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The use of human plasma as matrix for calibration standards in pre-clinical LC–MS/MS methods—A way to reduce animal use

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ABSTRACT

The option, for practical and ethical reasons, to replace animal plasma with human plasma for calibration standards was successfully applied to 73 analytical methods developed in our laboratory during the last years. The animals used for obtaining blank plasma could then be reduced with a number corresponding to about 25% of mice or 5% of rats in ordinary one-month toxicology studies. This is of important public concern and also in accordance with the 3R-strategy. The methods were successfully validated for determination of drug concentrations in plasma from rat, dog, mouse, rabbit and cynomolgus monkey. Reproducibility of study samples from dosed animals was established, showing a mean accuracy of 100.8% with a CV of 7.2% ($n = 1339$). The purpose of this paper is to present a scientific basis for the alternative approach to adopt human plasma matrix for calibration standards, which will reduce animal use, without compromising the quality of appropriately validated assays. Additional advantages are cheaper and simplified plasma maintenance and the possibility to validate methods for several species in the same analytical batch.

1. Introduction

In order to assess the safety of a new drug under development, toxicology and safety studies may be performed in several animal species. Claiming GLP-compliance for such studies implies that a thorough validation of bioanalytical methods has to be performed in accordance with authorial guidance [1,2]. At the same time, animal welfare is of important public concern and there is a strong need to refine, reduce and replace animal use. The expression Replacement, Reduction and Refinement, more known as the 3Rs, was established by Russell and Burch [3]. They addressed that all who are responsible for animal experiments have a moral duty to try to replace animal testing, reduce numbers of animals and refine performed experiments. Many organizations and companies have focused their attention on the 3Rs, which are internationally accepted and also incorporated in various laws. A potential way to reduce the use of animals in bioanalytical work is to use human tissue (plasma, blood or urine) as matrix in the preparation of calibration standards, as well as for the dilution of samples with concentrations above the upper limit of quantification. In this respect this paper will discuss issues, which can have an impact on accuracy and precision of bioanalytical liquid chromatographic methods with tandem mass spectrometry (LC–MS/MS). Authorial guidance is not explicit regarding the definition of a matrix, leaving room for interpretations, such as replacing animal plasma with human plasma as matrix for calibration standards in analytical method validations and for drug analysis in pre-clinical studies. This paper describes the benefits with such an approach.

2. Experimental

2.1. Equipment

A triple-quadrupole mass spectrometer with turbo-ion spray interface API 3000, 4000 or 5000 with electrospray ionization in positive mode and multiple reaction monitoring (AB Sciex, Concord, ON, Canada), and a Tecan liquid handling robot Genesis RSP 150 or Freedom EVO 150, Tecan, Switzerland and a BRAVO robot 96 LT, Velocity 11, CA, USA were used. Quantification was performed with Analyst 1.4 software (AB Sciex, Concord, ON, Canada). Other apparatus, chemicals and consumables were common commercial products from well-known manufacturers.

2.2. Analytical methods

Methods, defined as the analytical procedures for determination of the concentrations of one or several drug substances in a specific animal matrix, were developed for plasma samples from rat, dog, mouse, rabbit and cynomolgus monkey. The physical–chemical properties of the different drug substances varied a great deal with

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log D of −5.6 to 5.7 between octanol and water and with pK_a-values from less than 2 to 11. Sample preparation involved isolation of the analytes and their internal standards (IS) from the plasma matrix by liquid–liquid extraction, solid-phase extraction or protein precipitation. All methods but one utilized automated work-up procedures performed in 96-well plate format. Internal standards, labeled with stable isotopes (^{2}H, ^{13}C or ^{15}N) at 3–11 positions in the molecule, were used for all analytes but two, for which analog compounds were used as IS. The methods were developed for 25–100 μl plasma volume with K_2-EDTA, or in a few cases, heparin as anticoagulant.

2.3. Calibration and quality control

For preparation of calibration standards, blank samples and blank samples spiked with IS, drug-free human plasma with the same anticoagulant as for the study samples, was used as blank matrix. Prior to use the plasma matrix was centrifuged for 5–10 min at 2100 × g. Calibration standard samples in duplicates were prepared by the Tecan robot at six concentration levels from the expected range of concentrations. The calibration range, in general three orders of magnitude, covered the lower limit of quantification (LLOQ) up to the upper limit of quantification (ULOQ). The ranges varied between 0.00020–0.100 μM and 0.50–500 μM, with injection volumes of 5–20 μl. Study samples with concentrations above the ULOQ were diluted with human blank plasma and the procedure was verified during validation. Quantification was performed on peak area using a linear regression model with a $1/x$ or $1/x^2$ weighing factor.

For preparation of quality control (QC) samples drug-free plasma from the same species as for the study samples was prepared as stated above and spiked at three concentration levels, representing the low, medium and high ranges of calibration. Duplicate calibration standard samples and QC samples were analyzed from each concentration level, one sample placed in the beginning and the other one in the end of a batch of study samples. If more than 120 study samples were to be analyzed as one batch, QC samples were also dispersed in the middle of the batch.

2.4. Validation parameters

Method validation procedures were carried out according to official guidance and recommendations [1,2,4,5]. A full method validation was performed for the pivotal toxicology species in a drug project and in general a partial validation design for additional test species.

2.4.1. Accuracy and precision

Accuracy and precision were determined by analysis of six replicates of samples spiked at four concentrations, i.e. LLOQ, the low (3× LLOQ), medium and high (0.8× ULOQ) ranges of calibration. Analysis was performed in three separate analytical batches. In a partial validation design one analytical batch was performed. The within- and between-batch accuracy, reported as bias was recognized as the difference between the mean of a set of results and the nominal value and should be within ±15% of the nominal concentration at all levels (except 20% at LLOQ). The within- and between-batch precision, reported as coefficient of variation (CV), should be 15% or lower at all levels (except 20% at LLOQ). In early methods accuracy and precision were determined by analysis of five replicates at three concentrations (LLOQ, 3× LLOQ, ULOQ). For acceptance, the within- and between-batch CV had to be 10% or less (15% at LLOQ) and the bias ±10% or lower (15% at LLOQ).

2.4.2. Selectivity

The selectivity of the assay was assessed, using blank matrix from six different drug-free plasma samples from both humans and the relevant animal species, and tested with and without addition of IS. Selectivity was confirmed when no interfering peaks larger than 20% of the mean peak area for spiked samples at the LLOQ concentration level could be observed. For early methods selectivity was determined by using individual plasma from six humans and three animals.

2.4.3. Matrix effect on ionization

The relative matrix effect on ionization was assessed by using blank plasma from six individuals from both man and from relevant animal species. The blank plasma samples were processed and the extracts spiked with analyte corresponding to 3× LLOQ in plasma sample. The normal amount of IS was added to give a concentration representing the concentration in extracts according to the method assuming 100% recovery. The analyte to IS area ratio for the respective human and animal plasma samples was used to assess relative matrix effects. In order to be accepted the CV for each group of samples should be 15% or lower. In early methods the sample matrix effect on ionization was determined by comparing the response for the analyte (corresponding to a sample concentration of medium QC) added to extracted blank plasma samples with the response for the analyte added at the same concentration to elution buffer. The effect was estimated using six different samples from man and three from the relevant animal.

2.4.4. Carry-over

The impact of carry-over between injections was assessed in each validation batch by injecting extracts from two blank human plasma samples after the injection of the highest calibration sample (ULOQ). The analyte to IS area ratio response of the blank sample was compared with the mean area ratio response of the lowest calibration standard samples representing the LLOQ of the method. Target value of the peak area ratio following the high concentration sample at ULOQ was less than 20% of the response of the LLOQ level.

2.4.5. Stability

Stability of drugs in solutions and in plasma matrices from relevant species as well as extraction recoveries and release of compound adsorption to storage containers were established before start of method validation. Assessment of drug stability was verified during validation for the actual temperature and concentration range by analyzing spiked samples immediately and on subsequent days for the anticipated storage period. Attention was taken if a change of consistency appeared due to freezing conditions. The bias of the mean concentration ($n \geq 3$) should be less than ±15% of the nominal concentration for plasma samples (initial concentration for early methods) and ±5% for solutions.

2.4.6. Incurred sample reproducibility

A reproducibility test of incurred samples (ISR) was performed in order to discover possible matrix effects due to metabolites present in study samples from dosed subjects (incurred samples). Twenty to 40 samples, typically 5–10% of the study samples, were reanalyzed, in general two from each subject. One sample originated from a high concentration level and the other from the elimination phase, where metabolites are most likely to be found. For the ISR test to be acceptable two thirds of the results should be within 20% of the original ones. In addition an overall assessment of ISR results should be made. In early methods, ISR was not tested when using a partial validation design and the accuracy of the test was recognized as the mean difference between the repeated and the original results of a set of study samples and should be between 85.0 and 115% with a CV lower than 15%.

2.4.7. Extra validation experiments with human plasma

To support the use of human plasma as matrix in the calibration standards, the following validation experiments were performed in human plasma in addition to the validation experiments for the specific species: selectivity for six individuals; matrix effect in six individuals; long-term stability covering the intended storage time of the calibration samples; short-term stability (room temperature).

3. Results and discussion

3.1. Validation

The validation process is to secure the quality of the analytical method, a prerequisite for reliable concentrations in support of the interpretation of toxicological findings. Analytical methods should be thoroughly optimized prior to validation, as subsequent changes affecting matrix effects justifies revalidation. The usage of human plasma as matrix for calibration standards in pre-clinical bioanalytical methods is based on a more than 20 years long experience in our laboratory. However, the results presented in this paper are restricted to the work performed during the last few years with LC–MS/MS based methods, all validated according to official validation requirements [1,2,4,5], to secure a scientifically sound method. Seventy-three methods were developed and all (100%) successfully validated for the actual analytes and relevant species, using human blank plasma as matrix in the calibration standards. The success rate was independent of species analyzed or the type of clean-up process used. Of the 73 methods 8 were for additional test species beyond the pivotal toxicology species and were validated using a partial validation design. The acceptance criteria for accuracy, precision, selectivity, matrix effect, ISR and stability are described in Section 2.4. The 73 methods have been documented in 40 method descriptions including validation studies, giving an average of two species per method description and validation report. Factors, which can have a significant impact on assessing quality of the data generated in routine drug analysis, are further discussed.

3.2. Sample matrix effect

Using LC/MS–MS, matrix effect is one of the major unknown variables that adversely can affect the accuracy and precision of a bioanalytical method and should be addressed in method development and validation. The effect is due to an influence on the electrospray ionization efficiency and the observed signal of analyte can be either suppressed or enhanced. The severity and nature of the effect may be a function of the concentration of co-eluting components like e.g. endogenous compounds, metabolites or mobile-phase additives and can vary from sample to sample. Ion suppression or enhancement is frequently accompanied by significant deterioration of the accuracy and precision of the assay as demonstrated by Matuszewski et al. [6]. An estimate of the quantitative measurement of absolute matrix effect can be obtained by comparing the response of an analyte, spiked to a blank plasma extract after the work-up procedure, with the response of a neat solution directly injected into the LC system, i.e. in the absence of matrix ions. A qualitative finger-print of the matrix effects can be recorded by injecting a blank plasma extract during a post-column infusion of a neat solution of the analyte as LC-eluent. This enables identification of appropriate chromatographic conditions, where co-elution of analyte and suppressing or enhancing compounds is avoided. Our results for the validated methods indicated that any matrix effect on ionization of the analytes was consistent and did not compromise the quantification of the analyte.

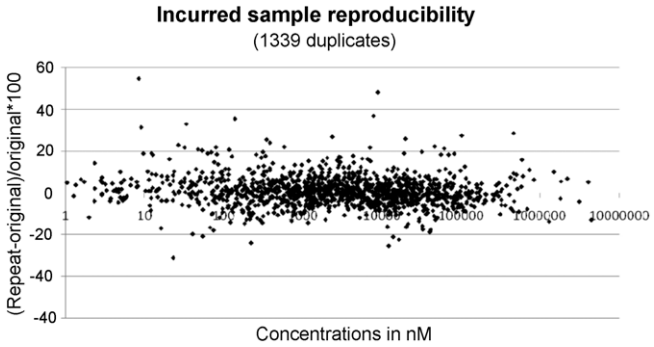

Fig. 1. A plot showing the agreement between the concentrations from duplicate incurred plasma samples ($n = 1339$), about 23 samples per analyte and method. Variability data is expressed as (repeat value − original value)/original value × 100.

3.3. Internal standard

Stable isotope-labeled analogs of the analytes are recommended as internal standards. An adequate number of labeling isotopes should be properly positioned so that no cross-contribution occurs between the ions designated for the drug and the IS. Due to nearly identical chemical and physical properties of the IS and the unlabeled analyte the IS is assumed to compensate for variability in sample extraction and LC–MS/MS analysis. An IS will generally diminish the influence of matrix effects on the accuracy and precision of the method. However, a full compensation is not always the case, especially not if the retention time differs slightly between the analyte and the deuterated IS due to its slightly different lipophilicity [7]. In the absence of significant matrix effects the analyte to IS peak area ratio should be constant. The extensive use of isotopically labeled IS in the presented methods is one likely cause of the high success rate of the performed validation experiments.

3.4. Incurred sample reproducibility

Matrix effects due to metabolites present in study samples from dosed subjects can be difficult to predict and compensate for in advance. A reproducibility test of incurred samples gives valuable information on the robustness of the method and should be performed as early as possible. This is a way to show that the matrix in the study samples does not give effects that differ from the matrices previously validated. Of the 65 methods that were fully validated 83% were tested for ISR, or for some early methods incurred sample stability (ISS) up to 8 months. The remaining 17% consisted of methods where the projects were put on hold or terminated before reanalysis of study samples. The results of the reanalysis of 1339 samples (mouse, rat, dog, or monkey plasma) analyzed with 54 methods, of which four comprised two analytes, are given in Fig. 1. The results are expressed as the variability (reassay value − original value/original value × 100) versus the original value (logarithmic scale). The variability of all the ISR tests appeared randomly over the concentration range studied and the results showed excellent reproducibility with a CV of 7.2% and a mean accuracy of 100.8%.

3.5. Benefits

There are several advantages of using human plasma as calibration standard matrix in pre-clinical methods. First, it is of ethical concern to reduce animal plasma consumption and by that minimizing the use of laboratory animals. In our laboratory an estimated blank plasma volume of 4 ml is needed for each analytical batch, comprising preparation of calibration standards and dilutions of samples with concentrations above ULOQ. An approximate plasma volume of 0.5 and 4 ml can be obtained from a mouse and

a rat, respectively, giving that each analytical batch will consume approximately 8 mice or 1 rat. This implies that the plasma consumption at analysis of concentrations in an ordinary one-month toxicity study corresponds to 25 and 5% of the mice and rats, respectively, used for the in vivo study. Second, the usage of one calibration standard matrix enables validation of several species in the same analytical batch, reducing the number of validation batches and thus the work effort. This compensates with full measure the effort to carry out some extra validation experiments with human plasma on selectivity, matrix effect and stability. Third, further efficiency is obtained by a reduced need of maintaining large volumes of different animal blank plasma matrices for use in routine analyses. Fourth, the cost for human plasma is 5–30 times lower compared with the price for animal plasma. For each analytical batch a volume of 2–3 ml of plasma was used for calibration standards, when automated sample transfer was used, which likely results in greater performance consistency over time. However, even larger plasma volumes were consumed as dilution medium for study samples with concentrations above ULOQ. Fifth, by using different matrices for calibration standards and QC samples possible individual based differences in the matrices, otherwise concealed when using the same batch of matrix, can be detected earlier during the method development process.

4. Conclusions

Considering that few candidate drugs proceed all the way to launch, measures should be taken to restrict the use of laboratory animals to necessary in vivo studies. This paper demonstrates that the use of human plasma matrix for calibration standards in pre-clinical analytical methods reduced the number of laboratory animals needed, without jeopardizing the accuracy and precision of the appropriately validated methods. Additional advantages are an efficient validation process as well as cheaper and simplified plasma maintenance. The purpose of the paper is to get a wider acceptance of this procedure and that regulatory authorities should take a proactive approach to encourage a science based approach to minimize animal use.

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