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Ion-suppression effects in liquid chromatography–tandem mass spectrometry due to a formulation agent, a case study in drug discovery bioanalysis

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Abstract

Liquid chromatography–tandem mass spectrometry (LC/MS/MS) has become the technology of choice for bioanalysis, due to its high selectivity and high sample throughput. However, concerns have grown that this technique may be subject to errors due to "invisible" interferences, in particular ion-suppression. Investigations on ion-suppression from formulation agents have only been published to a limited extent. Such effects can be of particular importance in pre-clinical discovery studies where drugs may be formulated with large amount of solubilisers and bioanalysis may use fast generic methods. In a preliminary pharmacokinetic study we observed strong ion-suppression from a polysorbate co-solvent, which, if undetected, would have given highly erroneous pharmacokinetic results and possibly could have led to the inappropriate elimination of a promising drug candidate. Different chromatographic methods were tested indicating that the separation step was essential in controlling these effects. A method based on matrix dilution is proposed to check for these effects during the use of discovery support methods, where full validation is not practical. Some excipients commonly used in formulations are polydispersed polymers, for which very limited pharmacokinetic information is available. Further investigation is needed to better understand the mechanisms of ion-suppression and the kinetics of the suppressing species to allow the development of new LC/MS/MS based analytical strategies, which will not be subject to such ionisation interferences.

Keywords: LC/MS/MS; Ion-suppression; Excipients; Formulation; Polysorbate; Tween; Reverse-phase HPLC; Bioanalysis

1. Introduction

The use of liquid chromatography–tandem mass spectrometry (LC/MS/MS) has expanded rapidly in bioanalytical laboratories over the last decade, and it has now become the main technique for drug quantification in biological samples to support pharmacokinetic studies. The inherent high selectivity and sensitivity of the technique has permitted analytical methods with increased throughput by allowing simplified sample preparation and very rapid chromatography to be used [1]. This has helped to remove "analytical bottlenecks" and allowed large numbers of pharmacokinetic studies to be completed earlier in the drug discovery process.

Originally LC/MS/MS was thought to be easily applicable to virtually any situation with minimum chromatographic separation. In recent years more and more users have come to realise that LC/MS/MS can be subject to unseen interferences [2,3] and that variability in the sample matrix may affect the analytical methods. Whereas with detection techniques such as UV or fluorescence, interferences are visible as peaks, in LC/MS/MS interferences are not generally observed directly, but they can modify the analytical response in unexpected ways, by suppressing (or enhancing) the signal. This

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phenomenon is typically referred to as "ion-suppression" or "matrix effect". Different authors have discussed matrix effects due to endogenous species [4–6] and drug derived products such as metabolites [7]. Strategies have been proposed to study these effects, including post-column infusion methods [8], and examination of ion-suppression from a mechanistic point of view [9]. A recent paper from Matuszewski et al. [10] gives a good overview of matrix effects relevant to the validation of LC/MS/MS methods for clinical studies.

Studies of interferences in LC/MS/MS have focussed mainly on the effects of endogenous compounds ("matrix effects"), but recently more interest has been given to possible effects of formulation agents [11–13]. Formulation excipients such as polyethylene glycol (PEG), sometimes administered in large quantities with the compound, can create ion-suppression effects that are not present in control matrices. In our laboratories significant ion-suppression was observed with a formulation containing polysorbate 80 (Tween 80®) and this paper describes the investigation of this effect. Approaches were also developed to check for, and avoid, such effects in future methods.

2. Experimental

2.1. Materials

Methanol and acetonitrile of HPLC grade and formic acid of analytical grade were purchased from Riedel-de Haen (Milan, Italy). Ammonium formate of analytical grade was obtained from Carlo Erba Reagenti (Rodano, Italy). Sterile 5% Glucose solution was obtained from Bieffe Medital (Grossoto, Italy) and polysorbate 80 (Tween 80®), pharmaceutical grade, from Uniquema (Gouda, Netherlands). Water was purified in house with a Milli-Q system from Millipore (Milan, Italy). Compounds A and B were research compounds synthesised at Nerviano Medical Sciences Srl, the chemical structures of which cannot be shown for proprietary reasons.

Blank matrix (pooled rat plasma with sodium heparin as anticoagulant) was obtained from Harlan Sera Lab (S. Pietro al Natisone, Italy).

2.2. LC/MS/MS conditions

High-pressure liquid chromatography (HPLC) separations were performed on a system that consisted of a binary high pressure mixing pump, an on-line degasser and a column oven (1100 series; Agilent, Cernusco sul Naviglio, Italy), and an HTS-PAL (CTC Analytics, Zwingen, Switzerland) autosampler. Mobile phase A consisted of ammonium formate buffer (10 mM, pH 3.5). The buffer was prepared diluting a 1 M ammonium formate stock solution and adjusted to pH 3.5 with formic acid before bringing to the final volume. Mobile phase B was acetonitrile.

Table 1
Description of the HPLC "gradients" used in the analysis of the samples from a preliminary pharmacokinetic study on compound A

Time (min)	B (%)
Generic method[a]	
0	20
0.1	20
0.2	60
2.4	60
2.5	20
6.3	20
Specific method[b]	
0	32
2.5	32
2.6	85
4.5	85
4.6	32
6.5	32

[a] Zorbax SB-C8, 2.1 mm × 50 mm flow (μl/min): 300.
[b] Zorbax SB-C8, 4.6 mm × 75mm flow (μl/min): 1000.

Zorbax (Agilent) and Symmetry (Waters, Vimodrone, Italy) HPLC columns were used. The gradients and columns used for the different methods are summarised in Tables 1 and 2. The column oven was maintained at a constant temperature of 45 °C.

Table 2
Description of the HPLC "gradients" used in the systematic study of four different LC/MS/MS approaches

Time (min)	B (%)
Ballistic gradient[a]	
0	10
0.2	90
1.0	90
1.1	10
2.6	10
Long gradient[b]	
0	10
15	90
16.5	90
17	10
20	10
Short gradient[c]	
0	10
2.0	90
2.5	90
2.6	10
4.6	10
Isocratic separation[d]	
0	30
3.0	30
3.1	90
3.6	90
3.7	30
5.7	30

[a] Symmetry C8, 4.6 mm × 50 mm, 3.5 μm.
[b] Zorbax SB-C8, 4.6 mm × 150 mm, 3.5 μm.
[c] Zorbax SB-C8, 4.6 mm × 75 mm, 3.5 μm.
[d] Zorbax SB-C8, 4.6 mm × 75 mm, 3.5 μm.

The LC eluent was directly introduced into an API 3000 triple quadrupole mass spectrometer (MDS Sciex, Toronto, Canada) through a TurboIonSpray® source operated in the positive ion mode. When the eluent flow was 1 ml/min it was split to introduce only 250 μl/min into the MS. Source conditions were individually optimised for compounds A and B. All quantitative analyses and post-column infusion experiments were performed in the selected reaction monitoring (SRM) mode using a transition specific for each compound analysed. Full scan experiments were performed scanning the first quadrupole (*Q*1) from 200 to 2000 amu.

2.3. Standard and solution preparation

To prepare stock solutions compounds were dissolved in methanol to give a concentration of 1 mg/ml. Working solutions were obtained by dilution of the stock solution with methanol and used to prepare standards by addition to the matrix; the methanol content of the standards prepared in biological matrix (plasma) was not more than 5%. For the post-column infusion experiments working solutions of 100 ng/ml were used.

All dilutions were prepared using glass volumetric flasks and volumes were measured with positive displacement micropipettes (Gilson, Milan, Italy).

Solutions of polysorbate 80 were prepared by weighing this material into a volumetric flask and then bringing to volume with methanol.

2.4. Post-column infusion experiments

Post-column infusion experiments were conducted using the approach described by Bonfiglio et al. [8]. The experimental set-up used is presented in Fig. 1. A T-junction was placed between the HPLC and the MS source (after the MS flow splitter) and the compound of interest, monitored by SRM, was introduced at 5 μl/min into the LC eluent. The compound solution was prepared at 100 ng/ml, placed in a 1 ml glass syringe (Hamilton, Bonaduz, Switzerland) and delivered with a syringe pump (Harvard Apparatus, Edenbridge, UK).

A blank matrix extract, or another test solution not containing the compound of interest, was injected by the autosampler into the HPLC. The response was monitored continuously to produce a profile of matrix effect.

2.5. Animal treatment and sample preparation

Sprague–Dawley rats (Charles River, Calco, Italy) were used to obtain the plasma from vehicle treated animals. The formulation vehicle (20% polysorbate 80 in 5% glucosate) was administered at 2.5 ml kg^{-1} via the tail vein. After 5 min the animals were sacrificed under deep ether anaesthesia and the blood collected from the abdominal aorta into heparinised tubes. The samples were centrifuged at 1200 × *g* for 10 min at 4 °C to obtain plasma.

Samples from a pharmacokinetic study with compound A, standards prepared in blank matrix, and samples taken from vehicle treated animals, were all analysed in the same manner. In 1.5 ml Eppendorf tubes, 200 μl of methanol were added to 25 μl of plasma, the samples were vortex mixed and centrifuged at 21,000 × *g* for 5 min at 6 °C. The supernatant was transferred to a polypropylene 96-well plate for injection into the LC/MS/MS system.

For dilution tests, the plasma samples were diluted with blank plasma prior to protein precipitation.

2.6. Software and data analysis

Analyst 1.1 Software (MDS Sciex, Toronto, Canada) was used to control the LC/MS/MS system and to acquire and analyse the data.

Data obtained for the pharmacokinetic (PK) study were imported into the Watson 6.2 (InnaPhase, Philadelphia, USA) LIMS system, which was used to calculate pharmacokinetic parameters using non-compartmental analysis (NCA) methods.

3. Results and discussion

3.1. Definition of issue and impact

Plasma samples from a PK study with compound A, consisting of an intra-venous (IV) and an oral (PO) dose, each in three individual animals, were analysed using two different

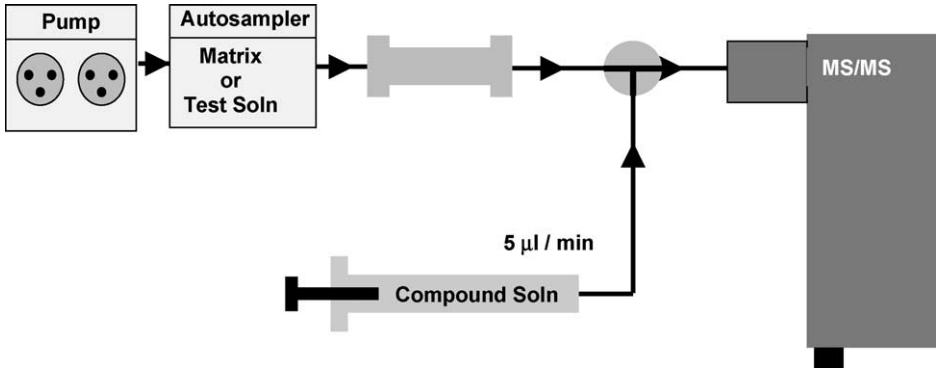


Fig. 1. Schematic for the post-column infusion analytical set-up.

methods. One was a "generic" method, commonly used for discovery research studies, in which the column is flushed with a mobile phase containing a low percentage of organic modifier after the injection and the analytes are then eluted with a fast gradient, the other method used an isocratic separation followed by a wash step (for columns and gradient profiles, see Table 1).

Plasma concentration–time curves obtained with the two methods are presented in Fig. 2. For the oral dose group the results were similar (the differences observed being well within normal analytical error), however the results obtained for the IV dose group were quite different. Using the generic fast gradient method the calculated concentration levels were much lower than with the isocratic method in the samples collected up to 3 h after dosing, whilst for later time points the results were essentially equivalent.

PK parameters calculated from these results are presented in Table 3. The area under the curve (AUC) for the IV dose obtained using the plasma concentration levels generated with the isocratic method were about double that calculated from the generic method results, and the corresponding clearance values were approximately one half. The half-lives (t 1/2) were similar with both methods. At the preliminary stage of the drug discovery process the clearance is often a primary PK parameter used for selecting compounds to study further. A high clearance value can be considered a significant liability for the molecule's potential, and could result in the compound being down prioritised or even dropped.

Table 3
Summary of basic pharmacokinetic parameters obtained for compound A according to two analytical methods

Parameter (units)	Generic method	Specific method
AUC $_{0-\infty}$ (ng h/ml)	2340	4320
CL (h^{-1} kg^{-1})	4.4	2.3
T 1/2 (h)	3.1	2.5
F (%)	1.6	0.9

In addition, whilst the AUC in the oral dose group was equivalent in both sets of results, the calculation of bioavailability (F%) was altered by the different AUCs obtained for the IV dose. In this case, the bioavailability was low for both methods and would not have influenced any decision made for this compound. However, an "apparent" doubling of bioavailability could have an important impact in case of compounds with higher F, perhaps erroneously allowing a compound to be regarded as "bioavailable" at this stage of screening.

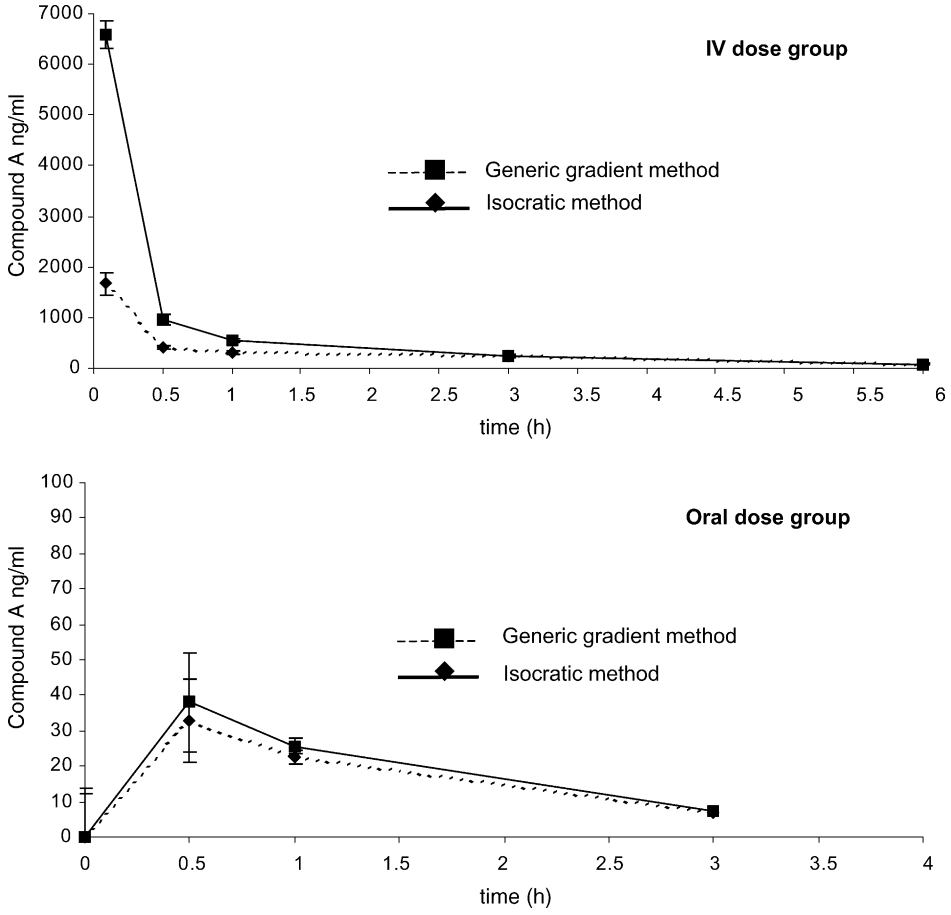


Fig. 2. Mean plasma concentration–time curves: IV and oral profiles for compound A obtained with two analytical methods. The error bars represent the CV between three individual animals.

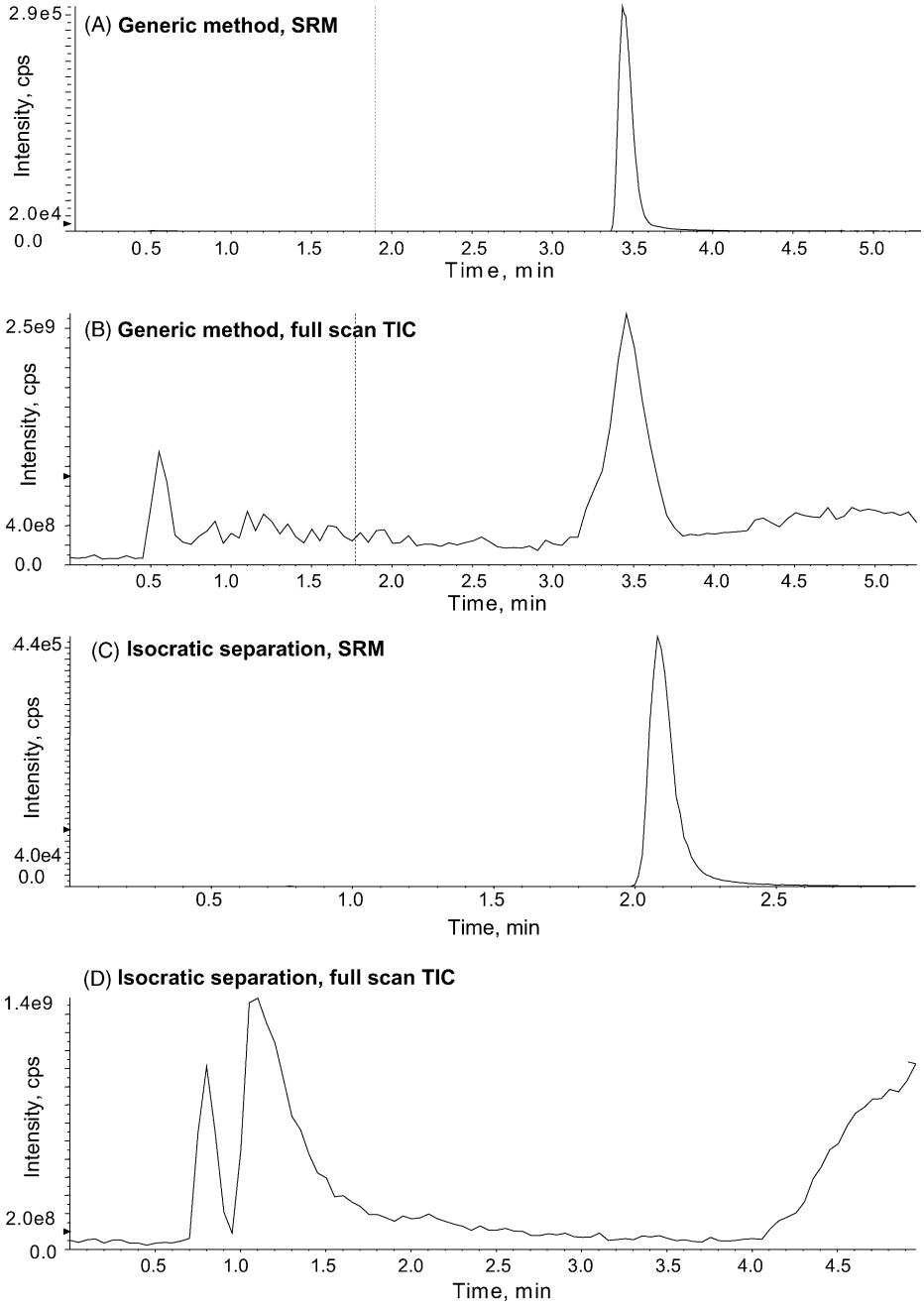


Fig. 3. Comparison of the generic gradient and specific isocratic methods. For each method the analyte specific chromatogram (SRM trace, A and C) and a full scan chromatogram (TIC trace, B and D) are presented.

An early time point sample from the IV dose group was analysed and full scan data acquired (Fig. 3) using the isocratic and generic methods. Using the generic method a signal is observed around the void time followed by a bulky wide peak between 3 and 4 min, which co-elutes with the analyte of interest (SRM trace). The combined mass spectra for this LC peak has many mass peaks at low and high masses with regular spacing (44 amu) between most peaks (data not shown). With the isocratic method, on the other hand, a wide peak is seen eluting soon after the void volume but the chromatogram returns to baseline before the elution of the analyte of interest; the change in signal seen in the trace after 4 min is due to the high concentration of acetonitrile during the wash step. Using this LC method the combined mass spectra at the retention time of the analyte peak is attributable to the analyte's molecular ion.

This suggests that the lower response for the analyte observed with the generic method for early time points is due to ion-suppression from the co-eluting material. The spectrum for this material appears consistent with that of an artificial polymer [12], most likely originating from the formulation

agent, as compound A was administered in a 20% polysorbate 80 in 5% glucosate solution.

3.2. Application of different separation methods

The effect of the chromatographic conditions on ion-suppression was further investigated, analysing solutions and samples containing compound A with four different HPLC methods.

As a strategy, modification of the chromatographic method appeared to us as the most attractive option. Use of different ionisation sources has also been suggested as an approach to avoid such problems. Atmospheric pressure chemical ionisation (APCI) rather than electrospray has been described as less succeptible to ion-suppression effects, at least for Sciex API instruments and differences in ion-suppression effects for different instruments and ion sources geometries has been discussed in detail in the literature [2,6,9,10]. In preliminary experiments, we tested the APCI source (Sciex Heated Nebulizer) and observed it suffered from ion-suppression when using the "generic" gradient, even though to a lesser extent than with the electrospray ion source. In general, electrospray is favoured for our discovery bioanalytical work, because it has generally better sensitivity, wider applicability, and as many of our investigational drugs form N-oxides, which can revert to the parent drug in the APCI ion-source.

Three types of model samples were prepared spiking compound A at the same concentration (10 μg/ml) in three different matrices: methanol (control), blank plasma (blank matrix control) and plasma obtained from animals treated with the polysorbate 80 vehicle (see Section 2.5). Four HPLC methods were tested—a "ballistic" gradient, a short gradient, a long gradient and an isocratic separation. Details of the systems are summarised in Table 2. All columns were operated at 1 ml/min.

Fig. 4 presents the results obtained with the ballistic gradient for compound A, spiked into blank plasma and into plasma from vehicle treated animals. The peak area observed for the latter sample is only about 30% of that of the former; indicating a reduction in response due to ion-suppression of about 70%. Whilst there is a strong interference from the matrix this is not "visible" in any way in the chromatographic trace obtained with MS/MS detection.

The results obtained with the four methods are summarised in Table 4. Ratios of the peak areas obtained with the different samples were calculated as a measure of ion-suppression: response ratio 1 represents the ratio between the response of the analyte in blank plasma and in methanol (effect of the blank matrix), and response ratio 2 that between the response in plasma from vehicle treated and control animals (effect of the vehicle). For compound A no ion-suppression was seen in the blank plasma matrix with any method: the peak area was always approximately the same for the compound prepared in either blank plasma or methanol. Suppression effects were only observed with the plasma of vehicle treated animals. Suppression was observed with the ballistic gradient and, to a lesser degree, the "long" gradient system. We originally thought that using a longer separation

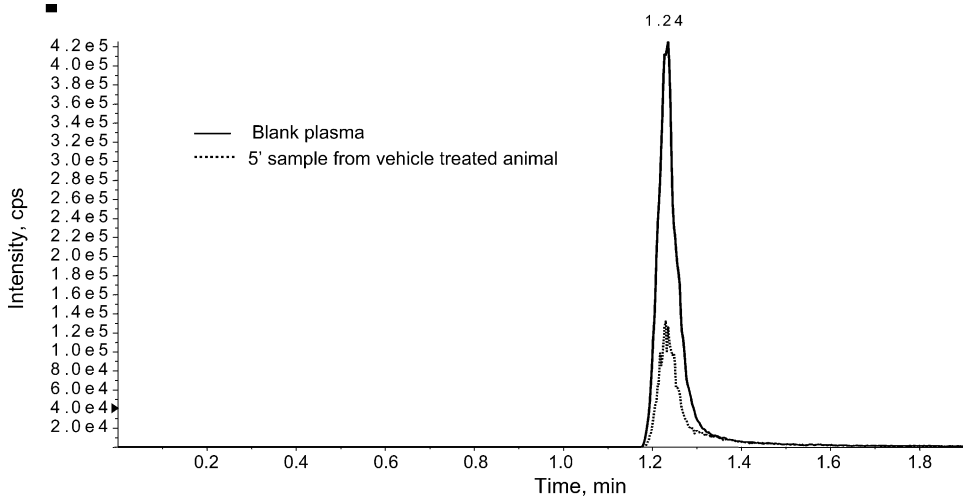


Fig. 4. Comparison of peaks obtained for compound A spiked in two different matrices using a ballistic gradient method.

Table 4
Comparison between the response of analyte A obtained in different matrices with different methods

Method	Analysis time (min)	Retention time (min)	Response ratio 1 (blank/MeOH)	Response ratio 2 (vehicle/blank)	Generic?
Ballistic gradient	3	1.3	1.0	0.3	Yes
Short gradient	5	2.5	1.0	1.0	Yes
Long gradient	20	7.5	1.0	0.8	Yes
Isocratic	6	2.5	1.0	1.0	No

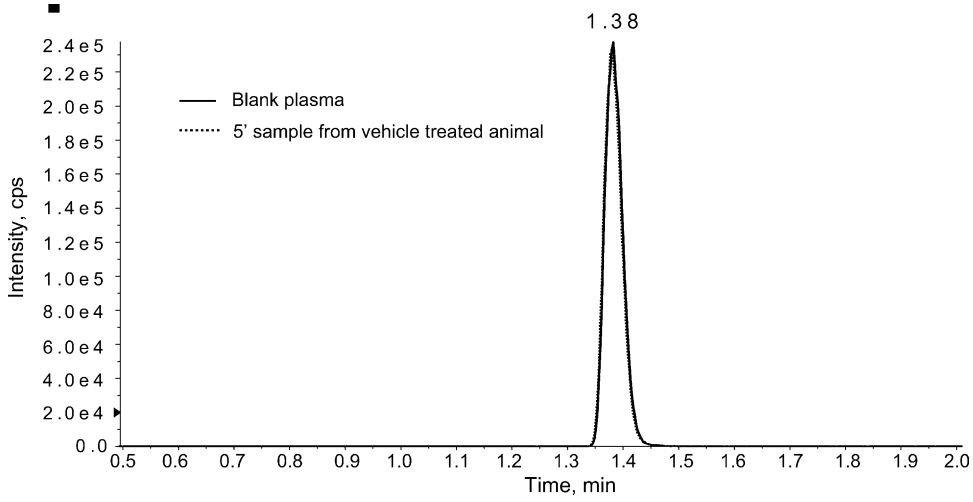


Fig. 5. Comparison of peaks obtained for compound B spiked in two different matrices using a ballistic gradient method.

should offer better resolution of the analyte from interferences, however this was not the case. Separation methods need to be chosen with care and practical tests are necessary to ensure that they are free from interferences. No suppression was observed using either the "short" gradient or with the isocratic method. In general, resolution of closely eluting species should be maximal under isocratic conditions. The empirical investigation described here shows that in this case the "short" gradient efficiently resolved compound A from the interfering material whilst with the "long" gradient it still suffered from suppression, this slightly surprising result may be due to slow elution of the formulation agent from the longer column using a shallow gradient profile.

The ballistic gradient experiment was repeated with another research molecule, compound B, for which no ion-suppression could be observed (Fig. 5). The absence of ion-suppression of compound B with the ballistic gradient appears to be due to the different chromatographic properties of this molecule. Compounds A and B have *c* log *P* values of 2.37 and 3.43, respectively, this difference in polarity makes compound B elute later than compound A and this appears sufficient to separate it from matrix interferences in all of the chromatographic systems studied.

3.3. Characterisation of the ion-suppression effects by post-column infusion

Use of post-column infusion techniques to investigate matrix effects in LC/MS/MS analysis have been widely reported in the literature [5,8,9,12,13]. This approach was applied using the three test matrices and the same four HPLC systems as described in the previous section; results are presented in Fig. 6A–D. The data obtained with methanol injections are not shown for clarity, as in all cases they showed a regular

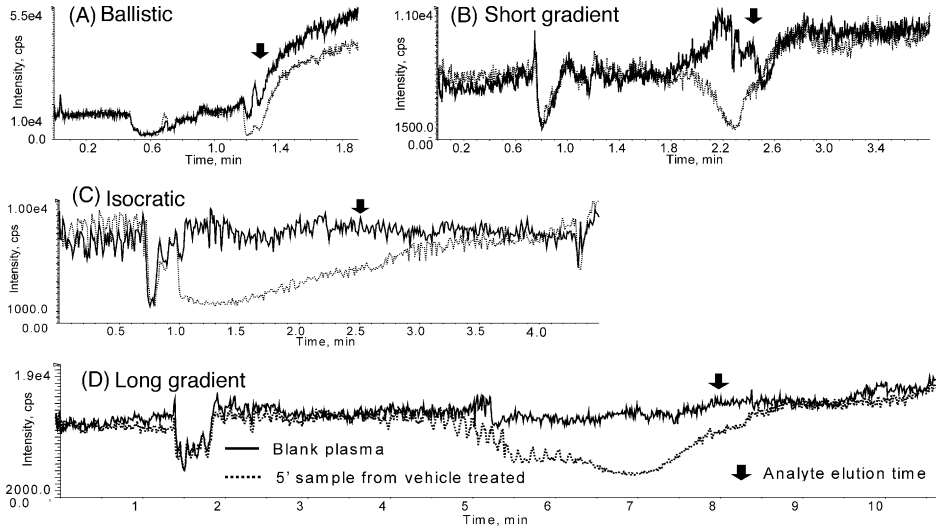


Fig. 6. Use of post-column infusion of compound A to characterise the suppression effects. (A–D) Present the post-column infusion experiments for four different chromatographic methods.

baseline, with response increasing as the acetonitrile content of the mobile phase entering the MS increased.

Separate "zones" of suppression could be observed in the chromatograms. For example, in the long gradient analysis (Fig. 6D) there is a first area of suppression around the void volume and a second one between 5 and 8 min. The first suppression area is common to both plasma samples, but not seen for methanol (data not shown), and probably originates from non-retained endogenous material. The second area of suppression is observed only for plasma obtained from animals treated with vehicle and not for control plasma, indicating this is due to the formulation. The suppression profile is sharper for the ballistic and short gradients (Fig. 6A and B), wider for the long gradient (Fig. 6D), and with a sharper onset and long "tail" for the isocratic method (Fig. 6C).

Although the post-column infusion approach appears qualitatively informative it does not provide quantitative information. In these experiments, one example was found in which the post-column infusion approach suggested the possibility of suppression when quantitatively none was observed. With the isocratic method (Fig. 6C) compound A elutes at 2.5 min, at which point the post-column infusion profile would suggest some suppression (dotted line lower than full line), but none was observed by comparing peak areas (Table 4). Defining a completely "matrix ion-suppression free zone" as suggested by Hsieh et al. [5] may not be necessary to

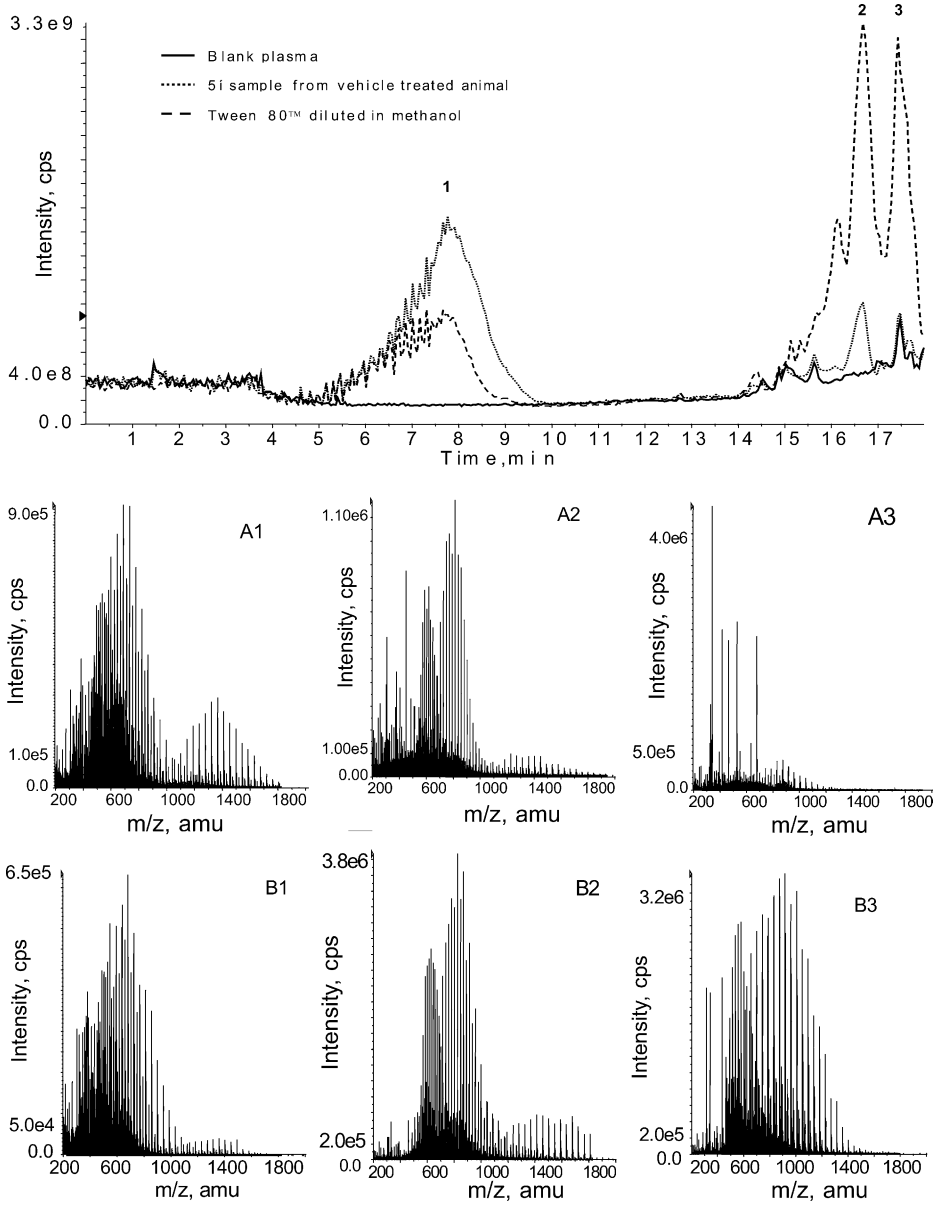


Fig. 7. Full scan experiments for Tween 80 "separation" in different samples. A blank plasma, a Tween 80 methanolic solution and an extract from plasma of an animal treated with Tween 80 vehicle are presented. Spectra obtained from "peaks" 1–3 for vehicle treated animal (A1–A3) and Tween 80 diluted in methanol (B1–B3) are presented below the chromatograms.

avoid ion-suppression, and could make method development unnecessarily cumbersome. Comparing areas for control and "worst-case" samples appears to be a better option.

3.4. Preliminary characterisation of responses from the polysorbate 80 material

Extracts of plasma samples containing polysorbate 80 were analysed in full scan experiment to obtain total ion current (TIC) profiles and full scan spectra (Fig. 7). These analyses were conducted using the long gradient conditions (Table 2) but with a Zorbax C18 column (4.6 mm × 50 mm). Plasma extracts from animals treated with polysorbate 80 and a methanol solution of polysorbate 80 were analysed together with a control plasma extract. The polysorbate methanolic solution was prepared diluting the excipient in methanol at a concentration of 25 mg/ml. This would correspond to the initial concentration in the blood, considering a volume of distribution equal to total blood volume: 2.5 ml kg^{-1} dose of a 20% polysorbate 80 solution to rats with a blood volume of approximately 20 ml kg^{-1} [14]. This approximate calculation is also supported by Tellingen's work on polysorbate 80 pharmacokinetics [15].

The resulting TIC chromatograms, and full scan mass spectra of selected peaks, are consistent with the nature of the polysorbate 80 materials and results reported by other authors [16,17,20]. Polysorbate is subject to rapid esterase-mediated breakdown in rodent plasma [15,16]. As the results in Fig. 7 indicate, even 5 min after IV administration the TIC chromatograms and full scan data show distinct differences from the formulated polysorbate material. The clear implication of these results is that in vitro experiments, spiking polysorbate 80 into plasma, cannot be used to fully assess for potential ion-suppression effects from such materials. A potential additional problem is that polysorbate 80 material can vary according to supplier and batch.

3.5. Implications for method validation and dilution check for discovery bioanalysis

Given that ion-suppression can result in large errors in quantitative LC/MS/MS, checks for this effect should be performed during method development and validation [18].

Matuszewski and co-workers [2,10] have studied the problem of ion-suppression as it relates to clinical studies, where they indicate inter-individual variability as a major potential source of error. The latter article [10] describes a comprehensive yet practical approach to cover the issue of matrix effect during bioanalytical method validation. It focuses on how to test a wide enough range of matrix sources to ensure that inter-individual variability will not significantly affect the results.

However, these approaches would not reveal the type of ion-suppression problem observed in the present case, where analyses were not affected by blank plasma matrix, but by the formulation material in which the drug was administered. In addition, the effect was not independent of drug administration, as the formulation appeared to undergo metabolism, which enhanced the ion-suppression effects. Consequently, checks for ion-suppression effects due to the formulation by spiking blank plasma, may not fully mimic the situation of real samples. To test fully for vehicle related interferences, it may sometimes be necessary to obtain plasma from animals, or human subjects, treated with the formulation [19]. When this is not practical, an alternative could be to use the real samples themselves with a "dose addition" or over-spike approach into the incurred samples, in the same manner as standard addition methods used in other analytical fields, such as the analysis of antibiotic residues in wastewater [21].

Whilst including such additional experiments for validated methods is possible, it is not, however, practical when supporting early discovery studies. We therefore devised a quick test to detect risks of ion-suppression, which is applicable when running discovery support assays. This test was based on the observation that, when diluting samples in which ion-suppression occurred, the measured concentration for diluted samples was usually higher than that for the undiluted samples; in contrast it remained unchanged when suppression was absent. To perform this test the samples from the earliest IV time point (usually 5 min) were analysed undiluted and diluted at two dilution levels (typically 1:5 and 1:10) with blank plasma. Examples of results for this test are presented in Table 5. Compound A was subject to ion-suppression (a generic gradient method was used) and the calculated concentrations for the diluted samples were higher than those for the undiluted samples, indicating that ion-suppression was occurring. Compound B was not subject to ion-suppression and the calculated concentrations did not change with dilution (differences of less than 15% are not considered significant).

This test appears to be very useful in revealing ion-suppression issues related to IV formulations and can be included in a typical analytical batch requiring little additional work and no delay in proceeding with analysis of study samples. It is now used routinely in our discovery bioanalytical group as an acceptance criterion for new methods. It has been extended to allow reporting of results for samples where suppression was detected using the results for diluted samples,

Table 5
Summary table of dilution tests

Animal number	Dilution factor		
	None	5	10
Dilution test for compound A[a]			
1	8080	17200	20100
2	5940	14500	13600
3	8150	19300	17900
Dilution test for compound B[a]			
1	2440	2620	2430
2	3990	4560	4330
3	2740	2810	2830

[a] Calculated concentration (ng/ml).

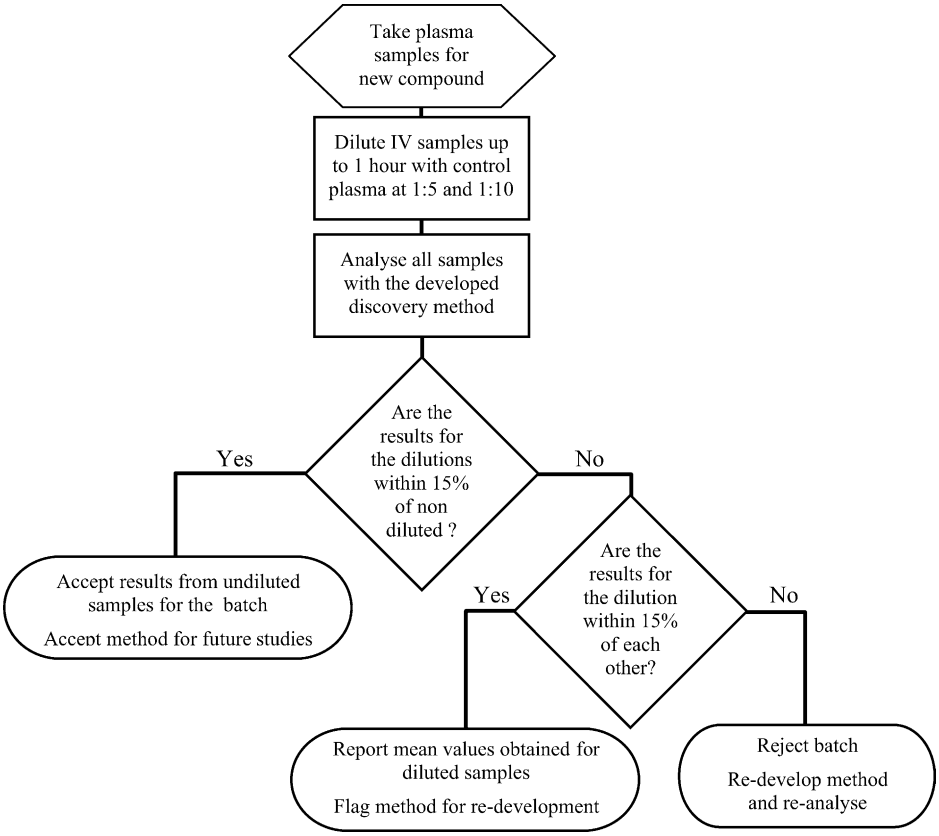
when two dilution levels agree with each other, following the process outlined in Scheme 1.

3.6. Approaches to avoid ion-suppression from formulation agents

In the literature a number of approaches are presented to avoid ion-suppression effects. One possibility would be to eliminate interfering species during sample preparation, and different authors have studied extraction approaches as they relate to matrix effects [8,12,13]. Liquid–liquid extraction (LLE) has been reported to be efficient in removing PEG400 and Tween 80 [12,13], but it does not always offer sufficient recoveries [12]. This limitation is not entirely surprising as more polar analytes, which co-elute with formulation excipients on reversed-phase columns, are less likely to partition into the extracting organic solvent. Solid phase extraction (SPE), generally favoured because it is easier to automate, has been reported to be limited in its ability to clean samples from interfering species [8,12,13]. However, most of these experiments have been done using a reversed phase mode of retention for both the SPE and HPLC separation, the use of orthogonal retention modes (e.g. polar SPE and reversed phase HPLC) may hold more promise. Alan Dzerk and his group, at MDS Pharma Services, succeeded in removing polysorbate material from plasma samples using a dual column approach, in which an ion-exchange column was coupled directly to a reversed phase analytical column [personal communication].

Temesi and et al. [20] from Astra Zeneca presented an alternative way of solving the problem of ion-suppression from formulation agents: designing a simpler excipient. They synthesized and characterised a single PEG form (PEG 414). As a formulation agent this material has properties essentially identical to PEG 400 but, being a single compound rather than a polydisperse polymer, it is easier to deal with from an analytical point of view.

As detailed method development is not possible for every individual compound in the drug discovery process setting, the challenge for bioanalysis in this context is to devise techniques that are generally applicable to most molecules or at least be relevant to classes of investigative compounds. Consequently, the risk of inaccurate results must be efficiently managed [22], whilst the approach must remain practical enough to allow fast development of new methods. Considering sample preparation techniques, simple protein precipitation methods are almost always used, as the development of more selective extraction procedures is not considered practical in the discovery context. When ion-suppression is found this can usually be overcome by refinement of the HPLC separation to obtain extra selectivity. Taking into consideration all these factors we have devised an approach to try and minimise the risk of ion-suppression effects in the discovery



Scheme 1. Decisional tree for dilution checks during first sample bioanalysis of a new discovery compound (it is assumed the concentrations in samples are within the standard curve range).

setting. It focusses especially on IV co-solvents as these will always be present in some plasma samples at high concentrations and bioanalytical errors caused can lead to inaccuracy in estimates of clearance and bioavailability. The main factors of this approach are:

(a) HPLC separation by isocratic elution with adequate retention ($k' > 2$), but with a gradient step after the analyte is eluted to "wash" the column with a high organic composition so as to avoid built-up of endogenous material on the column with repeated injections. The ability of the column to separate material with similar polarity is always degraded with gradient elution, especially with rapid or ballistic gradients.
(b) Limit the type of co-solvents used so that analysts can become familiar with the characteristics of a particular material, for example, in our laboratory generally only polysorbate 80 and PEG 400 are used as co-solvents. As shown in this publication the mass spectrometry and chromatography of polysorbate 80, which is the most commonly used by our discovery groups, has been investigated. More polar analytes requiring lower organic content in the mobile phase are much more likely to co-elute with excipients, and consequently extra care is required.
(c) Use the "dilution check" as described in section 3.5. Compared with tests such as those requiring infusion of analyte [5,8,9,13] this check can be included in routine analytical batches with minimal extra effort, and has the advantage of using actual study samples, which consequently contain relevant concentrations of analyte and formulation.

4. Conclusions

Ion-suppression is a major risk of error in quantitative LC/MS/MS. This type of interference is particularly difficult to identify because it does not present any visible "symptoms" and must be tested for specifically. Although most work on ion-suppression in bioanalysis has been concentrated on the interference from endogenous species, formulation agents can have dramatic effects and these studies are generating growing interest. Classical approaches to method validation are not sufficient to demonstrate the absence of this type of ion-suppression effects. In particular in a discovery setting, where formulation excipients are used in larger quantities and compounds are selected for their pharmacokinetic characteristics, inaccurate quantification due to formulation interferences may lead to the elimination or retention of candidates on false grounds.

The chromatographic separation remains the main step at which those effects can be controlled but it requires specific method development, not always compatible with a fast turnaround environment.

The issue can be identified using a test based on the dilution of real samples, with the blank matrix used to prepare the standards in a typical batch of analyses, that is easy to apply and can therefore be used in a discovery bioanalysis setting. In regulated environments, where full validation is required, a more complete and rigorous set of experiments should be applied.

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