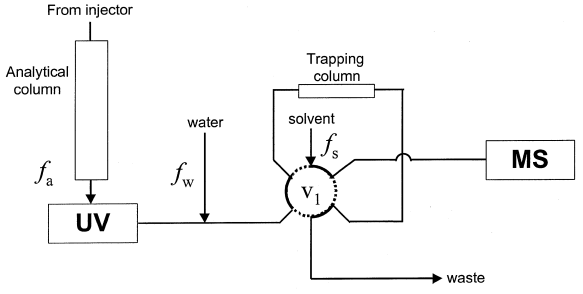

Fig. 1. Schematic diagram of the peak trapping apparatus. Legend: MS=mass spectrometric detector; UV=UV–visible absorbance detector; f_a=flow-rate of HPLC mobile phase; water=weak solvent delivered by high-pressure pump to the mixing tee; f_w=flow-rate of weak solvent; solvent=strong elution solvent delivered by a high-pressure pump to the six-port valve (v_1); f_s=flow-rate of strong solvent; ---------=position A of the six-port valve; ———=position B of the six-port valve (see text).

The strong solvent pump was deactivated, and the sample was injected on the analytical column.

The eluent from the analytical column (at flow-rate f_a) mixes with water or weak solvent (delivered at flow-rate f_w) in a static mixing tee downstream from the UV flow cell to reduce the solvent strength of the mobile phase. The combined flow (f_a+f_w) was directed to a six-port valve that directs the flow to either waste or the trapping column. When a peak of interest elutes from the analytical column the valve was switched from position A to position B to capture the analyte. Once trapped, materials were washed with water (or other weak solvent) for up to 30 min by bypassing the flow of the HPLC pump and cycling the six-port trapping valve to flush the trapping column of any buffer or other mobile phase additives.

After washing, the isolated compound was eluted from the trapping column using an appropriate strong solvent by deactivating the weak solvent and HPLC pumps and activating the strong solvent pump (at flow-rate f_s) with the six-port valve in position B (as shown in Fig. 1). The position of the trapping valve is then switched to position A so that the contents of the trapping column are flushed to the mass spectrometer for analysis.

2.4. Mass spectrometry instrumentation

Compounds eluted from the trapping column were analyzed by ESI using an HP 1100 MSD detector (Agilent). Where noted, the trapping column was transported to another laboratory for analysis using a TSQ 7000 triple-quadrupole mass spectrometer (Thermo Finnigan, San Jose, CA, USA).

3. Results and discussion

The peak trapping technique used in this work is a column-switching technique that uses a high-efficiency separation in the first dimension (i.e., the analytical method), and a small trapping column in the second dimension to collect a single analyte from the sample. Once a component is isolated on the trapping column its detectability may be enhanced in a number of ways including: (1) concentrating the analyte by eluting it in a small volume, (2) improv-

ing ionization efficiency by modifying the composition of the elution solvent, and (3) optimizing ion source parameters that affect sensitivity (e.g., flow-rate). Linder et al. demonstrated that post analytical-column addition of a weak solvent reduced the eluent strength sufficiently to allow trapping on a secondary column for further analysis by HPLC–UV [6]. Enhanced mass spectrometric detectability by means of solvent switching and peak focusing using a trapping column has been described previously for use with continuous-flow, fast atom bombardment ionization [7,8], and particle beam LC–MS [9].

Experiments were conducted to evaluate several parameters affecting peak trapping and mass spectrometric detection for model pharmaceutical compounds (tolbutamide, nortriptyline, and indomethacin) including: the size of the trapping column, f_w, f_s, mode of elution, and elution solvent composition. Enhancement of mass spectrometric detectability was explored by using elution solvents at various flow-rates comprised of different organic solvents, with some containing additives known to facilitate atmospheric pressure ionization. HPLC parameters such as f_a were presumed to be fixed to simulate a prescribed HPLC method.

3.1. Choice of trapping column

The factors that affect peak trapping include the retentivity of the trapping column, and the strength of the eluent entering the trapping column. In order to achieve a high capacity factor, *k*, in the trap we used Waters Symmetry C_{18} stationary phase because it is a high surface area silica (335 m^2/g) with high carbon load (19%). The column configurations chosen to determine retentivity were a 20×3.8 mm I.D. column packed with 5 μm particles, and a 10×2.1 mm I.D. column packed with 3 μm particles. Because of the small peak volumes expected, all tubing was 0.12 to 0.17 mm I.D. to minimize band dispersion between instrument components.

The data in Table 1 show that *k* for tolbutamide on the large and small columns is fairly consistent at 20 and 17, respectively, which was expected given that both contain a very similar stationary phase. Based on these data we would expect both columns to perform well as trapping columns. We hypothesized that the smaller trapping column with smaller particles would have less band broadening during elution with a corresponding increase in mass spectrometric signal intensity. However, tolbutamide was not trapped successfully on the small (10×2.1 mm I.D.) column.

Table 1
Retention of tolbutamide on trapping columns of different dimensions[a]

Dimensions (mm)	d_p (μm)	Nominal volume (μl)	t_R (min)	*k*	Pressure (bar)
20×3.8 I.D.	5	136	1.6	20	28
10×2.1 I.D.	3.5	21	0.3	17	73

[a] Columns are Waters Symmetry C_{18}. Mobile phase is 1.0 ml/min water–acetonitrile–TFA (60:40:0.05) plus 0.5 ml/min water (f_w). Nominal volume is estimated as $0.6\pi r^2 L$. The small column was not used for peak trapping because of inadequate trapping volume at a reasonable pressure.

To better understand the retention behavior of the two trapping columns we calculated the number of column volumes that flowed through each during trapping. Because the small trapping column volume is about 20 μl, the combined solvent flow-rate (analytical plus weak diluting solvent) of 1.5 ml/min is equivalent to about 75 column volumes per minute. If the trapping event is 0.5 min in duration, the *k* of the analyte must be very high (>50) to be retained adequately. In contrast, the larger column has a volume of about 135 μl, so the 1.5 ml/min flow-rate represents only about 12 column volumes per minute. In a 0.5 min trapping event, approximately six column volumes have flowed through the column, allowing successful trapping of the analyte at lower values of *k*.

The issue of insufficient column volume for the smaller column can be addressed by increasing f_w to produce an acceptable value of *k*. Because this increase in flow-rate generates prohibitive back pressure in the small column, this configuration can only be employed if the diluted eluent is first collected in a large sample loop, and then introduced to the small trapping column at a lower flow-rate as described by Asakawa et al. [8]. Taking all of these factors into consideration, the 20×3.8 mm I.D. (large) trapping column is better suited for trapping peaks directly from typical analytical columns because of its higher volume and lower back pressure,

allowing a wider range of analytical flow-rates and adjustments of f_w to optimize trapping efficiency.

3.2. Choice of elution solvent

Peak trapping enables the analyst to select a more optimal elution solvent for mass spectrometric detection. The magnitude of mass spectral response obtained for a molecule using electrospray ionization is affected by solution chemistry, as well as the efficiency of nebulization, desolvation, and ionization [10]. Under the conditions prescribed by a given HPLC method, the ionization process may not be optimal and detectability may be poor.

Several solvent mixtures were evaluated for the elution of the tolbutamide and indomethacin from the trapping column. Table 2 shows the relative mass spectral response obtained for the test compounds in the various solvents. A reduced response is seen when using a mixture of acetonitrile and water as the elution solvent as would be expected with no acid available to protonate the compounds for ESI^+ detection. Minimal responses were also seen for the solvent containing TFA, consistent with suppression of ionization due to ion-pair formation. Higher intensity signals were obtained using solvent systems containing methanol rather than acetonitrile. This behavior is attributed to proton affinity differences between the two solvents. Proton affinities of solvents can be a particularly important factor in dictating mass spectral response when analytes possessing relatively low proton affinities (gas-phase basicities) are competing with solvents possessing higher proton affinities [11]. Solvents containing formic acid and formate provided better response than similar solvents containing acetic acid or acetate. This is consistent with the observations reported by Temesi and Law [10], but may be instrument dependent. Based on these data, an elution solvent containing water, methanol, and formic acid was used for further studies.

The amount of methanol in the elution solvent was also studied by determining the mass spectral response for the test compounds (tolbutamide and indomethacin) when eluted from the trapping column. Optimization of this experiment is complex because of the inter-relationships of solvent composition on compound retention, efficiency of nebulization, desolvation, and ionization. The results, shown in Fig. 2, indicate that the maximum mass spectral response was obtained at an elution solvent composition of approximately 80% methanol, which was then used in further studies.

3.3. Elution flow-rate

ESI response exhibits a dependence on flow-rate due to the efficiency of nebulization and desolvation. Generally, ESI parameters are optimized for a single HPLC mobile phase composition and flow-rate,

Table 2
Effect of eluting solvent composition on the MS response of test compounds tolbutamide (at $[M+H]^+$ 271 u) and indomethacin (at $[M+H]^+$ 358 u)[a]

Ionizing solvent	% of maximum tolbutamide response	% RSD of response ($n=3$)	% of maximum indomethacin response	%RSD of response ($n=3$)
Water–MeOH, 0.1% formic acid	100	2	100	1
Water–MeOH, 10 m*M* ammonium formate	89	3	76	1
Water–MeOH, 0.3% acetic acid	80	4	65	2
Water–acetonitrile, 0.1% formic acid	77	2	69	3
Water–MeOH, 10 m*M* ammonium acetate	77	2	57	5
Water–acetonitrile, 10 m*M* ammonium formate	60	3	42	3
Water–acetonitrile, 10 m*M* ammonium acetate	58	5	37	8
Water–MeOH, 0.05% TFA	52	3	74	4
Water–acetonitrile, 0.3% acetic acid	52	3	40	0.3
Water–acetonitrile, 0.05% TFA	27	6	28	4
Water–acetonitrile	7	6	2	10

[a] The test compounds were eluted from the trapping column with the 40:60 (v/v) mixtures of the ionizing solvents described.

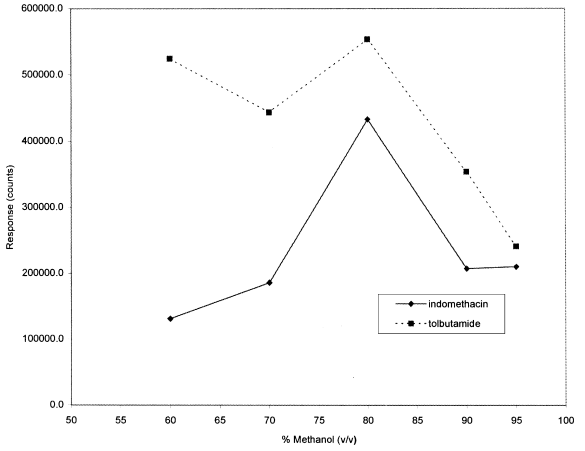

Fig. 2. Effect of eluting solvent strength on the MS response of tolbutamide and indomethacin. Solvents contain water and methanol in the ratios shown. Each solvent contained 0.1% formic acid.

despite the fact that the changing composition of the mobile phase can have a significant impact on the ionization response during a gradient elution experiment. Peak trapping allows the elution flow-rate to be controlled and optimized independently of the HPLC method parameters. Several injections of tolbutamide were trapped individually on the 20×3.8 mm I.D. trapping column and eluted at various flow-rates with methanol–water–formic acid (80:20:0.1). As seen in Fig. 3, the mass spectral response profile height is slightly higher at the high elution flow-rates (1–2 ml/min). While a decrease in flow-rate might be expected to decrease, significantly, the mass spectral signal because of a reduction in the amount of analyte being delivered to the mass spectrometer per unit time, the data indicate that the response is not diminished significantly at even 0.1 ml/min. This

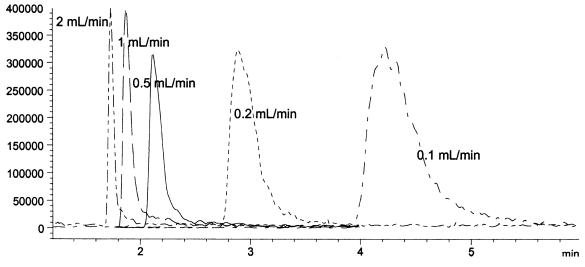

Fig. 3. Effect of elution flow-rate on peak response. Tolbutamide (50 ng) captured on trapping column and subsequently eluted at various flow-rates with water–methanol–formic acid (20:80:0.1).

behavior is consistent with increased ESI efficiency due to smaller droplet formation and more efficient desolvation, both of which are favored by lower eluent flow-rates. The source parameters in this experiment were not optimized for each flow-rate and further signal gains may be possible.

Because the analyte may be eluted from the trapping column at a low flow-rate, the duration of the elution event and subsequent mass spectral characterization can be extended (to the advantage of the analyst). Instead of being limited to capturing just a few mass spectra, the mass spectrometric analysis can be expanded to include ionization optimization, polarity switching, post column addition of ionization-enhancing reagents, collision energy optimization, and full MS–MS characterization of parent and fragment ions. The utility of such extended data acquisition times has been demonstrated for nanoelectrospray ionization [12–14].

3.4. Back flushing vs. forward flushing

Ideally, reduction of solvent strength should cause the compound being isolated to collect at the head of the trapping column with very little penetration into the stationary phase bed. Since one objective of the trapping experiment is to maximize mass spectral response, elution of the trapped compound into the mass spectrometer ion source should be accomplished with the smallest possible solvent volume. Reversing the eluent flow (back flushing) may seem the most effective way to achieve this objective. The response obtained by back flushing was compared to forward flushing tolbutamide from the trapping column (Fig. 4). The results indicate that while this component may elute faster when back flushing is employed, the mass spectrometric signal was not enhanced appreciably. Thus, while the baseline peak width for the elution of tolbutamide was decreased by approximately 50% by back flushing the trapping column, the peak signal actually decreased by 28%. The suppression of the MS signal seen when back flushing the trapping column for the tolbutamide peak may well be compound dependent. If the compound eluted before the trapping column was equilibrated with strong solvent, nebulization and desolvation may have been less efficient in the more aqueous solvent that was flushed off the trapping

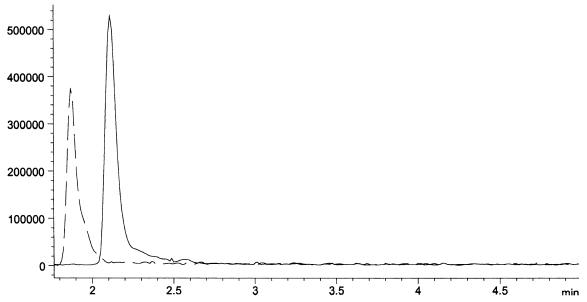

Fig. 4. MS response for $[MH]^+$ of tolbutamide (m/z 271) obtained with forward flushing (solid line) and back flushing (dashed line) the trapping column. A 50-ng amount loaded on trapping column, elution starts at 1.6 min. Elution solvent is water–methanol–formic acid (40:60:0.1).

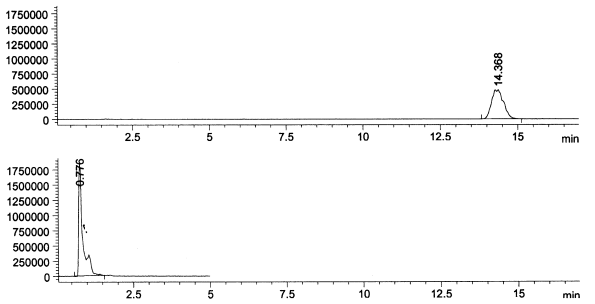

Fig. 5. Mass chromatogram for $[MH]^+$ of nortriptyline (m/z 264) using a TFA MP method (see text) on the analytical column (upper trace), and after elution from the trapping column with water–methanol–formic acid (10:90:0.1). Elution of the peak trap begins at 0.5 min, thus the of the peak eluted from the trap is 0.28 min. Peak response (height) is increased by 280%.

column with the analyte. Additionally, ionization may have been suppressed because the weak solvent flushed from the trapping column was not acidic. Since no advantage was gained by back flushing the trapping column, the remainder of the experiments employed elution in the standard direction of eluent flow.

3.5. Peak focusing

The ability to detect a compound in HPLC analyses ultimately depends on the height of the chromatographic peak above the baseline or background. For a chromatographic peak containing a given mass of material, reducing peak volume will result in a corresponding increase in peak height at a constant flow-rate. Thus, broad, late-eluting peaks in an isocratic chromatographic method may be concentrated on the trapping column and eluted in a smaller volume resulting in an enhanced signal response.

To demonstrate that peak focusing is a useful aspect of peak trapping and can enhance mass spectral sensitivity for broad peaks, 50 ng of nortriptyline was chromatographed using a mobile phase of water–acetonitrile–TFA (74:26:0.05). Those conditions produced a broad peak eluting at 14.4 min. The compound was chromatographed a second time, trapped and then eluted using a strong solvent with a retention (see Fig. 5). The peak response for the late eluting peak was enhanced by 280% by peak trapping. This effect was partially due to the enhanced ionization due to the change of solvent, resulting in a 33% increase in peak area, and partially due to peak focusing, with a 57% decrease in w_h.

3.6. Recovery

The intent of peak trapping in this work was to improve mass spectrometric response and qualitative characterization of trace mixture components. Quantitation was not the objective. Nevertheless, the recovery of the peak trapping and elution process was evaluated for a test compound.

The UV chromatographic response for 100 ng of nortriptyline injected on the analytical column was monitored as the compound was captured on the trapping column (first pass). The trapping valve was then configured as a sample loop injector, to elute the contents of the trapping column as a sample back through the analytical column to be monitored with UV detection (second pass). In this manner, the UV response of the compound eluted from the trapping column was compared to its response when it was eluted directly from the analytical column. By this means the recovery of the test compound (nortriptyline) was determined to be approximately 85%, as shown in Fig. 6. Recovery may be estimated for any unidentified analyte in the same manner.

This recovery efficiency compares favorably with that expected for a typical manual isolation technique such as solid-phase extraction, and is suitable for the intended purpose. Explanations for low recovery values could include incomplete capture of the peak

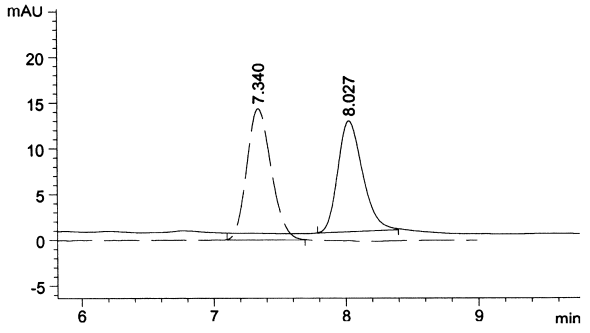

Fig. 6. Recovery of nortriptyline after peak trapping. Peak (100 ng of nortriptyline) at 7.3 min detected after the first pass through the analytical column. Peak displayed at 8 min represents the nortriptyline after being trapped and eluted back through the analytical column for detection (second pass). Recovery determined form peak areas was approximately 85%.

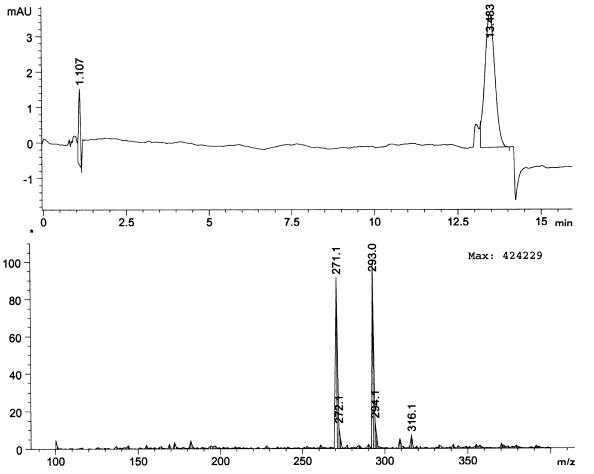

Fig. 7. Trapping of test compound tolbutamide from a mobile phase containing 70 m*M* phosphate buffer. Upper panel: UV chromatogram shows compound eluting from the analytical column at approximately 13.5 min. Perturbations in the peak shape and baseline are due to pressure disturbances associated with actuation of the trapping valve before and after the peak. Lower panel: mass spectrum obtained for tolbutamide after trapping, washing with water (5 ml) and subsequent elution into the mass spectrometer with water–methanol–formic acid (20:80:0.1) at a flow-rate of 1 ml/min.

(due to delayed actuation of the trapping valve), bleed through on the trapping column during trapping or wash step, or some level of irreversible adsorption.

3.7. Elimination of mobile phase additives

Non-volatile buffers, such as sodium phosphate salts or ion-pair reagents, are commonly used in HPLC methods. These additives have a deleterious impact on the mass spectrometer when they precipitate on the electrostatic elements of the instrument [15]. Occasionally, the HPLC method may be modified to allow direct LC–MS detection by employing volatile mobile phase additives, but changing the chromatographic conditions may confound peak identification as peak elution order may also change. We evaluated peak trapping as a means of eliminating non-volatile buffers and thereby isolate and identity a peak from a chromatographic separation that was not directly compatible with mass spectrometric detection.

A tolbutamide sample (50 ng) was injected on the analytical column and eluted with a mobile phase containing 70 m*M* phosphate buffer. The peak was trapped, washed, and eluted for mass spectral detection. Displayed in Fig. 7 is the mass spectrum obtained for tolbutamide after peak trapping. The data demonstrate the feasibility of peak trapping for removing incompatible mobile phase additives in order to obtain quality mass spectra.

3.8. Ionization suppression

In typical reversed-phase HPLC separations, acidic mobile phases are used to protonate free silanols in the stationary phase and thus reduce interactions with polar analytes such as amines and carboxylic acids. While this strategy enhances chromatographic performance it will also suppress the ionization of acids thereby hindering mass spectral detection. In addition, in certain instances TFA exhibits considerable ion-pairing with amines as noted earlier. When these strongly bound ion pairs are desolvated they carry no net charge and thus are undetectable by the mass spectrometer.

In a recent decomposition study, a compound was thought to form diflurobenzoic acid, but peak identity could not be verified by LC–MS due to presence of TFA in the mobile phase. Table 3 shows the suppression of the negative ESI response for di-

Table 3
Suppression of negative ion formation in ESI[a]

TFA concentration (μM)	MS response (arbitrary units)	Signal suppression (%)
0	10 031 083	0
4	3 864 857	61.5
7	1 151 571	88.5
35	10 586	99.9
70	2031	100
350	0	100

[a] Responses of the $[M-H]^-$ ion of difluorobenzoic acid as a function of TFA concentration in the mobile phase. Analyte signal is completely suppressed in the presence of as little as 350 μM TFA.

fluorobenzoic acid with micromolar levels of TFA present in the mobile phase. At a concentration of 35 m*M*, TFA reduced the intensity of the $[M-H]^-$ signal by three orders of magnitude. Still, such compounds may be detected after peak trapping from the TFA-containing mobile phase with adequate washing. Fig. 8 shows the mass spectrum of difluorobenzoic acid obtained after being trapped and washed with 30 ml of water. This experiment demonstrates the utility of peak trapping to remove mobile phase components that suppress ionization.

3.9. Multiple peak collections

Molecules exhibit disparate responses between UV and mass spectrometric detection due to their intrinsic physical properties. In cases where the UV absorbance of a molecule is substantial, and its solution-phase or gas-phase basicity (or acidity) is modest, little or no mass spectral signal is obtainable for a minor peak in an HPLC method. One approach to increase sensitivity to concentrate the analyte of interest by trapping the compound from separate repetitive HPLC injections on the trapping HPLC column. Subsequent elution of the combined sample from the trapping column should lead to enhanced mass spectral response.

Fig. 9 shows the mass spectral response from tolbutamide collected on the trapping column from a single HPLC injection (1×) and then from five successive injections (5×) on the analytical column. Control of the trapping valve was performed by the HPLC software such that multiple collections were performed unattended. In this example the 5×

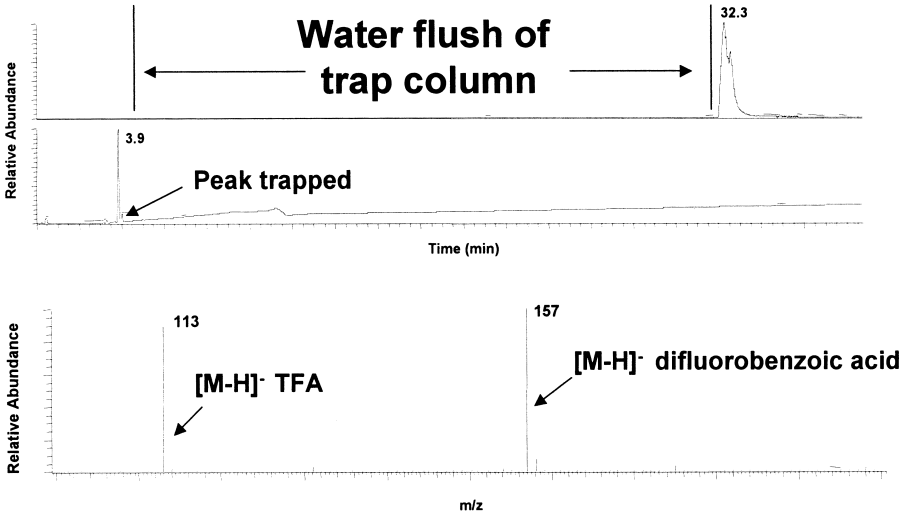

Fig. 8. Upper panel: HPLC peak (lower trace) eluted from analytical column using a TFA-containing mobile phase (3.9 min, UV detection) was trapped and washed with water for 30 min at 1 ml/min. Mass chromatogram (upper trace) shows the MS response from the peak eluting into the MS from the trapping column after a total time (wash+elution) of 32 min. Lower panel: ESI mass spectrum of the peak eluting at 32 min showing the $[M-H]^-$ ion for difluorobenzoic acid (m/z 157).

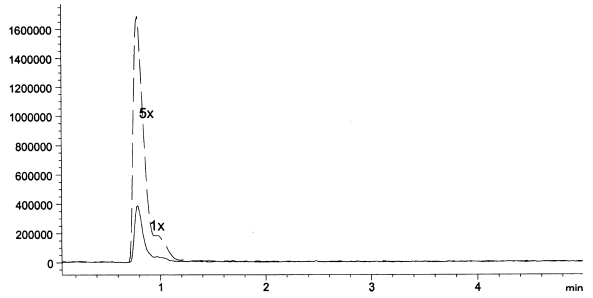

Fig. 9. Elution profiles for the test compound tolbutamide demonstrating the capacity of the trapping column to retain multiple collections from the analytical column prior to elution into the mass spectrometer. Traces show the responses (mass chromatograms for m/z 271, $[MH]^+$ for tolbutamide) from one (solid line) and five (dashed line) repetitive trapping cycles.

sample produced a nearly proportional (factor of 4.5) increase in the mass spectrometric signal. In the 5× experiment approximately 88 column volumes of HPLC mobile phase and weak solvent were directed through the trapping column without significant loss of analyte. It is important to use a trapping column of appropriate scale, and an f_w of adequate volume, for multiple peak trapping without eluting the analyte from the trapping column.

3.10. Interlaboratory analyses

Peak trapping may be useful in isolating and transporting samples between laboratories so that they may be analyzed using sophisticated analytical techniques such as MS–MS, nuclear magnetic resonance (NMR), and Fourier transform (FT) IR, which are often required for the structural elucidation of an unknown compound. Isolating these unidentified compounds in a separate (off-line) step may make this process more efficient. In particular, LC–MS–MS can often be inefficient for the identification of a minor HPLC impurity because a large fraction of the analysis time may be spent waiting for the peak of interest to elute, relative to the small amount of time available to interrogate the compound in the mass spectrometer.

We evaluated the feasibility of using the relatively simple HPLC peak-trapping apparatus to collect samples, and then transported them for analysis by a relatively sophisticated LC–MS–MS instrument in another laboratory. An injection of tolbutamide was trapped from the analytical column using the phosphate mobile phase on the 20×3.8 mm I.D. trapping column. The column was rinsed with 0.1% formic acid and transported to another building for LC–MS–MS analysis using a TSQ-7000 triple-quadrupole mass spectrometer. A summary of the HPLC–trap-MS–MS experiment is shown in Fig. 10. Collision-induced dissociation (CID) was carried out to generate structurally diagnostic fragment ions for the

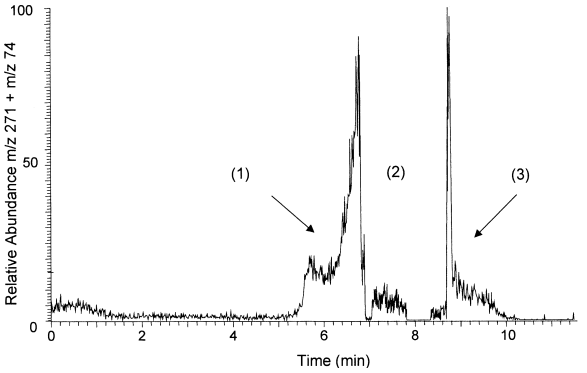

Fig. 10. Elution profile for tolbutamide after trapping and physical transport of the column (interlaboratory) prior to elution and analysis. Regions of the profile are; (1) flow-rate of 100 μl/min until elution from trapping column begins, as monitored by triple quadrupole MS, (2) flow-rate reduce to 50 μl/min as CID conditions and EM voltage are optimized, (3) CID experiment ends and flow-rate re-set to initial value and elution is completed. Reduced flow-rates at the time of elution allows sufficient time for mass spectral conditions to be optimized or for multiple experiments to be carried out.

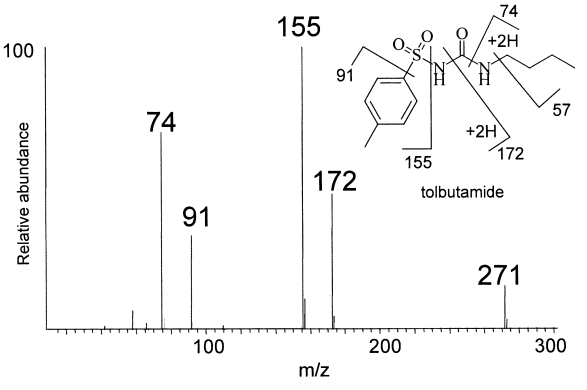

Fig. 11. Product ion MS–MS spectrum obtained for m/z 271 at the elution time of tolbutamide displayed in Fig. 10.

tolbutamide $[MH]^+$ ion. Low flow-rates were used (50–100 μl/min) so that modification of ionization conditions and other experimental parameters was possible during the course of the experiment. The product ion spectrum and proposed fragmentation of tolbutamide is shown in Fig. 11.

These data demonstrate the feasibility of inter-laboratory analysis using peak trapping. By submitting unknown samples on trapping columns, extensive mass spectral characterization was possible with efficient use of instrument time.

4. Conclusions

HPLC–MS analyses sometimes compromise mass spectral response in favor of chromatographic optimization. Mobile phase composition and pH have a marked affect on atmospheric pressure ionization (both ESI and APCI) efficiencies. Since conventional compound isolation can be tedious, an on-line approach to isolating and detecting compounds of interest has been explored. Chromatographic peaks may be trapped upon eluting from an analytical HPLC column, washed and then eluted into the mass spectrometer with solvents and at flow-rates that favor optimal mass spectral response.

Applications of peak trapping demonstrated in the present work include: (1) analysis of compounds separated using a mobile phase containing non-volatile additives at high concentrations, (2) analysis of organic acids separated using low-pH mobile phases, and (3) multiple collections of a low-level compound of interest. The analysis of organic acids using peak trapping has been of practical significance in a decomposition study where peak identity could be confirmed as difluorobenzoic acid. The technique appears to be well suited to the on-line isolation of impurities separated by HPLC for optimal mass spectrometric detection.

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