Ramifications of Pump-Switching Step Gradients for Bioanalytical LC/MS/MS Assays

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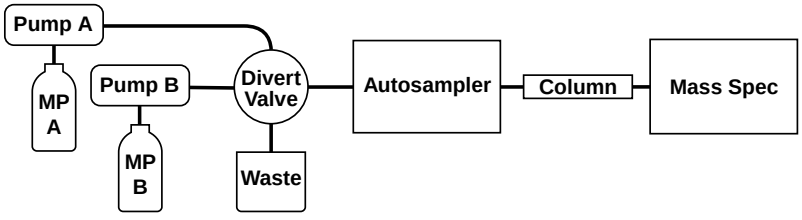
Two separate isocratic LC pumps were configured to perform fast, step gradients for bioanalytical LC/MS/MS assays, simultaneously reducing analysis time and ion suppression, and improving peak shape. Although the technique offers significant advantages for bioanalytical LC/MS/MS assays, it also presents some practical challenges. A newly designed LC pump, the BAS PM-92, is shown to be ideally suited for pump-switching step gradients.

Mobile phase gradients are useful for LC/MS/MS assays when multiple target compounds exhibit rather different chromatographic retention, as is often the case for drugs and metabolites. There are drawbacks to conventional gradients, however, especially for the low flow rates of typical electrospray assays. First, if the volume of the mixer is significant, sample analysis time is extended. Second, electrospray requires a pulseless pump for optimum performance. Large volume pulse dampers cannot be used for gradients, as their volumes are even larger than the mixer. These disadvantages can be avoided by using two separate isocratic LC pumps to perform fast, step gradients (***F1***). Pump-switching step gradient assays can be very efficient and ideal for high-throughput bioanalytical LC/MS/MS assays, however there are a number of aspects about the technique which must be considered for it to be practical.

Bioanalytical pump-switching step gradient assays are performed by injecting samples onto a reversed phase column equilibrated with a relatively weak mobile phase. The analytes of interest are retained while more polar potential interferences from the biological sample elute with or near the initial void. Although electrospray MS/MS is a very selective technique, this step is necessary to minimize ion suppression. Shortly after injection, a second LC pump is switched in-line, delivering a stronger mobile phase to the column to elute the analytes of interest. In practice, it is wise to make the initial mobile phase as strong as possible, such that the analytes of interest are still retained on the column. The second mobile phase should be as weak as possible, such that the analytes of interest still elute in a reasonable time with adequate shape. This will minimize co-eluting interferences, as will be demonstrated.

F1

Instrumentation configuration for pump-switching step gradient assays.

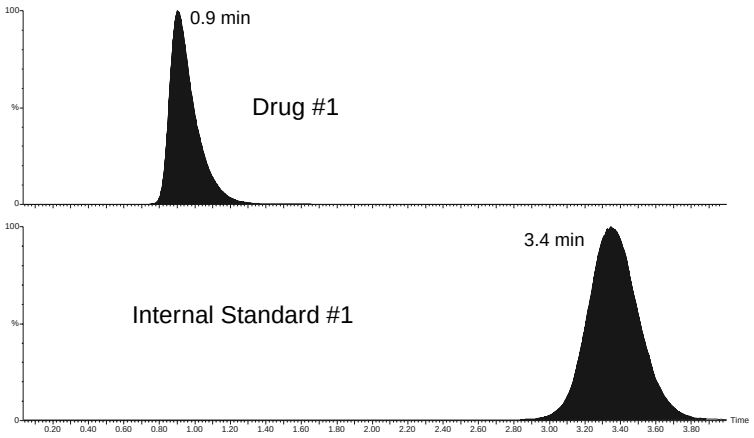


Experimental

The focus here is on a general technique. As such, certain details about the specific methods, compounds and instrumentation used have been omitted for clarity. In each example, mass spectrometers (Sciex API 3000's and Micromass Quattro LC's) were operated in the positive ion, multiple reaction monitoring (MRM) mode. The MRM mode is the MS/MS mode whereby various precursor ions of specific mass-to-charge (m/z) ratios are selected to undergo collisionally activated dis-

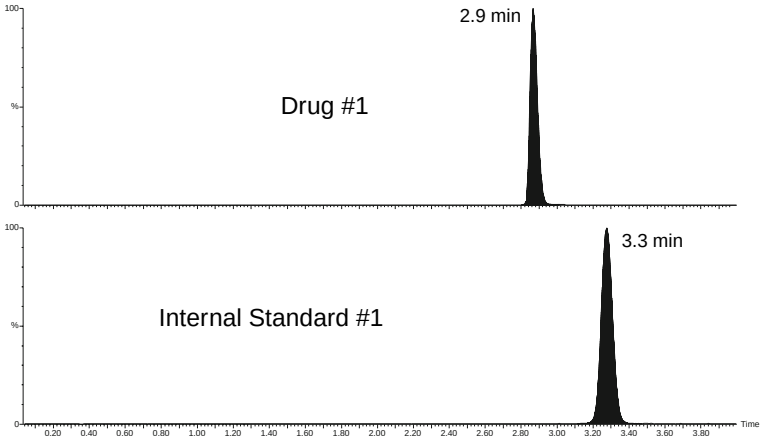
sociation (CAD) by acceleration into a cell pressurized with a neutral gas such as argon or nitrogen. Specific product ions from such collisions are then monitored. The column was a Zorbax Eclipse, XDB-C8, 50x2.1 mm, with a mobile phase flow rate of 200 µL/min. Specific mobile phases used are listed with each chromatogram. The pumps are manufactured by Bioanalytical Systems, Inc. (BAS), and are described in more detail later. A BAS Sample Sentinel autosampler injects samples and controls pump switching. The analytes from biological samples were extracted by conventional means, the details of which are not germane to this discussion.

F2



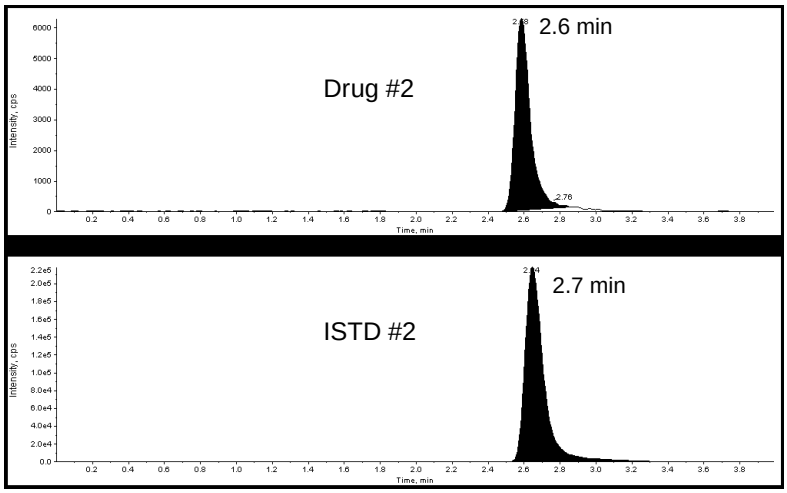
An isocratic separation using 46% methanol in water with 0.3% formic acid. Several problems are noted: the drug elutes too near the void and the internal standard (ISTD) peak is too broad. Attempts to increase retention of the drug by decreasing the solvent strength (% methanol) resulted in unacceptably long retention of ISTD.

F3



A pump-switching step gradient changing from mobile phase A (30% methanol in water, 0.3% formic acid) to mobile phase B (60% methanol in water, 0.3% formic acid) at time = 2 minutes. Several improvements are noted: sharp peaks, well-separated from the void are seen, and total analysis time remains short allowing for multiplexing. The analysis time could be decreased further by increasing the flow rate of mobile phase A.

F4



Assay of drug in monkey plasma sample spiked with calibration standard (100 ng/mL). A pump-switching step gradient was used. Mobile phase A is 30% acetonitrile in water, 1% isopropanol, and 0.1% formic acid. Mobile phase B is 55% acetonitrile, 1% isopropanol, and 0.1% formic acid. Switch between mobile phases occurred at time = 1 min. Samples were prepared by protein precipitation, and the method was validated over the range from 10 to 1000 ng/mL in plasma. Note that the drug and the internal standard are different than those shown in F2 and F3.

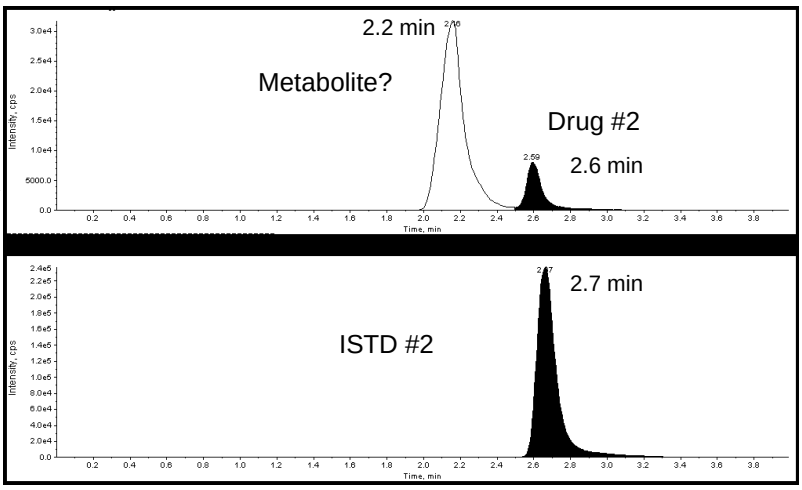
Results and Discussion

Assay #1 was a 4-minute, isocratic LC/MS/MS assay for Drug A and ISTD A that suffered from Drug A eluting too near the void, where ion suppression was a problem (***F2***). Isocratic efforts to move Drug A away from the void with a weaker mobile phase resulted in unacceptable band broadening and very long retention of ISTD A. Use of the pump-switching step gradient technique (***F3***) resulted in much sharper peaks for both Drug A and ISTD A. Both peaks eluted well-separated from the void, with a run time similar to that of the initial isocratic attempt. In fact, analysis time can be further reduced by increasing the flow rate of the initial mobile phase (MP A). The short time interval over which both peaks elute invites multiplexing, where multiple LC systems share a common detector, which can increase throughput even more. The pump-switching step gradient assay was successfully validated for Drug A in human plasma to support clinical trials.

Assay #2 was a pump-switching step gradient assay successfully validated for Drug B in monkey plasma (***F4***). However, during an actual monkey study, authentic samples yielded a significant "drug" peak prior to, though resolved from, the actual drug peak (***F5***). Remember that in MRM experiments, all peaks appearing in the same mass chromatogram arise from precursor ions of the same chosen m/z fragmenting to product ions of another selected m/z. This extra "drug" peak was not observed in spiked samples or in non-dosed monkey samples. It most likely resulted from the conversion of a metabolite, which eluted off the column prior to the drug in the ion source. Upon conversion to drug in the ion source, the compound behaves just as the actual drug does. This illustrates the importance of chromatography, and avoiding "sec-

F5

Dosed monkey sample (106 ng/mL in plasma). Pump-switching step gradient as in F4. Note that the extra (metabolite) peak only appears in the sample obtained from dosed monkeys, and likely results from a metabolite undergoing decomposition in the ion source to yield the parent drug.



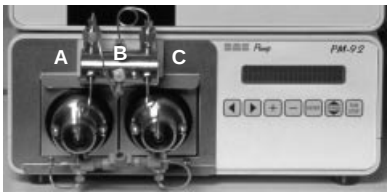
F6

BAS PM-92 pump front panel.

A- left pump head transducer

B- system pressure transducer

C- right pump head transducer



ondary voids" in pump-switching step gradient assays. Making the second mobile phase (MP B) too strong, can yield very sharp peaks, but may also result in co-eluting interferences, and/or ion suppression.

Another word of caution concerning pump-switching step gradients involves the dead volume within some autosampler injectors. The BAS Sample Sentinel utilizes a Rheodyne valve with relatively small sample loops, and negligible dead volume. However, when these same assays were performed in the 96-well format, a Shimadzu SIL-10Advp Autoinjector was used. Instead of filling a sample loop that is then rotated into the high pressure mobile phase stream, the Shimadzu Autoinjector actually places the sampling needle itself into the high pressure port. The volume of this needle/sample loop assembly is about 120 µL. This is relevant because with pump-switching step gradients, especially at low flow rates, one also has to think about the total dead volume following the pump-switching valve. For instance, an assay run with a BAS Sample Sentinel and a sample loop required pump-switching to occur at 2.0 minutes into the run. If that same assay were run with a Shimadzu SIL-10Advp Autoinjector, the pump-switching should occur about 30-40 seconds earlier, if the LC flow rate was 200 µL/min, to account for the extra pre-column dead volume.

Initially, the pump-switching step gradient assays were successfully performed using conventional LC pumps with cam-driven heads and large volume pulse dampers, such as the BAS PM-80. These pumps were used isocratically allowing the use of their pulse dampers for electrospray assays. The main drawback of using conventional pumps for pump-switching step gradients was that system pressures for the two constant-flow pumps had to be carefully matched. Recall that at any point in time, one pump is flowing to waste while the other is directed toward the analytical column and mass spectrometer. The two mobile phases typically have different viscosities, making system pressure matching even more difficult. If system pressures are not matched, upon pump-switching the large volume of mobile phase in the pulse damper can compress rather than be delivered to the analytical column. At the typical LC flow rates for electrospray LC/MS/MS assays, such mobile phase compression changed analyte "retention times" nearly one minute in an assay that was only 4 minutes long. The bigger problem was retention time reproducibility between systems, when system pressures were not matched.

The newly designed BAS PM-92 pumps solve this problem, yet deliver pulseless flow, making them ideally suited for pump-switching step gradient assays. The PM-92 pumps have dual, ballscrew-driven heads, each with separate pressure transducers (***F6***). At the end of its delivery stroke, a pump head quickly retracts, refills and rapidly pre-pressurizes to match system pressure. This not only obviates the need for a pulse damper, but also provides real-time compensation for backpressure changes due to mobile phase viscosity, compressibility, temperature, bubbles, etc. There is no need to worry about matching the pressures between two pumps because there is very little mobile phase volume to compress upon switching. When two such pumps are used with switching techniques, they can also be operated at different flow rates, reducing assay development time and increasing throughput. The PM-92 pumps are compact and light, so having an extra LC pump on the LC/MS/MS system is not at all inconvenient. They can be purged so quickly that changing mobile phases is a snap, making developing pump-switching step gradient assays that much faster.

Conclusion

Pump-switching step gradients offer significant advantages for bioanalytical LC/MS/MS assays. They can decrease analysis time while also decreasing ion suppression. Analytes of interest elute as sharp peaks with easily controllable elution times, such that the technique can be ideal for use in multiplexing multiple LC systems to a single mass spectrometer.

Although MS/MS certainly adds specificity, chromatography remains very important for bioanalytical pump-switching step gradient assays. The initial mobile phase is used to retain the analytes of interest while more polar biological moieties

that could cause ion suppression are eluted. The second mobile phase is used to resolve the analytes of interest from the "secondary void" which may contain related compounds that can also interfere and/or cause ion suppression. Simple adjustments to mobile phases and switching times can help chromatographically resolve metabolites and other related substances of interest.

The new PM-92 LC pump from BAS offers significant advantages for pump-switching step gradient LC/MS/MS assays. It offers pulseless flow without a pulse damper. There is also no need to match pump backpressures when using two PM-92 pumps for pump-switching step gradient assays. Finally, since each pump operates independently and isocratically, each pump can deliver at different flow rates. This increased flexibility can reduce analysis time as well.