# Drug Bioanalysis by LC-MS: some pragmatic solutions to commonly occurring problems

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The use of atmospheric pressure ionisation (API) LC-MS/MS has been very successful in drug bioanalysis. Despite this, many challenges, such as those presented by (a) sample matrix and mobile phase effects on ionisation, and (b) the determination of endogeneous analytes, remain. An aim was therefore set to investigate these problems with a view to attempting to find simple but effective solutions to them. It was found that late-eluting interferences could be dealt with by a change of mode of ionisation, that an order of magnitude improvement in limits of detection could be had by the post-column addition of an appropriate solvent and that ion enhancement could be induced by surfactants. In the determination of endogeneous analytes it was demonstrated that the use of non-matrix calibration standards and the use of a surrogate matrix were both useful approaches.

**Keywords: drug bioanalysis, LC-MS, matrix effects, endogeneous analytes**

## Introduction

The use of atmospheric pressure ionisation (API) interfaces for coupling LC with quadrupole mass spectrometers overcame many of the problems associated with earlier designs of interface and gave rise to analytical methods with good sensitivity, selectivity and robustness [1]. Accordingly since their introduction in the early 1990s LC-MS has become the predominant technique used in the field of drug bioanalysis, Drug bioanalysis using LC-MS/MS has enabled higher throughput assays to be developed. However, while the impression may even have been created that it is now relatively routine and may be dealt with by a de-skilled workforce, drug bioanalysis in fact remains a highly challenging discipline especially given the need to often develop robust assays down to pg/ml level concentrations of analyte in complex sample matrices. In a series of studies carried out over a period of time, it was found that difficulties could still be encountered which involved the ionisation process itself or the quantitative determination of endogeneous analytes. An aim was therefore set to investigate these problems and attempt to find simple solutions to them.

## Instrumentation

The systems used for the discussed applications consisted of a PE Series 200 Micro-pump (Perkin Elmer, Thornhill, Ontario, Canada), an online DGU-14A degasser (Shimadzu, Kyoto, Japan), and a PE Series 200 Autosampler (Perkin Elmer, Thornhill, Ontario, Canada) connected to a Perkin Elmer API 4000 triple quadrupole mass spectrometer. Chromatographic system control, data acquisition and analysis were performed by means of Analyst software version 1.2 (Applied Biosystems – MDS Pharma, UK).

## Matrix-Related Suppression of Ionisation caused by Co-Eluting Interferences

As suggested above, commonly reported problems in drug bioanalysis by LC-MS include the occurrence of matrix effects - a change in response of the target analyte(s) as a result of the presence in the samples of interferences. The critical problem associated with matrix effects is the lack of robustness of the method, as the degree of suppression (or enhancement) would be expected to vary considerably from sample to sample. This may be dealt with by the use of an isotopically-labelled internal standard but this is not always possible.

Recent FDA guidelines [2] gave recommendations on how matrix effects may be measured. However, during method development it is useful to qualitatively assess the likelihood of matrix effects occurring by employing post column infusion of the analytes of interest with injected matrix blanks. Data from such a procedure is shown in Figure 1. In this illustrative example, the infused line has levelled off (after initially dropping around the column dead volume at 0.5 min, where poorly retained polar matrix components elute) and therefore no ion suppression would be expected at the analyte retention time. However on the basis of experience gained over a range of studies, it is suggested that the matrix effect trial using post column infusion should be performed with great care using at least six separate sources of individual matrix and it should be performed for all analytes being investigated, including the internal standard and metabolites.

While interferences often arise from compounds in the sample which co-elute with the analyte during each chromatographic run, late-eluting interferences may also be an issue. These occur as a result of compounds which are retained on the HPLC stationary phase for

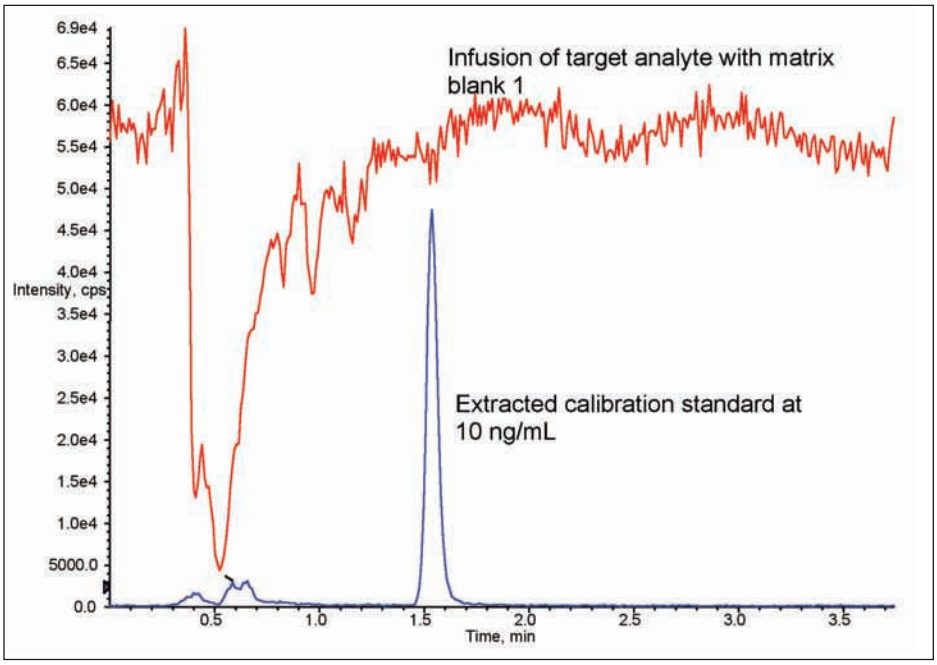


*Figure 1. Ion suppression profile for matrix blank (target analyte) using TurbolonSpray. The target analyte was added to the system by infusion and matrix blank 1 was injected (red). This has been superimposed over an extracted calibration standard (blue) to illustrate the analyte retention time. The infused line has levelled off (after initially dropping around the column dead volume at 0.5 min, where poorly retained polar matrix components elute); therefore no ion suppression would be expected at the analyte retention time.*

a longer period than the target analyte(s) and than the chromatographic run time. In such cases the post-column matrix blank infusion strategy, described above, run with just a few samples would probably not indicate the potential for interference and the problem might only be observed later in the form of irreproducible calibration runs.

Late-eluting interferences may be dealt with in several ways. Greater sample clean-up may be adopted to remove the interfering compounds from the samples [3]. The chromatography may be modified to ensure that the interferents no longer elute at the same time as the analytes of interest [4]. A post-sample gradient may be used to ensure that the late-eluters are removed from the analytical column before the introduction of the next sample [5]. However it was found that in several cases it was possible to eliminate the matrix effects by the simplest means possible, i.e. by changing the mode of ionisation from pneumatically assisted electrospray to atmospheric pressure chemical ionisation (APCI). Although in some cases APCI may be susceptible to significant matrix effects [6] it is generally considered to have reduced and in many cases no matrix effects [7]. The change from pneumatically- assisted electrospray to APCI often reduces or eliminates matrix effects as the mode of ionisation is completely different; therefore the chemical properties that are responsible for causing the matrix effect may not be relevant.

In general, ion suppression in electrospray ionisation can be considered to be caused by the presence of non-volatile solutes in the mass spectrometer source spray, which alter the droplet solution properties, thereby altering the response that would be expected for the target analyte [8]. Mass spectrometric conditions are routinely optimised using a "pure standard solution" (i.e. a solution containing only a suitable solvent and the target analyte). Re-optimising the mass spectrometer conditions with pooled extracted samples is an additional step that, although time-consuming, can potentially remove or reduce any problems associated with the sample matrix.

### Impact of Surfactants on the Mobile Phase

A method for the determination of deltamethrin in bovine and ovine tissues had been developed and validated to comply with recognised regulatory requirements for the monitoring of residue levels of agricultural chemicals in animal tissues [9]. During the validation process 'nominal recovery' values (i.e. apparent recovery as opposed to the actual recovery) in excess of 100% had been observed in liver tissues for both species (when the actual recoveries in muscle, kidney and skin/fat had been in the region of 70% - as confirmed by absolute recovery determinations undertaken as part of the validation). Nominal recoveries of over 100% can be the result of methodological errors or as a result of ion enhancement, the latter being the opposite of ion suppression in that there is a greater degree of ionisation for analytes in the sample than in well-resolved peaks in standard solutions. As this phenomenon of ion enhancement is not frequently observed, this was considered sufficiently unusual to warrant further investigation.

It had been postulated that the reason for this phenomenon was that the high concentrations of surfactants that are present in the liver [10] affect the ionisation process, by affecting the properties of the solvent, interferents and/or analyte in solution, thereby allowing the enhancement of ionisation relative to that for samples extracted from other tissues. The mechanism for this may have been that the purpose of the main group of liver surfactants – the bile salts – is to emulsify fats and oils into smaller droplets, which can then be broken down enzymatically. The presence of these fats and oils may have been affecting the ionisation process, such that there is a lesser degree of ionisation in the absence of these surfactants.

It was possible to confirm that the 'nominal recoveries' were higher from liver extracts and, further, that significant increases in mean nominal recovery in muscle could be observed following addition of sodium dodecyl sulphate. However, while it was clear that ion enhancement could be a problem in LC-MS assays involving liver extracts, the effects observed were not sufficient to suggest that surfactant addition could be used in a controlled fashion to improve limits of detection.

### Post-Column Modification of Mobile Phase

Optimal LC conditions for the separation of enantiomers on immobilised-protein chiral stationary phases (CSP) are achieved using predominantly aqueous mobile phases (on occasion with less than 2% v/v organic modifier). However, when such mobile phases are used with mass spectrometric detection, sensitivity is very poor, due to inadequate evaporation of the mobile phase within the LC-MS interface. A simple and convenient solution to this problem was found to be the post-column addition of organic modifier, which enhanced the ionisation process to give reproducibly and reliably improved sensitivity of detection. Using this approach, it was possible to develop a rapid, sensitive method for the determination of the enantiomers of dihydropyridine calcium channel blockers (Figure 2) in plasma samples. In an illustrative example using an α1-acid glycoprotein (AGP) CSP, the required limits of quantification validated to internationally recognised standards were achieved. Importantly, when

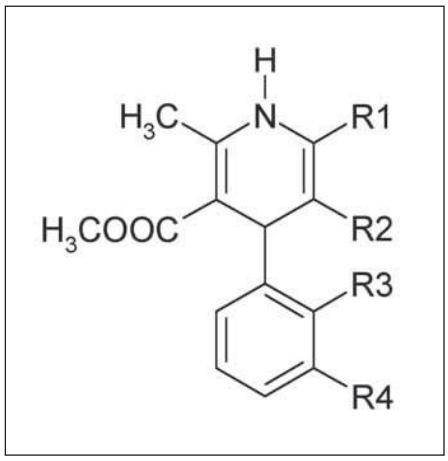


*Figure 2. Generic dihydropyridine calcium channel blocker structure*

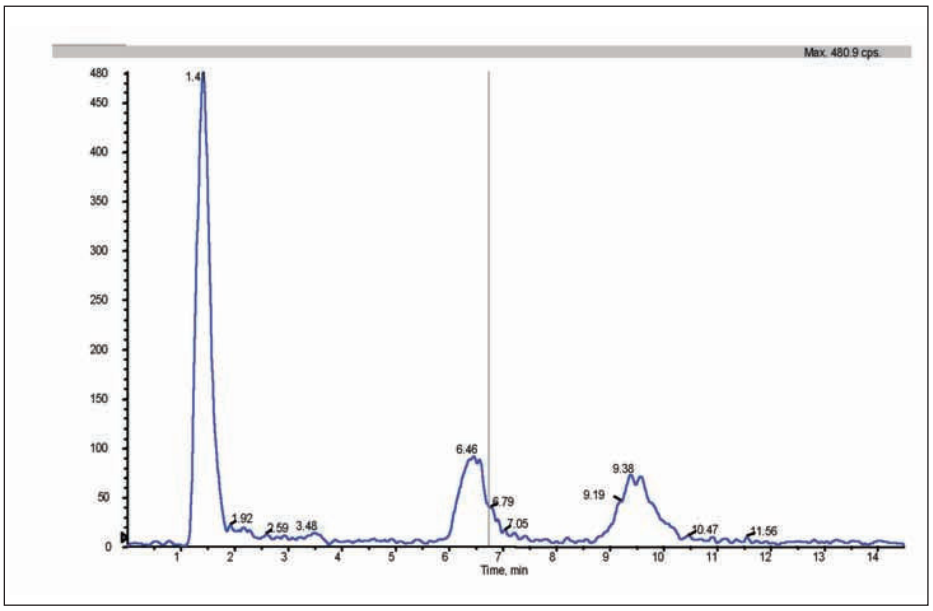


*Figure 3. Example ion chromatogram obtained at the LLOQ (1 ng/mL). A Chiral-AGP (4.0 (id) x 100 mm) column was used with mobile phase ammonium acetate (10 mM) - propan-1-ol - acetic acid (1000:15:0.8, v/v/v) at a flow rate of 0.9 mL/min through the column; acetonitrile was added via a post-column mixing tee following which a splitter was used to ensure that the optimum flow rate for a low limit of detection entered the MS (in this case, 0.9 mL/min)*

using this approach, it is not necessary to attempt to adjust the optimal conditions for chiral separation. These may be retained without compromise as it is not necessary to make changes to the mobile phase for the separation in order to improve limits of MS detection given that the mobile phase change is carried out post-column. Further studies confirmed the generality of this post-column addition of organic modifier, with subsequent flow splitting, approach for basic chiral drugs with typical improvements in sensitivity of around an order of magnitude.

### Quantitative Determination of Endogeneous Analytes

Difficulties also arise in LC-MS when determining endogenous analytes. There are a number of reasons for the requirement for a robust method for the determination of an endogenous analyte. One of the principal ways of determining the effectiveness of a therapy can be by observing the effect of a dose of a drug substance on an endogenous compound in a biological system (i.e. a biomarker [11]). In order to do this effectively a method for the quantitative analysis of the biomarker would be required. Similarly biomarkers may be used as an indication of a disease state, where their presence/absence and concentration may provide an indication of the presence or progress of a disease.

The main problem associated with the quantitative analysis of endogenous analytes is associated with the provision of "control" matrix, i.e. a supply of the matrix to be analysed that does not contain the analytes under investigation. There are a number of strategies available to overcome this problem:

a) The use of a "surrogate" matrix, i.e. a matrix that is as similar to the sample matrix as possible, but that does not contain the target analyte [12]. The similarity of a surrogate to the sample matrix can be extremely good, but the main disadvantage of this approach is that there are many examples where a truly representative surrogate cannot be obtained.

b) The use of non-matrix standards, where the calibration standards (and possibly quality control samples) are prepared in the injection solvent used for the analysis, without any matrix present at any time in the sample preparation procedure [13]. This is an extremely simple approach, but suffers from the difficulties associated with sample extraction recovery and matrix effects.

c) The adoption of the standard addition method for sample and standard preparation. Using this methodology a standard solution (i.e. a solution containing a known concentration of the analyte) is added to the unknown solution or sample matrix so that the endogenous amount can be accounted for in the analysis [14] [109]. Knowledge of how the response changes before and after adding the standard solution allows extrapolation to determine the concentration initially in the sample. In many cases this can provide an excellent strategy for these analyses, as any matrix-related (extraction and ionisation) effects are minimised. The main disadvantage is that the response of an analyte may not be linear across the entire range, particularly if the endogenous level selected is relatively high within the dynamic range investigated.

d) For many matrices, e.g. plasma, charcoal-stripping may be utilised. This involves many compounds within the matrix being removed by passing the matrix over charcoal [15]. The main disadvantages of this technique are that it can result in incomplete removal of the analyte and it can be an expensive and time-consuming technique.

e) The use of a surrogate analyte, where for example a deuterated version of the target analyte is used to generate the calibration curve (rather than as an internal standard) and the sample concentrations of the target analyte are read off this calibration curve [16]. Difficulties in obtaining pure deuterated analyte, or the preference to use the deuterated version of the analyte as an i internal standard make this a rare strategy of choice.

Of the approaches described above, the use of non-matrix calibration standards for the successful development and validation of a method for the analysis of indolyl 3 acryloylglycine (IAG) in human urine has already been reported [17]. However, for the determination of endogenous testosterone in human serum the use of gelding serum (i.e. serum from a horse that has been castrated, in which case the serum should be testosterone-free) as a surrogate matrix proved to be more appropriate. The lower limit of quantification (LLOQ), which was defined as the lowest QC at which accuracy was within 20% of nominal and precision was no greater than 20% [18], was 50 pg.mL-1 (Figure 4). This range was suitable for

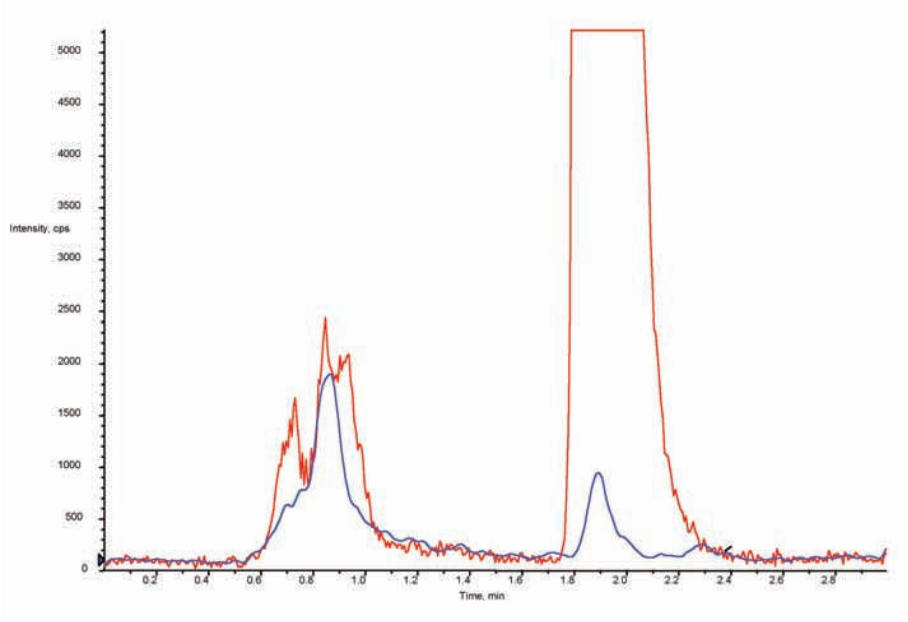


*Figure 4 Multiple reaction monitoring (MRM) chromatogram resulting from the analysis of a quality control sample at 50 pg.mL-1 HPLC system: PE Sciex API 3000 and PE Series pumps, SIM for testosterone and internal standard. Column Genesis C18 (50 x 4.6 mm id, 3 µm particles) with mobile phase methanol - ammonium acetate (pH native; 10 mM), (75:25, v/v) at flow rate 1.0 mL.min-1. The blue trace shows the response for testosterone at the LLOQ, with the red trace showing the deuterated internal standard.*

the analysis of both "normal" and clinically suppressed levels of testosterone in samples from prostate cancer studies.

## Conclusions

Although the use of LC-MS is very widespread in drug bioanalysis, there still remain quite a few difficulties that may be encountered. For example, carry-over, analyte adsorption and increasing time pressures may also cause problems. However, for the sample matrix and mobile phase effects on ionisation, and determination of endogeneous analyte issues that were faced over a range of studies here, it was possible to apply solutions that were pragmatic and relatively simple. In general, it may be said that knowledge of the nature of the sample undergoing analysis, the required analytical conditions, and, where required, careful application of one of the approaches described will be very helpful in attempts to develop robust LC-MS methods for drug bioanalysis.

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