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Short communication

Effect of liquid chromatography separation of complex matrices on liquid chromatography–tandem mass spectrometry signal suppression

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Abstract

The effect of liquid chromatography separation on liquid chromatography–tandem mass spectrometry (LC–MS–MS) signal response for the characterization of low-molecular-mass compounds in a complex matrix was investigated. Matrix induced signal suppression appears throughout the entire LC–MS–MS analysis of wheat forage extract, with greatest suppression occurring at early retention times. Experimental results show that co-elution of matrix components and analytes from the LC column may be most strongly attributed to column overloading rather than similar analyte and matrix retention behavior. As a result, two-dimensional (LC–LC) separation can be a highly effective approach to address signal suppression effects for the quantitative LC–MS–MS analysis of complex matrix samples.

Keywords: Signal suppression; Liquid chromatography–mass spectrometry; Hydroxyfenozide; Methoxyfenozide; G-Fenozide

1. Introduction

Liquid chromatography electrospray tandem mass spectrometry (LC–MS–MS) is a powerful analytical characterization technique that combines selective LC separation with highly selective and sensitive mass detection by electrospray ionization tandem mass spectrometry [1–4]. The on-line coupling of efficient LC separation with soft ionization mass analysis has allowed this technique to be especially useful for the characterization of complex sample mixtures in various matrices. However, one significant drawback of electrospray mass spectrometry is that the ionization source is highly susceptible to matrix signal suppression effects. LC–MS–MS signal response obtained from standard and matrix samples may differ significantly. As a result, matrix signal suppression presents a significant challenges for quantitative LC–MS applications involving physiological and environmental samples [5–8].

The electrospray ionization process and ion suppression effects have been extensively investigated [9–11]. Matrix signal suppression is believed to result from competition between matrix components and analyte ions in the sprayed solution for access to the droplet surface for gas-phase emission [11]. As a

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result, the rate of analyte ion formation and signal response from the characterization of single component samples will drastically differ from those obtained in the presence of matrix contaminants. For this reason, efficient sample clean-up (separation of matrix components from the target analyte) is essential to maintain high sensitivity and signal reproducibility for qualitative and quantitative LC–MS–MS applications.

A number of approaches have been developed to compensate for signal suppression effects in LC–MS–MS analyses by addressing issues associated with the electrospray ionization source. One approach involves the use of valve switches to divert the LC matrix effluent to waste and minimize the build-up of non-volatile contaminants in the ionization source. The application of internal standards has been especially useful in addressing quantitative signal reproducibility issues between standard and matrix analyses [12,13]. Signal response can be enhanced by the introduction of additives (e.g., propionic acid, ammonium formate) into the mobile phase [14,15]. However, none of these approaches can fully address both quantitative signal reproducibly and sensitivity when characterizing complex matrix samples.

The most direct means of obtaining maximum sensitivity and signal reproducibility is through comprehensive sample clean-up and purification. Although LC is a highly effective separation technique, LC separation alone may be insufficient to address LC–MS–MS signal suppression effects. LC separation is often supplemented with additional clean-up procedures to ensure the removal of all signal suppressing matrix components from the sample. These procedures may include off-line liquid–liquid partitioning, open column separation, on-line solid-phase extraction (SPE) or dual pre-column separation methods [2,7,16].

In this communication, the effect of LC separation on LC–MS–MS matrix signal suppression was investigated for applications involving low-molecular-mass compounds in a complex matrix. LC separation of matrix extract and factors contributing to the co-elution of signal suppressing matrix components and target analytes were examined. A detailed report of this study will be presented elsewhere [17].

2. Experimental

2.1. Materials

Hydroxyfenozide, methoxyfenozide, G-fenozide, and wheat forage matrix were obtained from Rohm and Haas (Philadelphia, PA, USA). Structures of the analytes are shown in Fig. 1. Solvents used in the extraction and clean-up procedure were HPLC grade and obtained from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Sample preparation procedures

Standard sample solutions were prepared by dissolving 10 mg of analyte in 100 ml acetonitrile–water (1:1, v/v). Lower concentration standards were prepared by serial dilution.

Wheat forage was used to prepare the matrix samples. A 2-g amount of wheat forage was mixed with 150 ml of extraction solvent (methanol–0.10 *M* HCl, 90:10) and shaken for approximately 30 min. The extract was separated by vacuum filtration; the filter cake was rinsed with 50 ml of extraction solvent. The filtrate was transferred to a 500-ml separatory funnel and underwent liquid–liquid partitioning (LL) with hexane. This procedure involved shaking the matrix extract with 100 ml of *n*-hexane. The polar phase of this LL matrix extract was collected (stock solution).

Preparation of the matrix sample involved drying a 20-ml aliquot of the stock solution under nitrogen, followed by redissolving the sample with 2 ml of acetonitrile–water (1:1). To avoid the recovery issue and focus on signal suppression effects, samples were spiked with the standard analyte solution after extraction/clean-up procedures.

2.3. LC–MS–MS analysis

LC was carried out with a HP-1100 system (Hewlett-Packard, Wilmington, DE, USA); injection volume was 5–100 μl. Analytes were chromatographed with a Hewlett-Packard Zorbax C_{18} column (25 cm×3 mm, 5 μm) column. The solvent gradient was composed of water (phase A) and acetonitrile (phase B). The flow-rate of the mobile phase was 0.5 ml/min. The initial gradient was 80% A and was

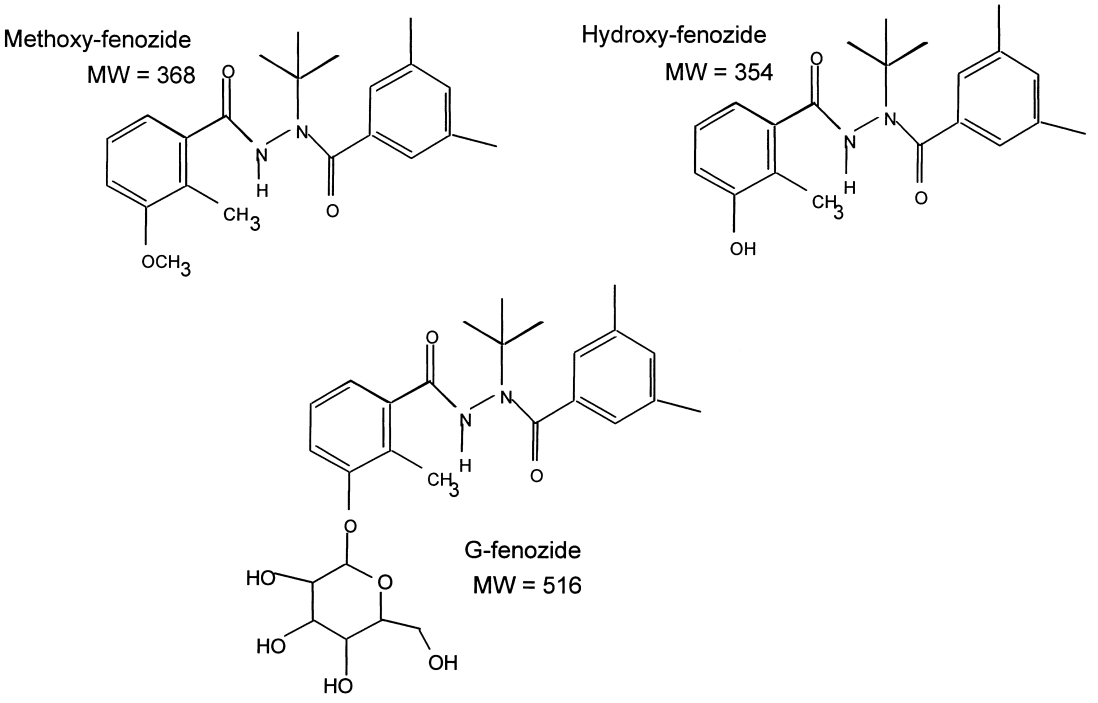

Fig. 1. Structures of analytes.

decreased to 50% at 6 min. Phase A was decreased to 30% at 10 min and decreased to 5% at 16.5 min. Phase A was returned to 80% at 20 min. Quantitative LC–MS–MS analysis employed valve switches to channel effluent from the first ca. 8 min of the analysis into waste. Effluent from the LC system was split to allow a flow-rate of ca. 50 μl/min into the ion source. Methoxyfenozide, hydroxyfenozide and G-fenozide elute at ca. 15, 13, and 9 min, respectively.

Post-column infusion experiments were performed by infusing a standard analyte solution (ca. 0.2 μg/ml) with a syringe pump into the LC effluent prior to electrospray ionization (ESI) MS analysis. The set-up has been described elsewhere [18,19]. The flow-rate of the infused solution was ca. 20 μl/min. During infusion of the standard solution, a blank wash (ACN–water, 1:1, v/v) or blank matrix extract was injected for LC–MS analysis. Valve switches were not used. Extracted ion chromatograms from the wash and matrix injection were compared to evaluate the extent of signal suppression with respect to LC retention time.

To avoid nominal mass interference and focus on signal suppression effects, LC–MS–MS analysis was performed on the API-365 triple quadrupole ESI mass spectrometer (Perkin-Elmer, Foster City, CA, USA) with a turbo-ionspray source (temperature set to 400°C). Analysis was performed in both negative and positive-ion modes using multi-reaction monitoring. For negative-ion acquisition, the instrument parameters were −3400 V, −30 V and −200 V for the spray, orifice and ring voltages, respectively. For the positive-ion mode, the instrument parameters were 4200 V, 20 V, 180 V for the spray, orifice and ring voltages, respectively.

3. Results and discussion

Although a highly effective separation technique, LC alone does not provide sufficient clean-up of complex matrix samples to address LC–MS–MS signal suppression effects. Fig. 2 shows the extracted ion chromatograms obtained from the analysis of both standard and matrix samples containing G-

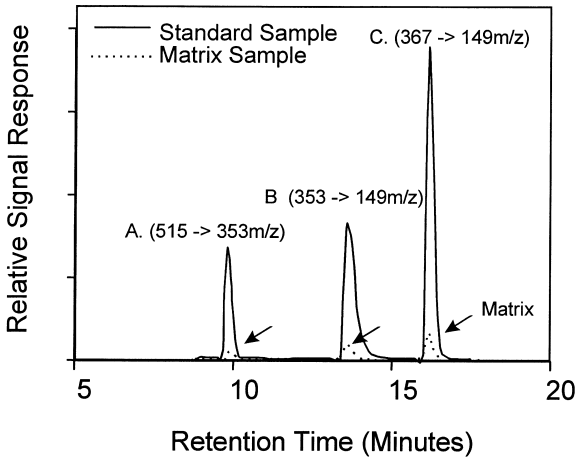

Fig. 2. Extracted ion chromatogram from the negative-ion mode LC–MS–MS analysis of 0.02 μg/ml (A) G-fenozide, (B) hydroxyfenozide, and (C) methoxyfenozide acquired from standard and matrix samples. Injection volume is 50 μl.

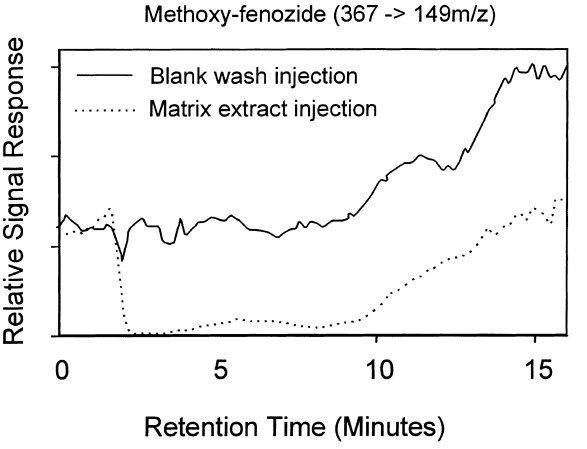

Fig. 3. Negative-ion mode LC–MS–MS extracted ion chromatograms from the post-column infusion of standard sample during the injection of blank wash (ACN–water) and matrix extract. Injection volume is 50 μl.

fenozide, hydroxyfenozide and methoxyfenozide. Signal responses from matrix sample injections were a factor of ca. 10 lower than those obtained from standards.

The LC separation efficiency of signal suppressing matrix components was visualized by a technique involving post-column infusion of the standard analyte solution [18,19]. Continuous post-column infusion of the standard into the LC effluent allows all components in the LC effluent to be consistently ionized with an identical quantity of the standard target compound. The separation of matrix components and the extent of signal suppression of the target analyte can be visualized by monitoring the response of extracted ion chromatograms obtained from the injection of blank wash (ACN–water) and blank matrix extract.

Fig. 3 shows the extracted-ion chromatograms of methoxyfenozide, introduced post-column, during the analysis of a wash injection and a blank wheat forage extract. The chromatogram obtained from the matrix extract injection shows a drastic decrease in signal response early in the analysis, relative to the blank wash injection. The greatest extent of suppression was observed at ca. 2 min. The analyte signal response from the matrix sample was a factor of ca. 10 lower than the wash injection for approximately the first ca. 8 min of the analysis. After ca. 8 min, the signal response gradually increases with respect to retention time. Signal response of the matrix injection at ca. 15 min was ca. 60% of that obtained from the wash injection. All compounds characterized by this LC separation method elute between ca. 9 and 15 min (see Experimental). The drastic signal suppression observed early in the analysis suggests that matrix components contributing the greatest signal suppression are significantly more polar in nature than the analyte.

The sample injection volume used for the LC–MS–MS analysis can also have a significant effect on the extent of matrix signal suppression. LC–MS–MS analysis was performed on spiked wheat forage matrix samples, with progressively increasing sample injection volumes. Table 1 summarizes the signal response of various analytes obtained from matrix samples, expressed as a percentage of that obtained

Table 1
Relative[a] signal response from various injection volumes for LC–MS–MS analysis of wheat forage matrix sample

Analyte	5 μl	10 μl	50 μl	100 μl
Methoxyfenozide	88.8	91.3	57.5	55.8
Hydroxyfenozide	91.8	92.5	58.5	44.8
G-Fenozide	63.6	65.2	30.1	27.2

[a] Signal response is expressed as a percentage of that obtained from standard samples, 100% is indicative of no signal suppression.

from standard sample injections. Data were acquired using negative-ion mode analysis. Increasing injection volume caused a drastic decrease in the matrix/standard signal response ratio for the various analytes. For the analysis of methoxyfenozide in the matrix, a signal response of ca. 88%, relative to standard sample analysis, was observed using a 5 μl injection volume, while ca. 55% was observed using a 100 μl injection volume. Similar results were observed for analyses performed using the positive-ion mode.

The LC co-elution of analyte and matrix components is often believed to result from similar retention behavior or stationary phase interaction. Lower injection volume would reduce the amount of matrix that would co-elute with the target analyte. However, we hypothesized that another possibility can account for co-elution and signal suppression effect. The LC–MS–MS signal suppression may be attributed to overloading of the LC column. The concentration of the injected matrix may be beyond the column's capacity to provide efficient separation, resulting in the continuous elution or tailing of various matrix components throughout the course of the entire analysis.

To elucidate the effects contributing to analyte/matrix co-elution and LC–MS–MS signal suppression, off-line two-dimensional (2-D) LC–MS–MS analyses were performed on wheat forage matrix samples. Several LC fractions of the analyte (±0.5 min around peak) were collected, dried, re-dissolved to the concentration of the originally injected sample, and re-injected using an identical LC–MS–MS separation and analysis method. Results from the negative-ion mode analyses are shown in Table 2. The signal response of methoxyfenozide acquired from conventional or one-dimensional (1-D) LC–MS–MS analysis of the wheat forage matrix samples was ca. 75%, relative to standard sample analyses. Significantly greater signal suppression (ca. 3% relative to standard sample analyses) was observed for G-fenozide. Signal suppression effects were significantly reduced when applying the off-line 2-D LC separation. The signal responses for the off-line 2-D LC–MS–MS analyses of methoxyfenozide and G-fenozide were ca. 99% and 83% relative to standard samples (Table 2). Similar effects were also observed for positive-ion mode analyses. (It should be noted that data shown in Tables 1 and 2 were acquired from matrix extract prepared from different batches. For this reason, attention should be directed to the general trends observed in each respective table).

Table 2
Relative[a] signal response obtained from 1-D and 2-D LC–MS–MS analysis of wheat forage matrix sample (injection volume=50 μl)

Analyte	1-D	2-D
Methoxyfenozide	75.2	99.2
Hydroxyfenozide	61.6	94.1
G-Fenozide	3.69	83.5

[a] Signal response is expressed as a percentage of that obtained from standard samples, 100% is indicative of no signal suppression.

Matrix components, having similar retention behavior to the target analyte, may contribute to overall signal suppression. However, the results from the 2-D LC–MS–MS experiments prove that the primary factor accounting for co-elution of the analyte with signal suppressing matrix components is column overloading. If the co-elution of analyte and matrix components were primarily contributed by identical retention behavior with the LC stationary phase, signal suppression effects should be similar for the 1-D and 2-D LC analyses. Signal suppression attributed to column overloading also agrees with the reduced signal suppression effect accompanying low injection volumes. Lower injection volumes likely reduce the loading of matrix components onto the column, minimizing tailing and column overloading effects, improving the overall separation of matrix and analyte. Column overloading would account for the continuous elution of signal suppressing matrix components throughout the LC–MS–MS analysis, as shown in Fig. 2.

To acquire sensitive and reproducible LC–MS–MS data, other separation techniques (e.g., liquid–liquid partitioning, open column separation, and SPE) are used in conjunction with LC for the analysis of complex matrix samples. However, such analytical methods are considered complex and time consuming. The results from this study show that sample preparation methods focusing on the removal of polar matrix components may be useful in addressing LC–MS–MS signal suppression effects associ-

ated with the analysis of wheat forage and other matrices. The application of on-line 2-D LC or other multi-dimensional separation techniques (online SPE–LC, dual pre-column separation), may be a highly effective approach to obtaining sensitive and reproducible quantitative information when characterizing complex matrix samples without comprehensive and time consuming sample preparation. Although this study primarily involves the application of LC–MS–MS, the results should be applicable to LC–MS methods. A detailed report of this study will be submitted elsewhere [17].

4. Conclusions

In this report, the effect of LC separation on matrix signal suppression for the LC–MS–MS characterization of low-molecular-mass compounds in complex matrix were investigated. Signal suppression or co-elution of matrix and analyte from LC can be primarily attributed to column overloading with matrix components rather than identical analyte–matrix retention behavior. Therefore, the application of 2-D (LC–LC) separation methods can be a highly effective and efficient approach to compensate for LC–MS–MS and LC–MS signal suppression effects for quantitative characterization of complex matrix samples.

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