Quantitative determination of endogenous compounds in biological samples using chromatographic techniques

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Chromatographic methods are increasingly being used for the quantitative determination of endogenous compounds in biological samples. This article presents an overview of the specific issues, which have to be taken into account for the development, validation and application of these methods. The usual lack of analyte-free samples of the biological matrix implies that alternative strategies for calibration have to be followed. This article compares and discusses the advantages and disadvantages of two strategies – the use of the authentic analyte in a surrogate matrix and the use of a surrogate analyte in the authentic matrix. In addition, it highlights important aspects of the validation of chromatographic methods for endogenous analytes.

Keywords: Biological sample; Biomarker; Calibration; Chromatography; Endogenous compound; Method development; Quantitative determination; Surrogate analyte; Surrogate matrix; Validation

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1. Introduction

Since the beginning of their application several decades ago, chromatographic techniques have played a predominant role in the quantitative determination of drugs and their metabolites in biological samples. Typically, these compounds are xenobiotic and, as such, do not normally occur in the biological matrix of interest. This makes development and application of analytical methods relatively straightforward, because reference samples, such as calibration and quality-control (QC) samples, can easily be prepared by spiking analyte-free aliquots of the matrix with known amounts of a reference standard. In this way, unknown analyte concentrations in samples taken from dosed subjects can be determined by reference to spiked calibration samples with accurately known concentrations.

As in many other fields of analysis, demonstrating the validity of bioanalytical methods has become increasingly important. Over the past 15 years, bioanalytical method validation has been the subject of much debate, and many papers, guidelines and commentaries on this topic have appeared (e.g., [1–11]). As a result, the validation principles for bioanalytical methods using chromatographic techniques are now reasonably well established. The general approach is that important validation parameters (e.g., selectivity, precision, accuracy and stability) are determined using spiked samples and that these are used as a model for so-called incurred samples (i.e. samples taken from human or animal subjects after administration of a particular compound). The underlying assumption is therefore that both an analyte-free matrix and a well-characterized reference standard of the analyte are available.

The quantitative determination of endogenous (i.e. naturally occurring) compounds in biological samples is more complicated, both analytically and from a validation point of view. It is often difficult, if not impossible, to obtain analyte-free samples of the authentic biological matrix or samples with accurately-known analyte concentrations, so the preparation of

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reference samples has to be addressed in a different way and, as a consequence, validation also becomes less straightforward.

Endogenous compounds have been measured in clinical laboratories for many years and with a variety of techniques, but interest in the determination of these compounds, now often referred to as biomarkers, as part of the development process of new drugs, is relatively recent. Often, critical decisions about the continuation of a drug-development program are based on concentrations of one or more well-selected biomarkers, as these give crucial information about the efficacy and the safety of a drug. To ensure adequate confidence in the results, the availability of accurate analytical methods is essential and, for low molecular-weight analytes, this has led to an increasing desire to apply chromatographic techniques rather than traditional ligand-binding assays, because of their better analytical performance.

The validation of chromatographic methods for endogenous analytes has so far been hampered by the absence of official guidelines. Many researchers would want to apply the method-validation principles for drug assays, in particular those issued by the US Food and Drug Administration [8] also to their methods for endogenous analytes, in order to ensure results with a comparable level of quality, but it has to be noted that these principles were not primarily meant for endogenous compounds and, in many cases, cannot be directly applied. It is the aim of this article to review and to discuss the specific analytical issues that play a role in the development, validation and application of chromatographic methods for endogenous analytes, by presenting selected applications from the scientific literature as well as unpublished results from the author's laboratory.

2. Quantitation using authentic matrix and authentic analyte

An essential part of method development is the selection of a proper way to prepare calibration and QC samples. Ideally, these samples are aliquots of the authentic biological matrix containing an accurately known concentration of the authentic analyte. For endogenous compounds, the authentic biological matrix typically contains an unknown concentration of the analyte, making it unsuitable for the preparation of reference samples. A well-known approach to circumvent this problem is the application of the method of standard addition. By adding increasing concentrations of the analyte to individual aliquots of the sample of interest, a calibration curve is created and the endogenous concentration in the sample is determined from the intercept of this calibration curve. Although this approach has been applied widely and for many years and certainly has attractive aspects (e.g., it rules out matrix effects), it generally requires more sample volume and analysis time than can be afforded in contemporary bioanalysis. There is therefore an increasing need to apply other methodologies.

Occasionally, samples of the authentic matrix can be found with negligible levels of the analyte. Compounds, which are readily oxidized, for example, quickly disappear from the biological matrix, if no stabilizing precautions are taken. If necessary, the remaining traces of the analytes can be removed by further incubation of the samples, where the presence of oxygen and elevated temperature help speed up depletion. A notable example is ascorbic acid, for which human plasma is essentially analyte-free after storage for 96 h at ambient conditions [12]. Likewise, the catecholamines adrenaline, noradrenaline and dopamine are oxidized to the corresponding quinone forms and eliminated from untreated plasma and urine when kept at 37°C for a few days [13]. However, there is a risk associated with this approach. Next to the fact that the oxidized product might interfere with analyte detection, it might also be converted back to the analyte. Biological samples for oxidizable compounds are usually stabilized with an anti-oxidant and, under these conditions, reductive back-conversion is not unlikely, thus increasing the risk for erroneous results. In our laboratory, this was demonstrated for ascorbic acid. Analyte-depleted, stabilized plasma was spiked with ascorbic acid at its lower limit of quantitation of 1 μg/ml and when a five-fold excess of the oxidized form dehydroascorbic acid was added, an increase in analyte concentration of 35% was found. At the physiologically more relevant ratio of 15 μg/ml ascorbic acid : 1.5 μg/ml dehydroascorbic acid, this effect was negligible [14].

Endogenous analytes may also be removed from biological samples by the action of enzymes. Plasma or serum from many species shows some form of *in vitro* esterase or lactonase activity. To give an example, species such as rat, mouse and rabbit show a high level of plasma carboxylesterase activity, an enzyme that is virtually absent in human plasma [15]. Nevertheless, enzymatic hydrolysis of esters also occurs in human plasma and serum (e.g., by the action of human serum albumin [16]). The removal of endogenous esters and lactones can therefore be effected by incubation of the sample, especially when kept at 37°C. Similarly, more specific enzymes cause the conversion of some analyte groups: (cytidine deaminase, which is also present in plasma and serum, catalyzes the hydrolytic deamination of the nucleoside cytidine into uridine [17] and incubation of whole blood at 37°C for 2 hours results in the complete removal of plasma thymidine, apparently by the action of intracellular enzymes [18]). In general, after enzymatic depletion of endogenous analyte levels, the biological sample is stabilized by the addition of a specific enzyme inhibitor (e.g., sodium fluoride in the

case of esterases or tetrahydrouridine in the case of cytidine deaminase) and can be spiked with known amounts of the analyte to serve as a reference sample.

In some cases, analyte levels in an authentic matrix are sufficiently low because of species, gender, age or diurnal variations. The endogenous anti-oxidant ubiquinone, for example, mainly occurs in human plasma in the form with 10 isoprene units (Q-10), while in rat plasma this form is present at much lower concentrations and the form with nine isoprene units (Q-9) is most abundant [19]. The concentrations of male and female sex hormones can obviously vary considerably between biological fluids obtained from men or women, but they also change with age and depend on the stage of pregnancy and the menstrual cycle (e.g., concentrations of testosterone in plasma of young girls or post-menopausal women can be a factor 20–30 lower than in young adult males [20]). Large concentration differences due to diurnal effects do not occur often. Melatonin levels in plasma at night exceed those during day time typically 3–4-fold [21], but, for most other compounds, effects are less substantial.

Finally, the concentrations of some endogenous compounds decrease as a result of illness or are suppressed as the effect or side-effect of drug treatment. To mention just two examples:

plasma cortisol concentrations decrease considerably after administration of synthetic corticosteroids (e.g., by more than 70% after prolonged daily administration of prednisolone [22]); and,

up to 50% suppression of the levels of estrogen in plasma is effected by administration of drugs inhibiting aromatase, an enzyme involved in the biosynthesis of estrogens [23].

Altogether, there are many ways, both *in vivo* and *in vitro*, to reduce the concentrations of endogenous compounds in biological samples and the overview given above is by no means exhaustive. However, in practice, it is often difficult to find an appropriate sample of the authentic matrix with a sufficiently low analyte concentration to serve as a truly blank matrix and alternative ways have to be found to prepare calibration or QC samples.

3. Quantitation using surrogate matrix and authentic analyte

If no analyte-free samples of the authentic matrix are available, calibration standards can be prepared by spiking the analyte in some sort of artificial or surrogate matrix. Surrogate matrices can vary widely in complexity. In its simplest form, a surrogate matrix is pure water or a buffer, such as phosphate-buffered saline (PBS), which is frequently used for plasma and serum analyses because of its similar pH (7.4) and ionic strength (150 mM). Often, bovine (BSA) or human serum albumin (HSA) is added to PBS at a concentration of 40–60 g/l to take the protein content of the biological matrix into account and increase the solubility of hydrophobic analytes. If a closer correspondence to the actual biological matrix is desired, more complex synthetic solutions can be prepared (e.g., those presented in Table 1 for the relatively simple matrices, urine [24] and cerebrospinal fluid [25]).

Table 1. Typical composition of synthetic urine [24] and CSF [25]

Synthetic urine		Synthetic CSF	
14.1 g/l	NaCl	7.6 g/l	NaCl
2.8 g/l	KCl	0.15 g/l	KCl
17.3 g/l	urea	0.17 g/l	$CaCl_2 \cdot 2H_2O$
0.6 g/l	$CaCl_2$	0.24 g/l	$MgCl_2 \cdot 6H_2O$
0.43 g/l	$MgSO_4$	0.92 g/l	$NaHCO_3$
0.05 %	ammonia	0.15 g/l	KH_2PO_4
0.02 M	HCl	0.72 g/l	glucose
		1.7 g/l	sodium lactate

Plasma has a more complex composition – because of its variety of proteins and lipids – and is more difficult to mimic. Several commercial suppliers offer ready-to-use synthetic surrogate matrices, the exact nature of which is often kept unknown. Examples include:

SeraSub, a protein-free substitute for plasma or serum, and UriSub, a synthetic urine, both marketed by CST Technologies (USA); and,

a serum substitute containing relevant concentrations of human albumin and globulins (Irvine Scientific, USA).

Alternatively, many endogenous analytes can be removed from the authentic biological matrix – along with other small organic compounds – by stripping with activated carbon. A typical procedure for this is to add activated carbon to the matrix and mix it for a several hours to days, followed by centrifugation and subsequent filtering of the matrix through micro-porous membranes. Special care should be taken that all carbon particles are effectively removed before the stripped matrix is spiked, because added analytes will readily bind to remaining traces of carbon, and that will result in decreased concentrations of dissolved analyte. A disadvantage of the carbon-stripping method is that it is not universally applicable. Compounds, which are bound to plasma lipoproteins (e.g., sterols), are not removed [14], and also for other compounds the depletion may not be complete. This is exemplified in Fig. 1 for progesterone in stripped plasma, which was obtained from a commercial supplier and claimed to be analyte-free. In addition, batch-to-batch variations may occur, which could very well be related to the analyte concentration before stripping. Obviously, it is advisable to start with a sample having a relatively low concentration of endogenous analyte.

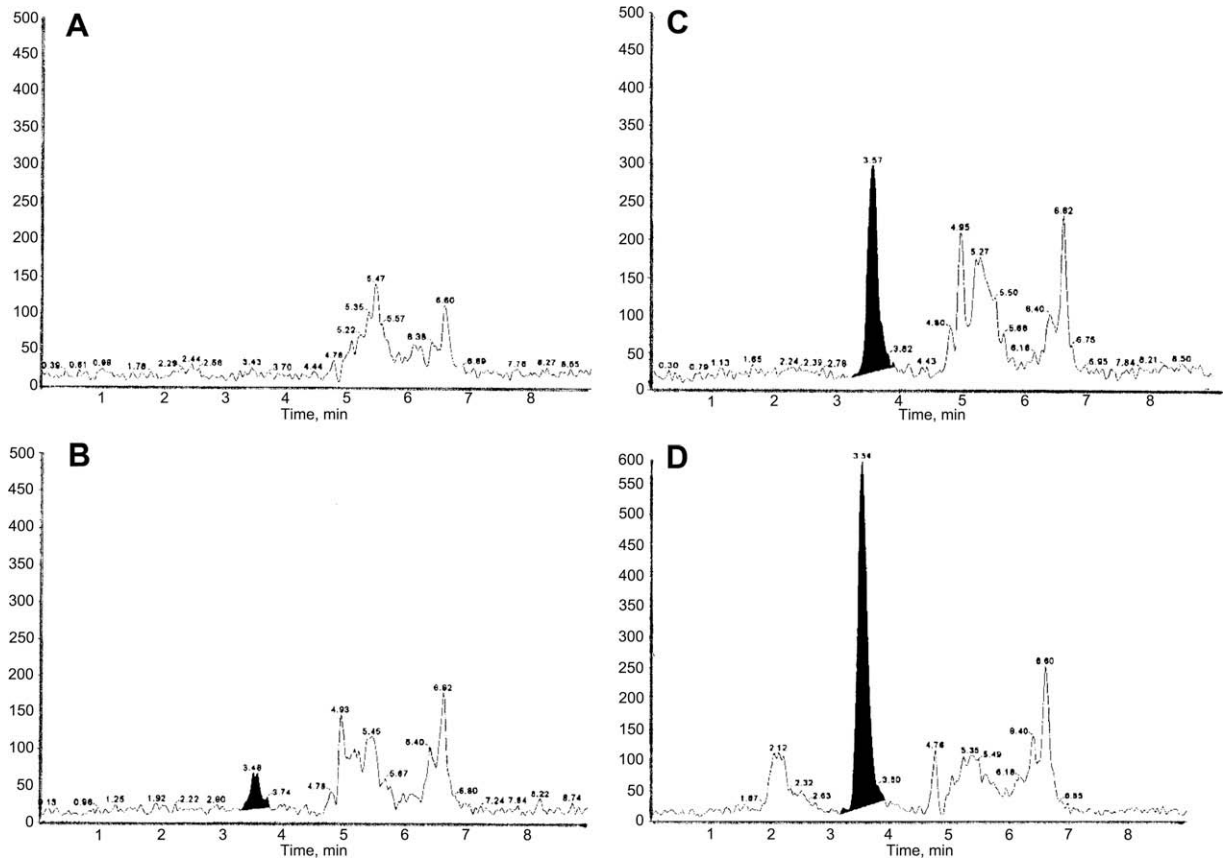

Figure 1. LC-MS/MS chromatograms showing the incomplete stripping of progesterone from plasma with activated carbon; mass transition for progesterone in: (A) buffer; (B) stripped plasma; (C) stripped plasma spiked at 20 pg/ml; and, (D) authentic plasma at a low endogenous concentration (66 pg/ml) [14].

A more selective way to remove endogenous analytes from biological matrices is the use of affinity extraction. By leading a biological fluid through a cartridge containing sorbent-bound antibodies directed towards the analyte, in principle, the only compound being removed is the analyte itself, perhaps along with a small number of structurally-related compounds. Although this approach comes close to the ideal situation, as it yields a virtually authentic biological matrix, which is free of the endogenous analyte, it is costly, and it may be time consuming and I have come across no examples of the determination of small molecules by chromatographic techniques.

Some issues related to the use of surrogate matrices have to be taken into account when developing a method. First, analyte solubility in a synthetic aqueous matrix may be limited. Non-polar analytes, in particular those normally present in the lipoprotein fraction of plasma, may be partly insoluble in a synthetic matrix, even in the presence of proteins. This could lead to partial precipitation of the analyte, adsorption to the surface of sample tubes and other unwanted effects, which will result in biased and irreproducible results. Perhaps the most extreme example is cholesterol, whose solubility in water (5 μM) is about 1000-fold lower than its typical concentration in plasma (5 mM). For analytes of this kind, the addition of lipids or micelles to a synthetic matrix may help improve solubility.

Another issue worth considering is that analyte extractability may be different in authentic and surrogate matrices. In plasma, many endogenous compounds have their own specific carrier proteins, to which they are usually more strongly bound than to a more general binding protein (e.g., albumin, which is often used in synthetic matrices). That this effect can be dramatic is nicely illustrated by testosterone. In plasma, this compound is both strongly bound to sex hormone-binding globulin (SHBG) and to a lesser extent and much more loosely to human serum albumin. By fine tuning the extraction conditions, total testosterone or only albumin-bound testosterone can be extracted from plasma [26].

For a proper assay performance, it is important that a similar extraction yield be obtained for the surrogate and the authentic matrix. A simple way to investigate this is to prepare a series of calibrators in the surrogate matrix and in the authentic matrix and determine the slopes of the calibration curves. Of course, similar slopes indicate

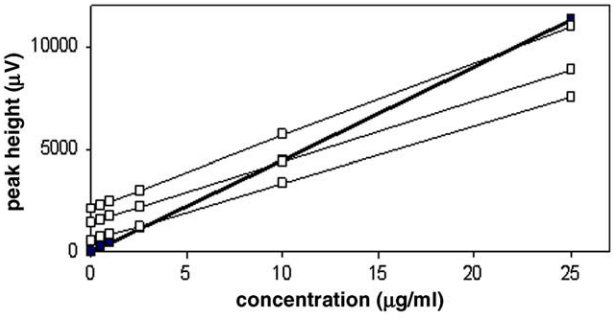

Figure 2. Calibration curves for carboxymethyllysine in protein hydrolysate of three different plasma samples (□) and in synthetic amino-acid solution (■) (Derived from [26]).

a comparable extraction yield. If the slope in the surrogate matrix clearly deviates from the one in the authentic matrix, application of the method will give incorrect results and, to prevent this, extraction conditions should be adapted or another surrogate matrix should be selected. A similar effect can arise from differences in derivatization yield, as illustrated by Fig. 2. The amino acid carboxymethyllysine, which was derivatized with fluorenylmethoxycarbonyl chloride (FMOC) prior to HPLC analysis with fluorescence detection, showed a ca 30% lower derivatization yield in different batches of the authentic matrix (plasma protein hydrolysate) than in a synthetic matrix, which was an aqueous amino-acid solution. This problem could not easily be solved and was addressed by applying the method of standard addition for quantitation [27].

Summarizing, an ideal surrogate matrix is completely analyte-free and is identical to the authentic matrix in terms of analyte solubility and extractability. For compounds that are strongly bound to specific plasma proteins, this means that a stripped matrix would probably show a better performance than a synthetic one, whereas, for analytes with a better solubility in water, a relatively simple synthetic matrix might be sufficient. If no aqueous surrogate matrix can be found to fulfill all criteria, calibration standards could be prepared in an organic solvent. In this case, there is a fair chance that calibrators and biological samples will be pre-treated differently (e.g., that the biological matrix, but not the synthetic one, will be subjected to an extraction). If so, care should be taken that the analyte-extraction yield is complete to avoid quantitative differences between both types of matrix. An example from our own laboratory is the method for plasma cholesterol, which is quantified by reference to a calibration curve prepared in isopropanol. The analyte-extraction recovery from plasma is around 100%, which guarantees proper quantitation, even though the calibrators are not extracted [14].

The surrogate-matrix approach has been widely applied for the determination of endogenous compounds in biological samples, in particular in the field of clinical chemistry. A variety of chromatographic techniques has been, and is still being, applied, but, over the years, preference has clearly shifted from gas chromatography (GC) to liquid chromatography (LC) and from conventional detection modes [e.g., ultraviolet (UV) detection] to mass spectrometry (MS) or tandem MS (MS/MS), a development that is also seen in other analytical fields. In addition, for assays with MS or MS/MS detection, there has been a strong trend towards the use of stable-isotope forms of the analytes as internal standards. Since their physico-chemical properties are supposed to be almost identical to those of the unlabelled substances, the assumption is that they provide a close to ideal correction for analytical variability. This approach is often called ''isotope dilution mass spectrometry'' (ID-MS) [28], although this term has found little use outside the field of clinical chemistry.

A variety of surrogate matrices is routinely being used and a selected number of recent applications are discussed below, a full overview being outside the scope of this article. For polar analytes, pure water can be a suitable surrogate matrix, as was shown in an LC-MS/MS method for amino-acid arginine and two methylated products in plasma, urine and cell-culture medium [29]. The suitability of the surrogate matrix was demonstrated by the identical slopes of nearly all calibration lines in water and in the biological matrices. For one analyte, a significantly higher slope was found in cell-culture medium than in water, and, since this was the only analyte for which no isotope-labeled internal standard was available, it was concluded that the use of such internal standards helps to make quantification independent of the matrix.

A simple PBS solution was used for the preparation of calibration samples in an LC-MS/MS method for urinary oxalate and no differences were found between the two types of matrix [30].

For the determination of the more hydrophobic 25-hydroxyvitamin D in serum by LC-UV, 50 g/l HSA in water was selected. The recovery from serum and from the surrogate matrix after an extraction with hexane was 85–105%, demonstrating the equivalence of both matrix types [31].

An example of the successful use of carbon-stripped serum as a surrogate matrix was given in a paper about testosterone measurement by ID-LC-MS/MS [32]. Again, extraction recoveries of the analyte from the surrogate and the authentic matrix were comparable, but an interesting difference was found between the recovery results for male (81–90%) and female (96–107%) sera.

Finally, for the quantification of a series of lipid-soluble vitamins in human plasma by LC with UV and fluorescence detection, calibration samples were prepared in ethanol, probably because of the limited analyte solubility in aqueous solutions [33]. The extraction recov-

eries were close to 100% for all analytes, which justified the use of an organic solvent as the calibration matrix.

4. Quantitation using authentic matrix and surrogate analyte

A novel and very interesting approach is the application of surrogate analytes. For methods employing MS or MS/MS detection, quantitation of an endogenous analyte can be performed by reference to a calibration curve constructed from samples of the authentic matrix spiked with a stable-isotope-labeled form of the analyte – the surrogate analyte – which does not occur naturally in the matrix. The assumption is that the physico-chemical properties of authentic and surrogate analytes are similar – that they differ in molecular mass only – and that, therefore, the extraction recovery, chromatographic retention and detection properties are identical, or at least that possible differences are small and constant. This approach should not be confused with the much more common use of isotope-labeled compounds as internal standards, as discussed in the previous section. An isotope-labeled surrogate analyte is used instead of the authentic analyte in the calibration standards and a third compound is added to both calibration and study samples to act as internal standard. Of course, this internal standard has to differ from the surrogate analyte and can be either a non-endogenous structural analogue or another stable-isotope-labeled form of the analyte.

Quantitation is performed by LC-MS or LC-MS/MS analysis of solutions of authentic and surrogate analyte at equal concentrations and determination of a response factor (RF), which is the ratio of the responses found for surrogate and authentic analyte, theoretically equal to 1. This RF is subsequently incorporated into the regression equation of the calibration curve for the surrogate analyte and authentic analyte concentrations are calculated as:

$$\text{concentration analyte} = \frac{\text{response analyte}^{*}\text{RF} - \text{b}}{\text{a}}$$

with a and b being the slope and intercept of the calibration curve, respectively, and the response typically being the peak area ratio of the analyte over an internal standard [34].

The feasibility of this approach has been shown in a number of applications. One of the first papers reporting the use of a surrogate analyte described a method for leucine-analogue α-keto isocaproic acid (KIC) in rat plasma and brain homogenate [34]. Calibration curves were prepared using a triply deuterated form of the analyte (KIC-d_3) and a structural analogue, ketocaproic acid (KC), was used as the internal standard, which was added to calibration standards as well as study samples. RF values of 0.95–1.06 (average 1.02) were found and

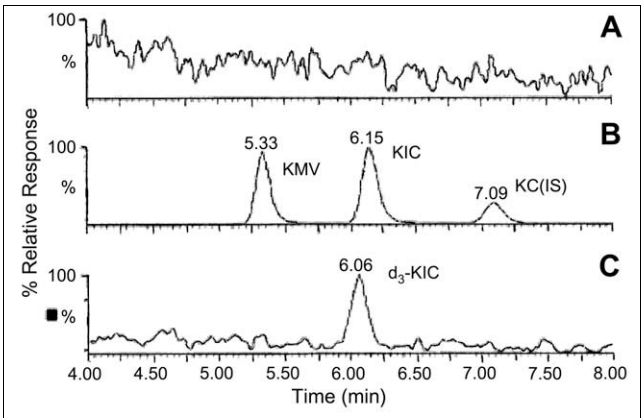

Figure 3. LC-MS chromatograms: (A) blank rat plasma; (B) rat plasma spiked with internal standard, KC; and, (C) rat plasma spiked with surrogate analyte, KIC-d_3. Masses monitored for KIC-d_3 (A) and (C), and for KIC (B) [27].

used to calculate analyte concentrations. Fig. 3 shows typical chromatograms for the surrogate-analyte approach; in an unspiked sample, no response was observed for KIC-d_3, while, in the same sample, a large peak was found for endogenous KIC, along with peaks for the isobaric internal standard and another endogenous compound.

In a method for mevalonic acid (MVA) in plasma, two surrogate analytes, MVA-d_3 and MVA-d_4, were tested and endogenous MVA was determined by calculation against calibration standards containing one of the surrogate analytes in authentic plasma. The results were compared to those obtained using the surrogate-matrix approach (authentic MVA in water) and found to be equivalent [35]. In all cases, a third stable-isotope analogue, MVA-d_7, was used as the internal standard. No response factor was included in the calculation, but apparently the authentic analyte and the surrogate analyte have an identical analytical behaviour in this application.

Likewise, endogenous sorbitol and fructose were quantitatively determined in human erythrocytes [36] and nerve tissue [37] by reference to a calibration curve of the $^{13}C_6$-forms of the analytes, using sorbitol-d_2 and fructose-d_3 as internal standards, respectively. For both sorbitol and fructose, peak area ratios of authentic and surrogate analyte over internal standard were found to be equal and thus it was concluded that the use of a surrogate analyte results in a reliable quantitation of the authentic analytes.

For a successful application of the surrogate-analyte approach, a number of requirements should be fulfilled.

First, the mass difference between surrogate and authentic analyte should be sufficient, in order not to cause responses from the naturally-occurring ^{13}C isotopes of the authentic analyte to appear in the

chromatograms of the surrogate analyte. Typically, a mass difference of at least 3 amu will suffice, but this of course depends on the structure and the mass of the analyte.

Second, the analytical equivalence of surrogate analyte and authentic analyte should be actually investigated and not be based on a theoretical assumption. In particular, the value of the response factor ought to be given appropriate attention: it should be constant over the entire calibration range [34] and not depend on the nature of the sample or change over time. This cannot be taken for granted, as there are cases known in which the relative response for unlabelled and deuterated forms of analyte were found to vary significantly from one batch of matrix to the other [35]. The exact cause of this deviation is unknown, but it may be related to the phenomenon that the properties of deuterated compounds somewhat differ from those of their unlabelled analogues. A slightly different chromatographic retention can be found when the number of deuterium atoms increases and this may induce differences in ionization efficiency in the mass spectrometer. Surrogate analytes containing ^{13}C-, ^{15}N- and/or ^{18}O- atoms do not seem to have this problem, at least not to the same extent, and would generally be preferred.

In any case, the influence of matrix variability on the response factor should be carefully evaluated and, if necessary, the sample clean-up or chromatographic separation should be adapted to reduce its effect. There are indications that atmospheric pressure chemical ionization (APCI) is less susceptible to matrix effects than electrospray ionization (ESI). For this reason, the use of APCI may also be helpful to minimize the differences between authentic and surrogate analytes [38]. Clearly, a single determination of the response factor at one concentration in an academic solution is an oversimplification, which could jeopardize the accuracy of the results.

Differences in response factor have been noted when changing from one MS instrument to the other, which is probably due to differences in MS calibration and other parameter settings [39]. The response factor should therefore be re-established when transferring a method to another mass spectrometer, even if it is from the same brand. Related to this, it is advisable to check the constancy of the response factor when using an instrument over a prolonged period of time, since it is likely that its value will be affected by fouling and subsequent cleaning of the ion source.

As yet, there is insufficient information to evaluate properly whether the surrogate-matrix approach or the surrogate-analyte approach gives the best results. Probably, the similarity of a surrogate analyte with the authentic one is often greater than the similarity of surrogate and authentic matrices. However, it is unclear if this means that the analytical performance associated with the surrogate-analyte approach is necessarily better.

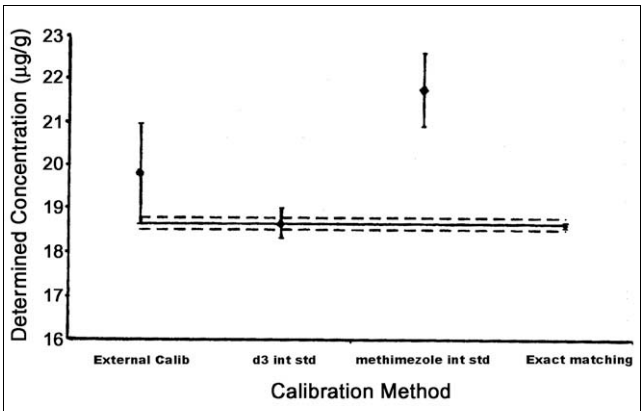

Figure 4. Comparison of different calibration methods for the quantitation of creatinine in serum. Solid horizontal line is the key comparison reference value [33].

An interesting comparison of different calibration methods was made for the determination of creatinine in human serum [40]. In the surrogate-matrix approach, a calibration curve of creatinine in water was used for quantitation, without using an internal standard (referred to as external calibration) and with either a stable isotope (creatinine-d_3) or a structural analogue (methimezole) as internal standard. Precision and accuracy of these methods were compared with those of a form of the surrogate-analyte approach, called exact matching, which used the creatinine-d_3 response to calculate endogenous creatinine concentrations. As shown in Fig. 4, the best results were obtained using the exact-matching approach, although the performance of the method using a surrogate matrix and a stable-isotope internal standard was also quite acceptable.

5. Validation

In contemporary drug development, the extent to which a biomarker assay should be validated is dictated by the intended purpose for which the results will be used. For example, this means that, for exploratory investigations, a limited validation would suffice, whereas methods for biomarkers of key importance would require a much more rigorous validation. For details of this so-called fit-for-purpose strategy, the reader is referred to a thorough paper by Lee et al. [41].

Many aspects of method validation are similar for endogenous and non-endogenous compounds. Those features that are specific for endogenous analytes are summarized in Table 2 and briefly discussed below.

Most importantly, the objective of each method validation should be to guarantee the quality of the results of the authentic analyte in the authentic matrix. Surrogate matrix or surrogate analyte should therefore, in principle, only be used for the preparation of calibration

Table 2. Typical execution of method validation for endogenous compounds

Parameter	Number of concentration levels	Matrix / Analyte	Note
Calibration	8	Surrogate / Authentic or Authentic / Surrogate	
Precision and accuracy	4: LLOQ	Surrogate / Authentic or Authentic/Surrogate	
	low	Authentic / Authentic	Unspiked
	medium/high	Authentic / Authentic	Spiked
Dilution	1	Authentic / Authentic	Diluted with surrogate matrix
Recovery	3	Authentic / Surrogate	
Stability	2	Authentic / Authentic	Unspiked

samples. Whenever possible, the actual validation samples should be aliquots of the authentic matrix, unspiked or spiked with known amounts of the authentic analyte. Validation of a method using only surrogate matrix or only surrogate analyte, as is sometimes seen, does not properly reflect the situation in actual study samples and should be avoided as much as possible.

Assessment of precision and accuracy is performed by analyzing multiple aliquots of unspiked and spiked samples of the authentic matrix against a surrogate calibration curve. Analysis of the unspiked sample will give the mean endogenous background concentration and, for obvious reasons, only precision and no accuracy can be determined for this sample. In preparing the spiked samples, care should be taken that the added amount of analyte is high enough to result in a concentration that can be statistically distinguished from the endogenous concentration. Assessment of precision and accuracy at the lower limit of quantitation (LLOQ) and the upper LOQ (ULOQ) may not be important, as frequently these concentrations are outside the physiologically-relevant range. If necessary, samples spiked in surrogate matrix or with surrogate analyte could be analyzed as an alternative. The above approach is illustrated in Table 3, which shows precision and accuracy results for plasma β-sitosterol [42]. Calibration samples were spiked in a surrogate matrix (0.9% sodium chloride and 4.5% BSA in water) over the concentration range 0.300–15.0 μM. Precision and accuracy were determined (n = 18 in three runs) at five concentration levels using unspiked plasma with the lowest endogenous level available and the same plasma sample, which was either diluted with proxy matrix or spiked to obtain relevant concentrations. The highest concentration was above the ULOQ and therefore diluted with proxy matrix prior to analysis.

Since the varying composition of the biological matrix might affect method performance, it is useful to investigate the occurrence of matrix-induced variability by performing similar experiments on a number of samples of the authentic matrix, obtained from different donors.

Since the concentration of an endogenous analyte in an unspiked sample is unknown if it is not equal to zero, in most instances it is impossible to directly establish the extraction recovery for authentic analyte in authentic matrix. As the best approximation, the increase of analyte-detector response after spiking the biological matrix can be related to the corresponding response of the same analyte concentration in a standard solution. Alternatively, the recovery can be determined for the authentic analyte in a surrogate matrix or, preferably, for a surrogate analyte in the authentic matrix.

In order to mimic the actual situation in study samples as much as possible, stability experiments should preferably be performed with unspiked, rather than spiked, samples. If a surrogate matrix is used for calibration and one wishes to avoid fresh preparation of calibrators on each day of analysis, it is important that stability also be demonstrated for the analyte in the surrogate matrix, as this could differ from stability in the authentic matrix.

It is important to be sure that a chromatographic peak represents only the analyte of interest. For endogenous analytes, the assessment of selectivity is complicated by the usual absence of analyte-free matrix. Particularly for relatively unselective detection methods, such as UV absorbance, it is desirable that peak purity be carefully

Table 3. Precision and accuracy for total β-sitosterol in plasma

Plasma sample	Nominal concentration (μM)	Mean concentration found (μM)	Bias (%)	CV (%) within-run / between-run / total
Unspiked, diluted four-fold	0.961	1.02	+5.9	2.7 / 1.0 / 2.8
Unspiked	n.a.	3.85	n.a.	1.8 / 1.7 / 2.5
Spiked with 4.00 μM	7.85	8.09	+3.1	1.3 / 1.6 / 2.1
Spiked with 8.00 μM	11.9	12.3	+3.8	1.6 / 1.8 / 2.5
Spiked with 20.0 μM, diluted two-fold	23.9	25.9	+8.7	1.7 / 1.8 / 2.5

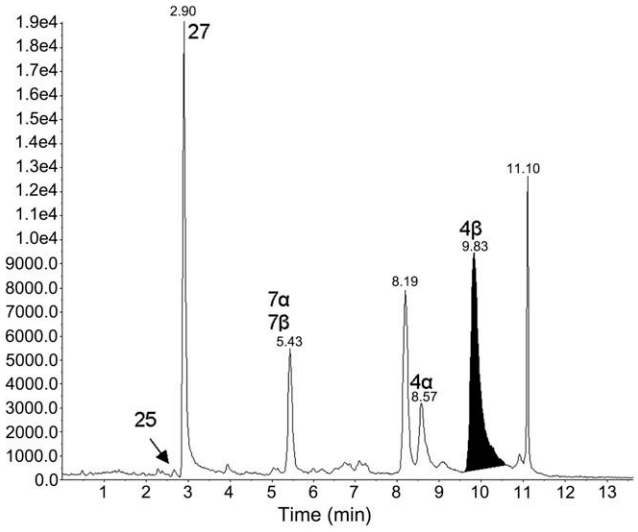

Figure 5. LC-MS/MS chromatogram of human plasma showing the mass transition for 4β-hydroxycholesterol and a number of isobaric endogenous compounds, among which are 4α-, 7α-, 7β-, 25- and 27-hydroxycholesterol [14].

investigated as part of method validation. This could be done by analyzing a number of matrix samples, obtained from multiple donors, using a more discriminative detection system, such as a diode-array detector or a mass spectrometer. The ultimate proof of selectivity is obtained by application of accurate-mass MS, although such instrumentation is probably not available in every laboratory. The interference of closely-related endogenous compounds is always a risk, even if a selective detection method such as MS/MS is used. An illustrative example is the occurrence of a variety of oxysterols in plasma, which all have an identical molecular weight and could interfere with each other's detection. In such a case, sufficient resolution of the chromatographic peaks is essential (see Fig. 5).

6. Conclusion

The quantitative determination of endogenous compounds in biological samples by chromatographic techniques presents a number of complications, because of the typical lack of analyte-free matrix. The examples described in this article show that, as a rule, these complications can be satisfactorily dealt with.

Two main approaches for a proper calibration exist, if no analyte-free samples of the authentic matrix can be found:

(1) the use of a surrogate matrix containing the authentic analyte, which is generally applicable; and,
(2) the use of a surrogate analyte spiked into the authentic matrix, which can only be used for methods with MS detection.

If, by fine-tuning experimental conditions, it can be assured that analyte solubility and extractability in calibration samples and in authentic matrix are identical, the method should produce reliable data and acceptable validation results should be found.

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