Matrix effects: The Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry

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Abstract

High-performance liquid chromatography coupled by an electrospray ion source to a tandem mass spectrometer (HPLC–ESI–MS/MS) is the current analytical method of choice for quantitation of analytes in biological matrices. With HPLC–ESI–MS/MS having the characteristics of high selectivity, sensitivity, and throughput, this technology is being increasingly used in the clinical laboratory. An important issue to be addressed in method development, validation, and routine use of HPLC–ESI–MS/MS is matrix effects. Matrix effects are the alteration of ionization efficiency by the presence of coeluting substances. These effects are unseen in the chromatogram but have deleterious impact on methods accuracy and sensitivity. The two common ways to assess matrix effects are either by the postextraction addition method or the postcolumn infusion method. To remove or minimize matrix effects, modification to the sample extraction methodology and improved chromatographic separation must be performed. These two parameters are linked together and form the basis of developing a successful and robust quantitative HPLC–ESI–MS/MS method. Due to the heterogenous nature of the population being studied, the variability of a method must be assessed in samples taken from a variety of subjects. In this paper, the major aspects of matrix effects are discussed with an approach to address matrix effects during method validation proposed.

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Introduction

The development of atmospheric pressure ionization techniques (electrospray, atmospheric pressure chemical, and atmospheric pressure photo-ionization) has enabled the coupling of high-performance liquid chromatography with...
mass spectrometry (HPLC–MS). The use of hydrophobic separation combined with selective mass spectrometric detection makes this a versatile analytical tool. Electrospray is currently the most widely used ionization source. The electrospray interface produces singly or multiply charge ions directly from an aqueous–organic solvent system by creating a fine spray of highly charged droplets in the presence of a strong electric field, with the assistance of heat or pneumatics. The successful formation of ions using electrospray ionization requires two steps: the transfer of the compound of interest into the gas phase and the addition of a charge to the analyte if it is not already in a charged state [1–4].

HPLC–MS systems using an electrospray ion source coupled with tandem mass analyzers (HPLC–ESI–MS/MS) have been applied to a wide variety of studies in pharmaceutical analysis and life sciences. With HPLC–ESI–MS/MS now considered the benchmark for measurement of drugs and their metabolites in biological matrices [5], the high selectivity of tandem mass spectrometry, with successive mass filtrations, leads to little or no observed interference even though there may be relatively high concentrations of coextracted and coeluted matrix components present. These characteristics have led to a growing trend of high-throughput analysis that incorporates little or no sample preparation and minimal chromatographic retention [6–8].

With HPLC–ESI–MS/MS having these characteristics of high selectivity, sensitivity, and throughput, it is not surprising that this technology is being increasingly used in the clinical laboratory. A recent review by Dooley [9] reported an exponential growth from 1991 to 2001 in clinical chemistry papers that mention tandem mass spectrometry. It is now accepted that HPLC–ESI–MS/MS is the method of choice for screening for inherited metabolic disorders [10], but it can be expected that many more applications will follow. While many biochemical markers such as steroids, fatty acids, amino acids, catecholamines, and thyroxine have been measured by this analytical technique [11–15], specific examples of clinical laboratories applying HPLC–ESI–MS/MS to drug quantification are for the therapeutic drug monitoring of immunosuppressant drugs [16] and protease inhibitors [17], and toxicological investigations [18,19].

While HPLC–ESI–MS/MS offers much promise for clinical laboratories, one issue that must be addressed in method development, validation, and routine use is matrix effects. Matrix effects are the alteration of ionization efficiency by the presence of coeluting substances. A recent paper by Annesley [20] highlighted the importance of understanding matrix effects in clinical mass spectrometry applications, and although critical to the success of an HPLC–ESI–MS/MS analytical method, few published methods adequately address this problem [21]. The aim of this report is to provide an overview of matrix effects and from a clinical laboratory perspective show how this issue should be addressed for quantitative HPLC–ESI–MS/MS methodologies.

What are matrix effects?

Matrix effects occur when molecules coeluting with the compound/s of interest alter the ionization efficiency of the electrospray interface. This phenomenon was first described by Tang and Kebarle [22] who showed that electrospray responses of organic bases decreased as the concentrations of other organic bases were increased. The exact mechanism of matrix effects is unknown, but it probably originates from the competition between an analyte and the coeluting, undetected matrix components.

King et al. [23] have shown through a series of experiments that matrix effects are the result of competition between nonvolatile matrix components and analyte ions for access to the drop surface for transfer to the gas phase. Although they conclude that the exact mechanism of the alteration of analyte release into the gas phase by these nonvolatile components is unclear. They postulate “...a likely list of effects relating to the attractive force holding the drop together and keeping smaller droplets from forming should account for a large proportion of the ionization suppression observed with electrospray ionization”. Depending on the environment in which the ionization and ion evaporation processes take place, this competition may effectively decrease (commonly known as ion suppression) or increase (ion enhancement) the efficiency of formation of the desired analyte ions present at the same concentrations in the interface. Thus the efficiency of analyte ions to form is very much dependent on the matrix entering the electrospray ion source.

Matrix effects are also compound dependent. Bonfiglio et al. [24] reported that the chemical nature of a compound has a significant effect on the degree of matrix effects. In a study of four compounds of different polarities under the same mass spectrometric conditions, the most polar was found to have the largest ion suppression and the least polar was affected less by ion suppression. These findings of differential matrix effects have important ramifications particularly when selecting a suitable internal standard for quantification purposes. For example, if a drug and a glucuronide metabolite were quantified by internal standardization against a close analogue of the parent drug and matrix effects were slightly different between samples, then the change in ionization of the more polar glucuronide metabolite would probably not be compensated by the internal standard. Thus if there are multiple analytes to be quantified, with varying degrees of polarity, there may be requirements for multiple internal standards [25].

The importance of matrix effects on the reliability of HPLC–ESI–MS/MS has been shown in terms of accuracy and precision [26], and when ion suppression occurs, the sensitivity and lower limit of quantification of a method
may be adversely affected [27]. Thus to develop a reliable
HPLC–ESI–MS/MS method, experiments should be per-
formed to understand these matrix effects.

How can matrix effects be assessed?

The two main techniques used to determine the degree of
matrix effects on an HPLC–ESI–MS/MS method are
postextraction addition and postcolumn infusion. The
postextraction addition technique requires sample extracts
with the analyte of interest added postextraction compared
with pure solutions prepared in mobile phase containing
equivalent amounts of the analyte of interest [20,21,26–28].
The difference in response between the postextraction
sample and the pure solution divided by the pure solution
response determines the degree of matrix effect occurring to
the analyte in question under chromatographic conditions.

An example of calculations from postextraction addition
experiments is shown in Table 1. These data are adapted
from a study by Buhrman et al. [27] who showed using this
technique the various amounts of matrix effects that may
occur using different sample preparation methods of a
platelet-activating factor receptor antagonist from human
plasma. The example shown is for the liquid–liquid
extraction of this compound using hexane. The matrix
effect was determined as −26%. This value represents a loss
of 26% of the analyte signal (ion suppression) due to
alterations in ionization efficiency. A calculated value of 0%
would represent no matrix effects, the ideal scenario.
Additional data from this experiment can be obtained from
the comparison of the ion intensity obtained from extracts
with analyte added preextraction with pure solutions to give
an overall value termed “process efficiency” (Table 1).
Process efficiency represents the combination of matrix
effects and recovery of the analyte from the matrix by the
sample extraction process. The absolute recovery in the
above example is 63% (4050/6390 × 100%), but the
process efficiency is only 47%.

Low process efficiency can be deleterious to the accuracy
and in particular the lower limit of quantification of a
method [26]. Fierens et al. [29] reported preliminary data on
the measurement of urinary C-peptide by HPLC–ESI–MS/
MS. Using aqueous standards, it was determined that by
direct injection of urine a limit of detection of 0.2 ng could
be achieved. Comparing these standards with urine, it was
found that the signal was suppressed by 70–85%. Thus
without removing these matrix effects, via sample prepara-
tion, the desired lower limits of detection could not be
attained.

The postextraction addition technique can be considered
a static technique that only provides information about
matrix effects at the point of elution of the analyte of
interest. A more dynamic technique for determining matrix
effects is postcolumn infusion [23,24,30–32]. An infusion
pump is used to deliver a constant flow of analyte into the
HPLC eluent at a point after the chromatographic column
and before the mass spectrometer ionization source (Fig. 1).
A sample extract (without added analyte) is injected under
the desired chromatographic conditions and the response
from the infused analyte recorded. The postinfusion
technique enables the influence of the matrix on analyte
response to be investigated over the entire chromatographic
run. One point taken into consideration is that the
concentration of the infused analyte should be in the
analytical range being investigated as too much added
analyte may overwhelm the electrospray’s ability to
generate ions and thus give misleading results.

Results of postcolumn infusion experiments enable the
scientist to evaluate the influence of different sample
extraction techniques on matrix effects, the appropriate
analytical column, where matrix effects occur and are absent
during a chromatographic run, the mechanistic aspect of
matrix effects, and the influence of mobile additives on
response. As an example, Fig. 2 shows a comparison of an
injection of (A) mobile phase, (B) a whole blood sample
prepared by protein precipitation, and (C) a whole blood
sample by solid phase extraction [33]. The analyte
sirolimus, an immunosuppressant drug (50 μg/L), was
infused (10 μL/min) postcolumn under the chromatographic
conditions described in Taylor and Johnson [33]. Sirolimus
was monitored by selected reactant monitoring using the
following mass transition: m/z 931.6→864.6. The retention
time of sirolimus under these chromatographic conditions
would be approximately 6 min. For mobile phase injection
(Fig. 2A), no change in signal was observed throughout the
chromatogram. For the sample treated with acetonitrile to
precipitate proteins (Fig. 2B), the signal is suppressed for
the majority of the chromatogram. While the sample
prepared by solid phase extraction (Fig. 2C) showed
minimal suppression in the solvent front but constant and
maximum signal at the retention time of the analyte.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Calculation and result (%)</th>
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<tbody>
<tr>
<td>Preextraction addition matrix effect</td>
<td>(6390 – 8580)/8580 × 100 = −26</td>
</tr>
<tr>
<td>Postextraction addition process efficiency</td>
<td>4050/8580 × 100 = 47</td>
</tr>
</tbody>
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Data modified from Buhrman et al. [27].
minimize their presence. The source of these interfering matrix components must also be considered. The interference may come from the current sample being injected, a previously injected sample (as a late eluting interference), or build-up and overload of the analytical column [25]. Two approaches to remove or minimize matrix effects are modification to the sample extraction methodology and improved chromatographic separation [34]. These two parameters are linked together in developing a quantitative HPLC–ESI–MS/MS method.

There are several sample preparation options that have been used for many years with HPLC and studies have evaluated the degree of matrix effects for these different techniques [24,27,29,30,32]. It is considered that protein precipitation using an organic solvent or dilute and shoot are the “dirtiest” sample preparation techniques and thus produce the most matrix effects compared to solid phase or liquid–liquid extraction [24,30]. More recently, on-line two-dimensional chromatography, using a column-switching device, has been shown to provide an alternate sample preparation that gives minimal matrix effects [35]. A clear advantage of this technique is the ability to automate the analytical process but column breakthrough will limit how much crude sample can be loaded. The selection of which extraction technique to use for an application is not just limited to providing the cleanest sample but linked also to the chromatographic separation when optimizing a method.

The majority of matrix effects occur in the solvent front of a chromatographic run [30]. Thus intuitively if the analytes can be retained chromatographically to some degree then matrix effects can be minimized. Data obtained from postcolumn infusion experiments can provide data on where matrix effects are occurring. Thus using these data to move the analytes away from these affected areas provides for a more stable method. These changes may limit the aim of high-throughput analysis. An alternative approach is to apply a rapid gradient or “ballistic gradient” to separate the analytes from the solvent front while maintaining high throughput [6].

It has been shown, for specific compounds, that atmospheric pressure chemical ionization is less prone to matrix effects than electrospray [36,37]. The presence of excess reagent ions to produce charged species means the ionization process of atmospheric pressure chemical ionization is less susceptible to matrix effects. Therefore, the problem of matrix effects can be removed by changing the ionization source, although some differences in matrix effect for atmospheric pressure chemical ionization have been found between manufacturer’s ion sources [37]. Another limitation being that the analytes must be thermally stable and readily ionized by this technique.

Method validation

The experiments described above can be considered as semiquantitative and confirm the presence or absence of matrix effects and aid in minimizing their influence on results. But these data do not provide evidence that a validated analytical method is acceptable in terms of these effects. While the US Food and Drug Administration states that there is a need to investigate matrix effects for HPLC–MS methodologies [38], there is no clear guidance on how this should be performed during method validation. The experiments described in the following are from a recent paper by Matuszewski et al. [21] and the author’s personal experience.
Most if not all method validations are performed using standard and quality control samples prepared from a pooled source of matrix (whole blood, urine, etc.). In such a case, the matrix is homogenous and thus can be assumed that prepared samples will have the same matrix effects. Using these homogenous samples for validation does not take into account the inter- and intrapatient matrix variability that is present in the clinical setting. It is highly likely that an extract of a plasma sample collected from a burn’s patient will be of a different composition to a recent liver transplant recipient and certainly to a healthy subject and thus may have variable matrix effects. This interpatient variability is a typical problem faced but seldom addressed by the clinical scientist establishing an HPLC–ESI–MS/MS method.

Matuszewski et al. [21] have elegantly shown the influence of different subjects on assay imprecision for a quantitative HPLC–ESI–MS/MS method by comparing standard samples prepared from one pool of plasma with that from multiple sources. Standard curves were prepared in pooled plasma and also different individuals, subjected to the same liquid–liquid extraction and chromatographic conditions with multiple extractions performed for each concentration ($n = 5$). For the standards prepared from pooled plasma, across an analytical range of 0.5–100 µg/L,
the variability at each concentration in terms of coefficient of variation was 4.6–11.3%. This variability would be considered acceptable for method validation based on current opinion [39]. For the standard samples prepared from different lots of plasma, across this same concentration range, the variability was between 11.6% and 23.8%. A significant increase in variability due to intersubject differences in matrix effects was reported; with this decrease in performance, the method would now fail validation. It can be speculated that many HPLC–ESI–MS/MS quantification methods may fall into this same scenario if validation is solely based on one pooled matrix.

It is clear that at some stage during method validation the variability of a method must be assessed in samples taken from a variety of subjects in the patient population of interest. Further, it has been shown that endogenous sources of matrix effects may come from the types of specimen containers and preservatives within these containers [37]. Thus matrix effect studies during validation must be carried out in the collection tube used for the method to be established. If different types of anti-coagulants are used, all of these should be evaluated.

While it is not practical to prepare all calibration standard and quality control samples from individual sources, some assessment of patient variability must be undertaken. The following approach has been used in the author’s laboratory. This protocol assesses the performance of a method using blood from subjects who are the subject of the clinical investigation being studied (i.e., transplant recipients, HIV patients, uremic patients, etc). Three quality control concentrations, over the analytical range, in replicates of five are prepared using different individuals for each aliquot (i.e., each individual is used only once). The analyte of interest and internal standard are added pre- and postextraction and prepared in pure solution according to the protocol of Buhrman et al. [27], previously described above. Using this protocol of 45 injections, matrix effects, absolute recovery, process efficiency, and most importantly intersubject variability can be calculated. As an example, data from an HPLC–ESI–MS/MS method for the measurement of cyclosporin in whole blood that used solid phase sample preparation are shown in Table 2 [40]. Three concentrations of cyclosporin at 30, 400, and 1500 μg/L were assessed using blood obtained from transplant recipients not receiving this drug. Matrix effects were minimal with less than 7% ion suppression. The intersubject variability, expressed as coefficient of variation, was <3% for the three concentrations. These data compared favorably with the variability obtained from pooled blood of <5% coefficient of variation (data not shown). The results of this experiment suggest that matrix effects will have minimal influence on the results of this method. If the intersubject variability was greater than 15%, changes to the extraction procedure and chromatography would have to be undertaken. It is hoped that this type of procedure will be common practice in the validation of HPLC–ESI–MS/MS methods.

### Conclusions

HPLC–ESI–MS/MS is a powerful tool for the scientist to utilize for quantitative clinical investigations. This technique is not a “turn key” solution to analytical problems, as matrix effects can be its’ Achilles heel. But by careful assessment of matrix effects and judicial use of the appropriate sample preparation coupled with adequate chromatography, HPLC–ESI–MS/MS can provide a robust analytical platform. Clinical scientists in developing methods must acknowledge matrix effects and build assessment systems into their validation protocols. If these procedures are not performed, there can be doubt in the validity of results produced.

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### References


