A Novel Dynamic Flush Method to Reduce Column-Related Carryover

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Column-related carryover affects both accuracy and precision in liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis. In this work, a novel straightforward dynamic flush method to reduce the column-related carryover was developed by alternating the column flow direction of liquid chromatographic separation with a Valco switching valve and pristine instrument control software. By alternating the column flow direction, a fresh inlet is always in line to accommodate sample injection and stacking. In addition, the contaminated column inlet from the previous run is switched to the outlet position for a flush with a gradient during the next sample run. In this way, the column-related carryover can be reduced effectively without additional blank runs. It also minimizes the carryover risk between the adjacent unknown samples. The column-related carryover of the tested “sticky” compounds was reduced by 52.3–94.4% compared with the non-dynamic flush method under the same experimental conditions. The performance and reproducibility of high-performance liquid chromatography (HPLC) separation in terms of the retention time shift and peak shape are not compromised under the dynamic flush even after over 300 consecutive injections. The described novel method is simple and easy to implement for compounds with column-related carryover.

Introduction

Liquid chromatography-tandem mass spectrometry (LC–MS/MS) has become a routinely used powerful analytical tool which provides a wide dynamic range, superior sensitivity, selectivity and speed for analysis (1). However, mainly due to the required wide dynamic range and higher throughput for sample analysis, the performance of an LC–MS/MS method could be intriguied by factors associated with instrument hardware and/or the nature of samples. Sample carryover is one of the major problems that are commonly encountered.

Sample carryover is defined as the response detected from a previous sample injection during the analysis of the immediately subsequent sample. It can be column related or non-column related; either could drastically increase the risk for skewing biochemical results (2). Non-column-related carryover happens more frequently and normally results from inadequate setup or flush of the autosampler injection needle assembly, transfer tube or injection valve. Over the years, numerous detailed approaches have been published to address this type of carryover issues (3–5). Column-related carryover is associated with the separation columns or pre-column. Although it happens less frequently, it is a problem much harder to mitigate, and the last resort employed is to curtail the dynamic range of the assay or to transform an assay into a dual range one (6).

Column-related carryover in LC/MS/MS analysis is in general a compound-dependent problem and may be associated with the unique chemical characteristics of the analyte (2). In our experience, the problem often occurs for small-molecule compounds rich in hydroxyl or boronic acid groups, preassembly due to strong interaction of the compound with the packing material or the inlet filter/frit. Choosing different columns sometimes can alleviate the on-column carry-over problem. However, the most often used method to shrink the dynamic range of the assay is to run one or multiple blank injections with appropriate solvents to flush out the adsorbed analyte. This method unavoidably increases the running time, i.e. reduction of throughput for a batch with a decrease of column lifetime.

In this work, a novel straightforward dynamic flush method was developed to address the column-related carryover by alternating the column flow direction of liquid chromatographic separation with a Valco switching valve and associated control software. By alternating the column flow direction, a fresh inlet is always in line to accommodate sample injection and stacking. On the other hand, the contaminated inlet from the previous run is switched to the outlet position for a flush with the gradient during the next sample run. In this way, the column-related carryover can be reduced effectively without additional blank runs. It also minimizes the carryover risk between the adjacent unknown samples. The method described is basically to back-flush the analytical column after each sample injection without interrupting a run. Based on the extensive literature search, to date, the described dynamic flush method has not been reported yet.

Experimental

Instrumentation and reagents

Rat and mouse K2EDTA blank plasmas were obtained from Bioreclamation (Hicksville, NY, USA). Bufexamac and 4-hydroxy coumarin were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Proprietary compounds Velcade, MLN2238, ML001 and ML002 were synthesized in house.

A SCIEX API-4000 mass spectrometer (Thornville, Ontario, Canada), two Shimadzu LC-10ADvp pumps with a SCL-10A controller (Shimadzu Scientific Instruments, Columbia, MD, USA) and an LEAP autosampler (LEAP Technologies, Carrboro, NC, USA) were used for the described experiments. A Valco switching valve (Valco Instruments Co. Inc., Houston, TX, USA) with associated controlling hardware was used to alternate the column flow direction.

Sample preparation, extraction and analysis

The stock solution of the testing compound was prepared at 1 mg/mL in either 50% of acetonitrile in water (ACN/water,
50:50 v/v) or dimethyl sulfoxide (DMSO). The stock solution was spiked into dipotassium ethylenediaminetetraacetic acid (K₂EDTA) plasma to prepare standard and quality control (QC) samples in the target concentration ranges from 2 to 500 nM for the proprietary compounds and 1–2500 nM for bufexamac and hydroxycoumarin.

Two sample preparation methods were used for this research. Plasma protein precipitation was applied to bufexamac and hydroxycoumarin. Specifically, a 50μL aliquot of plasma samples, standards and QCs was transferred onto a 96-well plate and mixed with 200μL of acetonitrile containing 150 nM carbutamide as a generic internal standard (IS). The mixed samples were vortexed for 5 min and centrifuged at 3000 rpm for 10 min. The supernatant of samples (~150μL) was transferred onto a fresh 96-well plate and evaporated under a stream of nitrogen. The dried samples were reconstituted in 150μL of 10% acetonitrile with 0.1% formic acid and an aliquot of 20μL was injected onto the LC/MS/MS for analysis.

For the proprietary compounds Velcade, MLN2238, ML001 and ML002, a liquid–liquid extraction was employed. Specifically, a 50μL aliquot of samples was transferred into the wells on a 96-well plate. An aliquot of structural analog IS solution at a concentration of 50 nM in ACN/H₂O 50:50 v/v containing 0.1% formic acid was added to the wells, except for the control blank samples for which only the solvent was added. For all samples, 100μL of 0.5 N hydrochloric acid was added to each well, followed by addition of 500μL of methyl tert-butyl ether for extraction. After mixing, the plate was vortexed at a low speed for 5 min, then centrifuged at 3000 rpm for 10 min. The supernatant (~300μL) was transferred to a fresh 96-well plate and evaporated under a stream of nitrogen. The dried samples were reconstituted in 150μL of 40% acetonitrile in water supplemented with 0.1% formic acid.

A reverse-phase gradient HPLC method was employed to provide sample stacking and separation. Mobile Phase A includes 0.1% formic acid in water and mobile Phase B includes 0.1% formic acid in acetonitrile. The elution gradient is 35% B to 95% B in 1.75 min, held 95% B until 2.5 min and then ramped back to the initial condition of 35% B until 3 min, an event added at 2.9 min for triggering the switching valve to alternate the column flow direction. Analytes and the IS were ionized under a positive ion spray mode and detected through multiple-reaction monitoring (MRM).

**Control of dynamic flush**

A Valco valve was configured according to the user’s manual to a two-position single triggering mode which can be directly controlled through a vendor’s embedded program within the Analyst software. The connection and flow direction of the column are shown in Figure 1. A run starts with the valve at a random position (shown as Position A). After the interested peaks are eluted, the column is briefly washed at a high organic composition (95% Mobile Phase B). A triggering signal is then sent to toggle the valve (shown as Position B) and the washing will continue for about 0.1 min followed by a 0.5 min of equilibration with mobile Phase A before injection of the succeeding sample. The toggle of the valve before each injection continues throughout a run.

**Carryover test**

The total carryover of an LC–MS/MS system was tested by sequential injections of extracted plasma standard samples at an upper limit of quantification (ULOQ) and a control blank into an LC–MS/MS system. The level of total carryover is expressed as the analyte peak area ratio of control blank against either that from the ULOQ or the lower limit of quantification (LLOQ) sample. The test was performed with or without the dynamic flush. The column-related carryover test was conducted in two steps. An extracted ULOQ standard was first injected into the LC–MS/MS system for analysis. At the end of the run, the column was bypassed from the autosampler and directly connected to the HPLC gradient flow outlet. A new run is then initiated without sample injection. The analyte peak ratio of the “dummy” run against that of the ULOQ or LLOQ was used to describe the column-related carryover. The same tests were also conducted under dynamic flush conditions.

**Results and Discussion**

The total carryover is the amount of the analyte held in an LC system from a preceding sample that co-elutes into the very next injected sample. In general, the desirable level of carryover in regulated analysis is < 20% of the corresponding LLOQ level. In a discovery setting, the tolerance could be higher. To minimize the potential influence, after high concentration standards, QC samples, single or, if carryover persists, multiple blank injections were employed to wash out the retained analyte in the LC system. This approach is effective in most cases. However, for compounds with certain structural characteristics, the carryover is not easy to address maybe due to the very tight affinity of the compound onto the inlet frits or packing materials. For these compounds, back-flushing a column is most effective for carryover removal, which normally can only be performed offline. The dynamic flush setup shown in this study makes it possible to conduct in-line back-flush of the column without interrupting the normal sequence of a run.

Six selected compounds were used to test the dynamic flush setup, including bufexamac, a hydroxyamide derivative, hydroxycoumarin and four Millennium proprietary boronic acid derivatives. All six compounds were previously found with serious carryover issues in bioanalysis. Historically, for the analysis of the compounds, either a narrower dynamic range or multiple blank washes after high standards and QC samples were employed to minimize the overall carryover. Table 1 shows the results of characterizing the total carryover, the column-related carryover and
Table I. Assessment of the Total- and Column-related Carryover with and without Dynamic Flush (n = 3)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Total carryover</th>
<th>Column-related carryover</th>
<th>% Column carryover of total</th>
<th>Total carryover w/dynamic flush</th>
<th>% Reduction of total carryover by dynamic flush</th>
</tr>
</thead>
<tbody>
<tr>
<td>Budesonide</td>
<td>1.55</td>
<td>1.35</td>
<td>87.1</td>
<td>0.403</td>
<td>74.0</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>0.761</td>
<td>0.091</td>
<td>12.0</td>
<td>0.372</td>
<td>52.3</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>2.93</td>
<td>2.84</td>
<td>96.9</td>
<td>0.178</td>
<td>93.9</td>
</tr>
<tr>
<td>MLN2238b</td>
<td>2.34</td>
<td>2.01</td>
<td>85.9</td>
<td>0.353</td>
<td>84.9</td>
</tr>
</tbody>
</table>

Table II. Carryover Assessment of Plasma Samples with or without the Dynamic Flush

<table>
<thead>
<tr>
<th>Compound</th>
<th>w/o dynamic flush</th>
<th>w/dynamic flush</th>
<th>% Reduction of carryover by dynamic flush</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bortezomib</td>
<td>395</td>
<td>46.9</td>
<td>88.1</td>
</tr>
<tr>
<td>MLN2238b</td>
<td>541</td>
<td>61.5</td>
<td>89.6</td>
</tr>
<tr>
<td>ML001b</td>
<td>193</td>
<td>44.5</td>
<td>76.9</td>
</tr>
<tr>
<td>ML002b</td>
<td>492</td>
<td>18.7</td>
<td>96.2</td>
</tr>
</tbody>
</table>

*Mouse plasma; LLOQ: 2 nM; ULOQ: 500 nM; column: Hypersil C8, 50 × 2.1 mm (5 μm) from Thermo Fisher Scientific.

**Rat plasma; LLOQ: 2 nM; ULOQ: 500 nM; column: Luna C8, 50 × 3.0 mm (5 μm) from Agilent Technologies.*

*% total carryover reduced by dynamic flush

% Carryover to LLOQ | % Carryover to ULOQ |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>74.0</td>
<td>88.1</td>
</tr>
<tr>
<td>93.9</td>
<td>89.6</td>
</tr>
<tr>
<td>76.9</td>
<td>96.2</td>
</tr>
</tbody>
</table>

The effect of the dynamic flush on the carryover. The testing was performed in triplicates using the method described in detail in the Experimental section. The carryover was expressed as the ratio of the analyte response in the blank injection to a high standard which immediately preceded the blank. As shown in Table I, all tested compounds showed a very high total carryover, ranging from 0.781 to 2.93% of the corresponding ULOQ level. Except for hydroxycoumarin, all compounds showed a very high column-related carryover which ranged from 82.1 to 96.9% of the corresponding total carryover. When the dynamic flush is employed, also shown in Table I, the total carryover of all the compounds was significantly reduced by 52.3–94.4%.

To test the utility of the dynamic flush setup for real sample analysis in a drug discovery setting, in vivo plasma samples of the proprietary compounds were processed together with dual sets of calibration standards to bracket the samples and multiple levels of QC samples. Multiple solvent blanks were added to follow each high standard and QC samples in order to evaluate the carryover and the effect of the dynamic flush by using the mean of the two analyte response ratios after the two high standards against the response from the calibration standard at the LLOQ level. As shown in Table II, all four boronic acid compounds showed a very high carryover, ranging from 93 to 541% of the corresponding LLOQ. It took at least two additional blank injections to reduce the carryover to an acceptable level for discovery studies (data not shown). Nevertheless, when the dynamic flush system is employed, the carryover assessed by the response of the very first blank immediately after the high standard was significantly reduced by 76.9–96.2%. The absolute carryover ranges from 18.7 to 61.5% of the corresponding LLOQ sample which in general is acceptable for conducting discovery studies.

The initial concerns of using the dynamic flush were the potential retention time shift, analyte peak shape change and the life-time shortening of the analytical columns resulting from switching LC flow directions between samples. In our test of the system, we never observed performance deterioration of the LC separation due to the use of the dynamic flush on the columns we used. To further test the effect of the flush on lifetime of a column, we compared the chromatographic separation of six identical plasma samples of ML001 at the beginning (Set 1) and the end of a run (Set 2) with 300 consecutive injections of extracted plasma blanks in between. The mean retention time of the analyte peak remained the same for both sets at 1.42 ± 0.01 min (n = 6 for each set). The peak areas of the analyte ML001 in Sets 1 and 2 were 95200 ± 4100 counts (n = 6) and 98100 ± 3800 counts (n = 6), respectively, resulting in a mean percentage difference of 3.00% between the mean peak areas. The asymmetry factors of the analyte peaks were 1.65 ± 0.10 and 1.72 ± 0.09 for Sets 1 and 2 (n = 6 for each set), respectively. All the results indicate that the dynamic flush method did not affect the performance of the analytical column tested in this work. Nevertheless, when using the dynamic flush on a column not extensively tested, it is encouraged to keep close monitoring the column performance.

Conclusion

Column-related carryover is rare but if it happens, it is very difficult to mitigate. For the model compounds selected for testing, the column-related carryover is the predominate contributor to the total carryover (except for hydroxycoumarin), and the dynamic flush method was shown to be able to effectively reduce the carryover by 52.3–94.4%. In addition, in real plasma sample analysis, the dynamic flush method also showed an effective reduction of carryover by 76.9–96.2% for the tested compounds and the absolute carryover level was acceptable for discovery studies without requiring additional time-consuming blank injections or extension of running time. The setup is easy to assemble and implement using commonly available switching valves and controlling software. Moreover, the separation characteristics of the HPLC in terms of the peak shape, retention time and column lifetime were not affected. The method should be useful as an alternative approach to mitigate the difficult column-related carryover issue for certain classes of compounds.

Acknowledgments

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References

1. Ackermann, B.L., Berna, M.J., Murphy, A.T.; Recent advances in use of LC/MS/MS for quantitative high throughput bioanalytical support of drug discovery; *Current Topics in Medicinal Chemistry* (2002); 2: 53–66.


