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Review

Validation of bioanalytical LC–MS/MS assays: Evaluation of matrix effects

Ann Van Eeckhaut, Katrien Lanckmans[1], Sophie Sarre, Ilse Smolders, Yvette Michotte*

Vrije Universiteit Brussel, Department of Pharmaceutical Chemistry, Drug Analysis and Drug Information, Laarbeeklaan 103, 1090 Brussels, Belgium

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ABSTRACT

Liquid chromatography coupled to atmospheric pressure ionization tandem mass spectrometry is currently the method of choice for the quantitative determination of drugs in biological matrices. The advantages of this technique include high specificity, sensitivity and throughput. However, co-eluting matrix components, which are not observed in the chromatogram, can have a detrimental effect on the analysis, since they can cause ion suppression or enhancement of the analyte. The evaluation of matrix effects on the quantitative analysis of drugs in biological fluids is an important and sometimes overlooked aspect of assay validation. In this review, the influence of matrix effects on bioanalytical LC–MS/MS methods is discussed and illustrated with some examples. In addition, possible solutions to reduce or eliminate matrix effects are highlighted. A literature overview of validated LC–MS/MS methods published from January till June 2008 is also included. Although matrix effects are investigated in most papers, there is no consensus on how matrix effects should be evaluated during method validation. In addition, the definition of specificity should be changed for LC–MS/MS based methods.

Contents

1. Introduction

Liquid chromatography (LC) coupled by an atmospheric pressure ionization (API) source to tandem mass spectrometric (MS/MS) detection is currently considered as the method of choice for quantitative analysis of compounds in biological matrices [1,2]. The advantages of using MS/MS in the selected reaction monitoring (SRM) mode are mainly the increased specificity, sensitivity and throughput. The aforementioned arguments have led analysts to develop high throughput methods with little or no sample preparation and minimal chromatographic retention [1–3]. However, molecules originating from the sample matrix that coelute with the compound(s) of interest can interfere with the ionization

Abbreviations: API, atmospheric pressure ionization; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; HILIC, hydrophilic-interaction liquid chromatography; IS, internal standard; LC, liquid chromatography; LLE, liquid–liquid extraction; PPT, protein precipitation; SRM, selected reaction monitoring; SPE, solid phase extraction; SIL-IS, stable isotope labeled-internal standard; MS/MS, tandem mass spectrometry; UPLC, ultra performance liquid chromatography.

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* Corresponding author. Tel.: +32 2 477 44 29; fax: +32 2 477 41 13.
E-mail address: ymichot@vub.ac.be (Y. Michotte).
[1] Present address: Department of Clinical Chemistry and Radio-immunology, Toxicology, UZBrussel, Laarbeeklaan 101, 1090 Brussels, Belgium.

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process in the mass spectrometer [2], causing ionization suppression or enhancement [3–5]. This phenomenon, the so-called matrix effect, was first described by Kebarle and Tang in 1993 [6].

Better understanding of how matrix effects can compromise the integrity of bioanalytical methods has re-emphasized the need for adequate chromatographic separation of analytes from endogenous biological components in quantitative bioanalysis using LC–MS/MS [3]. The inherent specificity of LC–MS/MS methods results in chromatograms that do not present any apparent interference, although relatively high concentrations of matrix components are sometimes present [4].

Matrix effects cause a compound's response to differ when analyzed in a biological matrix compared to a standard solution. The difference may be described as suppression or enhancement according to whether the response is diminished or magnified. These unpredictable effects are a regular problem for API ionization sources [7–9]. Ionization effects theoretically occur in either the solution phase or the gas phase and the main cause is a change in droplet solution properties caused by the presence of nonvolatile or less volatile solutes that change the efficiency of droplet formation or evaporation, which in turn affects the amount of charged ions in the gas phase that ultimately reach the detector [7,10]. Many different mechanisms of ion suppression have been proposed, most of which are specific to the ionization technique used [1,11]. The two most popular API techniques for LC–MS/MS are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). In a paper by Jessome and Volmer [11] an overview is given of the different theories of signal suppression. At high concentrations ($>10^{-5}$ M), the linearity of the ESI response is often lost. This might be due to a limited amount of excess charge available on ESI droplets or saturation of the ESI droplets with analyte at their surfaces, inhibiting ejection of ions trapped inside the droplets. Endogenous compounds can outcompete analytes for the limited charge on the droplet surfaces [11–13]. Another theory considers the effects of an increase in viscosity and surface tension of the droplets caused by interfering compounds, reducing solvent evaporation and the ability of the analyte to reach the gas phase [11,13]. Finally, nonvolatile materials can decrease the efficiency of droplet formation through coprecipitation of the analyte or by preventing droplets from reaching their critical radius required for gas phase ions to be emitted [7,11,13]. In addition to the described condensed phase processes, analyte ions can also be neutralized in the gas phase via deprotonation reactions with high gas phase alkaline substances, leading to suppression of their response signal [11,13].

In some cases, less ion suppression is observed with APCI, because the ionization occurs in a different manner. Unlike ESI, there is no competition between analytes to enter the gas phase, because neutral analytes are transferred into the gas phase by vaporizing the liquid in a heated gas stream [7,11]. Nonetheless, ion suppression can also occur with APCI, which has been explained by considering the effect of sample composition on the efficiency of charge transfer from the corona discharge needle through to the analyte [11,14]. Another proposed mechanism of ion suppression in APCI is solid formation, either as pure analyte or as a solid coprecipitate with other nonvolatile sample components [7,11].

Response suppression or enhancement effects may be exerted by any co-eluting components entering the API source via the liquid stream [15]. Some mobile phase additives, like trifluoroacetic acid, are also known to affect the response [16]. Several ways to minimize or correct for ion suppression with trifluoroacetic acid have already been described, including the use of weaker acids as ion-pairing agent [10], the post-column addition of a mixture of propionic acid and isopropanol (so-called TFA-Fix) [17] or the addition of acetic acid and propionic acid to the mobile phase containing trifluoroacetic acid [18]. In addition, matrix effects can also be caused by exogenous materials, such as polymers contained in different brands of plastic tubes or Li-heparin, a commonly used anticoagulant [19]. In this paper we will focus on matrix effects due to the actual analyte matrix.

Careful consideration must be given to evaluating and eliminating matrix effects when developing an assay [11]. Taylor [2] even designated matrix effects as the Achilles' heel of quantitative LC–ESI-MS/MS methods. Disregarding sample clean-up will lead to poor performance, especially when complex matrices are involved and sensitive methods are needed. This review describes how matrix effects can be assessed and reduced. The evaluation of matrix effects during validation is also discussed. For this purpose, an overview is given of validated LC–MS/MS methods for the analysis of drugs in biofluids, published between January and June 2008. For earlier published methods, the reader is referred to some excellent reviews [3,4,16,20,21].

2. Assessment of matrix effects

There are two common methods to assess matrix effects: the post-column infusion method, defined by Bonfiglio et al. [22], and the post-extraction spike method, proposed by Matuszewski et al. [1,23]. The post-column infusion method provides a qualitative assessment of matrix effects, identifying chromatographic regions most likely to experience matrix effects (Fig. 1a). Briefly, an infusion pump delivers a constant amount of analyte into the LC stream entering the ion source of the mass spectrometer. The mass spectrometer is run in SRM mode to follow the infused analyte. Blank sample extract is injected on the LC column under conditions chosen for the assay. Since the analyte is infused into the MS at a constant flow, a steady ion response is obtained as a function of time. Any endogenous compound that elutes from the column and causes a variation in ESI response of the infused analyte is seen as a suppression or enhancement in the response of the infused analyte [16,22]. This approach, however, does not provide a quantitative understanding of the level of matrix effect observed for specific analytes. In addition, if several compounds are determined in one method, all compounds should be infused separately to investigate possible matrix effects for every analyte. Moreover, analytes are infused at concentrations higher than LLOQ. Therefore, matrix effects are not investigated for low concentrated samples. In contrast, the post-extraction spike method quantitatively assesses matrix effects by comparing the response of an analyte in neat solution to the response of the analyte spiked into a blank matrix sample that has been carried through the sample preparation process (Fig. 1b) [1,24]. Matuszewski et al. [1] defined the absolute matrix effect as the comparison of the signal response of a standard present in a sample extract from one single lot to the response of a standard in neat solution. However, even more important is the evaluation of the relative matrix effect, which is the comparison of matrix effect values between different lots of biofluids [1]. Matuszewski et al. [1] have therefore proposed that matrix effects should be investigated in biofluid samples from at least five different sources. In a more recent paper, they have suggested to use the precision of the calibration line slopes in five different lots of a biofluid as an indicator of relative matrix effects. The relative standard deviation should not exceed 3–4% for the method to be considered practically free from relative matrix effects [23].

Recently, Heller [9] presented a new concept, namely matrix effect maps, for visualizing the impact of various parameters on matrix effects associated with a given method. In this approach matrix effects are studied as a function of the amount of co-injected matrix extract. This is in contrast to the assessment methods described above, which evaluate the behavior of a given method with a fixed amount of co-injected matrix. In Heller's approach, two sets of mixtures were prepared in different formats and, to provide different matrix effect conditions for testing these mixtures,

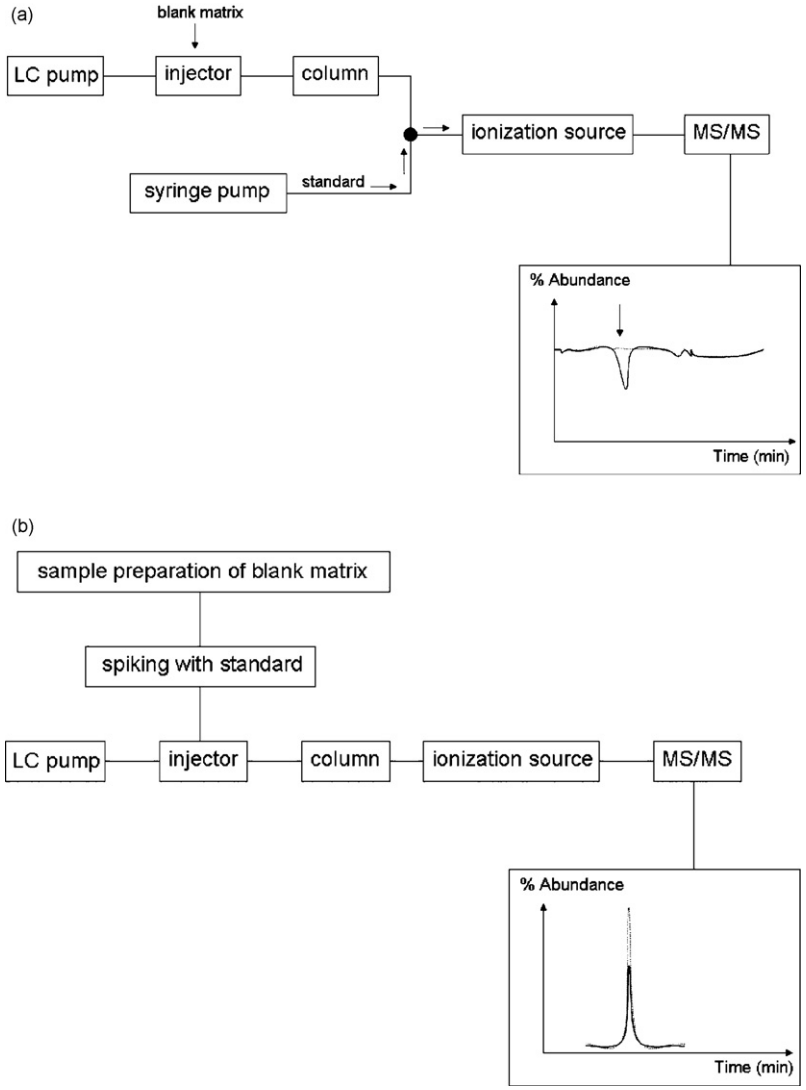

Fig. 1. Schematic overview of the two commonly used methods to assess matrix effects in LC–MS/MS. (a) The post-column infusion method. The dashed line represents the signal of the analyte. The full line is obtained when injecting blank matrix. The arrow indicates the region of ion suppression; (b) the post-extraction spike method. The dashed peak represents the standard in neat solution. The full peak is obtained with standard spiked in matrix post-extraction. A clear reduction of the peak area is observed, which indicates ion suppression.

chromatographic conditions were altered as well. Furthermore, operational variables like desolvation gas flow and temperature could also be included in these matrix effect maps. However, these issues remain to be fully tested. In the future, this approach could help to determine the ruggedness of a developed method.

3. Elimination or reduction of matrix effects

Different actions can be taken to overcome matrix effects. Matrix effects may be reduced by simply injecting smaller volumes or diluting the samples, which is useful as long as instrumental sensitivity remains adequate [9]. Other possibilities to reduce or eliminate matrix effects are the optimization of sample preparation and/or chromatographic parameters [3,4,15]. Another approach is the use of an internal standard (IS) to compensate for the alteration in signal. The use of lower flow-rates, flow splitting or the need to resort to standard addition is also described. If sensitivity is not an issue, an alternative ionization source, less sensitive to matrix effects, can be used, e.g. APCI [24] or electron ionization [25]. In many cases, several approaches are combined to achieve adequate quantitative results [15].

3.1. Sample preparation techniques

In general, matrix effects are directly related to an insufficient sample clean-up of the biofluid under investigation. Matrix effects may be reduced by simply injecting smaller volumes or diluting the sample. However, these solutions will clearly influence the sensitivity of the method and are therefore in many cases not appropriate [13]. Proper sample clean-up is therefore of primordial importance. The simplest and fastest method for preparing samples is protein precipitation (PPT). However, it does not result in a very clean extract. PPT is most likely to cause ion suppression in ESI, since this method fails to sufficiently remove endogenous compounds such as lipids, phospholipids, fatty acids, etc. Co-elution of these compounds with the compound of interest affects the ESI droplet desolvation process [7,8,16,24]. In comparison with PPT, the extracts obtained from solid phase extraction (SPE) are relatively cleaner [16,24]. Liquid–liquid extraction (LLE) often yields rather clean extracts, but the procedures are usually cumbersome and have many pitfalls. Multiple extraction steps are commonly needed to increase analyte recovery and to obtain cleaner extracts [11]. Supported LLE can be used to decrease sample preparation

time and improve analyte recovery. In this technique one liquid is immobilized in an inert medium packed into a polypropylene tube and the other immiscible liquid phase is percolated through the immobilized liquid in a manner similar to chromatography. Rapid extraction of analyte occurs during this intimate contact between the two immiscible phases. The solvent moves through the packing by gravity flow or by use of a gentle vacuum [26].

Dams et al. [8] have investigated the influence of the sample preparation technique and the biofluid on the presence of matrix effects in quantitative LC–MS/MS analysis of illicit drugs by post-column infusion experiments with morphine. Three biofluids (urine, oral fluid and plasma) were pretreated with PPT or SPE. Large differences in matrix effects were observed between both sample preparation techniques. They observed that sample preparation could reduce or even magnify matrix effects. Indeed, although matrix clean-up is more extensive with SPE, the preconcentration step increases the concentration of the target analyte together with the concentration of non-removed interfering substances from the biofluid and/or the sample preparation [8]. The presence of matrix effects also proved to be dependent on the biofluid analyzed. Matrix components, characteristic to each biofluid, interfered at different times and to a varying extent throughout the analysis [8]. Lindegardh et al. [27] have shown that matrix effects originating from their SPE extraction procedure rather than the plasma background were responsible for the ion suppression, observed in their study. Salts remaining from the buffers used in the SPE suppressed the signals for both piperaquine and its deuterated IS. This had however no effect on the quantification of piperaquine. On the other hand, triethylamine residues that remained after evaporation of the SPE eluate were found to suppress the signals for both analytes differently. Indeed, the deuterated IS was found to be less lipophilic than the parent compound and therefore it eluted slightly earlier in the chromatogram. In conclusion, the stable isotope labeled-internal standard (SIL-IS) failed to compensate for matrix effects if triethylamine was present in the sample which could lead to an underestimation of the true concentration by 50%. It is therefore important to carefully eliminate this compound by making sure the eluate is completely dry before reconstitution [27]. Chambers et al. [24] have compared various sample preparation techniques for plasma samples with respect to extract cleanliness, matrix effects and analyte recovery. Their results indicate that acetonitrile is a better choice of organic solvent than methanol for PPT. However, PPT caused significant ion suppression for many compounds and it was shown to be the least effective sample preparation technique. Both reversed-phase and cation exchange SPE resulted in significant lower phospholipid levels, a significant source of matrix effects in plasma samples, compared to PPT [24]. The most effective sample preparation technique was mixed-mode strong cation exchange SPE, which combines the retention mechanisms of reversed-phase and ion exchange. Excellent recoveries for several polar and non-polar analytes were obtained on first pass together with minimal matrix effects. LLE also provided clean final extracts. However, analyte recovery, particularly for polar analytes, was low.

Next to PPT, SPE and LLE, several other sample preparation techniques exist, such as microdialysis. Microdialysis is an *in vivo* sampling technique used in our laboratory to monitor changes in the composition of the extracellular fluid of different tissues and is based on the principle of dialysis through a semi-permeable membrane. It enables the collection of small, hydrophilic endogenous compounds, such as neurotransmitters and peptides, and exogenous compounds, such as drugs. Although microdialysates are protein-free aqueous solutions, they contain a large amount of salts (>150 mM) and other small molecules. These non-volatile compounds can cause ion suppression of the analytes of interest [20]. Different strategies can be applied to prevent salts from entering the ion source. An overview can be found in [20]. In

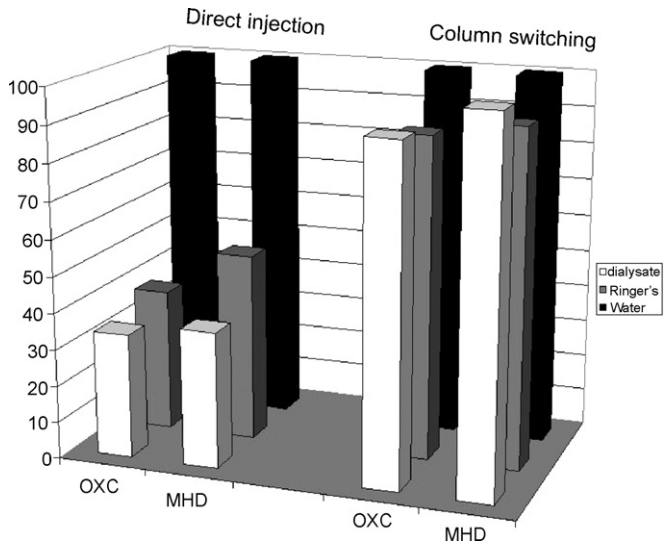

Fig. 2. Matrix effects after direct injection on the LC column or using the column switching technique of standards containing oxcarbazepine (OXC) and its active metabolite, 10,11-dihydro-10-hydroxycarbamazepine (MHD) in water, Ringer's solution or dialysate matrix. The responses of OXC and MHD are expressed as percentages, relative to the response of the compounds in water (reproduced with permission from [81]).

our laboratory it was shown that column switching is a prerequisite for the analysis of oxcarbazepine and its active metabolite in rat brain dialysates to minimize salt effects or matrix effects from other dialysate compounds [28]. Indeed, after direct injection, lower peak areas were observed for standards made in Ringer's solution compared to standards in water (Fig. 2). When spiking the analytes in blank dialysate matrix, this ion suppression was even more pronounced. These results suggest that, next to the salt effect, an additional matrix effect occurs due to endogenous compounds in the dialysate. Using the column switching technique, no differences were observed between standards in Ringer's solution or in dialysate matrix (Fig. 2). However, to obtain accurate results, Ringer's solution and not water needed to be used as solvent for the calibration standards since not all salts could be washed away using column switching.

3.2. Chromatograhic conditions

Improved chromatography is a straightforward way to separate interfering compounds from analytes. Gradient elution can help to wash the column after injection and prevent late-eluting compounds from the previous injection to interfere. If the analytes are ionizable, the pH of the mobile phase can have a significant impact on the retention, selectivity and sensitivity of the separation [24]. For example, Chambers et al. [24] have investigated the influence of mobile phase pH on matrix effects. Phosphatidylcholine containing phospholipids were monitored under both low and high pH conditions. Their results show that the least hydrophobic phospholipids, which elute early in the chromatogram, are independent of pH, while the more hydrophobic phospholipids require a longer organic hold at high pH to fully elute them [24]. In the same study, it was also shown that fast gradient LC promotes matrix effects by reducing chromatographic separation between analytes and endogenous compounds. If high throughput is required, effective sample pretreatment becomes critical, since the chromatographic conditions are not able to reduce matrix effects.

Another possibility is the use of a stationary phase with a different selectivity, for example hydrophilic-interaction liquid chromatography (HILIC). HILIC provides a complementary separa-

tion and thus greater freedom from matrix effects, compared to reversed-phase LC [9]. HILIC which combines the use of bare silica or polar bonded stationary phases and mobile phases with a high content of organic solvents, has been proven to be a valuable tool for the analysis of polar compounds in biological samples [29]. MS sensitivity can also be improved due to the higher content of organic solvent in the mobile phase.

Ultra performance liquid chromatography (UPLC) retains the practicality and principles of classical LC, but increases the speed, resolution and sensitivity of the method by using columns with small diameter (1.7 μm) particles and high pressures. The improved resolution might provide a benefit with respect to matrix effects, through improved separation from endogenous components [24]. Chambers et al. [24] obtained a statistically significant reduction in matrix effects under a variety of chromatographic conditions and with multiple basic analytes, using the UPLC technology.

3.3. Mass spectrometric conditions

Some studies have shown that APCI-MS is less susceptible to matrix effects than ESI-MS [7,8,15,23,30]. However, the occurrence of matrix effects has also been shown with APCI [8,14,15]. In addition, Mei et al. [19] have shown that for at least one vendor's design, the APCI interface was more susceptible to matrix effects than the ESI interface for the same instrument. Kelly et al. [31], for example, have evaluated both ESI and APCI ionization for the simultaneous analysis of 10 amphetamine-related analytes in meconium. APCI was selected on the basis of less matrix effects. Even with this source, matrix effects were observed for most analytes and internal standards at all concentrations tested, with values ranging from 85.2 to 149.4%.

Miniaturized ESI methods are proven to be more tolerant towards contaminations in the analyte solution. This was also shown in our laboratory. Dialysate matrix effects were estimated at different concentration levels of oxcarbazepine and its major metabolite, using a column switching microbore, capillary and nano-LC–MS/MS system [28]. No clear differences between Ringer's solution and dialysate matrix were observed at medium and high concentration levels of the different methods. Only at the lowest level of the microbore system, a significant matrix effect was observed. We have related this to the relatively high flow rate used at the microbore level. Since a lower flow rate reduces the size of the charged droplets, fewer droplet fission events and less solvent evaporation are required for ion release in the gas phase. This leads to a reduction in contaminant concentration [28,32,33]. Georgi and Boos [34] have observed ionization suppression caused by matrix effects after plasma or urine injection in a conventional restricted-access media-LC system. In contrast, assaying similar compounds with a capillary chromatographic setup, Santos-Neto et al. [35] showed that such matrix effects were not present. The authors attribute this to the better characteristics of ESI-MS/MS under low flow rate.

The extent of matrix effects also depends on the source design of the LC–MS system used. In some cases, problems observed with matrix effects can be solved by using a MS instrument from another manufacturer [9,15,19].

Regarding the ionization polarity, the negative mode is usually considered as more specific and consequently less subjected to ion suppression [13,15]. In practice, this is of course not possible for all analytes.

3.4. The use of an appropriate internal standard

As IS either a structural analogue or a SIL-IS can be applied. However, the ionization of the analogue IS and the analyte may be differently affected by the matrix. This can be solved by using a SIL-IS which co-elutes with the drug, since matrix effects should not affect the relative efficiency of ionization of the drug and its SIL-IS. SIL-IS are compounds in which several atoms in the analyte are replaced by their stable isotopes, such as ^{2}H (D), ^{13}C, ^{15}N or ^{17}O with ^{2}H being the most frequently used isotope. It is important that the mass difference between the analyte and the SIL-IS is at least 3 mass units [36,37], in order to avoid signal contribution of the abundance of the natural isotopes to the signal of the internal standard. If the compound and the standard are not separated adequately by mass, this will result in quadratic standard curves [38]. In general, a SIL-IS is considered to be ideal, since it shows almost identical behavior to the analyte of interest in sample pretreatment, chromatography, as well as in ionization [15]. However, issues like isotopic purity of compounds, cross-contamination and cross-talk between MS/MS channels, isotopic integrity of the label in biological fluid and during sample processing, etc. should be carefully addressed [1].

Both clomipramine and $^{2}H_5$-fluoxetine were evaluated as IS for the simultaneous analysis of five antidepressant drugs in human plasma [35]. Although acceptable results were obtained with clomipramine, the deuterated analogue allowed better calibrations for all compounds. Lanckmans et al. [39] showed that the use of a stable ($^{13}C_6$, ^{15}N) isotope labeled IS was necessary for the quantification of angiotensin IV in rat brain microdialysis samples. Indeed, the structural analogue norleucine[1]–angiotensin IV was not able to improve the accuracy and precision of the method. On the other hand, Jemal et al. [40] have demonstrated that under certain conditions the use of SIL-IS does not guarantee the constancy of the analyte/IS ratio. During method development for the analysis of mevalonic acid in urine, they observed an extensive matrix effect depending on the source of urine and the sample volume used for extraction. In addition, the analyte/IS response ratio changed from the expected value indicating that the response of the analyte and the IS were not affected to the same extent. Wang et al. [41] have recently shown that a deuterium labeled IS was not able to entirely compensate for the observed matrix effects. The replacement of the carbon bound hydrogen with deuterium slightly alters the lipophilicity of the molecule and hence a partial resolution of the analyte and its deuterated IS may occur in reversed-phase LC. If a large and sharp matrix suppression peak elutes at around the retention time of the analyte and IS, slight differences in retention may cause differential matrix effects [36,37]. The ^{13}C, ^{15}N or ^{17}O-labeled IS may be more ideal than the ^{2}H-labeled ones, since deuterium and hydrogen have greater differences in their physical properties than for example ^{12}C and ^{13}C [41,42].

Considering all the above described issues concerning the IS, it is of primordial importance to choose an appropriate IS during method development and to closely monitor the method performance in routine use, since only limited lots of biological matrix are tested during method validation [41].

Even if a SIL-IS is used, matrix effects should still be investigated. If ion suppression significantly reduces the signal of the analyte and/or of the IS, the signal to noise ratio may decrease to a point where accuracy and precision may be negatively affected [10].

Problems arise when more than one compound is determined in the same analytical method. A number of labeled IS identical to the number of compounds to be analyzed would in this case be required [4]. However, this is not always practically feasible.

4. Evaluation of matrix effects during validation of bioanalytical methods

Studies on matrix effects in quantitative bioanalysis revealed that the ion suppression or enhancement is frequently accompa-

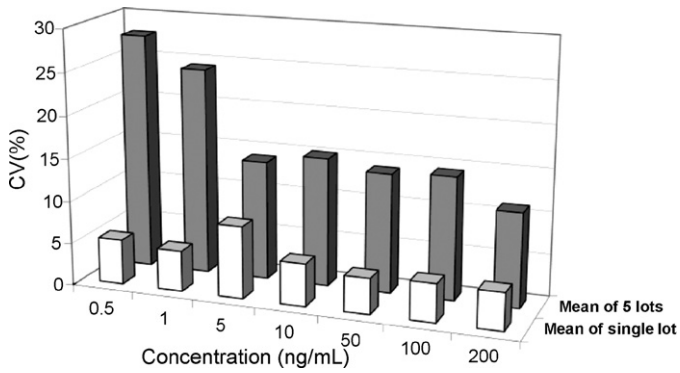

Fig. 3. Precision of a bioanalytical method at various analyte concentrations, determined in either a single plasma lot or five different plasma lots (reprinted with permission from Ref. [1]. Copyright (2003) American Chemical Society).

nied by significant deterioration of the precision and accuracy of the method. An example is shown in Fig. 3. The precision, expressed as relative standard deviation, is plotted as a function of the analyte concentration. While for a single plasma lot the precision is acceptable, this is not the case when five different lots of plasma are taken into account [1,15]. An absolute matrix effect on the other hand will primarily affect the accuracy of the method [1]. Even if matrix effects can be compensated by the use of an appropriate IS, efforts should be made to eliminate these co-eluting compounds, since their presence will reduce method sensitivity. When analyzing low concentrated samples, this can lead to false negative results.

Biofluids such as plasma and especially urine represent highly complex matrices. The composition of these biofluids can vary significantly between individuals and species, but also within an individual [34]. Most method validations are performed using calibration standards and quality control samples prepared from the same pool of blank matrix. Using these homogenous samples for validation does not take into account the inter- and intrapatient matrix variability [2]. Checking the quality of an assay using quality control samples, which are prepared in the same matrix as the calibration standards, will not reveal matrix effects observed in the incurred samples [43]. Repetitive analysis of incurred samples is one of the best strategies to evaluate any hidden analytical effect in the method. While it is not practical to prepare calibration standards and quality control standards for each individual matrix source, some assessment of patient variability must be undertaken [2]. For example, the strategy as proposed by Matuszewski et al. [23] could be followed (see II. Assessment of matrix effects). Similar strategies have been proposed by others [2,5]. Dewé et al. [43] proposed a partial within-animal calibration to almost eliminate the bias and to improve significantly the precision of the method.

The evaluation of matrix effects on the quantitative analysis of drugs in biological fluids is an important and sometimes overlooked aspect of assay validation [1]. The FDA guidelines on bioanalytical analysis explicitly require the evaluation of matrix effects [44]. As can be observed in the literature overview, most recent papers concerning quantification of drugs in biofluids with LC–MS/MS include the investigation of matrix effects. However, there is no consensus on how this should be performed during method validation [2]. In several cases, the addition of an appropriate IS compensates for the observed matrix effects. However when transferring a published method to their laboratory, users should bear in mind that matrix effects can occur using the same method but in a different setting or with a different matrix, or samples from a different subject. In addition, matrix effects can largely diminish the sensitivity of the method.

4.1. Literature overview

In Table 1 an overview is given of validated LC–MS/MS methods for analysis of drugs in biological fluids, published from January till June 2008 (a search was performed in PubMed and Web of Science). Our attention was especially focused on whether and how matrix effects were evaluated during the validation of these methods. If the percentage matrix effect or the region where ion suppression occurs was indicated in the paper, then this information was added to the table.

As can be seen from the table, different strategies to assess matrix effects were used. In some cases matrix effects were not investigated [45–49]. Analysts should bear in mind that endogenous compounds can induce matrix effects without being present in the chromatogram. Therefore, the definition of specificity as described in the FDA guidelines is not applicable for LC–MS/MS experiments. In the case that endogenous compounds are still present after the sample clean-up, quantification can be seriously hampered by matrix effects, especially if analytes are eluting early.

Although Kousoulos et al. [50] did study matrix effects, they did not specify the method used for this purpose.

De Meulder et al. [51] have developed an LC–MS/MS method for the simultaneous quantification of risperidone and the enantiomers of 9-hydroxyrisperidone in human plasma and urine. The selectivity of the method towards endogenous plasma and urine components was evaluated by analyzing six different sources of non-pooled, analyte-free matrix, unspiked and spiked at LLOQ level. Although the magnitude of the matrix effect was not determined, the accuracy and precision of the method at this level were found to be within the limits required by the FDA.

The standard addition method was used in two papers [52,53]. Byun et al. [52] have developed a method for the quantitative analysis of polyamines as carbamoyl derivatives in urine and serum samples. It is not clear from the paper how the calibration standards were prepared. Probably, these were not prepared in urine or serum, since these polyamines are present also in normal subjects. The matrix effects were observed to be less than 15% in both urine and serum samples. This relatively high deviation may be partly allocated to the fact that an analogue IS is used which is eluting at a different time point compared to several of the polyamines. The standard addition method was also used by Licea-Perez et al. [53], who have developed a UPLC method for the quantification of testosterone and 5α-dihydrotestosterone in human serum. Since testosterone en 5α-dihydrotestosterone are endogenous steroids, a surrogate matrix, more specifically double charcoal-stripped female human serum, was used for the preparation of calibration standards. Accuracy of the method was therefore determined using standard addition.

Several research groups have used the post-column infusion method to assess matrix effects [35,54–57]. In most cases, ion suppression was only observed in the beginning of the chromatographic run. As already mentioned above, the disadvantages of this technique is that no quantitative measure of matrix effects is obtained, all analytes need to be infused separately and matrix effects are not evaluated at LLOQ level.

Most researchers use the post-extraction spike method for the evaluation of matrix effects. However, some authors only investigated the absolute matrix effect and did not evaluate the effect of matrices from different sources [31,58–68]. Cao et al. [62] have quantified methyl protodioscin, a natural furostanol saponin with antitumor activity, in rat plasma after PPT. SRM was chosen for quantifying methyl protodioscin in ESI positive mode, while selective ion monitoring in negative ionization mode was used for the IS 17α-ethinylestradiol. Since matrix effects occur in the ionization source and not in the mass spectrome-

Table 1
Overview of validated LC–MS/MS methods for the analysis of drugs in biofluids, published between January and June 2008 (databases: PubMed and Web of Science).

Compound	Biological matrix	Sample clean-up	LC	MS	IS	Study of matrix effects	% Matrix effect or region of matrix effects	Reference
Domperidone	Human plasma	PPT	C18 150 × 2.1 mm	ESI-triple quad	Mosapride	Not studied	–	[45]
Nateglinide, cilostazol, 3,4-dihydrocilostazol	Rat plasma	LLE	C18 50 × 4.6 mm	ESI-quad-ion trap	Repaglinide	Not studied	–	[46]
Olmesartan	Human plasma	SPE	C18 50 × 4.6 mm	ESI-triple quad	Zisovudine	Not studied	–	[47]
Puerarin	Canine plasma	online SPE	C8 10 × 2.1 mm	ESI-linear ion trap	–	Not studied	–	[48]
Tamsulosin, dutasteride	Human plasma	LLE	C18 50 × 2.0 mm	ESI-triple quad	Finasteride	Not studied	–	[49]
Roxithromycin	Human plasma	LLE	C18 50 × 4.0 mm	ESI-triple quad	Clarithromycin	Not specified	5	[50]
Risperidone, 9-hydroxyrisperidone enantiomers	Human plasma and urine	mixed-mode SPE	Chiralcel OJ 50 × 4.6 mm	ESI-triple quad	$^{2}H_{2}$–$^{13}C_{2}$-risperidone racemic $^{2}H_{2}$–$^{13}C_{2}$-9-hydroxyrisperidone	Analysis of six different sources of non-pooled, analyte-free matrix, unspiked and spiked at LLOQ	–	[51]
Polyamines	Human urine, human serum	LLE PPT	C18 150 × 1.5 mm	ESI-quad-ion trap	1,6-Diaminohexane	Standard addition method	0.04–13.8, 2.4–14.7	[52]
Testosterone, 5α-dihydrotestosterone	Human serum	LLE, SPE	C18 (UPLC) 100 × 2.1 mm	ESI-triple quad	$^{2}H_{3}$-Testosterone $^{2}H_{3}$-5α-dihydrotestosterone	Standard addition method	Not specified	[53]
Amrubicin, amrubicinol	Canine plasma	methanol PPT pulse gradient	C18 (UPLC) 50 × 2.1 mm	ESI-triple quad	Doxorubicin	Post-column infusion method	<2 min	[54]
Ertapenem	Microdialysates collected from blood and muscle	microdialysis	C18 150 × 3.9 mm	ESI-triple quad	–	Post-column infusion method	1.4 min	[55]
Fluoxetine, imipramine, desipramine, amitryptiline, nortryptiline	Human plasma	RAM-ADS-C18	C18 AQ 85 × 0.25 mm	ESI-triple quad	$^{2}H_{5}$-fluoxetine	Post-column infusion method	No signal suppression	[35]
Lopinavir, ritonavir	Human plasma, seminal plasma, saliva and plasma ultrafiltrate	LLE	C18 125 × 4.0 mm	ESI-triple quad	A886093.0	Post-column infusion method	<10 min	[56]
Methadone enantiomers, EDDP, enantiomers	Human plasma	LLE	Chiral-AGP 50 × 2.0 mm	ESI-triple quad	Deuterated analogues	Post-column infusion of drug-free human plasma from 3 sources	<2 min	[57]
10 Amphetamine-related analytes	Human meconium	SPE	Ether-linked phenyl phase 150 × 2.0 mm	APCI-triple quad	Deuterated analogues if available	Comparison of analyte spiked post-extraction to analyte in solvent.	Max 49.4	[31]
EO9 (apaziquone), EO5a	Human plasma	LLE	C18 150 × 2.1 mm	ESI-triple quad	$^{2}H_{3}$-EO9, $^{2}H_{4}$-EO5a	Comparison of analyte spiked post-extraction to analyte in solvent.	19.2, 4.3	[58]
5-Fluorouracil	Human plasma	LLE	NH_2 (HILIC) 150 × 2 mm	ESI-triple quad	$^{15}N_{2}$-5-fluorouracil	Comparison of analyte spiked post-extraction to analyte in solvent.	9.8–25.7	[59]
Glimepiride	Human plasma	LLE	C18 50 × 4.6 mm	ESI-triple quad	Glibenclamide	Comparison of analyte spiked post-extraction to analyte in solvent.	8.9–11.6	[60]
Levocetirizine	Human plasma	LLE	C18 50 × 2.0 mm	ESI-triple quad	Fexofenadine	Comparison of analyte spiked post-extraction to analyte in solvent.	0	[61]
Methyl protodioscin	Rat plasma	PPT	C18 150 × 4.6 mm	ESI-ion trap	17α-Ethinylestradiol	Comparison of analyte spiked post-extraction to analyte in solvent.	Max 15	[62]
Metoprolol enantiomers	Human plasma	LLE	Chirobiotic T 250 × 4.6 mm	ESI-quad-ion trap	Racemic propranolol	Comparison of analyte spiked post-extraction to analyte in solvent.	10	[63]
Miltefosine	Human EDTA plasma	SPE	C18 150 × 2.0 mm	ESI-triple quad	–	Comparison of analyte spiked post-extraction to analyte in solvent.	39.6	[64]

Rimonabant	Human plasma	LLE	C8 100 × 4.6 mm	ESI-triple quad	Sitagliptin	Comparison of analyte spiked post-extraction to analyte in solvent.	Not specified	[65]
3 Trioxane antimalaria drugs	Rat plasma	LLE	CN 100 × 4.6 mm	ESI-triple quad	Trioxane analogue	Comparison of analyte spiked post-extraction to analyte in solvent	Not specified	[66]
Valproic acid	Human plasma	SPE	C18 non-porous silica 30 × 4.6 mm	ESI-triple quad	Betamethasone valerate	Comparison of analyte spiked post-extraction to analyte in solvent.	Not specified	[67]
7 Nucleoside/nucleotide reverse transcriptase inhibitors	Human plasma	PPT	C18 100 × 2.1 mm	ESI-triple quad	6-β-Hydroxy-theophylline	Post-column infusion method comparison of analyte spiked post-extraction to analyte in solvent.	0.7–1.4 min, <10	[68]
Buprenorphine, norbuprenorphine and their glucuronide conjugates	Meconium	SPE	ether-linked phenyl phase 150 × 2.0 mm	APCI-quad-ion trap	$^{2}H_{4}$-Buprenorphine $^{2}H_{3}$-norbuprenorphine	Comparison of analyte spiked post-extraction to analyte in solvent. Six different blank meconium samples were spiked	16.8–31.3, 14.0–44.9	[69]
Dexamethasone palmitate dexamethasone	Human plasma	PPT and LLE	C8 50 × 4.6 mm	ESI-triple quad	Tramadol hydrochloride	Comparison of analyte spiked post-extraction to analyte in solvent. Six different blank plasmas were spiked	4.9–9.6, 14.4–18.1	[70]
Doxazosin	Human plasma	LLE	Silica (HILIC) 50 × 3.0 mm	ESI-triple quad	Cisapride	Comparison of analyte spiked post-extraction to analyte in solvent. A pool of ten lots of drug-free human plasma was used	1.1	[29]
Etodolac	Human plasma	LLE	C18 50 × 2.0 mm	ESI-triple quad	Indomethacine	Comparison of analyte spiked post-extraction to analyte in solvent. Eight different blank plasmas were spiked	<10	[71]
Mirtazapine, 8-hydroxymirtazapine, demethylmirtazapine	Human plasma	liquid-phase microextraction	Chiralpak AD-RH 150 × 4.6 mm	ESI-triple quad	Haloperidol	Comparison of analyte in plasma and submitted to extraction procedure with analyte in solvent. Plasma samples from different sources were evaluated	Not specified	[72]
Molindone enantiomers	Human plasma	LLE	Chirobiotic TAG 100 × 2.1 mm	ESI-triple quad	$^{2}H_{8}$-molindone	Comparison of analyte spiked post-extraction to analyte spiked in blank water extract. Six different blank plasmas were spiked	<1	[73]
Pitavastatine, pitavastatine lactone	Human plasma, human urine	LLE	C8 50 × 2.1 mm	ESI-triple quad	i-Prolact	Comparison of analyte spiked post-extraction to analyte in solvent. Six different blank plasmas and urines were spiked	<10, <12	[74]
Pravastatine, 3-hydroxy isomeric metabolite, pravalactone	Human plasma	SPE	C12 150 × 2.0 mm	ESI-triple quad	Triamcinolone	Comparison of analyte spiked post-extraction to analyte in solvent. Six different blank plasmas were spiked	<15	[75]
Ritodrine	Human serum	Mixed-mode SPE	Silica (HILIC)	ESI-triple quad	Isoxsuprine	Comparison of analyte spiked post-extraction to analyte in solvent. Six different blank serums were spiked	<6	[76]

Table 1 (*Continued*)

Compound	Biological matrix	Sample clean-up	LC	MS	IS	Study of matrix effects	% Matrix effect or region of matrix effects	Reference
Tegaserod	Dog plasma	LLE	C18 250 × 4.6 mm	APCI-triple quad	Diphenhydramine	Comparison of analyte spiked post-extraction to analyte in solvent. Six different blank plasmas were spiked	Not specified	[77]
Triptolide	Human whole blood	LLE	C18 150 × 3.9 mm	APCI-ion trap	Prednisolone	Comparison of analyte spiked post-extraction to analyte in solvent. Six different blank plasmas were spiked	5.2	[78]
Trans, trans-muconic acid	Human urine	SPE	C18 150 × 4.6 mm	ESI-triple quad	$^{2}H_4$-trans, trans-muconic acid	Comparison of analyte spiked post-extraction to analyte in solvent. Five different blank urines were spiked	Max 40	[79]
Zoplicone, N-desmethylzoplicone, zoplicone-N-oxide	Human plasma	SPE	C8 150 × 4.6 mm	ESI-triple quad	Metaxalone	Comparison of analyte spiked post-extraction to analyte in solvent. Six different blank plasmas were spiked	6–7	[80]
Piperaquine	Human plasma	SPE	C18 50 × 2.0 mm	ESI-triple quad	$^{2}H_6$-piperaquine	Post-column infusion method Comparison of analyte spiked post-extraction to analyte in solvent. Six different blank plasmas were spiked	24	[27]

EDDP: 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; AGP: α-glycoprotein; RAM-ADS: restricted-access media–alkyl-diol sil.

ter, it is highly probable that the IS will not correct for matrix effects.

Le Saux et al. [68] have assessed matrix effects by both the post-column infusion method and the post-extraction spike method. Blank plasma from thirty healthy volunteers was evaluated. Ion suppression was observed in the 0.7–1.4 min interval. This observed ion suppression did not influence the quantification of the analytes, since they all eluted after this interval. This was confirmed by comparing the peak height of standards spiked to the thirty blank extracts with the peak height of standards in aqueous solution.

Proper investigation of matrix effects includes, in addition to the absolute matrix effect, the study of matrices from different sources [27,29,69–80].

For all studies using the post-extraction spike method, ion suppression or enhancement effects were observed within the range from 0 to 50%. In most cases, the use of an IS could correct for the observed matrix effects. If a SIL-IS is used, a deuterated analogue is mostly chosen, since they are commercially available [69]. Only in one paper a ^{15}N SIL-IS was used [59].

Lindegardh et al. [27] thoroughly investigated matrix effects during development and validation of their method for the analysis of piperaquine in human plasma. Estimations of the matrix effects were both obtained quantitatively as by visualization through the post-column infusion method. The ion suppression observed in this study was constant with very low variation between the six different sources of blank plasma. The deuterated IS did compensate fully for the observed ion suppression.

5. Conclusion

The quantitative analysis of biological samples with atmospheric pressure ionization coupled to tandem mass spectrometry is complicated by the presence of matrix components which can interfere with the assay, resulting in ion suppression or enhancement effects. The origin and mechanism of matrix effects are still not fully understood. Research into the fundamental mechanisms involved in the atmospheric pressure ionization processes is therefore of primordial importance.

It is generally accepted that matrix effect evaluation should be a mandatory part of the validation procedure of all LC–MS based methods. Consensus on how these matrix effects should be evaluated is obviously needed. It is important to keep in mind that matrix effects can be present, even though no peaks are observed in the blank chromatogram. To include this possibility, the definition of specificity concerning LC–MS/MS based methods should be changed.

In this review an overview is given of possible strategies to reduce matrix effects. Depending on the sample and the matrix, an adequate sample preparation technique should be chosen. Moreover, the use of an (stable isotope labeled) internal standard is highly recommended. Even if matrix effects can be compensated by the use of an appropriate IS, efforts should be made to eliminate co-eluting compounds, since their presence will reduce method sensitivity. When analyzing low concentrated samples, this can lead to false negative results.

Even results obtained with validated methods should be carefully checked, since the degree of ion suppression may be different in different lots of the same biofluid, originating from different subjects and over a prolonged period of time [1]. Special attention should be paid when the matrix used to prepare the calibration curve is not the same as for the unknown samples. Checking the quality of an assay using quality control samples, which are prepared in the same matrix as the calibration standards, will not reveal matrix effects observed in the incurred samples [43].

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