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Journal of Pharmaceutical and Biomedical Analysis 36 (2005) 1073–1078
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Elimination of autosampler carryover in a bioanalytical HPLC-MS/MS method: a case study

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Received 17 June 2004; received in revised form 10 September 2004; accepted 13 September 2004
Available online 22 October 2004

Abstract

A case study in identifying and eliminating the source of autosampler carryover in a bioanalytical HPLC-MS/MS assay is described. Through a series of systematic experiments, the carryover was traced to the injection valve and was eliminated by switching from a partial loop to a full loop injection, which provided more effective flushing of the sample flow path. The susceptibility of the HPLC system to carryover was demonstrated to depend on the absolute sensitivity of the detection method and the mass of analyte injected at the assay lower limit of quantitation (LLOQ).
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Keywords: Bioanalytical methods; HPLC/MS; Autosampler carryover

1. Introduction

Autosampler carryover is a common, often stubborn problem that can compromise the accuracy of HPLC assays. This is particularly the case for HPLC-MS/MS based bioanalytical methods used to support pharmacokinetics (PK) studies, in which carryover problems can be exacerbated by a number of factors, including low limits of quantitation (LLOQs), complex sample matrices, and wide dynamic ranges necessary for unknown samples that vary widely in analyte concentrations. Although some researchers argue that the effects of carryover in these assays can be ameliorated by arranging the run sequence based on expected analyte concentrations, this represents bias that could potentially be a concern from a regulatory perspective. This issue of bias has been recognized and mechanisms to eliminate or reduce such bias have been suggested [1] in which analysts are blinded as to the identity of the unknowns. Under blinded conditions, autosampler carryover could severely diminish the accuracy of an assay.

One approach to circumventing carryover is to narrow the dynamic range of the assay. This is undesirable, especially during dose escalation studies conducted early in phase I clinical trials, and/or for drug candidates that exhibit high peak to trough ratios, because many samples would require reanalysis after dilution, creating additional work and increasing the time required to generate data. Another potential problem with this approach is that analyte concentrations in post-dose samples often far exceed the upper limit of quantitation of the assay, in which case significant carryover might persist for numerous injections, potentially affecting subsequent unknowns or quality control samples. In light of these concerns, the preferred approach with respect to carryover is to identify its source and to eliminate it while maintaining as wide as possible a dynamic range.

This report describes a case study from our laboratories in identifying and eliminating autosampler carryover in an HPLC-MS/MS assay of a drug candidate (**I**) in human plasma. **I** is a weakly acidic compound with a pK_a of 5 and a molecular weight of 388. A partial chemical structure of **I** is provided in Fig. 1 showing the important phenolic hydroxyl group present in the molecule.

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doi:10.1016/j.jpba.2004.09.010

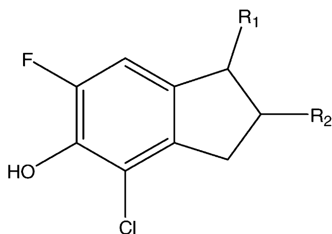

Fig. 1. Partial chemical structure of compound **I**.

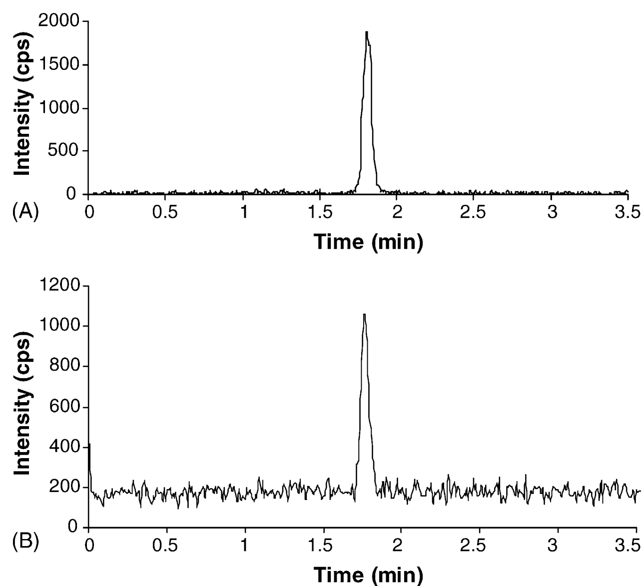

Fig. 2. Extracted ion chromatograms of 0.2 ng/mL extracted plasma standard (A) and a control plasma double blank (B) injected immediately after a 120 ng/mL plasma standard. Injection volume = 5 μL partial loop.

2. Experimental

Plasma samples were prepared by liquid–liquid extraction and the extracts were dissolved in mobile phase prior to injection onto the HPLC-MS/MS system. The standard curve range for **I** in human plasma was 0.2–120 ng/mL.

The HPLC system consisted of a Varian (Walnut Creek, CA, USA) ProStar 430 autosampler and a Perkin–Elmer (Norwalk, CT, USA) Series 200 Micropump. Separations were performed on a Thermo-Hypersil-Keystone (Bellefonte, PA, USA) BDS C-18 HPLC column (3.0 mm × 50 mm, 3 μm), which was used in conjunction with a guard column (3.0 mm × 10 mm) of the same packing material. The guard and analytical columns were thermostated at 30 °C. The mobile phase consisted of 0.1% (v/v) acetic acid in 40:60 (v/v%) water:acetonitrile and the flow rate was 0.6 mL/min.

An Applied Biosystems/MDS Sciex API-4000 (Concord, Ontario, Canada) triple quadruple mass spectrometer was operated in the negative ionization mode. Data was acquired in multiple reaction monitoring mode (MRM) with the ion transitions m/z 387 → 329 for **I** and m/z 394 → 327 for the internal standard (a deuterated analog of **I**) used for quantitation. The orifice potential was −80 V, the collision energy was −50 V, and the electron multiplier voltage was 2400 V. The curtain gas setting was 50 psi. For the Turbo ion spray (TIS) probe, the nebulizing (Gas 1) and Turbo (Gas 2) gases were set to 80 and 70 psi, respectively. The source heater was set to 400 °C and the needle voltage was −4500 kV. For the heated nebulizer (HN) probe, the heater was set to 400 °C, the nebulizer gas setting was 40 psi, and the needle current was 1 μA.

The same HPLC conditions were employed for both ion sources with the exception that the column ID was 4.6 mm and the flow rate was 1.2 mL/min with the HN probe. Unless otherwise indicated, all experiments described herein were performed using the TIS probe.

3. Results and discussion

3.1. Initial assessment of carryover

Initially, the autosampler was programmed for an injection volume of 5 μL (partial loopfill) and a needle wash volume of 1 mL between injections. The needle wash solvent was 0.1% (v/v) acetic acid in 20:80 (v/v%) water:acetonitrile. When a control plasma double blank was injected immediately following the upper limit of quantitation plasma standard (ULOQ = 120 ng/mL), a severe carryover peak was present that corresponded to approximately 0.1% of the ULOQ. The severity of this carryover peak became apparent by comparing its peak intensity relative to that of the LLOQ plasma standard. As shown in Fig. 2, the peak height of **I** in the plasma double blank was approximately 50% of the 0.2 ng/mL plasma LLOQ standard, an obviously unacceptable level of carryover.

3.2. Tubing connections/needle wash

As pointed out by Dolan [2], a common source of autosampler carryover is improper tubing connections at the injection valve. Improper connections can lead to unswept volumes, which act as reservoirs for analyte, resulting in carryover in successive injections. As tubing connections are relatively easy to check, this was a logical starting point. The sample needle tubing and syringe tubing were disconnected from the injection valve and fresh, straight cuts were made at the ends, after which the tubing was reinstalled in the valve ports. This had a significant positive effect, decreasing the carryover to approximately 20% of the LLOQ. Although improved, the extent of carryover was above what is generally considered acceptable for assays developed in our laboratories.

Alternative needle wash solvents were next investigated. Solvents consisting of acetonitrile, methanol, or isopropanol, neat, as well as in combination with various percentages of water, were evaluated. In addition, ternary mixtures of water, acetonitrile, and isopropanol were assessed. None of these

had a discernable effect on the magnitude of the carryover peak.

A key observation in the needle wash experiments was that increasing the volume of the needle wash from 1 to 2 mL after each injection (regardless of solvent composition) did not diminish the carryover peak. In light of these results, it appeared unlikely that the carryover was originating from the needle or from an unswept volume in the portion of the flow path flushed by the needle wash solvent. It was also concluded from these findings that further investigation into needle wash solvents and/or sequential washes with different solvents was not warranted.

To completely eliminate the sample needle as the potential source of carryover, an injection of blank mobile phase was made with the sample needle bypassed. Immediately after injecting a ULOQ plasma standard, the section of tubing connecting the needle to the valve was disconnected at the needle (refer to Fig. 3A) and inserted into a beaker of mobile phase. The autosampler was then programmed to perform an injection (5 μL—partial loopfill). The results of this experiment showed a carryover peak unchanged at approximately 20% of the LLOQ.

3.3. Evaluation of alternative rotor and stator materials

The results of the needle wash experiments suggested that the injection valve was the most likely source of the carryover. (Flow injection analysis ruled out the column as a possibility.) Thus, alternative rotor and stator materials were evaluated that could provide surfaces that were potentially less adsorptive of **I**. The standard stainless steel stator of the Valco valve was replaced with a polyaryletherketone (PAEK) stator and the standard Valcon H (a carbon fiber reinforced, PTFE lubricated composite) rotor was replaced with one made of Valcon E, a polyaryletherketone/PTFE composite (this rotor was designed to be used with the Valco PAEK stator). Unfortunately, under these conditions, the level of carryover increased 8-fold to approximately 160% of the LLOQ.

3.4. Autosampler injection/wash sequence

Before continuing the discussion of autosampler carryover in this method, a brief overview of the injection and wash sequences of the Varian ProStar 430 autosampler is in order. Although the foregoing discussion focuses on the ProStar 430, much will be applicable to other autosamplers of the same general design (i.e., those employing Valco or Rheodyne valves with external sample loops).

The injection sequence of the ProStar 430 autosampler is depicted schematically in Fig. 3. Initially the valve is in the INJECT position (Fig. 3A). The syringe aspirates a programmed flush volume (typically 30 μL) from the sample vial in order to fill the needle and connecting tubing volumes with sample. Next, the valve is switched to the LOAD position (Fig. 3B) and the programmed injected volume is aspirated into the sample loop. In full loop injection mode, the sample loop is quantitatively filled by flushing with excess sample volume (typically 2× loop volume). As indicated in Fig. 3C, the valve is then switched to INJECT and the sample plug flows onto the column while the needle wash solvent flushes the connecting tubing and sample needle.

3.5. Full loop injection

The results of the experiments conducted previously seemed to point to the stator surface between valve ports 4 and 5 as a potential source of residual **I** in the injection valve. In contrast to the other parts of the autosampler that are in the sample flow path, this region is not flushed with solvent between injections (ports 3 and 4 and associated tubing are flushed with needle wash solvent; ports 2 and 5, the sample loop and associated rotor groove is flushed with mobile phase when the valve is in the INJECT position). Instead, solution (sample dissolved in mobile phase) comes in contact with this part of the stator only when an injection is made. During a partial loopfill injection, after the initial flush step (valve in the INJECT position), the sample passes between valve ports 4 and 5 as it is drawn into the sample loop. As the loop fill step occurs, adsorbed analyte from the previous injection could be flushed into the sample loop as part of the injection plug, resulting in carryover. The situation is different in full loop injection mode. After the initial flush step, the sample loop is quantitatively filled by overfilling the loop with two or more times the loop volume of sample (e.g., 10 μL overfill for a 5-μL sample loop). In the case of full loop injection, analyte adsorbed on the stator surface could be flushed to waste during the overfill step. The partial and full loop scenarios described in this section are depicted in Fig. 4.

This hypothesis was tested by switching to full loop injection mode. Given that the MS/MS sensitivity for **I** was not limiting, a 2-μL sample loop was chosen as a compromise between loop overfill volume and the volume of sample consumed per injection. The autosampler was programmed to flush the loop with 10 μL of sample per injection, yielding an overfill of five times the loop volume. A shown in Fig. 5, carryover was not detectable under these conditions. Interestingly, the peak intensity for **I** with the 2-μL full loop injection was only slightly less than that observed for the 5-μL partial loop (Figs. 2A and 5A). This disparity was consistent when different 2-μL sample loops were evaluated. The less than proportional increase in response observed with the 5-μL partial loop injection was probably due to insufficient flushing of the needle and connecting tubing and/or the finite volumes of the valve ports and rotor. These effects were largely negated by the 5-fold overfill in full loop mode.

3.6. Effect of detection sensitivity on carryover

The autosampler carryover described above was made more problematic by the high sensitivity of the API 4000 mass spectrometer and the high ionization efficiency of **I** in TIS mode. The high absolute sensitivity of the mass spec-

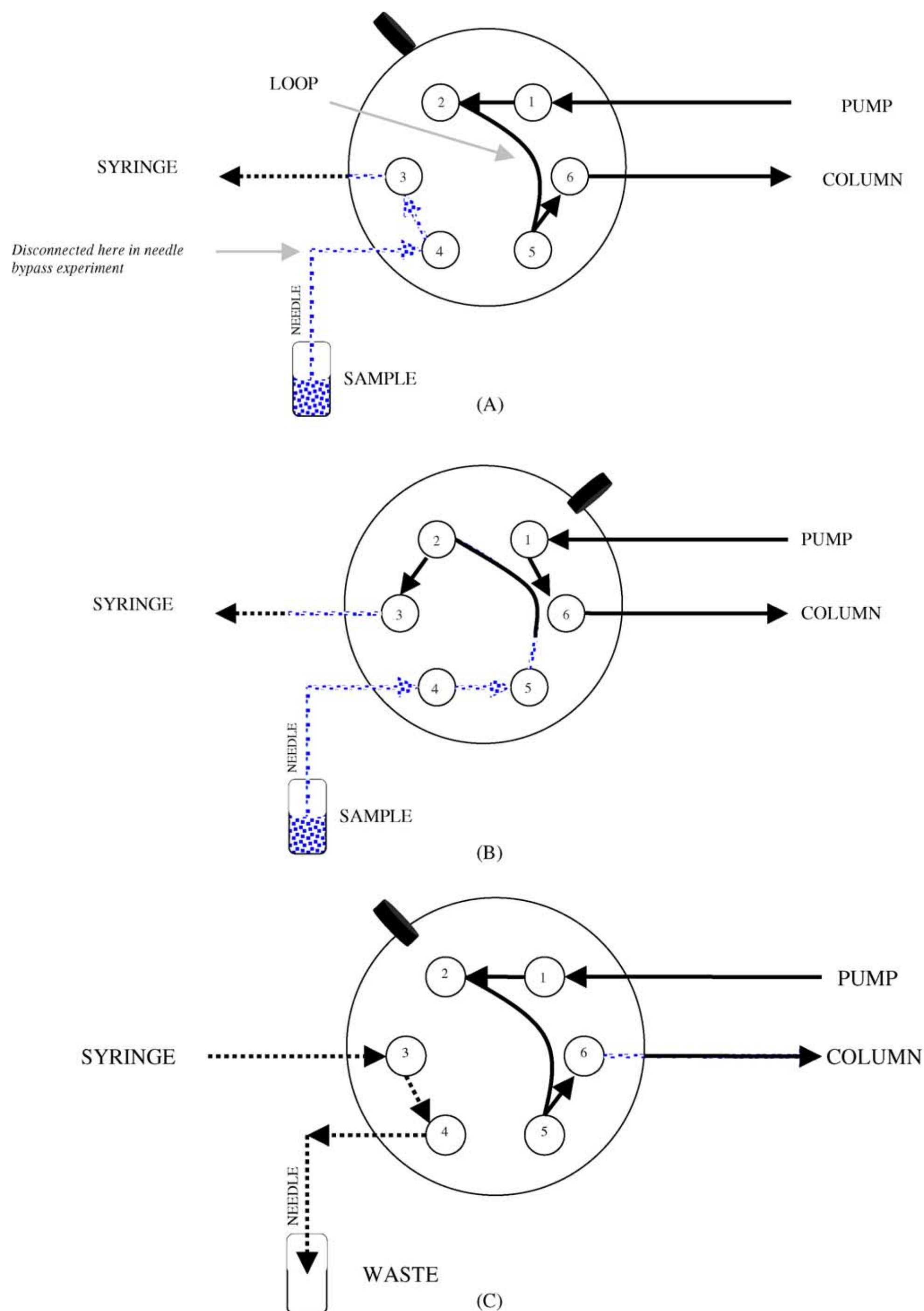

Fig. 3. Operational schematic of the Varian ProStar 430 autosampler. (A) Pre-injection flush step, valve is in the INJECT position, (B) loop fill step, valve is in the LOAD position (partial loop injection shown) and (C) inject/needle wash step, valve is in the INJECT position.

trometer for **I** is evidenced by the signal to noise ratio (>50) in the chromatogram shown in Fig. 5A. The mass of **I** injected in this chromatogram was approximately 280 fg. Under these conditions, a carryover peak one-fifth the intensity of the LLOQ would correspond to approximately 8.7×10^7 molecules of **I**, a relatively small number, potentially being adsorbed within the autosampler. In contrast to a system with this level of detection sensitivity, one might expect a reduced extent of carryover relative to the LLOQ for an HPLC system employing a less sensitive detector (i.e., a system on which a greater mass of analyte is injected at the LLOQ). The rationale for this prediction is that maintaining a constant ratio of carryover to LLOQ with a less sensitive detector would require significantly more analyte molecules to be adsorbed to a limited number of potential sites.

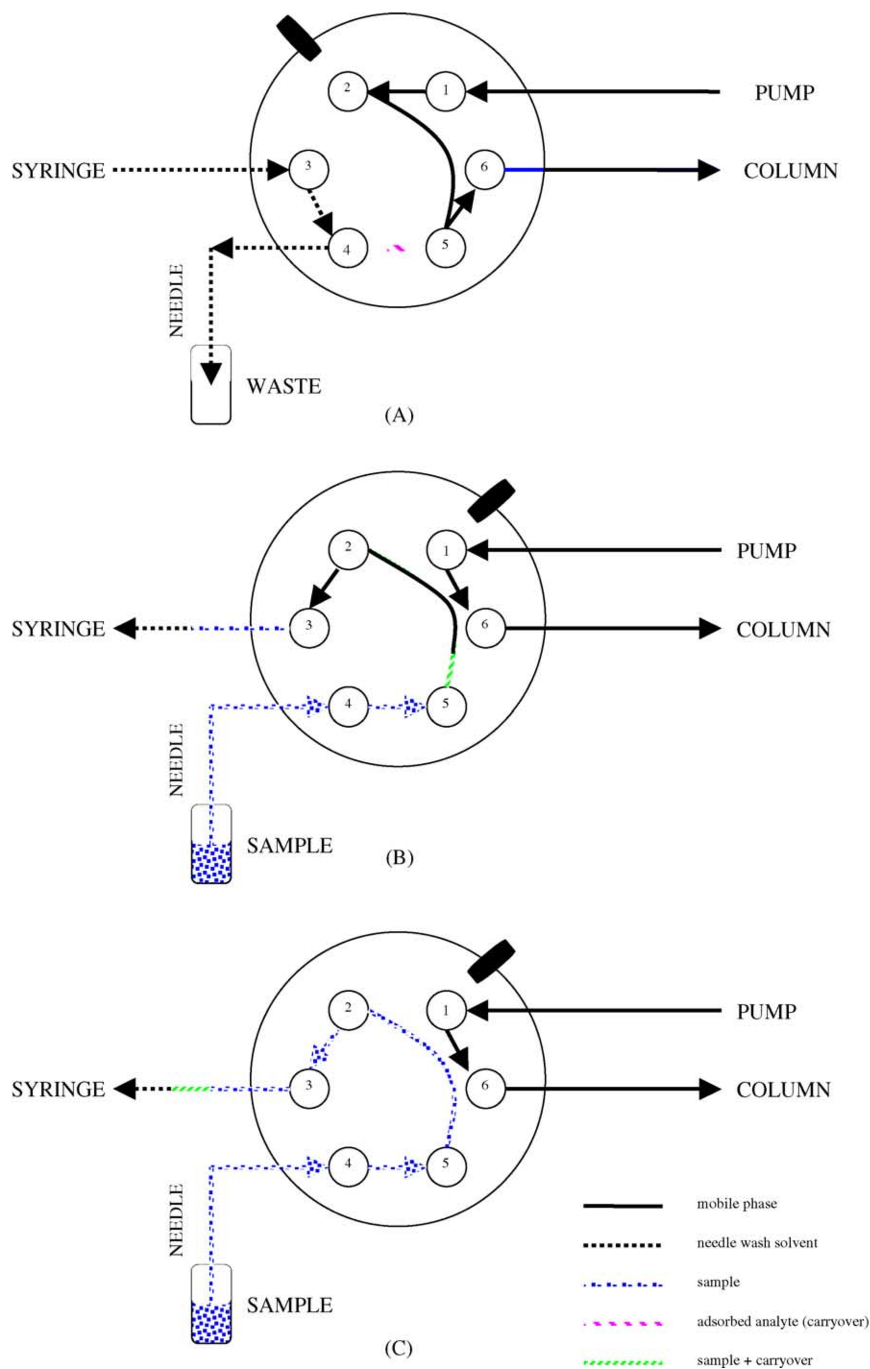

Fig. 4. Depiction of the potential for injection valve carryover in partial and full loop injection modes. (A) Residual analyte in valve following the injection of a concentrated sample, (B) partial loop injection—residual analyte is flushed into the sample loop resulting in a carryover peak and (C) full loop injection—residual analyte is flushed out of the sample loop during loop overfill.

To assess the severity of autosampler carryover of **I** as a function of detection sensitivity, the TIS probe in the mass spectrometer ion source was replaced with a HN probe. The MS/MS response for **I** using the HN probe was approximately 40-fold less than TIS. In the HN experiments, the concentration range of **I** in plasma was kept the same as the TIS method (0.2–120 ng/mL). To compensate for the decreased sensitivity of the HN probe, samples were concentrated (2.5×) following extraction and the injection volume was increased to 20 μL (partial loop mode). The same autosampler was used in both methods. Chromatograms of a plasma double blank (injected immediately after a ULOQ standard) and a LLOQ standard obtained with the HN method are shown in Fig. 6, in which it is evident that no detectable carryover was present under these conditions. The difference in sensitivity between the methods is apparent; the mass injected at the LLOQ was 6 pg for the HN compared to 0.28 pg for the TIS method.

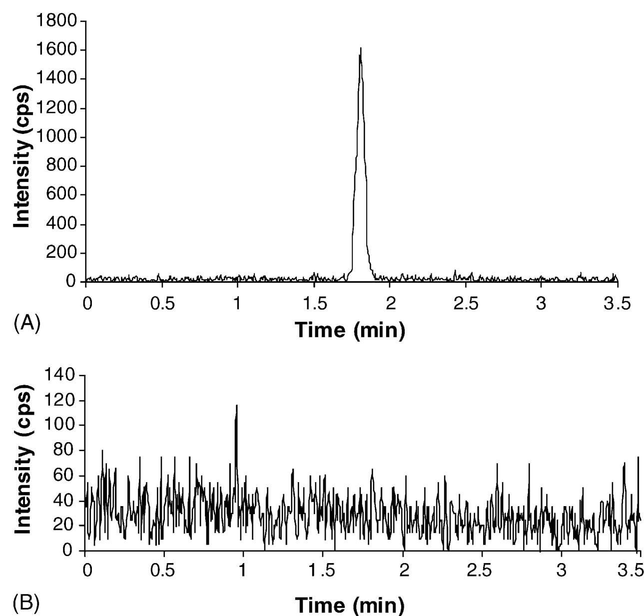

Fig. 5. Extracted ion chromatograms of 0.2 ng/mL extracted plasma standard (A) and a control plasma double blank (B) injected immediately after a 120 ng/mL plasma standard. Injection volume = 2 μL full loop. Mass of **I** injected in (A) = 0.28 pg.

These results clearly illustrate that the mass of residual **I** in the autosampler (i.e., the carryover) is not proportional to the concentration of **I** in the sample. Assuming that adsorbed **I** gives rise to carryover, the absence of detectable carryover in the HN method, despite the fact that the same autosampler, mobile phase, and needle wash solvent were used as in the TIS method, implies that the adsorption of **I** is saturable. Adsorption of this type can be described by a Langmuir isotherm. The Langmuir isotherm is useful in rationalizing why HPLC systems with less sensitive detectors are generally less susceptible to carryover. In these cases, it is more likely that the mass of analyte adsorbed when all the adsorption sites are occupied is below the limit of detection of the method. Obviously, the severity of carryover in any particular case is compound dependent, but in general terms, assuming that a given system is optimized (e.g., proper tubing connections and appropriate needle wash solvent), the system will be more susceptible to carryover the greater the absolute sensitivity of the detector.

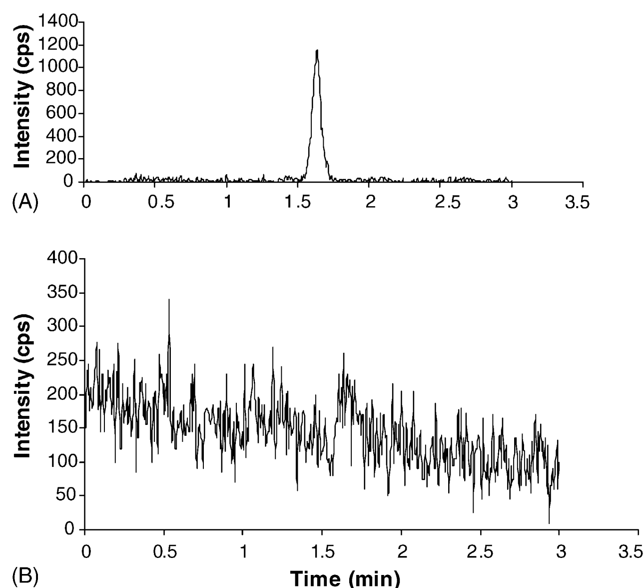

Fig. 6. Extracted ion chromatograms of 0.2 ng/mL extracted plasma standard (A) and a control plasma double blank (B) injected immediately after a 120 ng/mL plasma standard using a heated nebulizer (HN) probe. Mass of **I** injected in (A) = 10 pg.

It is worth reiterating that the range of plasma concentrations of **I** was identical in both the TIS and HN methods (0.2–120 ng/mL). Thus, the critical difference in the methods was not the concentration range of **I** in the plasma samples, but the range of masses of analyte injected onto the HPLC-MS system. These observations demonstrate that a key criterion with regard to the susceptibility of a method to carryover is the mass of residual analyte in a system relative to the mass of analyte injected at the LLOQ.

4. Conclusion

In this report, a case study in the elimination of autosampler carryover in a bioanalytical HPLC-MS/MS method was presented. Through a systematic set of experiments and a thorough understanding of the operation of the autosampler, a relatively straightforward solution was implemented by switching from a partial loop to full loop injection. Since these experiments were conducted, this approach has been successfully utilized in other assays in our laboratories that were prone to autosampler carryover.

It has been demonstrated in this work that autosampler carryover can be a greater concern in HPLC systems with highly sensitive detectors, for which the mass of analyte injected at the LLOQ is relatively low. As advances in technology continue to yield more sensitive mass spectrometers, controlling autosampler carryover will be an increasingly challenging aspect of the development of highly sensitive bioanalytical HPLC-MS methods.

References

[1] M.E. Swartz, I.S. Krull, Analytical Method Development and Validation, Marcel Dekker Inc., New York, 1997.
[2] J.W. Dolan, LCGC NA 19 (2001) 164–167.