Universal LC–MS method for minimized carryover in a discovery bioanalytical setting

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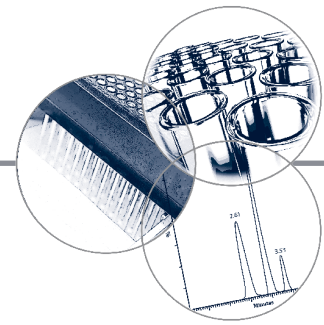

Universal LC–MS method for minimized carryover in a discovery bioanalytical setting

Background: A frequent impediment to accurate quantitation in bioanalytical LC–MS arises from carryover. For many new chemical entities in drug discovery carryover is not limited to the autosampler, but instead arises from several different sources. **Method:** We tested several different columns, injector wash sequences and gradient compositions to understand and eliminate these sources. In many instances carryover was dictated by the elution gradient and column as much as the autosampler hardware and wash protocol. **Conclusion:** Several trends were observed. First, different columns resulted in significantly different amounts of carryover (even for nominally the same column chemistry). Second, a continuous high organic wash of the column was not as effective at removing carryover as cycling between high and low organic mobile phases during the column wash. Combining our observations (column, gradient and autosampler configuration) we devised a short 3-min method that is appropriate for a diverse set of new chemical entities and minimizes carryover while still being sufficiently robust to use in a drug-discovery setting.

Quantitative analysis of biological samples by LC–MS is an established technique used throughout bioanalytical laboratories for the determination of analyte concentration [1,2]. The accuracy of a sample measurement can be affected by **carryover**, which is the contamination of a sample by the analyte of interest coming from a previous sample injection. Because carryover is a ubiquitous problem, the pharmaceutical industry and the US FDA have collaboratively addressed the issue by publishing best practices for minimizing carryover levels when validating a bioanalytical method [3,4]. It is recommended that following an injection at the method's ULOQ, the analyte peak area measured in a subsequent blank injection should be less than 20% of the analyte peak area at the **LLOQ**. While the allowable carryover under these guidelines depends on the 'worst-case scenario' established by the validated linear range, recent efforts to better evaluate carryover over the entire analytical sequence have been proposed [5,6].

There are many physical and chemical contributors to carryover, including adsorption of analyte onto surfaces (other than the column stationary phase) and trapping of analyte in dead volumes within system flow paths [7]. The autosampler is often assumed to be the main source of carryover due to the high concentration of analyte exposed to a large variety of surface materials and large number of interrupted flow paths [8]. Since the injector contains the only moving parts in the flow path, special attention is given to the friction generated in the injection valve and syringe during routine operation, which can degrade their respective surfaces leading to the formation of channels that can trap analyte and prevent its removal. Other parts of an LC–MS system can also contribute to carryover. Improper tubing connections create dead volumes that physically trap analyte, while the material the tubing is made of can chemically interact with analyte. Likewise, the same chemical and physical attraction of the analyte to the packing material and walls of the LC column can contribute to carryover. Even the ion source of the mass spectrometer can contribute to carryover via spray contamination of orifice surfaces and heating elements [9]. In effect, any portion of the LC–MS system that comes into contact with the analyte can potentially contribute to carryover.

Despite its numerous sources, strategies for carryover reduction are most often focused on rinsing the autosampler syringe and valve with compatible organic and aqueous solvents. For reverse phase LC–MS, cocktails of organic and aqueous solvents that are compatible with the mobile phase are most often used. Typically, the organic solvents are combinations of acetonitrile (ACN) or methanol with water (often acidic or basic), although other more

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Key Terms

Carryover: Presence of analyte from a previous sample injection that contributes to subsequent injections' analyte response.

LLOQ: The lowest level of analyte that can be quantitatively measured with acceptable signal-to-noise ratio, as defined by a bioanalytical method's linear calibration range.

Absorption, distribution, metabolism and elimination: The central descriptors for the time course of drug distribution in the body.

Saw-tooth wash: A type of chromatographic column cleaning step characterized by several rapid cycles from a high to low percentage of organic solvent; shown to be effective at reducing carryover.

aggressive solvents, such as tetrahydrofuran, isopropanol or dimethylsulfoxide (DMSO), have been used [10,11]. The success of specific wash solvents to reduce carryover often depends on the physicochemical properties of the analyte. Since most small-molecule drugs are relatively hydrophobic, organic solvents work well to solubilize and remove them from the system. Likewise, wash solvents modified with acid or base can offer improved carryover reduction for analytes with ionizable groups whose solubility may be affected by different pH. Other wash solvent additives, such as perchloric acid and trifluoroethanol, can remove analytes as an additional approach to tailor the wash solvent system to the desired analyte [7,12].

The reduction of carryover has been a driving force in autosampler hardware development. In general, there are two ways to introduce a sample into the sample loop of an autosampler, push-to-fill and pull-to-fill [8]. Between these two techniques, the potential for carryover has been shown to be greater with the push-to-fill technique due to a larger number of surfaces in contact with the sample that remain unswept by the mobile phase flow path [13]. To minimize these contributions to carryover, more rigorous active pumping of wash solvents through internal valve components and the use of polymeric coatings to reduce adsorption are now employed in push-to-fill autosampler designs [101–103]. Alternative strategies using modified push-to-fill techniques that separate the syringe barrel from the needle in conjunction with ultrasonic cleaning of the injection port have emerged [14,15].

Improvements in analytical instrumentation offer the bioanalyst an arsenal of tools to simplify bioanalytical method development and validation. In our discovery bioanalytical group we perform bioanalysis on new chemical entities (NCEs) to identify those compounds with the desired **absorption, distribution, metabolism and excretion** (ADME) profile (*in vitro* and *in vivo*) for the target we are investigating. The ADME profile is usually derived from a concentration versus time curve following the concentration of the analyte. The analyte–time profile is a critical set of data used to determine which NCEs to pursue for further optimization and which to abandon. It is imperative, therefore, to generate accurate and precise concentration data in order to make well-informed decisions. With ever-increasing throughput demands, bioanalysts' focus on method optimization to shorten cycle times while maintaining robustness and reliability [16]. From a chromatographic perspective, this goal is often achieved by running very short or ballistic-type gradients. In most instances, carryover assessment focuses on the autosampler while neglecting the HPLC column as a source. In this paper we examine the contributions of different columns on system carryover as well as the effectiveness of different gradients at reducing carryover due to the column. We have developed a generic method that utilizes a post-gradient **saw-tooth wash**, which effectively reduces carryover when applied to a diverse library of commercial drugs. Additionally, the effectiveness of a DMSO-based three-solvent active wash procedure was compared with a two-solvent dynamic load and wash (DLW) procedure in reducing carryover originating from the autosampler.

Experimental

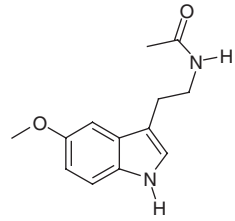
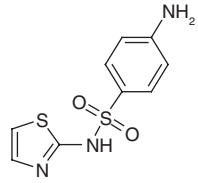
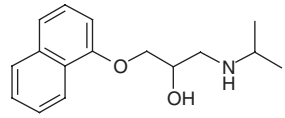
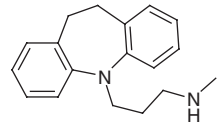
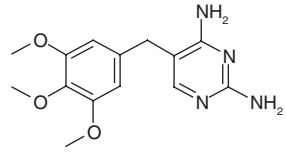
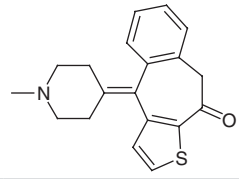
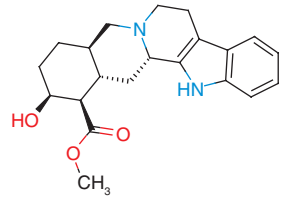
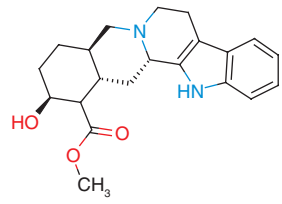
Chemicals & reagents

ACN, methanol and DMSO were obtained from Mallinckrodt Baker Inc. (NJ, USA). All solvents were high-purity LC–MS grade. Water was purified using a Barnstead NANOPure Diamond water purification system (IA, USA). All chemicals and commercially available drugs were obtained from Sigma Aldrich (MO, USA) at purities >95%, except for the proprietary compound VRT-X, a probe molecule synthesized and purified at Vertex Pharmaceuticals Inc. (MA, USA). The structures and identities of the diverse set of drugs are shown in Table 1. Blank rat plasma treated with K_2 EDTA was obtained from Bioreclamation Inc. (NY, USA).

Sample preparation

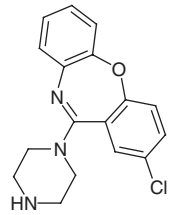
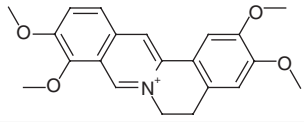
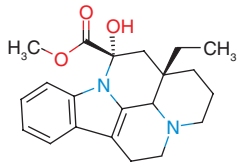
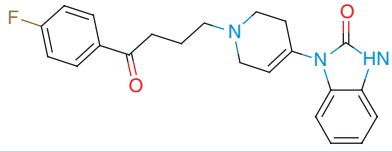
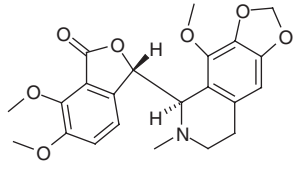
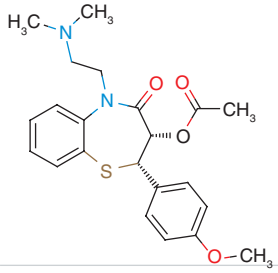
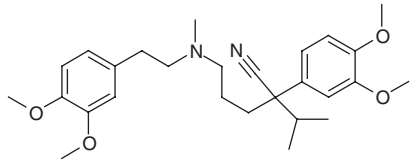
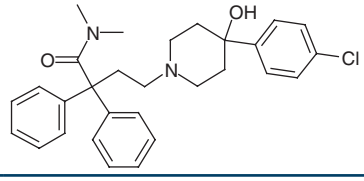
All compounds tested in this evaluation were initially prepared as 1 mg/ml stock solutions in DMSO. Subsequent dilutions were made in ACN to prepare working solutions at several concentrations. Working solutions of each compound were then separately spiked into blank rat plasma in a 1:10 dilution step to create plasma concentrations spanning from the ULOQ of our method at either 5000 ng/ml or 1000 ng/ml, to the LLOQ at 1 ng/ml. Plasma samples were extracted by protein precipitation in ACN at a 4:1 ACN:plasma ratio. Precipitated samples were shaken on a vortex mixer for 5 min and then spun down at 1209 g in an Eppendorf 5804R centrifuge for 20 min. Sample supernatant was transferred to shallow 96-well plates for injection on the LC–MS system.

Table 1. The diverse drug set used to evaluate the optimized generic method for carryover reduction.

Name	MW	MRM transition	cLogP	pKa	Structure
Melatonin	232.1	233.2→174.0	1.15		
Sulfathiazole	255.0	256.2→156.2	0.98	0.4, 2.0, 5.7	
Propranolol	259.2	260.0→116.2	2.58	9.7	
Desipramine	266.2	267.2→72.0	3.90	10.0	
Trimethoprim	290.1	291.2→230.2	1.28	7.2	
Ketotifen	309.1	310.4→96.0	3.35	7.2	
Amoxapine	313.1	314.2→271.2	3.08	1.0, 9.2	
Palmatine	352.2	353.2→337.2	-1.22		

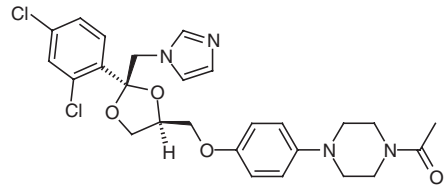
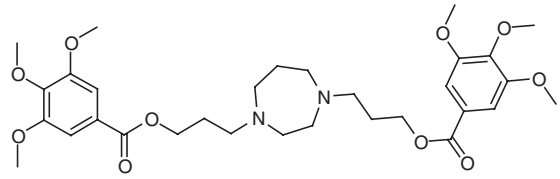
MRM: Multiple reaction monitoring.

Table 1. The diverse drug set used to evaluate the optimized generic method for carryover reduction (cont.).

Name	MW	MRM transition	cLogP	pKa	Structure
Yohimbine	354.2	355.2→144.2	2.10	7.7	
Corynanthine	354.2	355.2→144.2	2.10	7.7	
Vincamine	354.2	355.0→337.2	3.16	8.2, 10.5	
Droperidol	379.2	380.0→165.4	3.06	7.1	
Noscapine	413.2	414.2→220.2	2.58		
Diltiazem	414.1	415.2→178.2	3.65	8.2	
Verapamil	454.3	455.4→165.2	5.04	9.7	
Loperamide	476.2	478.2→267.4	4.77	9.4	

MRM: Multiple reaction monitoring.

Table 1. The diverse drug set used to evaluate the optimized generic method for carryover reduction (cont.).

Name	MW	MRM transition	cLogP	pKa	Structure
Ketoconazole	530.2	531.4→489.4	4.19		
Dilazep	604.3	605.6→195.2	2.88	5.5, 9.5	

MRM: Multiple reaction monitoring.

LC–MS

All analyses were carried out by LC/MS with a system configuration consisting of a CTC Analytics HTS PAL autosampler (Zwingen, Switzerland) coupled with an Agilent 1100 binary HPLC (CA, USA) and an API-4000 triple quadrupole mass spectrometer (AB Sciex, CA, USA). The PAL autosampler was outfitted with the four-solvent active wash system (4S-AWS) (Gerstel Inc., MD, USA). The PAL autosampler was also separately configured with either the standard two-solvent fast **wash station** (2S-FWS) or the DLW station for comparison to the 4S-AWS in carryover performance. Injection port cleaning with the 4S-AWS used an optimized three-step wash procedure that consisted of: six-times 50:50 DMSO:methanol; six-times methanol; and three-times 0.1% formic acid in water. The wash procedure for the 2S-FWS consisted of first cleaning the syringe with three-times 90:10 ACN:water and two-times 90:10 water:ACN, followed by repeating steps one and two to wash the injection valve. The wash procedure for the DLW consisted of 5-s wash times of both the valve and port; first with 90:10 ACN:water and then with 90:10 water:ACN.

To identify and minimize the contribution of the analytical column to carryover, several reverse phase columns and gradients were tested. The columns used in this study were the Xterra MS C_{18} (2.1 × 50 mm, 5 $_{\mu m}$, Waters, MA, USA), Venusil ASB C_{18} (2 × 30 mm and 2 × 50 mm, 5 µm, Bonna-Agela, DE, USA), Symmetry C_8 (2 × 30 mm, 3 µm, Waters), Unison-UK C_{18} and Unison-UK C_8 (2 × 30 mm, 3 µm, Imtakt USA, PA, USA), and Presto FF C_{18} (2 × 30 mm, 2 µm, Imtakt USA). The column characteristics are shown in Table 2. A number of gradients were tested that varied in the length, flow rate, and style of the wash step. A detailed description of the gradients used can be found in the discussion. For all experiments the mobile phase consisted of solvent A: 90% 10 mM ammonium acetate pH 4.5 with 10% ACN; and solvent B: 100% ACN. The flow rate was 800 µl/min unless otherwise noted.

The mass spectrometer settings were obtained by manually tuning the instrument to obtain optimal positive mode ESI multiple reaction monitoring (MRM) transitions for sensitivity. The MRM transitions for the commercial drug set are listed in Table 1. Sample injection and wash conditions were controlled with Gerstel Maestro PrepBuilder software. Data acquisition and peak area integration was performed using Analyst 1.4.1 software. To calculate carryover, peak areas of three LLOQ injections were averaged and compared with the peak area of a single blank plasma extract injection following a single ULOQ injection. This injection sequence was performed in triplicate (n = 3) for each condition tested, and all carryover was reported as the % average of the LLOQ. To measure carryover arising from the column only, the procedure was modified between the ULOQ and the blank extract injection steps. After running the ULOQ sample, the HPLC flow was disconnected from the injection valve and replumbed directly from the HPLC pump to the column. Subsequently, a blank injection was made to trigger the gradient and data acquisition start, which measured only the remaining

Key Term

Wash station: A generic term for an autosampler's hardware component utilized to remove remaining analyte from the injection needle, port and valve following sample introduction.

Table 2. Column characteristics.

Column	Chemistry	Diameter (mm)	Length (mm)	Particle size (µm)	Pore size (Å)	Endcapped	Carbon load (%)
Imtakt Presto FF	C_{18}	2.0	30	2	NP	Y	N/A
Imtakt Unison-UK	C_8	2.0	30	3	130	Y	N/A
Imtakt Unison-UK	C_{18}	2.0	30	3	130	Y	N/A
Waters Symmetry	C_8	2.1	50	5	100	Y	12
Agela Venusil ASB	C_{18}	2.1	50	5	150	Y	10
Agela Venusil ASB	C_{18}	2.1	30	5	150	Y	10
Waters Xterra MS	C_{18}	2.1	50	5	125	Y	15.5

analyte in the system from the column to the MS source, excluding any contribution from the autosampler.

Results & discussion

In the drug-discovery setting, bioanalytical LC–MS methods must provide accurate and precise data with reasonable throughput to keep pace with the large number of NCEs produced while still delivering reliable and accurate data. The robustness and timeliness of this data directly impacts project advancement by identifying compounds with desirable pharmacokinetic profiles. In an effort to increase throughput, run times are often shortened at the expense of separation efficiency and column equilibration. Often, these steps lead to increased carryover between injections, which negatively affects the accuracy and precision of the method. While carryover is most often attributed to inefficient cleaning of the injection port and valve on the autosampler, there is also a component derived from the column. Our goal was to identify column and gradient combinations that could minimize carryover and be generically applied to a diverse chemical set.

■ Gradient optimization

A graphical representation of the gradients used in this study is depicted in Figures 1A–D. For each gradient, the initial step consisted of a linear gradient from 5–95% B (organic) in 0.5 min. The length and mode of the column wash step was then varied to determine its effectiveness at cleaning the column and reducing carryover. In Gradient I, the wash step was held at 95% B for 0.7 min and then equilibrated at 5% B for 0.8 min for a total run time of 2 min. Gradient II had an extended wash time of 2.5 min at 95% B and equilibration at 5% B for 1.0 min, for a 4 min total-run time. Gradient III consisted of running Gradient I back to back to create a 4-min double-gradient method. Several saw-tooth-like washes were tested to replace the isocratic 95% B wash step with a series of rapid cycles of 95 to 5% B steps. An example of this 3 min run with three wash steps (Gradient IV) is shown in Figure 1C. Here, an individual step cycles from 95% B for 0.5 min to 5% B for 0.2 min. Additional 3 min runs were tested that increased the total number of saw-tooth steps while reducing the individual step wash time at 95% B. Gradient V included four steps that cycled from 95% B for 0.3 min to 5% B for 0.2 min. A 3 min five-step gradient was tested, but did not perform better than the three- or four-step gradients and was not included in the results. Additionally, a 3-min three-step saw-tooth gradient run at higher flow (1400 µl/min instead of 800 µl/min) during periods at 95% B (Gradient VI, not shown) was also tested.

The method previously used most often in our laboratory for routine analysis consists of Gradient I run on an Xterra MS C_{18} (2.1 × 50 mm, 5 µm) column at 800 µl/min. Using this column and flow rate we tested the gradients described herein for their effect on carryover of compound VRT-X, a probe compound known for its 'stickiness'. As shown in Figure 2, the effect of different gradients on carryover of VRT-X is pronounced, with reductions ranging from 60 to 80%, as exemplified by Gradients II and III. The carryover for Gradient I is 156% of the LLOQ peak area, nearly eight-times the best practice recommendation of 20%. By extending

the wash time of the column using Gradient II, the carryover can be reduced to 62% of LLOQ. It is possible to reduce the carryover to 29% of LLOQ using Gradient III. The total run time can be reduced to 3 min while maintaining similar carryover levels to the 4 min Gradient II if the wash step is changed from an isocratic to a saw-tooth-step process (Gradients IV and V). Utilizing either three or four saw-tooth steps does not statistically alter the carryover level. However, increasing the flow rate from 800 to 1400 µl/min during the wash steps (Gradient VI) is effective at further reducing carryover. As demonstrated by the >80% drop in carryover from Gradients I to III, it is clear that different column cleaning steps can significantly affect carryover levels.

Column optimization

The carryover following a 1000ng/mL injection of VRT-X on several different columns was determined using Gradient IV. The results are depicted in Figure 3. The Xterra MS C_{18} (2.1 × 50mm, 5 µm) suffered from the highest carryover levels at 57% of LLOQ. The lowest level of carryover was measured using the Presto FF nonporous C_{18} column (4.6 × 30 mm, 2 µm), at 15% of LLOQ. Based on the columns tested, the only column characteristic that correlated with decreased carryover was particle size. The seven columns tested had particle sizes ranging from 2 to 5 µm and showed a direct correlation between particle size and carryover level. This phenomenon may be due to the greater linear velocity of the wash solvent experienced by smaller particles than larger particles at a fixed column diameter and flow rate. The Presto FF C_{18} column had the smallest particle size as well as nonporous particles, which would further reduce the column volume, and exhibited the lowest carryover levels. Two Venusil C_{18} columns, identical except for column length (30 vs 50 mm), gave nearly identical carryover, indicating column length is not the main determinant of carryover. Similarly, two identically sized Unison columns packed with different chemistries (C_{18} vs C_8), also had similar amounts of carryover, indicating that in this instance the reverse-phase column chemistry is not significant in altering carryover level. A complete and fair comparison between % carbon load and carryover could not be made since the Imtakt brand columns do not report % carbon load. However, it can be noted that the Xterra MS C_{18} column had both the highest % carbon load and highest carryover. Differences in carryover between columns may also arise

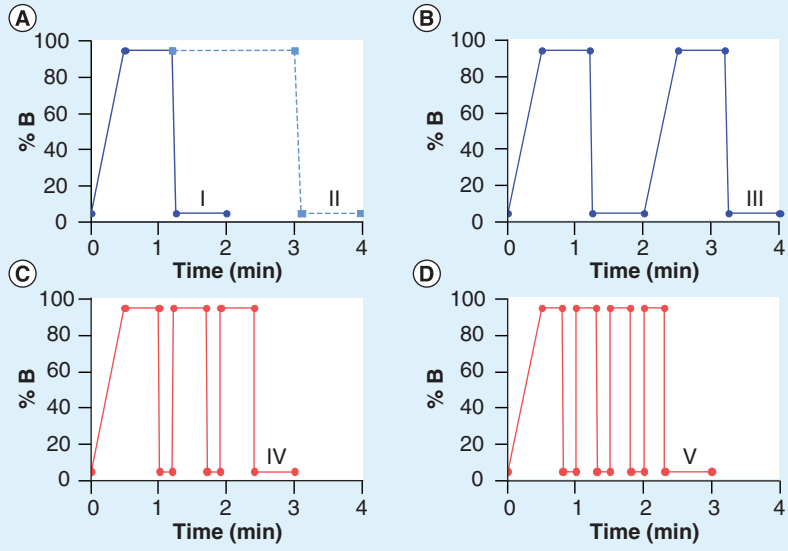


Figure 1. Gradient profiles. Several gradients with different wash types were developed to reduce carryover. **(A)** Gradients I and II represent single isocratic wash steps (2 and 4 min extended wash, respectively). **(B)** Gradient III represents a single gradient/wash run in series (4 min double gradient). **(C)** Gradient IV represents a single gradient with three wash steps (three-step saw tooth). **(D)** Gradient V represents a single gradient with four wash steps (four-step saw tooth).

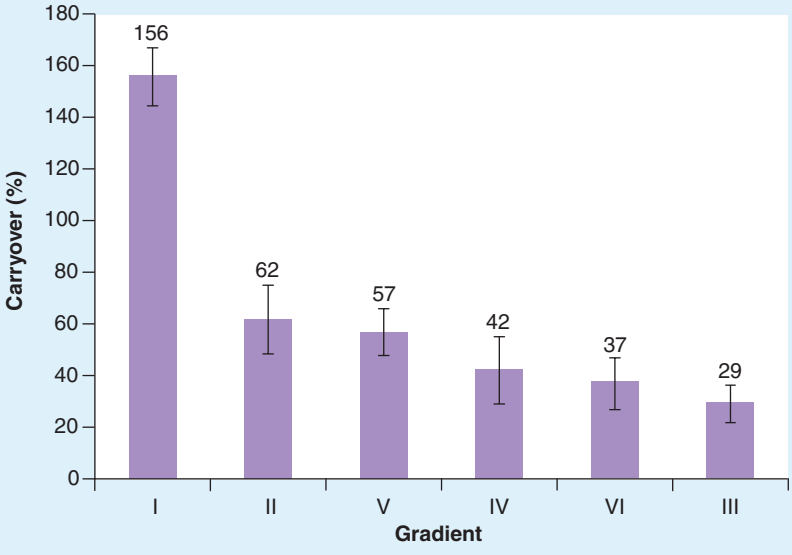


Figure 2. Effect of gradient type on column carryover. Six different gradient wash combinations were tested against the Xterra MS C_{18} column using the four-solvent active wash system autosampler. The carryover is expressed as percentage of LLOQ in blank injection following a 5000 ng/ml injection. The lowest carryover was observed when combining two gradient and wash steps together (Gradient III). Similar results could be obtained in shorter time by running a single gradient with several saw-tooth-wash cycles (Gradients IV and VI). Error bars represent the SD of three measurements.

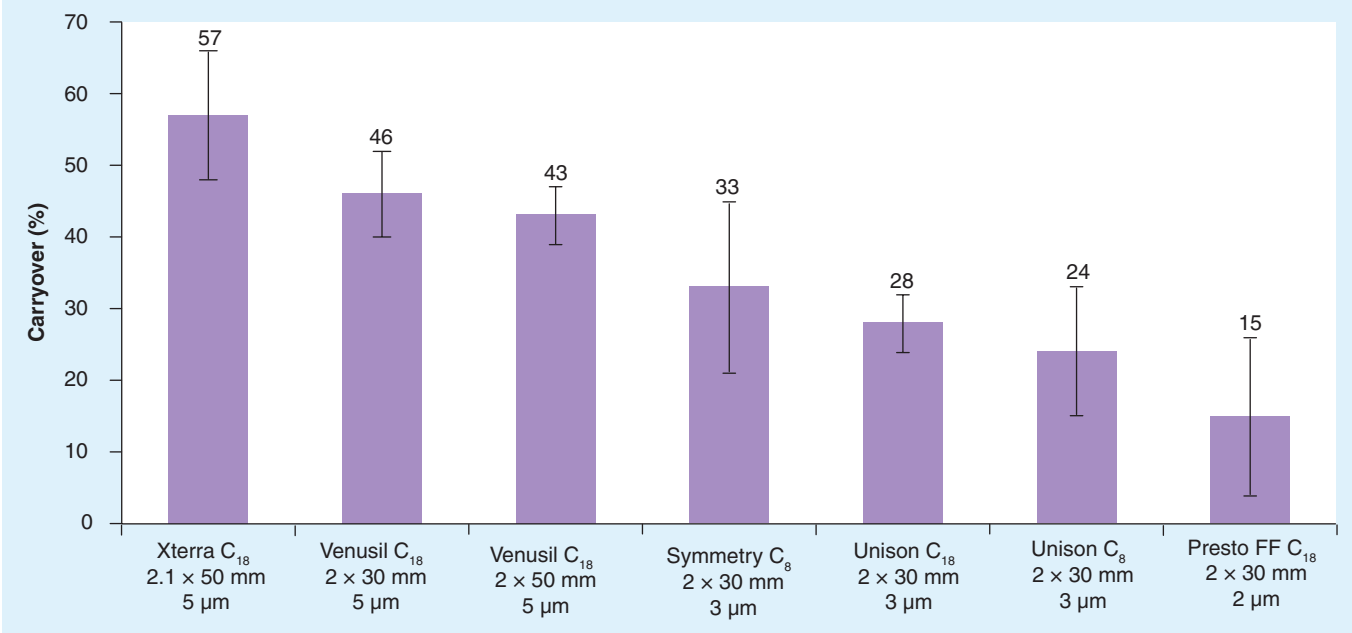


Figure 3. Effect of column type on column carryover. Gradient IV was run on all seven columns using the four-solvent active wash system autosampler to compare the carryover as a function of column type. The carryover is expressed as percentage of LLOQ in blank injection following a 5000 ng/ml injection. Note that five of the seven columns are endcapped C_{18}. Thus, even with nominally the same column chemistry, the carryover differed significantly. Error bars represent the standard deviation of three measurements.

from differences in hardware construction, specifically frit design, that are not related to the particle chemistry.

Carryover arising directly from the column, rather than the autosampler or MS source, was also determined. The contribution from each column on the overall carryover is depicted in Figure 4. In this experiment, a ULOQ injection was first run on each column. Before injection of the subsequent blank, the flow path of the LC system was redirected to bypass the autosampler and flow directly from the LC pump to the column. Since the autosampler was excluded from the flow path, the resulting peak resulted from any remaining analyte on the column. No analyte was detected in blank injections bypassing both the column and the autosampler, indicating that the MS source did not contribute to carryover (data not shown). The Presto FF C_{18} column was completely void of carryover while the carryover measured with the Xterra MS C_{18} was 33% of the LLOQ. When compared with the total system carryover of 57% on the Xterra MS C_{18}, as shown in Figure 2, the contribution to the carryover arising directly from the column is more than half (58%) of the total carryover. Since no single factor appears to dictate a column's likelihood to contribute to carryover, the most prudent course during method development is to experimentally determine carryover on several different columns and choose the column with lowest carryover that meets separation requirements.

In choosing the column to test with the diverse drug set, we chose the Imtakt Unison C_{18} over the Imtakt Presto FF C_{18}, even though the Presto had the lowest column only carryover in the head-to-head test. The Presto FF nonporous particles can only tolerate a small injection volume (1–2 µl) compared with the Unison C_{18} porous particles (10–15 µl) before peak symmetry will be affected due to overloading. The carryover due to the column measured with the Unison C_{18} was only 4%, which was an acceptable trade-off for better sample loading and sensitivity. The gradient chosen to test against the drug set was Gradient IV due to its balance of speed and effectiveness at reducing carryover.

■ Autosampler performance

In addition to optimizing the column and gradient, we also compared the carryover reduction performance of three wash stations for the PAL autosampler, namely, the 2S-FWS, the 4S-AWS and the DLW. The main differences between these wash stations, with regard to their ability to reduce carryover, are the wash solvent flow paths, the number of wash solvents and the wash solvent delivery rates. The 2S-FWS uses

the injection syringe to dispense wash solvent to the injection port. It is relatively slow and uses much less wash solvent compared with the 4S-AWS and the DLW, whose wash solvents are actively dispensed using separate pumps. The DLW has the fastest wash cycle time and has the fewest surfaces in contact with sample during injection for simplified cleaning.

A diverse chemical set of commercial drugs was chosen to validate the performance of the optimized column, gradient and autosampler wash stations. The selection of compounds for the test set aimed to cover a range of characteristics that might be produced in a medicinal chemistry discovery effort (Table 1). These included a range in molecular weight from approximately 230 to 600 amu, as well as a cLogP range of -1.22 to 5.04. The set contains a variety of chemical functionalities, such as aromatic/non-aromatic heterocycle, aromatic hydrocarbon, amine, amide, sulfonyl, methoxy, carbonyl, cyano and halogen groups. The drug set is comprised of neutral molecules, weak acids and bases with pKa values ranging from 0.4 to 10.5.

The reduction of carryover on the 4S-AWS autosampler using the optimized method (Unison C_{18}, Gradient IV) as compared with our routinely used method (Xterra MS C_{18}, Gradient I) on the 2S-FWS autosampler is shown in Table 3. Carryover was measured following injections of 5000 and 1000 ng/ml on both systems. On average, the reduction of carryover following the 5000 ng/ml injection was 87 compared with 61% for the 1000 ng/ml injection. These reductions incorporate the improvements made at all sources of potential carryover, including the autosampler design and wash-solvent optimization [16], plus the column and gradient optimization. Comparing the unoptimized 2S-FWS/Xterra C_{18}/Gradient I carryover between ULOQs of 5000 and 1000 ng/ml in Table 3, the carryover is reduced by an average of 84%, which is approximately proportional with the expected decrease in amount injected. This demonstrates that carryover can be reduced by injecting less concentrated samples. However, this approach will reduce the linear range of the method and the resulting carryover reduction may still be insufficient based on method requirements. By utilizing the optimized 4S-AWS/Unison C_{18}/Gradient IV with a 1000 ng/ml ULOQ, the additional 61% reduction of carryover may provide the necessary improvement to satisfy validation guidelines.

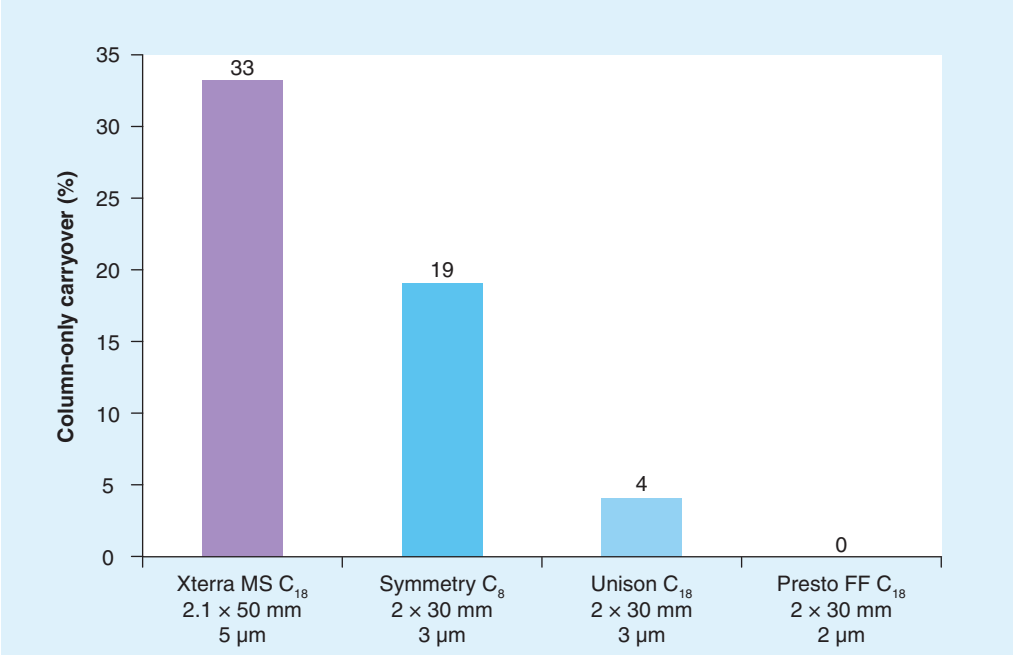


Figure 4. Column-only carryover. To identify which column contributed most to overall carryover, the carryover arising directly from each column without the autosampler was measured. The carryover is expressed as percentage of LLOQ in blank injection following a 5000 ng/ml injection. Column particle size and porosity appear to play a role in the level of carryover observed. The Presto FF C_{18} column had the smallest particles tested, which were also nonporous.

Table 3. Comparison of carryover reduction using 'optimized' versus 'unoptimized' methods following ULOQ injections of 5000 and 1000 ng/ml.

Compound	5000 ng/ml ULOQ			1000 ng/ml ULOQ		
	2S-FWS/Xterra C_{18}/Gradient I % LLOQ	4S-AWS/Unison C_{18}/Gradient IV % LLOQ	% reduction	2S-FWS/Xterra C_{18}/Gradient I % LLOQ	4S-AWS/Unison C_{18}/Gradient IV % LLOQ	% reduction
Melatonin	246	41	83	40	11	73
Sulfathiazole	365	9	98	17	16	6
Propanolol	1175	142	88	165	30	82
Desipramine	1186	53	96	153	41	73
Trimethoprim	119	19	84	32	23	28
Ketotifen	252	110	56	65	24	63
Amoxapine	1311	50	96	440	41	91
Palmatine	1350	162	88	114	57	50
Yohimbine	799	44	94	27	8	70
Corynanthine	202	17	92	26	6	77
Vincamine	142	51	64	45	15	67
Droperidol	1488	129	91	119	25	79
Noscapine	285	20	93	28	8	71
Diltiazem	1088	150	86	41	22	46
Verapamil	268	40	85	48	27	44
Loperamide	1410	42	97	64	25	61
Ketoconazole	687	90	87	150	33	78
Dilazep	534	77	86	149	95	36
Average	**717**	**69**	**87**	**96**	**28**	**61**

Using the optimized method of the 4S-AWS autosampler, Unison C_{18} 2 × 30 mm column, and Gradient IV, the average percentage reduction of carryover is 26% greater when injecting 5000 versus 1000 ng/ml on column. However, the average absolute carryover levels are lower by 41% for the 1000 ng/ml injection than the 5000 ng/ml injection (28 vs 69%). The unoptimized method consisted of the 2S-FWS autosampler, the Xterra MS C_{18} 2 × 50 mm column, and Gradient I. The carryover is expressed as percentage of LLOQ in blank injection following a ULOQ (5000 or 1000 ng/ml) injection.
2S-FWS: Two-solvent fast wash station; 4S-AWS: Four-solvent active wash system.

The comparison in carryover reduction using the same optimized column/gradient method on both the 4S-AWS and the DLW is shown in Table 4. The carryover (as a percentage of a 1 ng/ml LLOQ) of the chemical set following a 1000 ng/ml injection was measured on both autosamplers. The DLW was simpler to use and performed better at reducing carryover by about 20%. The overall performance of the DLW in regard to its speed, simplicity of use, and carryover reduction makes it superior to the 4S-AWS.

Using the same drug set tested in Table 4, a comparison was made between the carryover originating from the autosampler versus the carryover originating from the column and gradient choice. The results of the comparison are shown in Table 5. In our case, improvements to the column gradient combination resulted in a greater impact on carryover than changes to the autosampler. When testing the unoptimized column and gradient combination (Xterra C_{18}, Gradient I) against the best and worst autosampler wash stations (DLW vs 2S-FW), the average carryover levels for the drug set were 56 and 90%, respectively. The difference of 34% between the wash stations performance represents a 38% reduction in carryover. However, when utilizing the same DLW wash station and comparing the optimized and unoptimized column/gradient combinations, the Unison C_{18}/Gradient IV combination averaged 16% carryover and Xterra C_{18}/Gradient I combination average 56% carryover. The 40% difference between the two column/gradient combinations represents a 71% reduction in carryover by using the optimized column and gradient. Obviously, optimizing both the autosampler and column/gradient simultaneously would provide the greatest reduction in carryover of 82% (90 compared with 16%). Interestingly, the difference between the columns (Xterra C_{18} 56% vs Unison C_{18} 31%) when keeping the DLW and Gradient I constant correlates to a 45% reduction in carryover. The difference between gradients (Gradient I 31% vs Gradient IV 16%) when keeping the DLW and the Unison C_{18} constant correlates to a 48%

Table 4. Comparison of four-solvent active wash system and dynamic load and wash stations in carryover reduction following a 1000 ng/ml injection.

Compound	4S-AWS			DLW		
	2S-FWS/Xterra C_{18}/Gradient I % LLOQ	4S-AWS/Unison C_{18}/Gradient IV % LLOQ	% reduction	2S-FWS/Xterra C_{18}/Gradient I % LLOQ	4S-AWS/Unison C_{18}/Gradient IV % LLOQ	% reduction
Melatonin	40	11	73	37	0	100
Propanolol	165	30	82	232	30	87
Ketotifen	65	24	63	56	15	73
Corynanthine	26	6	77	24	4	83
Vincamine	45	15	67	27	4	85
Droperidol	119	25	79	134	29	79
Diltiazem	41	22	46	42	8	81
Loperamide	64	25	61	68	0	100
Ketoconazole	150	33	78	185	18	90
Dilazep	149	95	36	169	48	72
Average	**86**	**29**	**66**	**97**	**16**	**85**

The dynamic load and wash outperformed the 4S-AWS in reducing carryover for the diverse library set. The dynamic load and wash average percentage reduction was 19% better than the 4S-AWS, and the average absolute carryover was 13% lower. The carryover is expressed as percentage of LLOQ in blank injection following a ULOQ (1000 ng/ml) injection.
2S-FWS: Two-solvent fast wash station; 4S-AWS: Four-solvent active wash system; DLW: Dynamic load and wash.

reduction in carryover. These results highlight the level of chromatographic (column and gradient combination) contribution to carryover and underscore the importance of optimizing chromatographic performance for carryover during method development.

When optimizing a system to reduce carryover, it is critical to examine not only the autosampler (which is usually inspected carefully) but also examine the column and gradient used. As calculated from Figures 3 & 4, carryover from the column can account for 58% of the total carryover observed in our system. Thus optimizing the system as a whole (not focusing solely on the injector) leads to improved elimination of carryover.

Conclusion

This work examines the contribution of the analytical column and HPLC gradient to

Table 5. Effectiveness of autosampler, column and gradient optimization in reducing carryover.

Compound	% carryover remaining			
	(A) 2S-FWS/Xterra C_{18}/ Gradient I	(B) DLW/Xterra C_{18}/ Gradient I	(C) DLW/Unison C_{18}/ Gradient I	(D) DLW/Unison C_{18}/ Gradient IV
Melatonin	40	35	5	0
Propanolol	199	82	49	30
Ketotifen	65	53	36	15
Corynanthine	26	28	26	4
Vincamine	45	25	16	4
Droperidol	119	61	51	29
Diltiazem	41	32	23	8
Loperamide	64	49	16	0
Ketoconazole	150	114	30	18
Dilazep	149	84	58	48
Average	**90**	**56**	**31**	**16**

Carryover was measured on the diverse chemical set while testing the autosampler, column or gradient individually. Holding the column and gradient constant and comparing the 2S-FWS to the DLW autosampler (column A vs B) reduced carryover by 38%. Holding the gradient and autosampler constant and comparing the Xterra MS C_{18} to the Unison C_{18} column (column B vs C) reduced carryover by 45%. Holding autosampler and column constant and comparing Gradient I to Gradient IV (column C vs D) reduced carryover by 48%. Holding the autosampler constant and comparing the optimized column and gradient (Unison C_{18}/ Gradient IV) to the unoptimized column and gradient (Xterra MS C_{18}/Gradient I; column B vs D) reduced carryover 71%. Therefore, greater improvements in carryover reduction can be realized by optimizing column and gradient than by changing from the 2S-FWS to the DLW autosampler. Optimizing for all three components (column A vs D) has the greatest effect on reducing carryover (by 82%).
2S-FWS: Two-solvent fast wash station; DLW: Dynamic load and wash.

carryover in bioanalytical LC–MS analysis. We have demonstrated the impact of column choice on carryover level and identified a generic gradient utilizing a three-step wash procedure that drastically reduces carryover by 60–87%, depending on the autosampler wash station used. Both the 4S-AWS and DLW autosampler configurations were shown to be highly effective at reducing carryover when used in conjunction with optimized columns and gradients. Interestingly, residual analyte is more efficiently removed from the column with a saw-tooth-type wash (alternating between high organic and high aqueous) rather than a high organic continuous wash. For our diverse set of drug-like compounds, four cycles from high organic to high aqueous was sufficient to remove most residual compound (a fifth cycle resulted in negligible improvement). Based on this study, we conclude that in addition to careful examination of the autosampler, one must also focus closely on column type and gradient when trying to eliminate carryover.

Future perspective

While novel autosampler designs to reduce carryover are continually introduced, the same cannot be said for column design. Rather, other criteria (e.g. resolution, selectivity, speed, cost and robustness), are more important drivers than carryover for column improvements. This work demonstrates the importance of selecting the correct column and gradient to minimize carryover. We hope that future bioanalytical method developers will consider this component when validating novel methods. Due to the unique and specific nature of NCEs and their interaction with different column chemistries, it appears that carryover assessment of a chosen column and gradient will need to be performed during the validation process if carryover is an issue.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- A saw-tooth-wash cycle, in which organic solvent is rapidly cycled from high to low percentage, is more effective than continuous high percentage organic wash in reducing carryover.
- The analytical column has been identified as a major source of carryover, contributing over half of total carryover in some instances.
- The column contribution to carryover is highly column specific and must be determined on a case-by-case basis.
- Column and gradient optimization can reduce carryover two-times more effectively than autosampler optimization alone.
- The optimized LC–MS method described here reduced carryover by an average of fivefold for a diverse compound library and can be used as a generic discovery bioanalytical method.

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