Questions of Quality

The Next in Line?

Question: *What do chromatographers use every day even though there is little written on the subject?*
Answer: *Injection sequences.*

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You know, it's a funny world. You can inject samples into a chromatograph for many years, but when you perform a literature search to find the optimum sequence to inject sample extracts, there's nothing there. In contrast, we have many papers on experimental design for method optimization, method validation and, even, international agreements. However, when we come to something as fundamental as injecting samples for analysis in a chromatograph, there's not a specific paper.

Therefore, if nobody has written a paper discussing the order that samples should be injected, it can't be very important, can it? However, if you search a little deeper you'll find that what is available on injection sequences is hidden inside publications on other subjects, as we'll discover later in this article.

Injection sequences are something that we all take for granted. They may not be very important; we have done all the hard work with the sample preparation, and injection sequences are simply a means of transferring the sample extract to the instrument. They can be performed manually using a syringe, or automatically using an autosampler, or even a robot.

Dr Jekyll and Mr Hyde

So why should I raise something as apparently simple as the order in which you inject your samples into the chromatograph as a point for discussion? Well, as a small diversion, I'll let you into a secret. I lead a "Jekyll-and-Hyde" existence. On one hand, I'm a consultant and, on the other, I'm an assessor for NAMAS (National Accreditation for Measurement and Sampling, the UK implementation of ISO Guide 25) specializing, amongst other items, in chromatographic separations. I'll let you figure out which one is my Mr Hyde role. Anyway, in both capacities, I visit laboratories in different industries and have seen many variations in injecting samples in working practice. If you have only worked in one or two laboratories, there may not appear to be large, or even any, differences in injection sequences.

Yet, the order that you inject samples into your instrument can directly affect the quality and integrity of your analysis. Never thought about it? Why not?

The Main Players

First, let's have a look at the main sample types that we can inject into a chromatograph.
Standard: As chromatography is a comparative technique, we need a known standard to calibrate the method, check the system suitability parameters for that method or provide a reference for the retention time of an analyte.
Unknown sample: This is needed to determine analyte(s) either quantitatively or qualitatively in order to generate information to make a decision. Presumably, this is why we're analysing the sample in the first place, unless it's to play with the latest chromatographic toy!
Quality-control sample: This is an independently prepared sample of known concentration or amount to ensure that today's run of the method is producing acceptable results and that the method from day to day is under control.
Blank: This is used to check for any possible contamination and extraneous peaks. Blanks can also be used in the dual role of checking for contamination and as a marker at key stages of the analytical run.

There may be subsets of each of these types, but for ease of discussion and presentation (and also because I'm late making the deadline for this issue), we will stay with these four basic sample types.

However, there are several different approaches to injecting samples. I am not advocating any specific approach, as this will depend on a number of factors, such as the reason for the analysis, the analytical aims and the questions you need to

answer using the data generated by your analysis. There are also a number of practical considerations, such as autosampler capacity, run time and total analysis time, that must be taken into account when preparing the sequence for injection. For example, if you have an autosampler:

- Does the tray or carousel have open access?
- Is the instrument easy to use and programme?
- Is instrument control being used?
- Is the instrument integrated with the data system? (1)

Different Approaches to Injecting Samples

Have you ever looked at a map and wondered why places are named as they are? One can but wonder at the thoughts and mental state of the discoverers of such places as Cape Desolation, Table Mountain and Pratts Bottom. Applying the same logic, as there are no prior naming conventions here (with the exception of the rolling bracket), I claim the privilege of naming the following injection sequences and leave you to wonder at my mental state:

1. In God We Trust

This is the simplest approach to injecting samples (Figure 1). The standards are injected first, followed by the samples. There may be blanks to separate the samples before or after the standards. Quality controls are optional, which is a polite way of describing the blank look from a chromatographer following the question, "Are quality-control samples used?"

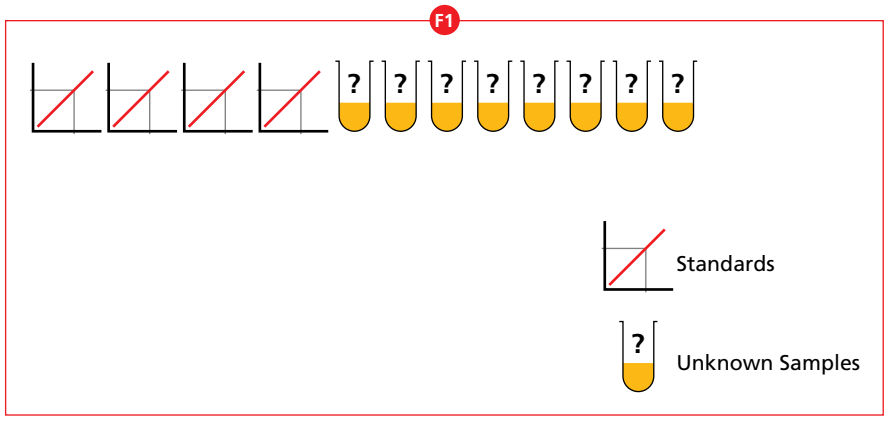

Figure 1: *The simplest approach to injecting samples.*

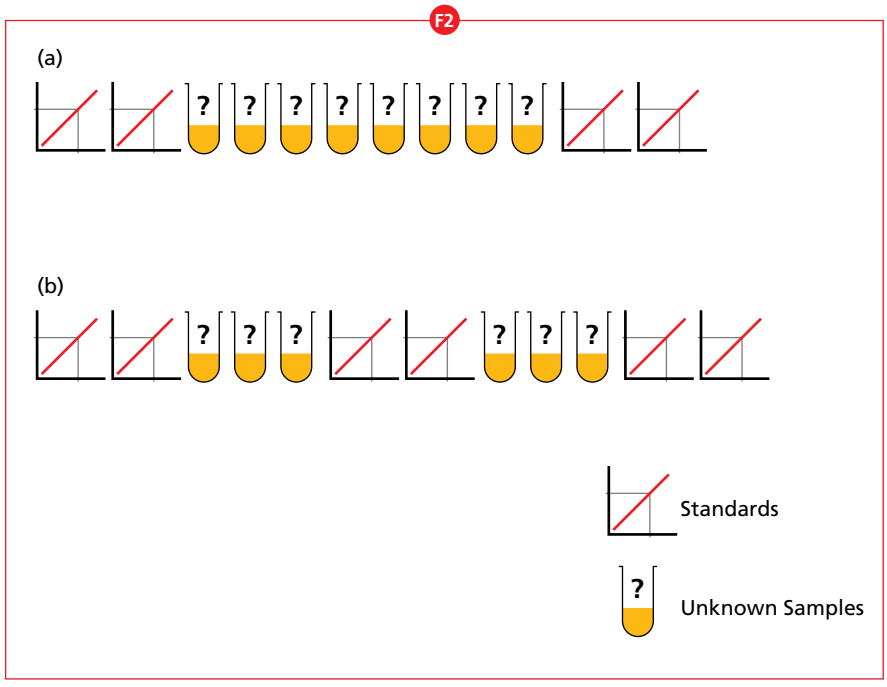

Figure 2: *Standards are run before and after unknown samples.*

OK, what do you think of this approach? The problem is that the assumption being made is that the performance of the chromatograph is constant throughout the run. Fair enough, let's look in more detail at what this implies.

Pump performance is constant and you have enough mobile phase. *OK?*
Injector or autosampler performance is constant. *OK?*
Chromatographic separation is constant throughout the analytical run. *Er?*
Laboratory temperature is constant over the run. *Er?*
Temperature of the column in the column-heating block is constant. *Er?*
Detector performance (gas chromatography, liquid chromatography or mass spectrometry) is constant throughout the run. *Well?*

Perhaps you can see why this approach is called "In God We Trust". The assumption that chromatographic performance and detector stability are constant throughout the run may be perfectly acceptable for relatively small numbers of samples, but consider using this approach for injecting samples when you either have a large number of samples or long run times. Almost certainly you will be running the process overnight, and it is then that problems with this approach to injecting samples will arise:

- Retention times can vary between the beginning and end of the analysis;
- Peak shape can change through the run as the temperature of the column or environment changes.

What do you think of the quality of the results that will be generated from an analytical approach that uses this injection sequence?

2. Belt and Braces

This is an enhancement of the "In God We Trust" injection sequence. Standards are split into two sets and run at the beginning and the end of the injection sequence. Figure 2(a) shows that we now run the standards before and after unknown samples, allowing us to generate information that starts to address issues such as the consistency of the detector performance and the chromatographic separation.

However, we still have some potential problems with this approach. These are most obvious when a large number of samples are to be analysed. If there are differences between the standards at the start and end of the sequence, how do you know when the problem began? This starts an interesting hunt to see how much salvageable data we can discover without

the need to reanalyse the samples:

- Do we look for the point when changes in retention time occurred?
- Do we look for the point when changes in peak shape occurred?
- How do we justify this approach from both a scientific and regulatory viewpoint?

It could be viewed as shutting the stable door after the horse has bolted.

This is where some laboratories modify the approach and introduce a third set of standards in the middle of the run (Figure 2(b)), so that there is a middle data set to compare and control the results. If the start and middle standard results are OK then, by implication, the samples run in between them should be OK too. The same approach is used for the middle and end sets of standards. At least half the data in a run should be OK using this injection sequence.

3. Rolling Bracket

Where closer control on quantification is required, a rolling bracket method is used. As shown in Figure 3, the samples and standards are interspersed and there is a higher proportion of standards to samples than in the first two examples. The standards that bracket the sample(s) are used to calculate the concentration or amount of analyte between them. There can either be an updated calibration function or the response function is recalculated between the brackets as the analysis progresses.

However, there are laboratory differences. The number of samples, or replicates of samples between the standards, can vary, as can the number of injections, but the essence of this approach is the same. The best use of this approach is the calculation of a fixed analyte amount or concentration, such as in quality-control laboratories, and it is perhaps best suited to analysing raw materials or finished products in which the analyte can be well specified. It is less suitable for determining analyte over a wide range of values. This is because the number of standards must increase in proportion to the range of analyte concentration or amount measured to be really effective.

4. Macho Chromatographer

No problems here. "Macho Chromatographer" can leap tall buildings in a single bound! Analysis is a challenge and as we don't wish to be biased, we will randomize all samples for analysis. Thus, we will eliminate all sources of analytical and human bias by relying on computer-aided randomization (the program is validated, of course) and then calculate the results after the analysis is complete.

In my humble and personal opinion, our job is difficult enough as it is. Why make it more difficult? Moreover, if you randomize the sequence, you can only detect gross problems with the run before the randomization is broken. Other more subtle issues only surface later. This approach, I suspect, would result in a greater incidence of repeat analysis than would be the case with the earlier sequences described above. However, I would be interested in hearing from readers and laboratories that use this approach so that they can present their case in more detail than I have given it here.

A Case Study — Blanks In Injection Sequences

Michael Awe, group leader in the department of Pharmaceutical Sciences of Fujisawa USA Inc., wrote (2) to add his comments to my "Questions of Quality" article on contamination (3), in which I discussed the regular injection of reagent and matrix blanks to help keep control over the presence of mystery peaks in sample chromatograms. Mike positions blanks selectively throughout a sequence of samples to solve mystery-peak and related system problems quickly.

Quoting directly from his letter: "Shown in Figure 4 is a sequence that is run in our laboratory for potency determination of routine pharmaceutical stability samples using external standard quantification. The standard diluent is the solvent used to prepare the standard preparation. System suitability testing is usually resolution and tailing factor checks. The 'Typical Sample' is placed before the first standard injections which will be used to bracket the sample injections. The 'Typical Sample' is a normal

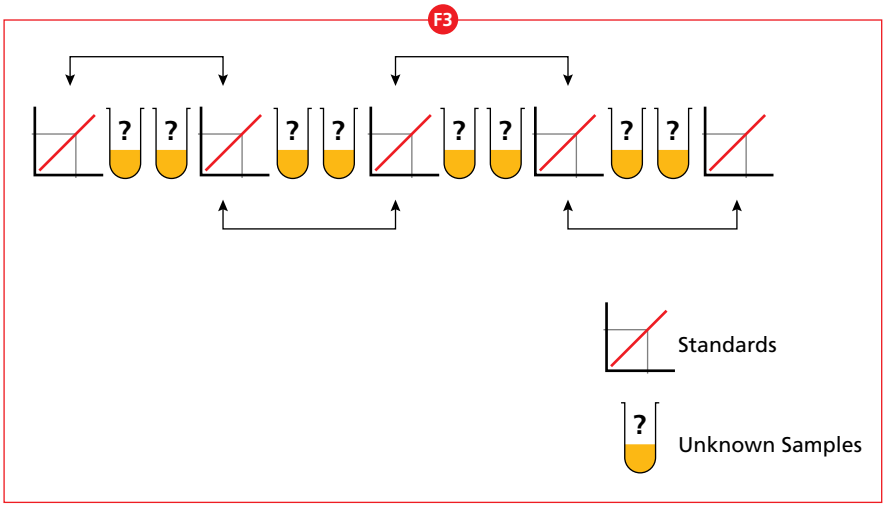

Figure 3: *Samples and standards are interspersed and there is a higher proportion of standards to samples.*

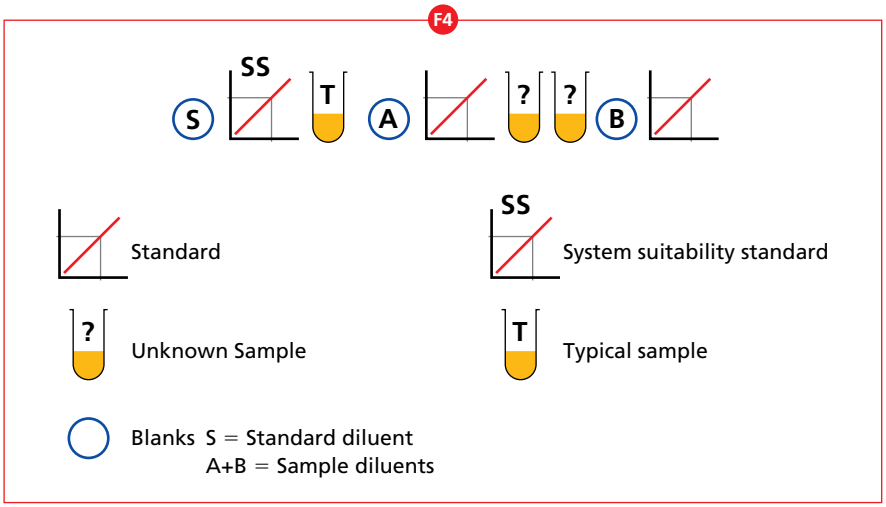

Figure 4: *A sequence for potency determination of routine pharmaceutical stability samples using external standard quantification.*

sample which will be analysed again within the standard bracket and is used here for qualitative and, occasionally as discussed below, for quantitative interpretations of the behaviour of the HPLC system.

As stated in the *LC•GC International* article (2), blanks should be analysed each time one runs a method. In our case, the close inspection of sample diluent chromatograms enables us to regularly perform three checks of system performance.

Checks 1 and 2 using sample diluent A: Unless otherwise noted in the method Standard Operating Procedure (SOP), this injection should be free of any peaks eluting at, or near, the main peak retention time (as defined in the just previously injected 'Typical Sample'). The presence of any peaks at the retention time of the main peak could be a sign of autoinjector carryover, indicating improper needle wash or autoinjection performance. If a peak is observed eluting at the main peak retention time, its area is compared to that of the main peak in the 'Typical Sample'. A judgement is then made regarding the significance of this interference. The presence of other peaks in the sample diluent, especially broader looking peaks, is most likely because of late eluting peaks present in the 'Typical Sample' injection, which eluted in the subsequent LC injection because the sample analysis time was too short. Granted, this problem of late eluting peaks should have been solved in the method development and validation, however, that work is usually done on samples from different sources, which may not be the same as the stability samples.

Check 3 using sample diluents A and B: The lack of any peaks at the main peak's retention time in these two injections ensures that the main peak in the all-important standard injections are not affected by "contaminants", for example, injection carryover or a late-eluting peak. This check ensures the accuracy of the response factor used for external standard calibration.

Note: A similar check of the standard diluent is also performed at the beginning of the sequence as shown in Figure 4.

A similar sequence is used to analyse for drug impurities, although usually area percentage, instead of external standard calibration, is performed. A sample diluent is injected after every twelve samples, providing a regular check for autoinjection carryover and the presence of unexpected late-eluting peaks.

In summary, we have found that systematically building blanks into our sequence of sample injections provides assurance that the HPLC system and the method are working as expected in the routine analysis of pharmaceutical samples."

Guidelines versus Regulations

In the discussions above, I have attempted to look at the science and not at regulations or guidelines. Some industries, notably pharmaceutical, are required to operate according to regulations. Calibration and testing laboratories can be certified under ISO Guide 25. What do these regulations and guidelines say explicitly about injection sequences?

The key paper by Furman et al. (4) outlines current US Food and Drug Administration (FDA) policy concerning chromatographic analysis for the pharmaceutical industry. It refers to injection sequences in the section on Initial Calibration: Linearity, where it states:

"Only after acceptable system precision with replicate injections of a standard solution is obtained, should sample analysis proceed."

This is reasonably explicit. Run the system suitability standards and determine if the system is suitable before committing your samples. However, recently, Pharmacopeial Forum (5) listed a proposed change for the US Pharmacopoeia General Chapter 627 on chromatography (6) and gave proposed guidance on setting up each injection sequence:

"Replicate injections of the standard preparation required to demonstrate adequate system precision may be made before the injection of samples or may be interspersed among sample injections. System suitability must be demonstrated thoughout the run by injection of an appropriate control at appropriate intervals. The control preparation can be a standard preparation or a solution containing a known amount of analyte and any additional materials useful in the control of the analytical system, such as excipients or impurities. Whenever there is a significant change in equipment or in a critical reagent, suitability testing should be performed before the injection of samples. No sample analysis is acceptable unless the requirements of system suitability have been met. Sample analyses obtained while the system fails requirements are unacceptable."

The new wording requires that system suitability be demonstrated throughout the run, not just at one point (1, 5). However, no specific injection sequences are mentioned, but it could be interpreted to imply a preliminary system suitability test before committing to the analysis, as well as throughout the run.

Should we have standardized injection sequences? Bill Furman, in a personal communication (1), has expressed his own opinion that an individual laboratory should state which characteristics of the LC system it is trying to control, select its own sequences of samples and standards for the injection sequence, and prepare an SOP or method report that describes the injection sequence. This may be more acceptable than devising standardized sequences and recommending that all laboratories use them. Moreover, the differences between individual chromatographic systems is a factor. Some work very reliably and would require little control, whilst others can be new or problematic and would need more control. How would standardized sequences aid quality assurance for these different cases?

This discussion illustrates that effective equipment qualification is necessary BEFORE method validation, to highlight potential problems with equipment. Furthermore, method validation cannot substitute for equipment qualification. There are a number of issues around this and method transfer that will be discussed in a forthcoming "Questions of Quality".

What Do You Think?

So far, these have been my views. What do you think about this issue? Do you care about injection sequences or are you content to inject samples in any order through an instrument?

Acknowledgements

I would like to thank Mike Awe and Bill Furman for their comments and help in preparing this article.

References

(1) W.D. Furman, personal communication.
(2) M. Awe, personal communication.
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(4) W.B. Furman, T. Layloff and R. Tetzlaff, *JOAC International*, **77**, 1314–1318 (1994).
(5) *Pharmacopeial Forum*, **23**(4), 4513–4514 (1997).
(6) *US Pharmacopoeia XXIII* (1995), United States Pharmacopoeia Inc.