Comparing ultrafiltration and equilibrium dialysis to measure unbound plasma dolutegravir concentrations based on a design of experiment approach

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Dolutegravir therapeutic drug monitoring (TDM) could be improved by measuring the unbound dolutegravir plasma concentration (Cu), particularly in patients experiencing virological failure or toxicity despite achieving appropriate DTG total plasma concentrations. Equilibrium dialysis (ED) is the gold standard to measure Cu, but ED is time consuming, precluding its use in clinical practice. In contrast, ultrafiltration is applicable to TDM, but is sensitive to numerous analytical conditions. In order to evaluate measurements of Cu by ultrafiltration, ultrafiltration conditions were validated by comparison with ED. DTG concentrations were measured by LC–MS/MS. Three ultrafiltration factors (temperature, duration and relative centrifugal force [RCF]) were evaluated and compared to ED (25/37 °C), using a design of experiment strategy. Temperature was found to influence Cu results by ED (p = 0.036) and UF (p = 0.002) when results were analysed with ANOVA. Relative centrifugal force (2000 g) and time (20 min) interacted to influence Cu (p = 0.006), while individually they did not influence Cu (p = 0.88 and p = 0.42 for RCF and time). Ultrafiltration conditions which yielded the most comparable results to ED were 37 °C, 1000 g for 20 min. Ultrafiltration results greatly depended on analytical conditions, confirming the need to validate the method by comparison with ED in order to correctly interpret DTG Cu.

Dolutegravir (DTG) is an efficient and well-tolerated antiretroviral currently used in antiretroviral therapeutic strategies1,2. Clinical studies have confirmed that the efficacy of DTG depends on drug plasma exposure (concentrations)3,4 and several efficacy-related targets have been proposed5,6. However, virological failures have been reported despite total concentrations (Ct) deemed to be efficient and close to the values recorded in HIV patients without virological failure5–7. Furthermore, the toxic neuro-psychiatric events described in a general HIV population cannot be explained by differences in DTG Ct8. Thus, therapeutic drug monitoring (TDM) which is open

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containing DTG-d4 and then diluted in water (a fifth dilution). Analytical validation of DTG unbound fraction into the LC–MS/MS system. Following dialysis, the remaining plasma was extracted with 200 µL of methanol dialysate was diluted with 200 µL of mobile phase containing 1 ng/mL of DTG-d4. Fifty microliters were injected.

Sorensen buffer preparation protocol is described in supplemental data 2). The resulting plasma and buffer (pH 7.4), in a chamber set to a nominal and controlled temperature of 37 °C and rotated at 12 rpm for 4 h (the Visking Dialysis Tubing membrane (molecular mass cut-off: 12,000-14000 Da, Medicell Membranes Ltd, London, UK). A 1 mL plasma aliquot was dialysed against 1 mL of Sorensen phosphate buffer (pH 7.4), in a chamber set to a nominal and controlled temperature of 37 °C and rotated at 12 rpm for 4 h (the Sorensen buffer preparation protocol is described in supplemental data 2). The resulting plasma and buffer dialysates were then promptly recovered from the Teflon cells and analysed by LC–MS/MS. Fifty microliters of dialysate was diluted with 200 µL of mobile phase containing 1 ng/mL of DTG-d4. Fifty microliters were injected into the LC–MS/MS system. Following dialysis, the remaining plasma was extracted with 200 µL of methanol containing DTG-d4 and then diluted in water (a fifth dilution). Analytical validation of DTG unbound fraction (fu, calculated as the ratio of unbound substance on total concentration; fu = Cu/Ct) through ED is described in detail in a previous study and summarised in a supplemental figure (supplemental data 3).

Ultrafiltration. Unbound-DTG determined using UF was measured in parallel to ED (on the same day). Prior to plasma UF, filters were pre-washed at 20 °C with LC–MS grade water for 20 min at 2000 g, according to the manufacturer’s recommendations. Five hundred microliters of either a DTG spiked sample or patient plasma was incubated in a Centrifree (Millipore, Billerica, MA), with a molecular weight cut-off of 30,000 Da. The optimal UF conditions (temperature, time and relative centrifugal force [RCF]) were selected based on results from the reference method (ED at 37 °C). After UF, DTG was extracted from the plasma ultrafiltrate as previously described for the ED dialysate in the corresponding ED section above (see supplemental figure from supplemental data 3).

Equilibrium dialysis vs. ultrafiltration. Plasma samples used to compare both methods were spiked with 1 mg/L of DTG, which approximates concentration usually observed. Following plasma spiking, samples were stored at 25 or 37 °C prior to analysis, depending on the temperature of the planned sample analysis (ED or UF, at 25 or
ED 37 °C (37 °C, 1,000 g, 20 min). Vuf was predicted according to the same method previously described. Both conditions: (1) “short-duration” UF (37 °C, 1000 g, 5 min) and (2) UF condition providing comparable results to those obtained with equilibrium dialysis for n = 5 samples each. CV coefficient of variation, ANOVA analysis of variance, min minutes; g gravitational constant, °C temperature, CI95 confidence interval, RCF relative centrifugal force.

Table 1. Design of experiment comparing 8 conditions of ultrafiltration and 2 conditions of equilibrium dialysis for n = 5 samples each. CV coefficient of variation, ANOVA analysis of variance, min minutes; g gravitational constant, °C temperature, CI95 confidence interval, RCF relative centrifugal force.

| Condition | T (°C) | Time (min) | RCF (g) | Mean unbound fraction (%) | Unbound fraction, CV (%) [95 CI] | ANOVA | Dunnett test (p value) |
|-----------|--------|------------|---------|--------------------------|---------------------------------|-------|----------------------|
| Ultrafiltration | | | | | | | |
| 1 | 25 | 10 | 1,000 | 0.80 | 14.85 [14.74; 14.96] | Reference group | 0.044 |
| 2 | 25 | 10 | 2000 | 0.93 | 5.30 [5.26; 5.34] | | <10−4 |
| 3 | 25 | 20 | 1,000 | 0.87 | 7.63 [7.57; 7.69] | | 0.0262 |
| 4 | 25 | 20 | 2000 | 0.63 | 29.42 [29.25; 29.59] | | p<10−4 |
| 5 | 37 | 10 | 1,000 | 0.58 | 7.22 [7.16; 7.27] | | 0.97 |
| 6 | 37 | 10 | 2000 | 0.52 | 7.14 [7.10; 7.18] | | 0.46 |
| 7 | 37 | 20 | 1,000 | 0.65 | 7.46 [7.42; 7.50] | | 0.997 |
| 8 | 37 | 20 | 2000 | 0.85 | 6.45 [6.45; 6.49] | | 0.007 |
| Equilibrium dialysis | | | | | | | |
| 9 | 25 | – | – | – | 1.56 | 4.96 [4.89; 5.03] | | <10−4 |
| 10 | 37 | – | – | – | 0.64 | 6.61 [6.56; 6.66] | | Reference group |

Comparison of dialysate and ultrafiltrate matrix. As the same extraction procedure was applied to dialysate and ultrafiltrate samples, both matrices were compared. For that purpose, blank ultrafiltrates and dialysates were spiked with 1.5 and 75 ng/mL of DTG, respectively and extracted. The final DTG concentrations measured were subsequently compared.

Ultrafiltration method evaluation. The precision and accuracy of UF was determined using two kinds of samples (spiked and patient plasma samples). Inter-day assays were performed on spiked and patient samples on four different days, both by UF and ED (as reference method). The ratio of UF/ED fu (R_{UF/ED}) was calculated for each sample. The accuracy, defined as (mean R_{UF/ED} − 1) × 100, and the precision, defined as (SD/mean R_{UF/ED}) × 100 (SD = R_{UF/ED} standard deviation), were calculated. To date, there is no official guideline to evaluate accuracy/precision of the ultrafiltration method. A consortium of pharmaceutical companies recommends a precision fu CV < 30%. Based on FDA bioanalytical guidelines (for analytical procedures), a value of 100 ± /− 20% was used as a criterion for accuracy. The accuracy of UF results was evaluated in relation to ED, which is the reference method to evaluate fu. It should be noted that the assumption of concordance between fu ED 37 °C/in vivo has not been verified using current procedures.

To evaluate DTG NSB, a LC/MS grade water solution was spiked with DTG at 1 mg/L and then serially diluted to yield samples of: 0.1, 0.01 and 0.001 mg/L. The didotegravir concentration was measured in five replicate samples of each of these individual dilutions. Assays were performed at 25 and 37 °C in parallel. Non-specific binding was calculated as follows: NSB(%) = (1 − ultrafiltrate concentration/initial concentration) × 100. The weight of the ultrafiltrate obtained was used to calculate V_{ultr}. The density of the ultrafiltrate was extrapolated by comparison to the weight of 100 µL of V_{ultr} which did not contain any DTG.

In order to achieve V_{ultr} lower than 50% of the initial plasma volume, a short-duration (five minutes) UF assay was evaluated, independently of the DOE assays. Five plasma samples were each spiked with 1 or 0.1 mg/L of DTG. Ultrafiltration was then performed on five repeats of each of these concentrations at the following two conditions: (1) “short-duration” UF (37 °C, 1000 g, 5 min) and (2) UF condition providing comparable results to ED 37 °C (37 °C, 1,000 g, 20 min). V_{ultr} was predicted according to the same method previously described. Both total and unbound DTG concentrations were measured.
IQR [0.77; 0.91] and m = 0.61% IQR [0.55; 0.75] as well as for UF at 25 and 37 °C, respectively; p = 0.002), regard-

lysed independently, no significant differences were observed in either Vuf (< 0.05. Statistical analyses were performed with the R software (3.5.1)22.

level of significance was set at p < 0.05. Statistical analysis. Analysis of variance (ANOVA) and a normality test were performed. Where the ANOVA test was statistically significant, Dunnett’s test was subsequently performed in order to identify a potential interaction. Results were expressed as median InterQuartileRange [25%; 75%] and 95% confidence intervals).

Whenever possible, results were associated with a paired t test and expressed as the mean and standard deviation (m ± sd). The relationship between two quantitative variables was assessed using Pearson’s correlation test. The influence of confounding parameters on fu results. In order to explore the effect of a first freeze/thaw cycle on DTG/protein binding, assays were conducted on five DTG TDM samples. We evaluated any changes in pH, before and after thawing. A pH meter from Siemens RapidPOINT® 500 controller was used to measure pH.

Unbound DTG concentrations were measured using the following UF protocol (37 °C, 1,000 g during 20 min; comparable to ED results).

Temperature (25/37 °C) is a parameter that may disrupt drug binding to plasma proteins12, as it is the case for calcium (Ca) binding to albumin18. This is particularly significant as a binding interaction (chelation) between Ca and DTG has been previously described13. In order to explain variations of DTG fu in ED according to temperature, pH and ionised Ca were measured on paired plasma samples (n = 5) at 25 and 37 °C. Plasma measurements were performed on the Siemens RapidPOINT® 500 controller. Sample temperature was maintained at 37 °C with a device routinely used for the determination of serum cryoglobulins. Ionised Ca is defined as free Ca which is able to bind to DTG.

Equilibrium dialysis and ultrafiltration of HIV patient samples. Once the conditions for achieving similar results between UF and ED were identified for spiked plasma, they were applied to HIV patient samples (n = 16). Immediately after being received at the laboratory, blood samples were centrifuged at 2000 g, + 20 °C for 15 min then the plasma was aliquoted and stored at − 20 °C until required for analysis.

Moreover, since samples (for analysis in a routine hospital laboratory) may not always contain the volume required (500 μl) to perform UF; the unbound form was also determined on duplicate 250 and 500 μL HIV patient plasma samples (n = 5). Assays for both volumes were performed in parallel on the same day.

This was a non-interventional study which did not require any additional procedures to be performed. Dolutegravir TDM and data collection were part of routine patient care. For these reasons, no Institutional Review Board or Ethics Committee approval was required, in accordance with French legislation governing biomedical research19,20.

Results

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Table 2. Results of mean ultrafiltrate volume under different temperature conditions. ANOVA analysis of variance, g gravitational constant, CV coefficient of variation..

| Condition       | Temperature | Volume (µL) mean; CV (%) | ANOVA  | Dunnett test (p value) |
|-----------------|-------------|--------------------------|--------|------------------------|
| 1 (1,000 g 10 min) | 25          | 252; 6.3                 |        | p = 0.006              |
| 2 (1,000 g 20 min) | 25          | 264; 9.9                 |        |                        |
| 3 (2,000 g 10 min) | 25          | 265; 13.1                |        |                        |
| 4 (2,000 g 20 min) | 25          | 316; 11.3                |        |                        |
| 5 (1,000 g 10 min) | 37          | 257; 6.1                 |        | < 0.001                |
| 6 (1,000 g 20 min) | 37          | 289; 7.7                 | p < 10^-4 |                        |
| 7 (2,000 g 10 min) | 37          | 296; 17.0                |        | 0.0421                 |
| 8 (2,000 g 20 min) | 37          | 351; 8.4                 |        | Reference group        |

Statistical analysis. Analysis of variance (ANOVA) and a normality test were performed. Where the ANOVA test was statistically significant, Dunnett’s test was subsequently performed in order to identify a potential interaction. Results were expressed as median InterQuartileRange [25%; 75%] and 95% confidence intervals). Whenever possible, results were associated with a paired t test and expressed as the mean and standard deviation (m ± sd). The relationship between two quantitative variables was assessed using Pearson’s correlation test. The level of significance was set at p < 0.05. Statistical analyses were performed with the R software (3.5.1)25.

Results

Equilibrium dialysis vs. ultrafiltration. Results of the full design of experiment (DOE) analysis, comparing ED and UF are presented in Table 1. Similar results were obtained with an UF of 20 min at 1,000 g and at 37 °C (condition 7). In addition to the statistical outcome, this condition has also been chosen to stress the similarity of the result with ED at 37 °C and the low variability between results.

Temperature variations yielded statistically different free fraction (fu) results for both ED (m = 1.56% IQR [1.48; 1.57] and m = 0.64% IQR [0.61; 0.65], for ED at 25 and 37 °C, respectively; p = 0.036) and UF (m = 0.88% IQR [0.77; 0.91] and m = 0.61% IQR [0.55; 0.75]) as well as for UF at 25 and 37 °C, respectively; p = 0.002), regardless of duration and RCF.

A comparison of UF temperatures revealed statistically significant differences in Vuf (m = 274 ± 28.2 µL and m = 298 ± 38.8 µL for UF at 25 °C and 37 °C, respectively; p = 0.036).

Furthermore, regardless of temperature conditions, the interaction of RCF (2000 g) and duration (20 min) of UF resulted in statistically different fu, compared to other conditions (p = 0.006 and < 10^-4, at 25 and 37 °C, respectively) (Table 1). Ultrafiltrate volumes were higher in UF conditions performed at 2000 g and for 20 min, compared to the Vuf of other conditions (Table 2). However, when RCF and duration of centrifugation were analysed independently, no significant differences were observed in either Vuf (p = 0.588 for centrifugation time 10 vs. 20 min and p = 0.882 for RCF 1,000 vs. 2000 g) and fu results (p = 0.42 for centrifugation time 10 vs. 20 min and...
\[ p = 0.88 \text{ for RCF 1,000 vs. 2,000 g). In this instance, combined variations of duration and RCF (2,000 g for 20 min) influenced V_{ul} and therefore the unbound fraction result, whereas individually, those conditions had no impact.}

In condition 8, the V_{ul} was higher compared to the other conditions (at both 25 and 37 °C; \( p < 10^{-4} \); Dunnett’s test with condition 8 as reference, \( p < 0.05 \); for condition 4, only a trend was observed with a mean difference of \( m = 0.035 \text{ mg} \)).

**Comparison of dialysate and ultrafiltrate matrix.** Analytical extraction of DTG from the dialysate or ultrafiltrate was comparable for both low DTG spiked (\( m = 1.70 \pm 0.05 \text{ ng/mL and } m = 1.78 \pm 0.03 \text{ ng/mL for dialysate and ultrafiltrate; } p = 0.095 \)) and high DTG concentrations (\( m = 80.83 \pm 1.36 \text{ ng/mL and } m = 81.93 \pm 2.84 \text{ ng/mL for dialysate and ultrafiltrate; } p = 0.578 \)).

**Ultrafiltration method evaluation.** Accuracy and precision of UF method were 121.5% and 27.2%.

Results revealed NSB ranging from 0.1% to 8.2% over the range of concentrations tested. NSB were comparable regardless of DTG concentration and/or temperature (\( p = 0.317 \)).

Five minutes UF led to lower V_{ul} than a UF duration of 20 min (\( m = 224.5 \text{ mL IQR[212.5; 239.4] and } m = 385.3 \text{ mL IQR[368.7; 417.3] for 5 min and 20 min respectively; } p < 10^{-4} \)). While a decrease in V_{ul} and a shorter duration of UF was associated with higher fu (\( u = 2.32 \text{ IQR[2.19; 2.45] and } m = 1.83 \text{ IQR[1.71; 1.90] for 5 min and 20 min respectively; } p < 10^{-4} \)). The relative V_{ul} and fu increase, from 5 to 20 min-conditions, was 43 and 21%, respectively. The variation of fu over time, with the same temperature and RCF condition, was not linear. Between 5 and 20 min, fu decreases and between 10 and 20 min, fu increases (results detailed Table 1; comparison of results from conditions 5 and 7, \( p = 0.036 \)).

**Influence of confounding parameters on fu results.** An increase in pH was observed after one freeze/thaw cycle (proportion of pH increase \( m = 6.28 \text{ IQR[3.71; 8.61]; pH level } m = 7.45 \text{ IQR[7.35; 7.55] and } m = 7.92 \text{ IQR[7.75; 8.15], before and after thawing, respectively; for pH level, mean and standard deviation of differences } m = 0.47 \text{ sd } 0.28; p = 0.006 \)). Unbound DTG concentrations measured before freezing ranged from 6.62 to 42.00 µg/L and remained unchanged after thawing (mean of relative differences \( m = 0.023; \text{ paired t-test } p = 0.586 \)).

At 25 °C pH was significantly lower, compared to measurements made at 37 °C (=+ 0.7% IQR[+ 0.5; + 0.8] for median pH increase; \( m = 7.53 \text{ IQR[7.45; 7.54] and } m = 7.57 \text{ IQR[7.49; 7.60] for median pH in plasma at 25 °C and } m = 0.01 \text{ respectively; } p = 0.001 \text{ paired samples t-test)}. Associated to lower pH, ionised Ca was significantly increased at 25 °C, compared to measurements made at 37 °C (\( m = −7.4 \text{ IQR[−8.5; −6.5] for median ionised Ca decrease } = 1.97 \text{ mM IQR[1.06; 1.11] mM and } m = 1.00 \text{ mM IQR[0.98; 1.04] for median ionised concentration in plasma at 25 °C and } m = 0.003 \text{ paired samples t-test).}

**Equilibrium dialysis and ultrafiltration on HIV patient samples.** The patient dosage regimen was only based on 50 mg once a day. The seventh condition (following UF conditions: 37 °C with settled centrifugation at 1,000 g for 20 min) was then applied to HIV patient samples. Free fraction results did not differ statistically between UF (37 °C, 20 min and 1,000 g) and ED (37 °C) (\( m = 0.44 \pm 0.06 \% \text{ and } m = 0.47 \pm 0.02 \% \text{ for ED and UF, respectively; } p = 0.685 \)). Comparison of 250 and 500 µL plasma volumes for UF did not reveal any statistically significant differences between fu results (\( m = 0.52 \pm 0.09 \% \text{ and } m = 0.60 \pm 0.13 \% \text{ for 250 and } m = 0.89, \text{ t-paired test)}. No relationship (\( p = 0.661 \)) was observed between fu and Ct (range of Ct: 0.8 to 6 mg/L).

**Discussion**

In our study, the two most commonly performed techniques for studying protein-drug binding were used and compared to determine the free DTG concentration. Equilibrium dialysis was used as the gold standard\(^{12,13}\) to evaluate and set the temperature, RCF and duration of centrifugation for UF.

Out of the three test parameters, temperature is the parameter most likely to influence fu results determined by UF\(^{12,23–31}\). However, because temperature could also modify the BE\(^{24}\), UF and ED were both compared in our study at two different temperatures, namely 25 and 37 °C. Since it allows approximation of the in-vivo condition, 37 °C is therefore considered as the reference temperature\(^{24}\). Consequently, 37 °C was the temperature selected for UF. Even if condition 4 (25 °C) provided results similar to those obtained with ED at 37 °C, this condition was rejected because (1) the temperature was not within the physiological range (37 °C), (2) the V_{ul} was >60% of initial plasma volume (i.e. it could create a disruption in BE during UF\(^{12}\)) and (3) this condition was associated with greater analytical variability.

Surprisingly and contrary to previous observations\(^{27,31,32}\) and results from our study (ED assays), increasing the temperature (from 25 to 37 °C) resulted in lower fu. Temperature is a parameter which conditions pH level and may thereby interfere with substance/protein BE\(^{13}\). In our study, pH fluctuations were not found to affect DTG binding. The increase in ionised Ca at 25 °C, compared to 37 °C may have altered the DTG/protein BE and consequently fu. Indeed, DTG like tigecycline\(^{33}\) chelates divalent cations (i.e. Ca, Mg, etc.). It may be hypothesised, that the increase in Ca in vitro may increase the amount of Ca bound to DTG. Consequently, DTG/protein BE is disrupted and the concentration of DTG-bound to protein is reduced. As the ED membrane between plasma/buffer compartments is permeable to DTG-Ca, this complex is also found in the buffer compartment. A new BE is achieved and the same amount of DTG/Ca is recovered on both sides of the membrane. The decrease in bound DTG associated with the increase of DTG in the buffer compartment may explain that fu at 25 °C is higher compared to 37 °C. In addition to the indirect effect observed in ED, temperature also
affected the UF process. This effect was particularly noticeable with conditions 4 and 8 (2000 g/20 min at 25 and 37 °C, respectively; higher fu at 37 °C compared to 25 °C). Indeed, unexpected effects of temperature on fu at those “extreme” conditions of UF were observed (fu decreased at 25 °C and increased at 37 °C) compared to other UF conditions and ED. As neither pH, ionised Ca nor NSB could explain these results, temperature may have a direct influence on the UF process. According to Poiseuille’s law and as illustrated by Cinar et al.34 with blood plasma, fluid viscosity decreases with increasing temperature. At 25 °C, condition 4 (“extreme” condition 2000 g/20 min) led to a larger Vuf compared to other conditions at the same temperature (1, 2 and 3). One can assume, as observed with 5/20 min UF assays, that such an increase in Vuf resulted in diluting the DTG contained in the ultrafiltrate. The fu decrease observed with condition 4, compared to other conditions, could therefore be explained by dilution of the ultrafiltrate. Conversely, at 37 °C, plasma viscosity is lowered and could explain even higher Vuf at condition 8 (2000 g 20 min), compared to the other conditions (both 25 and 37 °C). This Vuf greatly exceeded 50% of the initial plasma volume. The effect of such extreme UF on the BE is currently not well understood35. A disruption in DTG/protein BE could occur at condition 8, leading to an increased amount of DTG in the ultrafiltrate. As a result, the DTG fu also increases, as observed in our assays.

Besides temperature, RFC/duration interaction, which were poorly evaluated25,27-29,31, or a short duration (5 min) resulted in an increase of both fu and Vuf. Vuf has been suggested as an indirect flag of the effect of UF on DTG/protein BE32. However, only sparse information is available relating to fu variations for Vuf > 50% (comparing with the initial plasma volume) and only high Vuf (> 80%) should be associated with BE disruption. Moreover, fluctuations in duration did not consistently have the same effect on Vuf and fu. Thus, variations of Vuf/fu observed with DTG are comparable to those reported by Di et al.28 with vancomycin. These authors attributed this result to a BE disruption. Such a hypothesis is however not consistent with our results. Indeed, we found that fu decreased between 5 and 20 min while it increased between 10 and 20 min (at 1,000 g and 37 °C in both cases). The non-linear variation of fu over UF duration is therefore not consistent with a BE disruption at those “extreme” conditions of UF were observed (fu decreased at 25 °C and increased at 37 °C). Indeed, unexpected effects of temperature on fu were examined12,33. Non-specific binding of our UF method could be considered low, so there is no need to integrate a correction of the fu result38. This result could be attributed to the weak lipophilicity of DTG9,35. This result was comparable to that observed with our dialysis technique16. Moreover, NSB conditions leading to identical results as ED at 37 °C for whole substances, has been identified to date. Even if the concordance of fu from ED and in vivo cannot be verified, ED is considered to be the gold standard to determine fu. Thus, for an accurate interpretation of unbound DTG concentration results, an experimental comparison of UF vs ED appears to be essential. The predicted effects of temperature, RFC and UF duration on fu results are summarised in Figure S1.

Based on these results, UF conditions were set at 37 °C, for 10 min at 2000 g. Besides, as the effects of some conditions, such as high pH observed during prolonged sample storage, were not explored in this study, it is recommended to use fresh plasma to study DTG fu. To complete results from DTG spiked samples, unbound forms from HIV patient plasma samples were also measured by both ED at 37 °C and UF at pre-defined conditions. Patient results validated UF to confirmed determination of the unbound form of DTG by UF. Moreover, two volumes (250 and 500 µL) were evaluated and validated for routine use of low volume samples.

The extraction procedure with the spiked dialysate buffer or ultrafiltrate did not reveal any interference from the solution matrices. This validation was necessary since their composition was relatively different, in particular the dialysate. Sorensen buffer mainly comprises a phosphate buffer whereas UF is a plasma ultrafiltrate, which therefore has a lower phosphate content46. However, this validation method is not described in the few articles comparing the two techniques30 although phosphate buffer could be a major limiting factor for LC–MS techniques due to an ion-suppression phenomenon37. In addition, in our method validation process, the first freeze/thaw cycle led to an increase in plasma pH. However, such an increase did not impair DTG/protein binding, in contrast to previous observations12,23. Non-specific binding of our UF method could be considered low, so there is no need to integrate a correction of the fu result38. This result could be attributed to the weak lipophilicity profile of DTG35. This result was comparable to that observed with our dialysis technique16. Moreover, NSB were consistent, regardless temperature (25 °C or 37 °C) and/or DTG concentration. Conditions used during UF allowed accurate determination of fu, compared to ED results obtained when applying recommendations from pharmaceutical company consortium (precision CV < 30%)49.

Despite promising results from our DOE analysis, our study did have some limitations. Indeed, we did not explore the effect of higher total DTG concentrations on UF results. However, since (1) fu results did not vary with total concentrations in HIV patient samples and (2) albumin physiological levels (close to 650 µM)12 are more than 50 times higher than the DTG total concentration (9.58 µM for the maximal concentration generally observed, close to 4 mg/L), a protein-binding saturation at the standard DTG concentration is unlikely. Unfortunately, this result is not as powerful as performing a DOE evaluation and this interpretation should be considered with caution. Another limitation of our study was the evaluation of only two levels for each of the factors examined (temperature, RCF and duration). This approach does not allow the identification of non-linear relationships between parameters (e.g. temperature) and response (fu result), because results from only two levels per factor in a DOE cannot be extrapolated outside of the study conditions.

To date, few data are available on DTG unbound forms in HIV patients. Using an ED technique, at the same temperature, Imaz et al.11, found median fu values similar to ours (0.46% vs. 0.44%). In contrast, the Letendre et al.14 study which does not describe any device or analytical procedure used, observed a higher fu compared to Imaz et al.11 and our results (fu = 0.70% at the sixteenth week). Higher fu values from the Letendre et al.14 study were mainly driven by higher unbound concentration values compared to our results and results from Imaz et al.11, while their Ct values were comparable to our results. The discrepancy between published results is similar to that observed in our study between ED and UF without set conditions. This difference raises the issue of an appropriate interpretation of the unbound form of DTG. Indeed, unbound concentrations were measured
and used (1) to explain DTG tissue diffusion (e.g. CSF and genital organs) and/or (2) to evaluate drug efficacy by comparing an unbound concentration to an in vitro inhibitory concentration (typically IC<sub>50</sub>). As a consequence, if the unbound concentration is over-estimated, particularly if using an UF method, the truly effective concentration (unbound) in an anatomical compartment could be misinterpreted. This misreading could introduce a bias in the PK-PD analysis and therefore in the selection of dosing regimen for antiretroviral strategies.

While unbound concentrations have been deemed effective concentrations, PK/PD study is still confined to a relationship between total concentration and viral load, both in terms of kinetics of decrease or rebound of viral load during virological failure. This lack of interest in the unbound drug form mainly stems from pre-analytical and analytical constraints. Indeed, the measurement of unbound concentration requires a more technically intensive bioanalytical approach than determining total concentrations. Where studies require the unbound concentration to be measured, the most convenient method, namely UF, is predominantly chosen for the analysis. From an organisational point of view, this method is easy and quick to use. But, as stressed in our study, UF may exhibit several analytical disadvantages in the form of ultrafiltration issues involving set UF conditions or NSB. As has been recommended by several authors, it is imperative to validate UF conditions versus ED at 37 °C for each individual drug investigated. Many studies have to date explored the unbound form of ARVs, but technical procedures used to measure unbound concentrations are not always described and only a few of these studies compared UF to ED. The lack of validation of the UF procedure may potentially introduce a bias when interpreting results or when making comparisons with previously published data. Despite a call from Boffito et al., guidelines on the bioanalytical validation of unbound drug measurements, such as those established by the FDA or EMEA for total drug concentrations, still remain to be established. Recommendations should therefore focus on deficiencies which preclude standardised evaluation of unbound forms. This could be the first step in furthering PK-PD studies based on the pharmacologically active form of a drug.

**Conclusion**

Our study defines pre-analytical and analytical conditions which facilitate a more uniform measurement of free DTG concentrations between UF and ED. Temperature, duration and RCF were identified as interfering factors, thereby highlighting the merits of simultaneously validating UF and ED protocols for the investigation of individual drugs.

Received: 28 September 2018; Accepted: 30 June 2020

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Author contributions
D.M., D.C. and P.G. devised and designed the study. D.M. and M.A. performed the experiments. G.M.-B., T.L., V.G., E.C., P.D. and A.R. contributed technical support. D.M., F.F. and A.R. drafted the initial manuscript. T.L., V.G., E.C., P.D. and F.F. reviewed and revised the final manuscript. All of the authors approved the final manuscript submitted.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-69102-y.

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