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Comparison of the quantitative performances and measurement uncertainty estimates obtained during method validation versus routine applications of a novel hydrophilic interaction chromatography method for the determination of cidofovir in human plasma

F. Lecomte[a,*], C. Hubert[a], S. Demarche[a], C. De Bleye[a], A. Dispas[a], M. Jost[c], F. Frankenne[c], A. Ceccato[b], E. Rozet[a,1], Ph. Hubert[a]

[a] Laboratory of Analytical Chemistry, CIRM, Institute of Pharmacy, University of Liège, CHU, B36, Tour 4, Avenue de l'Hôpital 1, B-4000 Liège, Belgium
[b] Odyssea Pharma, Rue du Travail 16, B-4460 Grâce-Hollogne, Belgium
[c] Mithra Pharmaceuticals, Rue Saint-Georges 5, B-4000 Liège, Belgium

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ABSTRACT

Method validation is essential to ensure that an analytical method is fit for its intended purpose. Additionally, it is advisable to estimate measurement uncertainty in order to allow a correct interpretation of the results generated by analytical methods. Measurement uncertainty can be efficiently estimated during method validation as a top–down approach. However, method validation predictions of the quantitative performances of the assay and estimations of measurement uncertainty may be far away from the real performances obtained during the routine application of this assay. In this work, the predictions of the quantitative performances and measurement uncertainty estimations obtained from a method validation are compared to those obtained during routine applications of a bioanalytical method.

For that purpose, a new hydrophilic interaction chromatography (HILIC) method was used. This method was developed for the determination of cidofovir, an antiviral drug, in human plasma. Cidofovir (CDV) is a highly polar molecule presenting three ionizable functions. Therefore, it is an interesting candidate for determination by HILIC mode. CDV is an acyclic cytidine monophosphate analog that has a broad antiviral spectrum and is currently undergoing evaluation in clinical trials as a topical agent for treatment of papillomavirus infections. The analytical conditions were optimized by means of design of experiments approach in order to obtain robust analytical conditions. These ones were absolutely necessary to enable the comparisons mentioned above. After a sample clean-up by means of solid phase extraction, the chromatographic analysis was performed on bare silica stationary phase using a mixture of acetonitrile–ammonium hydrogen carbonate (pH 7.0; 20 mM) (72:28, v/v) as mobile phase. This newly developed bioanalytical method was then fully validated according to FDA (Food and Drug Administration) requirements using a total error approach that guaranteed that each future result will fall within acceptance limits of ±30% with a probability of 95% over a concentration range of 92.7–1020 ng/mL. A routine application of the cidofovir determination in two pre-clinical trials demonstrated that the prediction made during the pre-study validation was consistent by retrospective analysis of the quality control (QC) samples. Finally, comparison of the measurement uncertainty estimations calculated from the method validation with those obtained from the routine application of the method was performed, stressing that the estimations obtained during method validation underestimated those obtained from routine applications and that the magnitude of this underestimation was function of the cidofovir concentration. Finally, this new HILIC method is reliable, easily applicable to routine analysis and transposable at low cost in other laboratories.

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* Corresponding author at: Department of Pharmacy, Laboratory of Analytical Chemistry, Institute of Pharmacy, University of Liège, CHU, B36, Tour 4, Avenue de l'Hôpital 1, B-4000 Liège, Belgium. Tel.: +32 4366 4319; fax: +32 4366 4317.
E-mail address: F.Lecomte@ulg.ac.be (F. Lecomte).
[1] F.R.S.-FNRS post-doctoral researcher (Belgium).

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1. Introduction

Bioanalytical methods validation is of crucial importance in order to ensure that results generated by the analytical methods during their routine application will be trustable. Indeed, important decisions are taken with these results, e.g. determining the toxicity of a new active ingredient or the bioavailability of a new drug formulation, or the success or failure of clinical or pre-clinical trials. For validating bioanalytical methods the main regulatory document to follow is the United States Food and Drug Administration guideline [1]. However method validation only gives a relatively small evaluation of the real quantitative performances of the method as it is realized in a relatively short period of time with analytical runs length that are most often far shorter than routine ones. Therefore, the prediction of the results accuracy obtained in method validation studies should be confirmed by the analysis of the quality control (QC) samples integrated during the in-study routine application of the method. These QC samples can provide increased knowledge of the real analytical method quantitative performances and could allow to verify that the predictions of the results accuracy obtained during the method validation were adequate.

In order to increase the knowledge of the reliability of analytical results, another parameter is essential to estimate. This parameter is the measurement uncertainty of the analytical method. Measurement uncertainty is defined as "*a parameter associated with the result of a measurement that characterises the dispersion of the values that could reasonably be attributed to the measurand*" [2]. This parameter is usually a standard deviation, a given multiple of it, or the width of a confidence interval. This uncertainty expanded by a factor, e.g. 2, is interpreted as an interval in which the true value of the result of a measurement resides with a defined probability. For instance, when the coverage factor is 2, there is about 95% probability that the true measurement result is within this interval, assuming a normal distribution of the results. More detailed explanations about measurement uncertainty can be found in various guides or articles [3–6]. In bioanalytical method applications, it is quite rare that measurement uncertainty is evaluated, although usefulness of measurement uncertainty is more and more recognised [6–9]. Measurement uncertainty can be estimated from method validation studies [6–9]. This approach to measurement uncertainty evaluation is a top–down approach and is recognised as an efficient way to estimate measurement uncertainty for complex bioanalytical procedures [3,6,9–11]. However, again, the precision of the estimation of measurement uncertainty obtained from analytical method validation depends on the design of the method validation and thus may be relatively far away from the real analytical method measurement uncertainty.

In this work, we therefore propose to first compare the prediction of the results accuracy obtained during the validation of a newly developed bioanalytical method to its routine performance monitored through the QC samples included in the routine runs. Second, measurement uncertainty of the bioanalytical method will be estimated during the method validation step and it will be compared to measurement uncertainty estimates obtained during its routine use.

To carry out this comparative study, a novel hydrophilic interaction chromatography method with UV detection coupled with solid phase extraction for the determination of cidofovir (CDV) in human plasma was selected. Cidofovir is an acyclic cytidine monophosphate analog that has a broad antiviral spectrum [12–15]. Its main therapeutic target is the treatment of cytomegalovirus (CMV) retinitis in AIDS (Acquired Immune Deficiency Syndrome) patients [16,17]. Topical treatments with CDV have sometimes resulted in acute renal insufficiency but this side effect was not clearly attributed to cidofovir. It is suggested that topical CDV should be avoided on abraded skin or mucous membrane and should be carefully monitored [18]. CDV is currently undergoing evaluation in clinical trials as a topic treatment of papillomavirus infections [12,19]. Therefore, it was certainly interesting to develop a rapid and efficient chromatographic method for the determination of CDV in human plasma.

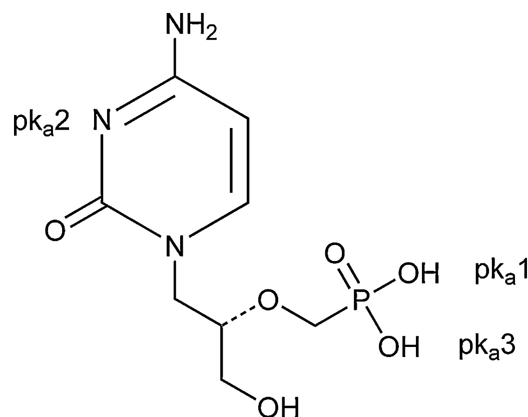

Fig. 1. Chemical structure of cidofovir.

In this context, it is important to note that cidofovir (Fig. 1) is a highly polar molecule presenting three ionisable functions whose pK_a values are 2.15 (phosphonic acid), 4.57 (cytosine) and 7.00 (phosphonic acid), determined at 25 °C [20]. Consequently CDV may be present as a zwitterion in aqueous media according to the pH value of this latter. Due to the presence of its three ionisable functions, CDV may be considered as an interesting candidate for hydrophilic interaction chromatography (HILIC). Indeed HILIC can be considered as a variation of normal-phase liquid chromatography (LC) particularly suited for compounds such as proteins, peptides, aminoacids, carbohydrates, nucleotides, nucleosides, and for separation of highly polar ionized compounds [21–26]. Some HILIC methods have been described in the literature for the determination of nucleoside analogs as antiviral or cytostatic drugs but never for the quantitative determination of CDV [27–29]. The development of a HILIC method for the determination of cidofovir in human plasma was thus an interesting challenge. Moreover the HILIC mode allowed an increase in the retention of CDV without derivatization or addition of an ion-pairing agent in mobile phase. The analysis of CDV in biological matrices has been described in the literature, mainly by reverse phase LC [13,16,17,20,30,31]. After plasma samples clean-up, these methods require either pre-column fluorescence procedure or use liquid chromatography with tandem mass spectrometry (LC–MS/MS). The derivatization process is time-consuming and the LC–MS/MS is not necessarily available in all laboratories thus making this latter one less accessible to analysts. Additionally, MS/MS detectors are generally used in order to increase the method selectivity and sensitivity. However these gains have to be compared to the quantitative performances of the method and especially to the ability of these LC–MS/MS to obtain accurate results. The quantitative requirements of the analytical method to be used in our study were to develop an analytical method able to quantify CDV in human plasma over, at least a concentration range of 100–1000 ng/mL. Results should be included in acceptance limits of ±30% around their target concentration level [32] with a probability of 95%. In order to evaluate the quality of the results generated by the two most recent LC–MS/MS methods [30,31], accuracy profiles were built based on the information available in their respective publications and following the recommendations of Hubert et al. [33–35]. These profiles were defined by requiring that each future result should fall within the ±30% acceptance limits with at least a probability of 95%. As can be seen on Fig. 2a, the LC–MS/MS method of Breddemann et al.

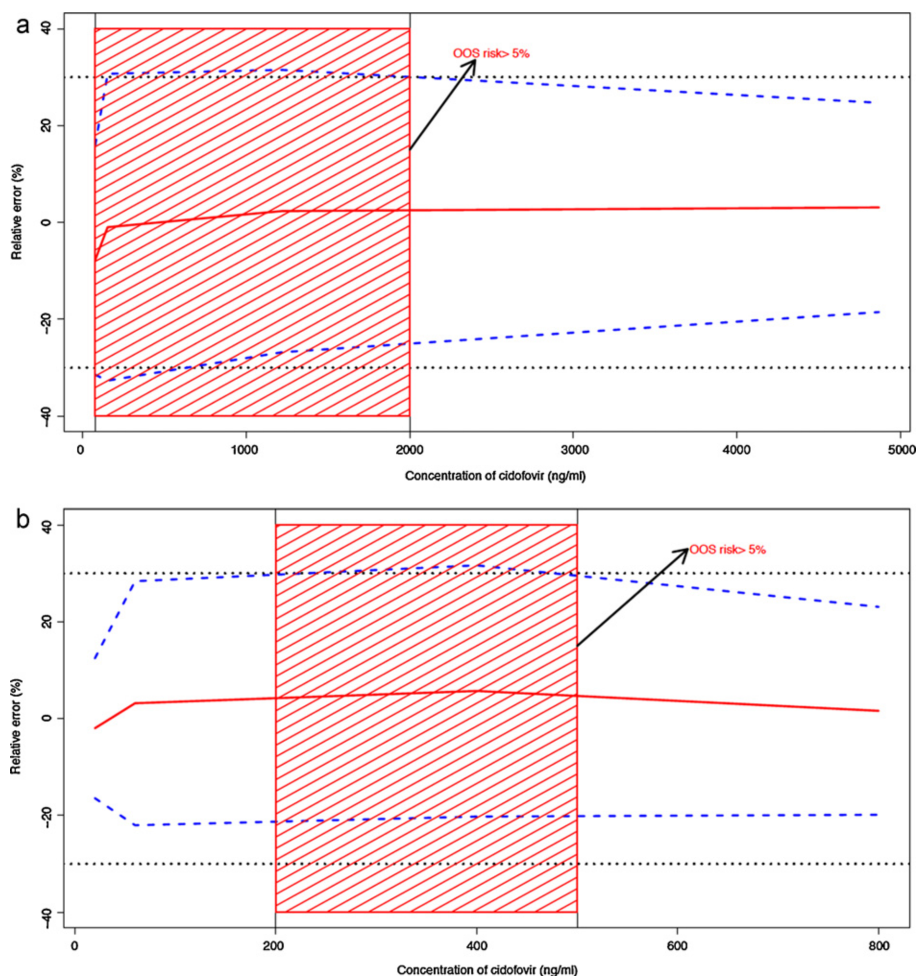

Fig. 2. Accuracy profiles for the quantification of cidofovir in plasma for (a) the LC–MS/MS method developed by Breddemann et al. [30] and (b) for the LC–MS/MS method developed by Momper et al. [31]. Continuous line: Relative bias; Dotted lines: ±30% acceptance limits; Dashed lines: 95% β-expectation tolerance limits; Hatched region: range of concentration levels where the risk of having results falling outside of the acceptance limits (OOS risk) is greater than 5%.

[30], is only able to provide accurate results 95 times out of 100 over the concentration range 2000–4870 ng/mL of CDV. Indeed, for concentration levels of CDV smaller than 2000 ng/mL, the risk to obtain results out of the ±30% acceptance limits is greater than 5%. This means that there are more than five chances out of 100 that future results will fall outside the acceptance limits recommended by the Food and Drug Administration (FDA) [32]. For the LC–MS/MS method of Momper et al. [31], the accuracy profile is also problematic over the concentration range of 200–500 ng/mL of CDV as shown on Fig. 2b. Indeed, into this range of concentration the risk to obtain results of inadequate quality is greater than usually required. These observations further support the need to develop a more reliable analytical method that meets the quantitative requirements needed to answer our specific analytical issue: the accurate quantification of CDV in human plasma in pre-clinical trials.

The newly developed method proposes an original solid phase extraction prior to hydrophilic interaction chromatography coupled with ultraviolet (UV) detector determination. UV detection allows quantify accurately CDV at concentration level close to 100 ng/mL. This concentration level was in full accordance with the requirements of this pre-clinical study.

In order to allow efficient comparison between validation and routine performances, the selection of the most robust analytical conditions for the new method was performed by means of design of experiments (DoE). In addition, the solid phase extraction efficiency has been improved by using a cation exchange sorbent. The analyte retention was increased so as to optimize the separation between CDV and endogenous compounds of human plasma. The method was then fully validated according to FDA requirements using a total error approach [32]. Using this latter, measurement uncertainty estimates were directly obtained from the method validation and used to predict the uncertainty of the routine results [36]. Finally, this HILIC method was then successfully applied in two routine pre-clinical trials where a further comparison of the prediction obtained during the method validation for the accuracy of the results as well as for the measurement uncertainty was performed.

2. Experimental

2.1. Chemicals

Cidofovir dihydrate was obtained from 2Y-Chem, Ltd. (Shanghai, China). Ammonium hydrogen carbonate, ammonium carbonate, meta-phosphoric acid, trifluoroacetic acid, acetonitrile, ammonium hydroxide (25%), formic acid, ortho-phosphoric acid (85% w/w) were all of analytical grade and were purchased from Merck (Darmstadt, Germany). Acetonitrile was of gradient grade from Merck. Ultrapure water was generated from a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Apparatus

Plasma sample preparation was performed by means of a robotic Multiple Probe SPE 215 System from Gilson (Villiers le Bel, France). The robotic system, controlled by Trilution^{TM} LH software from Gilson, is able to automatically prepare the samples before their injection in the LC system. The solid phase extraction (SPE) cartridges (SCX, HCX, SAX, HAX) were purchased from Biotage AB (Uppsala, Sweden). The cartridges Bond Elut Plexa PCX were obtained from Varian (Palo Alto, CA, USA).

The LC system used for this study was a Waters (Milford, MA, USA) 2695 Separations Module with Waters 2998 Photodiode Array detector. The system was controlled by Waters Empower^{TM} 2 software.

The LC columns tested in this study were: Alltima HP HILIC (150 mm × 2.1 mm i.d., 3 μm) preceded by Alltima HP HILIC guard column (7.5 mm × 2.1 mm i.d., 5 μm) from Grace (Columbia, MD, USA), YMC-Pack Amino-120 (150 mm × 2.1 mm i.d., 3 μm) preceded by YMC-Pack Amino-120 guard column (10 mm × 2.1 mm i.d., 3 μm) from YMC EUROPE GMBH (Dinslaken, Germany), and ZIC®-HILIC (150 mm × 2.1 mm i.d., 3.5 μm) preceded by ZIC®-HILIC guard column (20 mm × 2.1 mm i.d., 5 μm) from Merck SeQuant AB (Umeå, Sweden).

2.3. Standard solutions

For the method validation, calibration and validation standards of cidofovir were prepared by spiking blank human plasma. The calibration standards are used to set up the calibration model while the validation standards are used to estimate precision, trueness and accuracy of the method. The calibration standards were prepared at seven concentration levels, ranging from 50 to 1000 ng/mL while the validation standards were prepared at five concentration levels, also ranging from 50 to 1000 ng/mL. Four series ($p = 4$) were performed by injecting all the calibration standards in triplicate and the validation standards five times.

2.4. Sample preparation

In order to precipitate the plasmatic proteins 850 μL of plasma sample were mixed with 850 μL of aqueous meta-phosphoric acid (5%, w/v) [37,38]. After a brief stirring with a vortex mixer, the samples were left standing in the dark for 15 min prior to centrifugation at 7000 × g (6500 rpm) for 15 min. 1.1 mL of the supernatant were collected for sample handling by means of the robotic SPE 215 System. The supernatant was then loaded onto a Bond Elut Plexa PCX solid phase extraction cartridges packed with mixed-mode polymeric cation exchange sorbent (60 mg, 1 mL). The SPE cartridges were previously conditioned with 1 mL of acetonitrile and then with 500 μL of ammonium formate (pH 3.0; 20 mM). 250 μL of a mixture of acetonitrile–water (85:15, v/v) were then applied onto the cartridge in order to perform the washing step. The elution of the analyte was then obtained by dispensing 1.0 mL of mixture of acetonitrile–ammonium carbonate (pH 10.0; 20 mM) (70:30, v/v). All these operations were performed automatically. 300 μL of the resulting extract were then transferred manually to the LC autosampler.

2.5. Optimal HILIC conditions

The analytical column was an Alltima HP HILIC (150 mm × 2.1 mm i.d., 3 μm) protected by a Alltima HP HILIC guard column (7.5 mm × 2.1 mm i.d., 5 μm). The isocratic separation was performed at a temperature of 25 °C using a mobile phase consisting of a mixture of acetonitrile–ammonium hydrogen carbonate (pH 7.0; 20 mM) (72:28, v/v). This buffer was prepared by dissolving 20 mmol of ammonium hydrogen carbonate salt in 1.0 L water and adjusted with hydrochloric acid (6 M) to pH 7.0. The mobile phase was degassed by sparging with helium. The flow rate was set to 0.21 mL/min. The sample injection volume was set at 5 μL. UV detection was operated at 275 nm and the total run time was 10 min.

2.6. Computations

The e.noval software v3.0 (Arlenda, Liège, Belgium) was used to compute the validation results of the bioanalytical method as well as to obtain all the accuracy profiles. Designs of experiments as well as their statistical analysis were performed using JMP V8.0 (SAS Institute, Cary, NC, USA).

3. Results and discussion

3.1. Optimization of the chromatographic conditions

As previously mentioned, Cidofovir is a highly polar molecule presenting three ionizable functions. Therefore, it is an interesting candidate for determination by HILIC mode. Three stationary phases were initially tested to perform the LC analysis of cidofovir: a bare silica phase (Alltima HP HILIC), an amino modified phase (YMC-Pack Amino) and a zwitterionic modified phase (ZIC®-HILIC). For these preliminary runs the mobile phases were prepared using constant mixtures of acetonitrile and aqueous phases (80:20, v/v). The aqueous phases were trifluoroacetic acid 0.1% (v/v) (pH 2.0) or ammonium formate (pH 4.0; 20 mM) or ammonium hydrogen carbonate (pH 7.0; 20 mM) for each column under investigation. The pH values of these aqueous phases were selected according to the pK_a values of Cidofovir so as to modulate its global charge. The salt concentration of the buffers was set at 20 mM in order to obtain a suitable retention time (<10 min), the smallest peak width and a Gaussian peak shape.

In all conditions tested, the YMC-Pack Amino column exhibited too strong interaction with CDV, leading to excessively long runs and inadequate peak symmetry and height. On the contrary, weak retention and a lack of reproducibility of the retention time of CDV were observed on the ZIC®-HILIC stationary phase with the three mobile phases tested. Therefore two additional conditions were tested with this column. For this purpose, mobile phases were mixtures of acetonitrile–ammonium acetate (pH 5.6; 100 mM) (70:30, v/v) or acetonitrile–ammonium acetate (pH 6.7; 100 mM) (70:30, v/v), respectively [39,40]. The analyte retention times (*RT*) were adequate but the peak widths were not found to be acceptable (>90 s). These conditions were not kept for further investigation.

The results obtained with the Alltima HP HILIC column using mobile phases with low salt concentration were better in terms of peak width and retention time. After this preliminary screening step, this later column was selected for further chromatographic optimization by means of DoE.

Four levels full factorial statistical designs of experiments were then prepared to test the adequacy of each aqueous phase simultaneously with the column oven temperature and acetonitrile proportion in the mobile phase. The aqueous phases tested were trifluoroacetic acid 0.1% (v/v) (pH 2.0) or ammonium formate (pH 4.0; 20 mM) or ammonium hydrogen carbonate (pH 7.0; 20 mM). The experimental domain ranged from 25.0 to 35.0 °C and from 70 to 95% for temperature and acetonitrile proportion respectively. The responses modeled were the CDV peak width that should be minimized and the CDV retention time with an optimal target of 5 min to avoid a too long run time. Each aqueous phase tested the DoE involved 4^2 runs with two supplementary experiments at the center of the experimental domain.

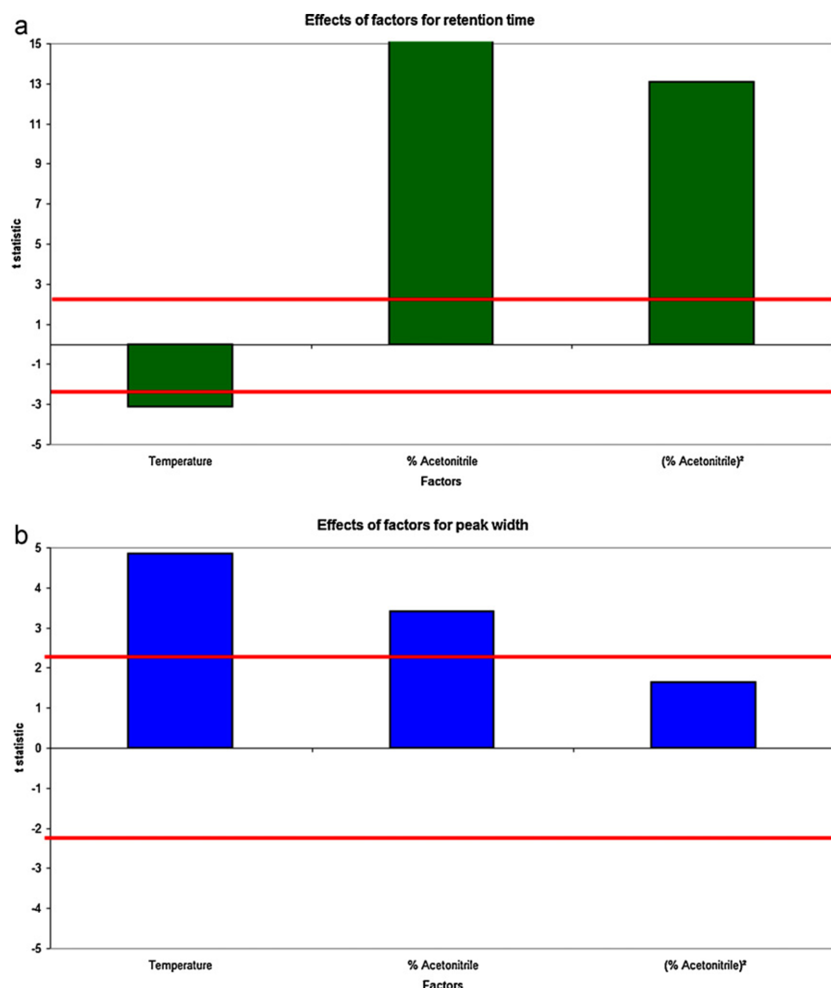

Fig. 3. Size of the effects for the significant factors used to optimize the chromatographic separation (a) for the retention time (*RT*) and (b) for the peak width.

The mobile phases containing trifluoroacetic acid 0.1% (v/v) (pH 2.0) or ammonium formate (pH 4.0; 20 mM) were discarded due to either non Gaussian peak shapes or broad peak width or high CDV retention times (*RT* > 10 min) in some conditions. These conditions were therefore rejected for further consideration.

For the remaining aqueous phase, the ammonium hydrogen carbonate (pH 7.0; 20 mM), the simplest model that best fitted the data (R^2 = 0.99 for *RT* and R^2 = 0.80 for *W*) was a response surface without the quadratic term for temperature and without interactions. Fig. 3a and b shows the significant effects (p-values < 0.05) of each factor on the two responses studied: retention time and peak width. As expected, it is the percentage of acetonitrile that has the strongest effect on the retention time and the temperature on the peak width. Following these observations, the optimal conditions for this HILIC method proposed by the DoE are 72% acetonitrile in mobile phase and the column oven temperature at 25 °C. The predicted cidofovir *RT* is 5.09 min (±0.87 min) and the predicted peak width is 65.96 s (±120.64 s). Fig. 4 shows a chromatogram performed at these optimal conditions. The experimental *RT* and peak width are 4.7 min and 67 s, respectively. As can be seen the pH 7.0 aqueous buffer provided the most adequate *RT* (closest to 5 min) and the thinnest peak width.

At pH 7.0 the CDV has a net negative charge and the silanol groups of the bare silica are ionised. The retention mechanism results from the interaction between the cidofovir and a partial electrical double layer on the surface of the stationary phase. Therefore an increase in salt concentration could complete the electrical

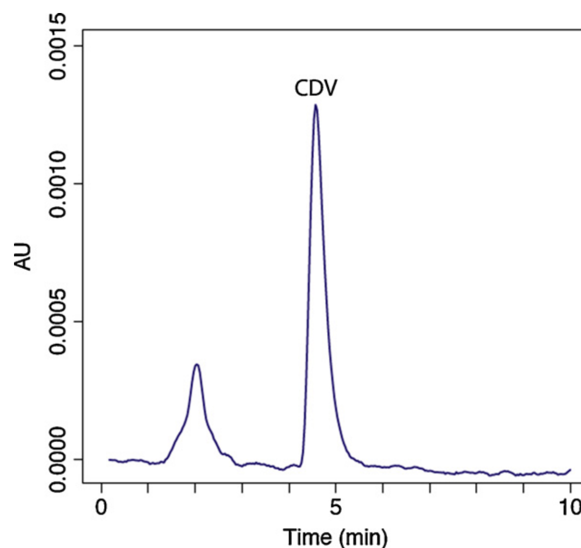

Fig. 4. Chromatogram showing cidofovir peak (CDV) at a concentration of 1000 ng/mL obtained using the optimal conditions predicted during the chromatographic conditions optimization. For the detailed chromatographic conditions see text.

double layer and reduce the electrostatic repulsion, thus resulting in stronger analyte retention [40]. Consequently, the optimal conditions resulting from DoE were kept as the final ones.

3.2. Optimization of the sample preparation

The protein precipitation is the initial step of the sample clean-up procedure. Different reagents (meta-phosphoric acid, trifluoroacetic acid and acetonitrile) were evaluated in order to remove protein and increase the solid phase extraction efficiency. Several SPE cartridges with either weak or strong cation as well as anion exchange sorbents (Plexa PCX, SCX, HCX, SAX, HAX) were tested according to the involved ionisable functions. Protein precipitation media, as well as nature, pH and obviously the volume of the conditioning, loading, washing and elution liquids used in the SPE process were also optimized. Based on preliminary screening experiments, the most promising cartridge was the Plexa PCX one. The optimal conditions for this cartridge type were then determined by using statistical designs of experiments. The factors and the experimental domain explored are: wash volume from 0.25 to 0.75 mL, elution volume from 0.25 to 1.0 mL and proportion of water in the wash liquid ranging from 0% (only acetonitrile) to 50%. The key response measured was recovery (%) that most closely approaches 100%. The design selected was a three level full factorial design involving 3^3 experiments with three supplementary center points. From the design the optimal conditions were obtained for the wash volume, proportion of water in the wash liquid and elution volume. The optimal extraction recovery obtained with the Bond Elut Plexa PCX cartridge with recovery values of more than 80%. The optimal sample clean-up procedure is previously described at Section 2.4.

3.3. Method validation

Validation should ensure that the analytical procedure is fit for its purpose [1]. In this application the aim of the developed method is to quantify cidofovir in plasma. A total error approach was used to demonstrate the fitness of the method using tolerance interval methodology and the accuracy profile as a decision tool [33–35]. The accuracy profile is a predictive tool that can be used to evaluate the capacity of the method to give future results within defined limits. The accuracy profile is obtained by linking on one hand the lower bounds and on the other hands the upper bounds of the β-expectation tolerance limits calculated at each concentration levels for the validation standards. As long as this accuracy profile stays within the predefined acceptance limits, the method can be considered as valid. Indeed, it guarantees that each future results obtained with the analytical method over the validated concentration range has at least a probability β (e.g. 0.95 or 95%) to be within these a priori set acceptance limits [41].

The same concept of accuracy profile was also used to select the most appropriate regression model for calibration, to determine the lower limit of quantification (LLOQ) and the range over which the method can be considered as valid. The acceptance limits were settled at ±30% according to the regulatory requirements [1,32] and the minimum probability β was set at 95%.

3.3.1. Extraction process efficiency

The recoveries of cidofovir were determined using blank plasma samples spiked at three different concentrations ranging from 100 to 1020 ng/mL [1,42]. The mean recoveries are shown in Table 1. Those recoveries were calculated by comparing peak areas of CDV from freshly prepared plasma samples treated according to the described procedure with peaks areas found after the direct injection on the analytical column of standard solutions at the same concentrations as required by regulatory guidance [1,42]. The recoveries were found to be constant (around 85%) over the entire range studied, demonstrating the overall extraction efficiency of the process [1].

Table 1
Extraction rate obtained for three different concentrations of cidofovir ranging from 100 to 1020 ng/mL considering the optimal extraction conditions.

Concentration (ng/mL)	Number of replicate (n)	Extraction rate ± SD (%)
100.2	3	84.9 ± 1.5
501	3	85.9 ± 0.5
1002	3	86.9 ± 2.1

3.3.2. Selectivity

Selectivity of the analytical method was assessed by analyzing six independent sources of plasma [1]. No endogenous source of interference was observed at the retention time of CDV. Typical chromatograms obtained with a blank plasma chromatogram, a plasma sample spiked with 100 ng/mL and a plasma sample spiked with 1000 ng/mL of CDV are presented on Fig. 5.

3.3.3. Analysis of the response functions

The response function of a LC method is an important criterion that must be considered in the validation of a method since it corresponds to the assessment of the relationship between the chromatographic response and the concentration of the analyte [43]. The approach based on the β-expectation tolerance intervals for total measurement error was used to determine the most appropriate response function for the determination of CDV in human plasma.

The optimal regression model should be the one that firstly allows to accurately quantify CDV over the widest concentration range and secondly provides the smallest bias over this concentration range. From each response function tested, the concentrations of the spiked plasma validation standards were back calculated in order to determine the upper and lower β-expectation tolerance limits at β = 95%. Fig. 6 illustrates the different accuracy profiles [33–35] obtained by analyzing the validation experiments with the different following regression models such as linear regression, weighted ($1/X$) linear regression, weighted ($1/X^2$) linear regression, quadratic regression, weighted ($1/X$) quadratic regression

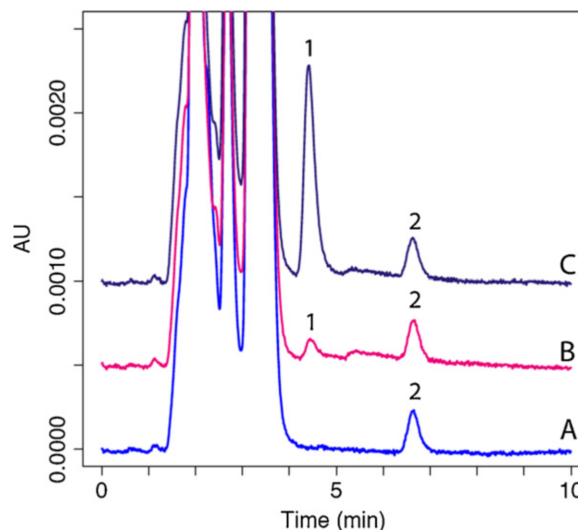

Fig. 5. Chromatograms of (A) a blank plasma chromatogram, (B) a spiked plasma at 100 ng/mL and (C) a spiked plasma at 1000 ng/mL of cidofovir. Peak identification: (1) cidofovir, (2) endogenous compound.

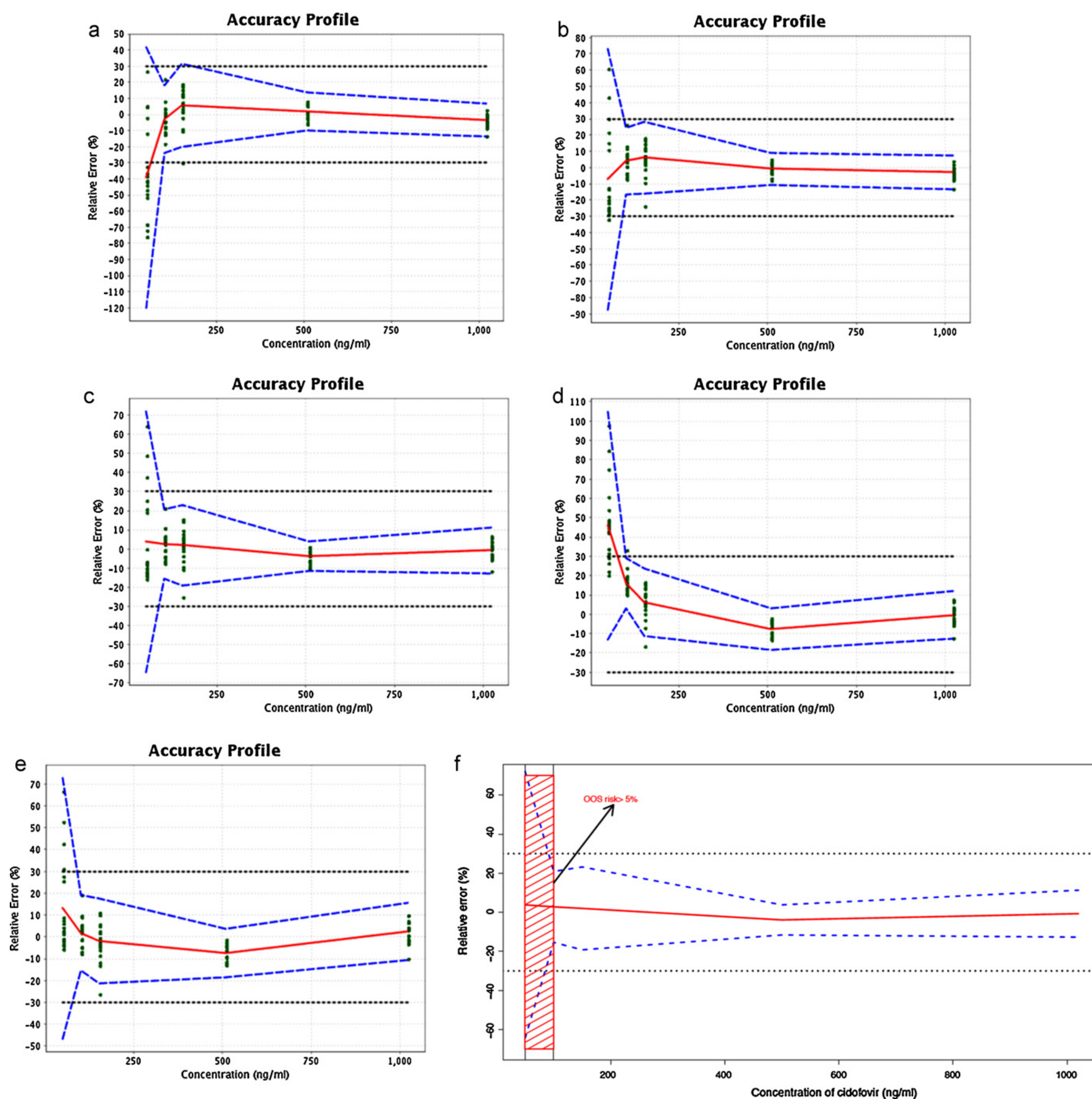

Fig. 6. Validation phase - Accuracy profiles for the quantification of cidofovir in plasma using (a) a quadratic model, (b) a weighted $1/X$ quadratic model, (c) a weighted $1/X^2$ quadratic model, (d) a linear model, (e) a weighted $1/X$ linear regression model and (f) a weighted $1/X^2$ linear regression model. Continuous line: Relative bias; Dotted lines: ±30% acceptance limits; Dashed lines: 95% β-expectation tolerance limits; Dots: relative back-calculated concentrations of the validation standards; Hatched region: range of concentration levels where the risk of having results falling outside of the acceptance limits (OOS risk) is greater than 5%.

and weighted ($1/X^2$) quadratic regression. The weight functions selected ($1/X$ and $1/X^2$) are in general the one providing the most efficient solutions. These weight functions are two special cases of the more general weight function $1/X^{\gamma}$ and if the previous weights do not provide acceptable solutions it is possible to estimate more precisely [43,44]. The acceptance limits were set at ±30%. As can be seen on Fig. 6, the only response function that complied with the defined criteria is the weighted quadratic model using the weight $1/X^2$. This model was therefore selected as the final calibration model. The responses functions obtained by applying this calibration model are presented in Table 2 for each series of experiments.

3.3.4. Trueness, precision and accuracy

Trueness [33,45,46] expressed in terms of relative bias (%) was assessed from the validation standards at five concentration levels of spiked plasma, ranging from 50 to 1020 ng/mL (Table 2). According to the regulatory requirements [1,32], trueness was found to be acceptable for CDV, since the relative bias values did not exceed the 15%, irrespective of the concentration level.

The precision of this bioanalytical method was then determined by computing the relative standard deviations (RSD, %.) for repeatability and time-different intermediate precision at each concentration level of the spiked plasma validation standards [1,33,35,46]. The RSD values presented in Table 2 are less than 15%

[1,32] for all the concentration levels tested except for the lowest one where the RSD values was found to be 25.6%.

The accuracy takes into account the total error, i.e. the sum of systematic and random errors related to the test result [33,45,46]. Total error was estimated using 95% β-expectation tolerance intervals at each concentration level of the validation standard. This interval defines a region where each future results generated by the bioanalytical procedure has 95% chance to fall. As shown in Table 2, the upper and lower 95% β-expectation tolerance limits (%) demonstrate that the method is accurate within the range 100–1020 ng/mL since the limits of tolerance of the errors (relative β-expectation tolerance limits) do not exceed the acceptance limits set to 30% [33–35,46]. At the lowest concentration limit (50 ng/mL), the accuracy of the method is clearly not suited to its objective. Indeed, at this concentration level, the calculated β-expectation tolerance limits are clearly outside the desired limits (±30%).

3.3.5. Linearity of the results

In order to demonstrate the linearity of the results [33–35,46], a regression line was fitted between the back-calculated concentrations of the validation standards versus the introduced concentrations applying a linear regression model. The equation of the regression line and its coefficient of determination (R^2) is given in Table 2.

3.3.6. LLOQ and LOD

The lower limit of quantitation (LLOQ) is defined as the smallest quantity of substance that can be quantitatively determined under the experimental conditions with well defined accuracy [47], i.e. taking into account the systematic and random errors [1,7,32]. Using the accuracy profile, the LLOQ was estimated as 92.7 ng/mL. The LLOQ was obtained by calculating the smallest concentration for which the β-expectation tolerance limits cross the acceptance limits [35,46]. The limit of detection (LOD) was estimated using the mean intercept of the calibration model and the residual variance of the regression and was evaluated to be 28.1 ng/mL [46].

3.3.7. Risk assessment

The risk of having future measurements falling outside the specified acceptance limits was evaluated using the β-expectation tolerance intervals obtained with the previously selected regression model. This risk is computed for each concentration level investigated, as the sum of the proportion of results effectively lying outside the upper acceptance limit on one hand and under the lower acceptance limit on the other hand [48]. The maximum risk tolerated was set to 5%, meaning that it is accepted that at most each future result provided by the developed method will have five chances out of 100 to fall outside the acceptance limits of ±30%. Fig. 7 illustrates the risk profile for CDV results obtained at each validation standard concentration level. The risk was clearly smaller than 5% over the valid concentration range. However, this risk was about 36% for the smallest concentration level (50 ng/mL) thus confirming the inaccuracy of the results generated by the bioanalytical method at this concentration level.

3.4. Comparison of routine performances versus validation predictions

The validated method was applied routinely to the quantitative determination of CDV in human plasma samples from a pre-clinical trial. Fig. 8 illustrates chromatograms obtained from incurred blank sample at T0 (pre-dose) and a real unknown sample from the same patient at T5. The estimate concentration level is equal to 184.9 ng/mL and demonstrating the good selectivity of the current method at low concentration levels (two times the LLOQ). In each trial apart from the calibration standards and the real unknown samples, each analytical run involved quality control (QC) samples prepared in blank plasma spiked with CDV in order to reach three

Table 2
Results of the validation of the HILIC method dedicated to the quantification of cidofovir in plasma samples.

Response function (p = 4; n = 5)	Weighted quadratic regression model calibration range (m = 7): 50–1020 ng/mL			
	Series 1	Series 2	Series 3	Series 4
X^2	2.11E-03	3.51E-03	1.41E-03	1.82E-03
X	13.35	12.00	12.44	11.41
Intercept	−604.10	−455.70	−513.90	−469.50
Weight	$1/X^2$	$1/X^2$	$1/X^2$	$1/X^2$
r^2	0.9799	0.9958	0.9906	0.9861
Trueness (p = 4; n = 5)		Relative bias (%)		
50.0 ng/mL		3.8		
100.0 ng/mL		2.8		
150.0 ng/mL		2.0		
500.0 ng/mL		−3.9		
1020 ng/mL		−0.7		
Precision (p = 4; n = 5)	Repeatability (R.S.D.%)		Intermediate precision (R.S.D.%)	
50.0 ng/mL	15.6		25.6	
100.0 ng/mL	5.6		7.5	
150.0 ng/mL	9.3		9.7	
500.0 ng/mL	2.8		3.4	
1020 ng/mL	3.2		4.7	
Accuracy (p = 4; n = 5)		β-Expectation tolerance limits (%)		
50.0 ng/mL		[−64.4; 72.1]		
100.0 ng/mL		[−15.4; 20.9]		
150.0 ng/mL		[−19.2; 23.2]		
500.0 ng/mL		[−11.6; 3.8]		
1020 ng/mL		[−12.7; 11.3]		
Linearity (p = 4; n = 5)				
Range (ng/mL)		[92.69; 1020]		
Slope		0.9861		
Intercept		1.355		
r^2		0.9956		
LOD (ng/mL)		28.09		
LLOQ (ng/mL)		100.0		

p: number of days (series) of analysis; n: number of repetitions per day of analysis; m: number of cidofovir concentration levels.

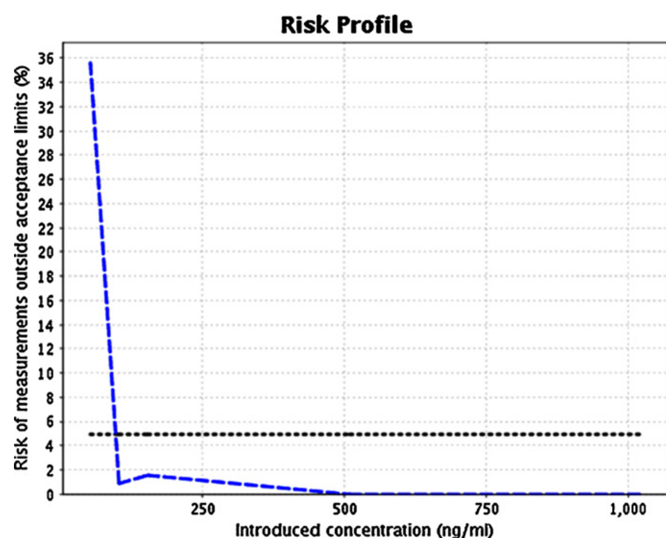

Fig. 7. Risk profile for cidofovir (dashed line) obtained by concentration level. The maximum tolerated risk is set at 5% (dotted line).

concentration levels: 150, 500 and 850 ng/mL according to FDA regulations [1] and each level was analysed in quadruplicates. The QC samples were worked in two groups of six QC samples in the analytical runs. For the first trial, ten routine runs (one run per day) were performed, leading to the analysis of 40 QC samples (spiked plasma samples) at each of the three concentration levels as well as 192 real samples. For the second trial, 15 routine runs were realized. The numbers of QC samples for this trial add up to 172 and the number of real samples to 252.

3.4.1. Assessment of the method validation prediction

In order to confirm and further evaluate the ability of the proposed method to provide accurate results, the prediction made during the validation step of the proposed analytical method was globally verified over both trials by counting the number of QC samples at each concentration level falling within the ±30% acceptance limits. Fig. 9 shows the result of each QC sample as well as the ±30% acceptance limits (dotted lines). As can be seen, for the two highest QC levels (Fig. 9b and c) all QC samples fell within the acceptance

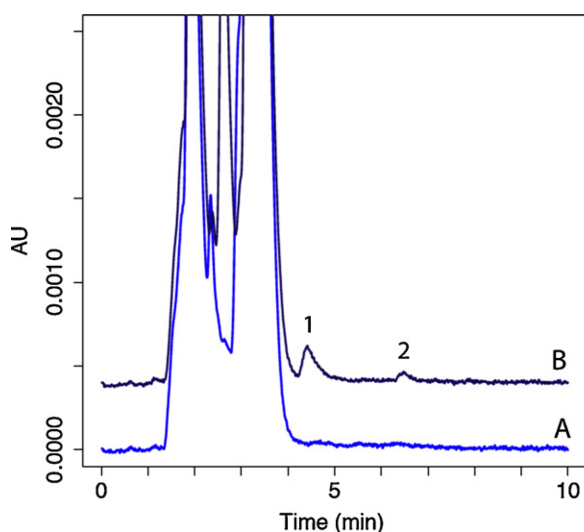

Fig. 8. Chromatograms of (A) real blank plasma sample at T0 (pre-dose), (B) real plasma sample back calculated at 184.9 ng/mL at T5 (post-dose). Peak identification: (1) cidofovir, (2) endogenous compound.

limits, thereby confirming the validation step prediction. For the smallest QC level (150 ng/mL; Fig. 9a) only two samples out of 106 at this level fell outside the acceptance limits of ±30% resulting in a proportion of 98.1% which is above the minimum requirement of 95% defined during the method validation. As shown on the right panes of Fig. 9a–c, the 95% kernel distribution densities of the QC results of both trials represented by the hatched regions are fully included within the ±30% acceptance limits for all the QC sample levels, thus justifying the validity of the developed assay.

It has to be noted that according to the FDA guidance on bioanalytical methods validation [1,32], it is acceptable that only 67% of the QC samples of each run must be included within the routine acceptance limits. The routine performance of this bioanalytical method demonstrates that this method is clearly appropriate for its final use since at least 95% of the QC samples are included in the routine acceptance limits.

3.4.2. Comparison of measurement uncertainty estimates

3.4.2.1. Measurement uncertainty estimates from method validation. While the method validation demonstrates the reliability of the results generated by the method, it is not sufficient to allow the correct interpretation and comparison of the results [7]. To achieve this, the measurement of the uncertainty of the assay needs to be estimated. Feinberg et al. demonstrated that the β-expectation tolerance interval used in the accuracy profile approach is directly related to the uncertainty of the measurements [36]. This allows obtaining estimates of the measurement uncertainty of the CDV assay without additional experiments as long as the method validation experimental design includes the major sources of uncertainty that will be involved during the routine application of the method. Estimates of measurement uncertainty were therefore obtained at each of the concentration levels of the validation standards and are given in Table 3. The expanded uncertainty is obtained by applying a coverage factor of $k = 2$ [4]. This corresponds to a 95% confidence interval around the results where the measurand may lie. Table 3 indicates that the relative expanded uncertainty of CDV is at most 20% over the concentration range validated. This maximum value is obtained for the 150 ng/mL concentration level. This means that with a confidence level of 95% the measurand is situated at maximum ±20% around the measured result. Table 3 also shows that the relative uncertainty is not constant over the valid concentration range fluctuating from 7% to 20%. Indeed, it can be seen that relative uncertainty is decreasing when concentration increase. This is dependency of uncertainty on concentration is nonetheless typical when working on a relatively large concentration range [49–52]. Therefore, in order to obtain estimation of the uncertainty of the routine results, the uncertainty was modeled as a function of the concentration over the valid concentration range using the following model:

$$\mathrm{Log}_{10}(u_c^2) = \beta_0 + \beta_1\ \mathrm{Log}_{10}(x) + \varepsilon, \tag{1}$$

where u_c is the uncertainty, x the cidofovir concentration in ng/mL and ε, the residual error is assumed normally distributed of 0 mean and of σ^2 variance: $N(0,\sigma^2)$. The choice of this model was made a priori as it has been observed in many situations that variance increases exponentially with the concentration, especially when working on a relatively large range of concentration [50–55]. In addition, the logarithmic transformation of the uncertainty function is also preferred in order to ensure that only positive values of uncertainties will be obtained.

Finally, the estimated measurement uncertainty for any concentration level included within the valid range is expressed by:

$$\hat{u}_c = \sqrt{10^{1.218} x^{0.4178}}. \tag{2}$$

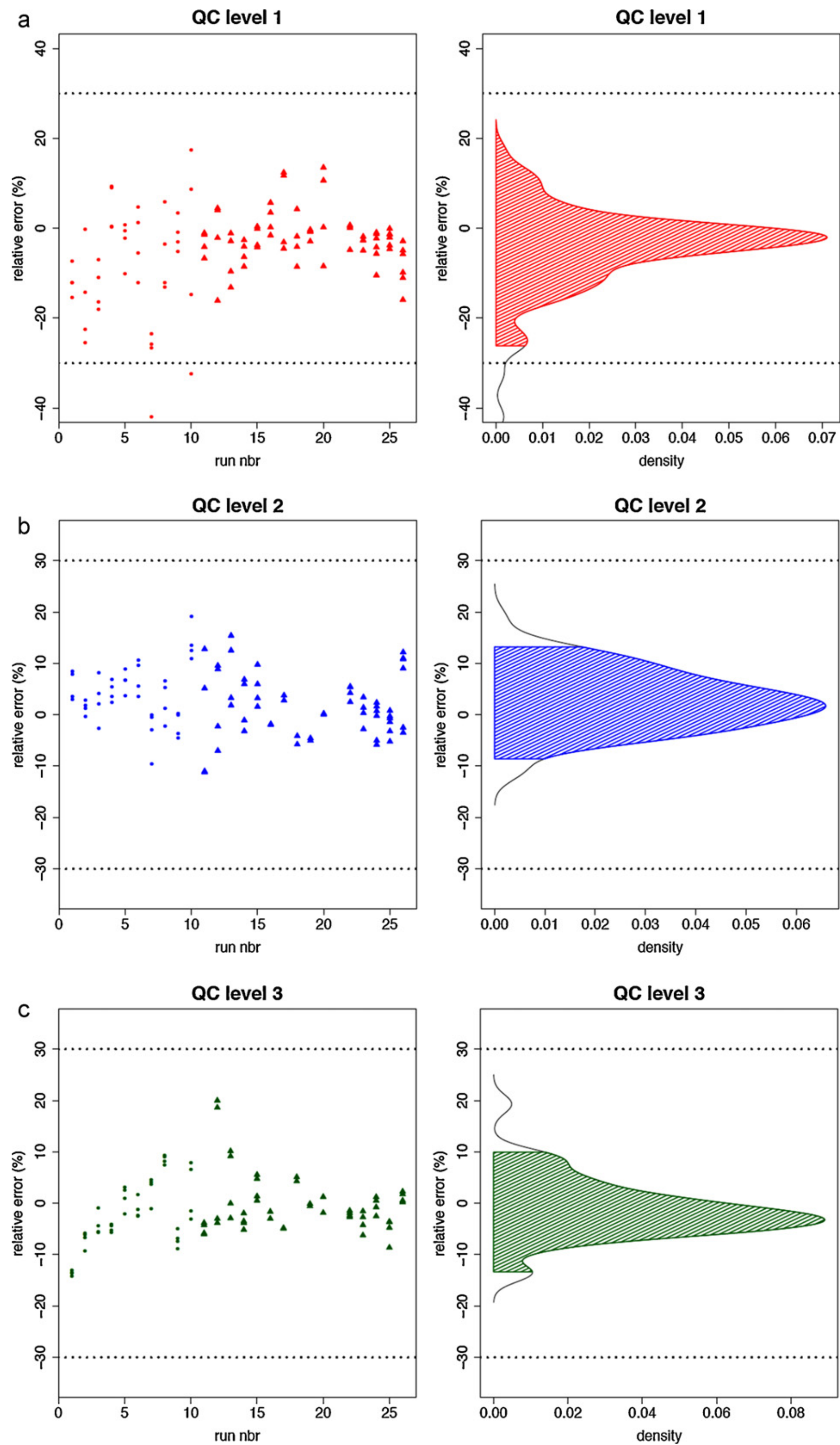

Fig. 9. Routine trials – On the left pane: Runs acceptance chart illustrating the results of each QC samples (dots: first trial; triangles: second trial) prepared with plasma spiked at three concentration levels of cidofovir: (a) 150, (b) 500 and (c) 850 ng/mL for both routine trials. On the right pane: kernel distribution densities of the QC samples for each concentration level. Dotted lines: ±30% acceptance limits; Dots and triangles: relative back-calculated concentrations of the QC samples for the first and second trial, respectively. Hatched areas: regions where 95% of the distribution of the QC results is observed using kernel densities.

Table 3
Measurement uncertainty estimations of cidofovir results at each concentration level investigated during the method validation (Val) using the selected regression model as well as at each concentration level of the quality control samples analysed during the routine use of the method for the two pre-clinical trials: Trial 1 and Trial 2. The expanded uncertainty was computed using a coverage factor of 2. The 95% prediction intervals of uncertainty were obtained from the method validation uncertainty model using Eq. (1) (see text).

Study name	Concentration level (ng/mL)	Uncertainty of the bias (ng/mL)	Uncertainty (ng/mL)	Uncertainty 95% prediction interval (ng/mL)	Expanded uncertainty (ng/mL)	Relative expanded uncertainty (%)
Val	50	5.46	14.11	–	28.23	55.5
	100	2.83	8.13	–	16.27	16.0
	150	3.89	15.34	[2.27;59.07]	30.68	20.1
	500	5.61	18.00	[3.01;73.57]	36.00	7.1
	1020	19.16	51.68	[2.92;94.55]	103.4	10.1
Trial 1	150	4.79	20.37	–	40.74	26.9
	500	9.35	34.05	–	68.1	13.5
	850	15.98	55.95	–	111.9	13.0
Trial 2	150	1.37	8.68	–	17.36	11.6
	500	4.83	30.53	–	61.05	12.2
	850	8.95	48.04	–	96.07	11.3

This equation will allow to provide a first estimation of the measurement uncertainty of results that would be acquired during the routine application of the method in the pre-clinical trial.

However, a key question that remains is to evaluate whether this first estimate of measurement uncertainty obtained from analytical method validation is sufficiently precise and represents adequately the real measurement uncertainty of the method.

3.4.2.2. Measurement uncertainty estimates from routine studies. To answer this question, measurement uncertainty estimation was obtained using the QC samples analysed during the two pre-clinical trials. Table 3 also gives these uncertainty values as well as the 95% prediction intervals of the uncertainty computed from the uncertainty model obtained during the method validation step. As can be seen the measurement uncertainty values estimated during the routine analysis from the QC samples are fully included in their respective prediction interval, indicating that the uncertainty model previously obtained is relatively adequate.

Finally, in order to further compare the estimations of measurement uncertainty obtained during the method validation with the one obtained during the routine use of the method, the following model was tested:

$$\mathrm{Log}_{10}(u_c^2) = \beta_0 + \beta_1\ \mathrm{Log}_{10}(x) + \begin{pmatrix} 1 \\ 0 \\ -1 \end{pmatrix} \beta_2 + \begin{pmatrix} 1 \\ 0 \\ -1 \end{pmatrix} \mathrm{Log}_{10}(x)\beta_3 + \varepsilon, \quad (3)$$

where u_c is the uncertainty, x the CDV concentration in ng/mL and ε is the residual error assumed normally distributed of 0 mean and of σ^2 variance: $N(0,\sigma^2)$. The coefficient β_2 models the effect of the method life cycle step: 1 is the coded value of the validation step, 0 is the coded value of the first routine study and +1 is the coded value of the second routine analysis step. The coefficient β_3 models the effect of the interaction between the concentration of CDV and the method life cycle step The effect of the method life cycle step (β_2) p-value is 0.089 showing that it has a non negligible importance for the estimation of measurement uncertainty. However we are not interested in comparing any of the steps studied but rather the comparison of interest is to evaluate whether the effect of method validation is different of the effect of routine analyses. To allow this evaluation a contrast t-test was realized whose p-value = 0.017, thus providing strong statistical evidence of the difference between the estimates of measurement uncertainty obtained from method validation step than from the routine use of the method. These results show that the method validation gives globally a smaller estimation of measurement uncertainty than those obtained during routine use of the bioanalytical method. This is illustrated on Fig. 10 which shows the relationship of uncertainty versus concentration using the validation standards for the validation study and the QC samples of each pre-clinical trial. Additionally, the p-value of the interaction (β_3) is 0.064 also indicating that the difference in measurement uncertainty observed between the life cycle steps depends highly on the concentration value of CDV. Here again we will test a contrast t-test to compare the effect of method validation to the simultaneously effect of the two routine trials. For this contrast, the p-value = 0.039 also giving statistical evidence that the difference in measurement uncertainty estimation between validation and routine both depends on the concentration of CDV but in different manner. As shown on Fig. 10, it can be seen that the underestimation of measurement uncertainty obtained during method validation is greater for higher concentration levels of CDV. For the lowest QC concentration level used in the routine applications, this underestimation is less evident. This is especially true when comparing the second pre-clinical trial uncertainty value to the one obtained from the method validation that are very close as shown on Fig. 10. Although the method validation involved four different runs of analyses over four different days and two operators, it nevertheless neglected some other sources of uncertainty that were included during the two routine studies. For the first

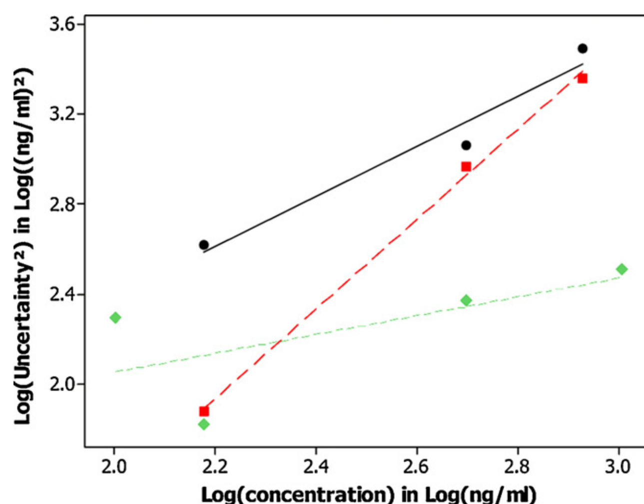

Fig. 10. Uncertainty models versus concentration of cidofovir in plasma obtained from the method validation experiments using the results of the validation standards (Val) and the quality control samples analysed during the first pre-clinical trial (Trial 1) and the second pre-clinical trial (Trial 2).

Table 4
Main different sources of uncertainty between the method validation phase and the two routine trials (Trial 1 and Trial 2).

	Validation	Trial 1	Trial 2
Time period (weeks)	1	2	4[a]
Operator	Op-1, Op-2	Op-1, Op-2	Op-1, Op-3
Pre-columns	PC-1	PC-2	PC-4 to PC-7
Analytical columns	AC-1	AC-1, AC-2	AC-3 to AC-6
Plasma pool	Pool-A	Pool-B	Pool-C, Pool-D

[a] One week of analyses per month during four months.

pre-clinical assay, ten consecutive days of routine use were performed. They involved the same two operators. However key differences with the method validation step can be reported and are summarized in Table 4. The first one is that another pool of plasma was used to prepare the calibration standards and the QC samples than the one used during method validation, therefore providing additional and different sources of uncertainty potentially arising from the bio-matrix even if the selectivity of the method was tested on six different sources of plasma during the validation phase. A second difference is that two pre-columns and two analytical columns were needed during this trial while only one of each was used during method validation. For the second pre-clinical trial, two operators were also involved. However one of them was not part of the method validation. The analysis of the samples of this second trial was realized during four months (one week of analyses per month) with two different pools of plasma thus also increasing the potential sources of uncertainty. Indeed, different batches of reagents, chemicals and solvents were needed. Additionally, six pre-columns were used together with three analytical columns further increasing the sources of uncertainty. It is evident from these observations that the sources of measurement uncertainty included during the validation of bioanalytical methods are not all those that can be encountered during the daily application of the method. However the underestimation of measurement uncertainty observed from the method validation in this case study has been shown to depend over the concentration of CDV. For the smallest QC level this underestimation was the smallest, even negligible for one of the routine applications.

4. Conclusions

By including design of experiments in the optimization strategy, an efficient novel chromatographic method for the determination of cidofovir in plasma has been developed. The HILIC separation was preceded by a solid phase extraction using mixed mode polymeric cation exchange sorbent necessary to clean up the biological samples and concentrate the analyte. This newly developed method was then fully validated according to FDA requirements by means of the Total Error approach that guaranteed that each future result will fall within acceptance limits of ±30% with a probability of 95% over a concentration range of 92.7–1020 ng/mL. This newly developed HILIC method has also been shown far more reliable over this range of concentration than two LC–MS/MS methods previously developed. Additionally, the routine application of the cidofovir assay in two pre-clinical trials demonstrated that the predictions made during the pre-study validation were very consistent. Actually the risk to observe results outside the ±30% acceptance limits was smaller than 5%. In order to ensure the trustiness of analytical results, measurement uncertainty was estimated. A comparison of measurement uncertainty estimates obtained from the method validation experiments versus those obtained during the two routine uses of the method was performed. This comparison indicated that method validation gave adequate measurement uncertainty estimations, allowing to predict correctly the results routine uncertainty. Nonetheless a slight underestimation of measurement uncertainty was seen for higher concentration levels indicating that although major sources of uncertainty were included into the method validation study, there still remained extra ones. These extra sources of uncertainty could nonetheless be included into the uncertainty estimates by using the QC samples included in each of the routine run performed during the analyses of the samples of the two pre-clinical trials. This emphasizes the need to build a validation protocol including the major sources of variations that can reasonably be encountered when using the method in routine analyses in order to allow method validation providing a good prediction of the analytical method's future behavior.

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