Trial@home for children: novel non-invasive methodology for the pediatric clinical trial of the future

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CHAPTER 13

Population pharmacokinetics of clonazepam in saliva and plasma - steps towards non-invasive pharmacokinetic studies in vulnerable populations

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Abstract

INTRODUCTION Traditional studies focusing on the relationship between pharmacokinetics (PK) and pharmacodynamics necessitate multiple blood draws which are too invasive for children or other vulnerable populations. A solution is to use non-invasive sampling matrices, such as saliva. The aim of this study was to develop a population PK model describing the relationship between plasma- and saliva clonazepam kinetics and assess whether the model can be used to determine trough plasma concentrations based on saliva samples.

METHODS Twenty healthy subjects, aged 18-30, were recruited and were administered 0.5mg or 1mg of clonazepam solution. Paired plasma- and saliva samples were obtained until 48h post-dose. A population pharmacokinetic model was developed describing the PK of clonazepam in plasma and the relationship between plasma and saliva concentrations. Bayesian maximum a posteriori (MAP) optimization was applied to estimate the predictive accuracy of the model.

RESULTS A two-compartment distribution model best characterized clonazepam plasma kinetics with a mixture component on the absorption rate constants. Oral administration of the clonazepam solution caused contamination of the saliva compartment during the first 4 hours post-dose, after which the concentrations were driven by the plasma concentrations. Simulations demonstrated that the lower and upper limits of agreements between true and predicted plasma concentrations were -28-36% with 1 saliva sample. Increasing the number of saliva samples improved these limits to-18-17%.

CONCLUSION The developed model describes the salivary-and plasma kinetics of clonazepam and can predict steady-state trough plasma concentrations based on saliva concentrations through Bayesian MAP optimization with acceptable accuracy.

Introduction

Traditional studies focusing on pharmacokinetics in plasma necessitate multiple blood samples and therefore may become too invasive for a pediatric population. However, the determination of pharmacokinetic-pharmacodynamic (PKPD) relationships between a drug and an endpoint is an important method for evaluation of drug effects and dose optimization. A possible solution is to determine drug concentrations in sampling matrices that can be collected in a non-invasive manner, such as saliva. Salivary kinetics have been determined in a wide range of compounds, and applications in the field of therapeutic drug monitoring (TDM) have been explored.[1-3] However, analyses have been mainly performed by calculating the plasma concentrations based on a constant ratio over time with the saliva concentrations. Nonlinear mixed effects models have potential advantages, such as accounting for a changing ratio over time and different sources of variability, in the evaluation of the population kinetics in plasma and saliva, and this approach could potentially also be used to improve the prediction of plasma concentrations when only saliva concentrations are available.

Clonazepam is a GABA-A positive allosteric modulator used to treat a range of clinical conditions, such as epilepsy, panic disorder, depression, and bipolar disease.[4,5] Although clonazepam has been prescribed less often in recent years[6], the drug is a candidate for rediscovery in other conditions. For example, pre-clinical evidence shows possible efficacy of clonazepam in ARID1B-related intellectual disability (ID).[7] Conducting clinical trials in a (pediatric) intellectually disabled population is challenging, as trial designs must be unobtrusive to motivate as many patients as possible for participation.[8,9] Inclusion of non-invasive pharmacokinetic assessments may help in this respect.

The aims of this study were to study the population kinetics of clonazepam, to determine the relationship between salivary- and plasma clonazepam concentrations and to investigate the performance of a population PK model describing this relationship to determine trough plasma concentrations based on saliva samples in patients treated with clonazepam.

Materials and Methods

Location and ethics

This study was conducted in preparation of a study researching the therapeutic effects of clonazepam on patients with ARID1B-related ID[7,10] at the Centre for Human Drug Research in Leiden, the Netherlands from June 2020 until July 2020. Ethical approval was obtained from the Beoordeling Ethiek Biomedisch Onderzoek Foundation Review Board (Assen, the Netherlands) prior to initiation of the study. The study was conducted in compliance with the Dutch Act on Medical Research Involving Human Subjects and Good Clinical Practice. Informed consent was obtained prior to study-mandated procedures.

Study design and sample collection

This was an open-label, single dose study in 20 healthy subjects aged 18-30. Exclusion criteria were history of disease- or use of medications that might interfere with saliva production, such as opiates and anticholinergics. Subjects were asked to refrain from alcohol- and caffeine use for 24 hours prior to drug administration until the end of the study, and from any nutrients with CYP-modulating activity for three days prior to drug administration. Subjects were administered a single dose of 0.5 mg (n=10), or 1.0 mg (n=10) clonazepam solution (Rivotril®) dissolved in lemonade, after which paired plasma- and saliva samples were taken at 0.5h, 1h, 2h, 4h, 6h, 8h, 24h and 48h post dose. Subjects thoroughly rinsed their mouth with water 10 minutes prior to saliva sampling. Saliva samples were obtained using the SalivaBio Infant Swab (Salimetrics, Carlsbad, CA, USA) according to the manufacturer's instructions.

Bioanalytical assay

Clonazepam (1 mg/mL, provided by Duchefa Farma (Haarlem, The Netherlands)), clonazepam-d4 (0.1 mg/mL, provided by LGC Standards (Luckenwalde, Germany)), methanol and acetonitril (both provided by Merck BV (Darmstadt, Germany)) were obtained. Assay validation was performed in accordance with European Medicines Agency (EMA) guidelines.[11] From the clonazepam solution, standards were prepared in saliva at the concentrations of 0.1, 0.5, 2.5, 5, 10 and 20 µg/L. The internal standard clonazepam-d4 was diluted with methanol to a final concentration of 30 µg/L. The lower limit of quantification (LOQ), low, medium, and high reference samples were prepared in saliva with a concentration of resp. 0.1, 1, 5 and 15 µg/L. For the measurement of the plasma samples Recipe ClinChek calibrators were used and controls with a linear range of 2 - 72.3 µg/L and the LLQ, LOW, MED and HIGH with a concentration of resp. 2, 5.25, 14.8 and 48.1 µg/L.

Sample preparation of saliva samples was performed by diluting each aliquot of 20 µL saliva with 20 µL internal standard solution in eppendorf cups, which were vortexed for 1 min and centrifuged at 13000 rpm for 5 min. The extract was transferred in vials with insert. For plasma samples, each aliquot of 50 µL plasma was diluted with 50 µL internal standard solution and 150 µl acetonitril in eppendorf cups. The eppendorf cups were vortexed for 1 min and centrifuged at 13000 rpm for 5 min. The extract was transferred in a vial with 150 µl water.

Analysis was performed via Liquid chromatography-mass spectrometry (LC-MS). Extracts (2 µL) were injected onto a Thermo Scientific Hypersil GOLD C18 column, with methanolic mobile phase gradient elution. Clonazepam was detected with a Thermo Scientific TSQ Quantiva triple quadrupole mass spectrometer with positive ionization. Ions monitored in the selected reaction monitoring mode were m/z 316-270 for clonazepam (at 2.97 min) and m/z 320-274 for clonazepam-d4 (at 2.96 min).

Pharmacokinetic modelling

A population PK analysis was performed with a sequential nonlinear mixed effects modelling approach using NONMEM® (version 7.3).[12] Structural plasma model selection was performed by fitting both 1- and 2-compartment models to the plasma concentrations over time. An allometric scaling component (normalized around 70kg) was included on clearance and inter-compartmental clearance, with an exponent of 0.75, and on all volume of distribution parameters, with an exponent of 1, to account for weight-based influences. As only oral data was available, no bioavailability component could be estimated. However, variability on the relative bioavailability (F_{PLASMA}) could still be explored on this parameter. Inter-individual variability following a lognormal distribution on population parameters were selected by a forward inclusion procedure ($p < 0.01$) and the residual error structure was introduced as proportional and checked for appropriateness with goodness-of-fit figures.

The empirical Bayes estimates of the developed plasma model were used for the development of the saliva model. Model structure selection was driven by exploratory figures of

the data and contained a constant plasma:saliva ratio, a non-linear plasma:saliva ratio, and a first-order elimination component to account for the contamination in the saliva compartment immediately after dosing. Inter-individual variability following a lognormal distribution were selected following the same procedure as with the plasma model. Improvements in model fit were judged on a decrease in objective function value (OFV) of 6.64 ($p < 0.01$) after inclusion of 1 parameter, numerical stability as judged by the relative standard errors (RSE) of parameters and shrinkage, and evaluation of goodness-of-fit figures.

After the saliva model was developed, both models were estimated simultaneously, and model predictions were assessed via a prediction-corrected visual predictive check (PCVPC) and the individual model fit over time in both matrices.

Simulations

R version 4.0.2[13] and the mrgsolve package[14] were used to simulate the predictive capability of saliva concentrations in the context of clinical trials. A simulation cohort (n = 2000) with a uniform distribution of age between 6 and 30 and corresponding weights (10TH-90TH centile[15]) was prepared, and twice daily administration of 0.015 mg/kg (max 0.5 mg per dose) was simulated for each subject. As inter-individual variability on the relative bioavailability (F_{PLASMA}) of clonazepam might be lower in the healthy population on which the model was built, this parameter was increased to a coefficient of variation of 50% to allow for the simulation of a wider range of trough concentrations and therewith provide a more conservative simulation. Simulated saliva samples were obtained at 5h, 6h, 8h, 10h and 11.5h post-dose. The accuracy of predicting the trough concentration (C_{TROUGH}) in plasma after dose 1 (based on samples obtained after dose 1) and at steady state (based on samples obtained during steady state) that could be obtained by using 1-5 saliva samples was assessed using Bayesian maximum a posteriori (MAP) optimization and traditional linear regression. An additional scenario (0 samples) without saliva sampling was simulated to establish baseline predictive capability of the model based on population parameters and the weight of a subject, but without any TDM sampling information.

Bayesian maximum a posteriori optimization

The simulated saliva concentrations of each simulated individual were used as input for the Bayesian MAP estimation. During this process, the optimal F_{PLASMA} was estimated for each individual within the constraints provided by the several levels of residual- and between-subject variability in the population PK model, based on the information provided by the saliva samples and covariates.[16] From the individual Bayes estimates, the corresponding plasma C_{TROUGH} after dose 1 (based on samples obtained after dose 1) and at steady-state (after 240 hours of twice daily dosing, based on samples obtained after dose 20) was calculated. As the true simulated Bayes estimates were known for an individual, the estimated C_{TROUGH} were compared with the true plasma C_{TROUGH} to evaluate the predictive performance of this approach. Predictive performance was quantified for each sampling scenario using the root mean squared prediction error (RMSPE), which is a predictive error expressed in the original units (ug/L). Additionally, the average bias and limits of agreement (LOA) of the predictions, expressed as percentage, were calculated according to the methods of Bland and Altman.[17]

Linear regression

Traditionally, plasma:saliva relationships are calculated as a constant ratio or linear regression formula. In order to compare these traditional methods with Bayesian optimization, a linear relationship between the two matrices was estimated via a linear mixed model based on the plasma- and saliva samples obtained during the study. In the model, saliva concentration was considered as fixed effect and subject as random intercept. The derived equation was used to predict plasma concentrations in the simulation cohort after dose 1 based on a single saliva trough samples obtained either at 11.5h post-dose after dose 1, or at 11.5h post-dose at steady state.

Results

Of the 20 subjects included in the study, 9 subjects were male, and the average age was 22 years. Other baseline characteristics are displayed in *Table 1*. Of the 160 saliva samples taken during the study, 154 provided enough volume for analysis. All 160 plasma samples were collected successfully and none of the post-dose saliva or plasma concentrations were below the LOQ. The concentration-time profiles of clonazepam in plasma and saliva are displayed in *Figure 1.* Plasma concentrations showed variability in the C_{MAX} and t_{MAX}, with some subjects immediately reaching the C_{MAX} at the first sample (30 minutes) after dosing. Salivary concentrations were high and could not be correlated with plasma

concentrations directly post-dose, which indicates that clonazepam contamination was present in the saliva. However, the salivary concentration decreased exponentially and appeared correlated with plasma concentrations after 4 hours post-dose.

Figure 1. Individual-and mean (SD) concentration-time profiles of clonazepam in plasma and saliva.
A: Plasma concentration over time for the 0.5mg dose group (left panel) and 1.0mg dose group (right panel).
B: Saliva concentration over time for the 0.5mg dose group (left panel) and 1.0mg dose group (right panel).
Individual concentration-time profiles are displayed as light gray lines. The bold line and dots represent the mean (± SD) concentration on each timepoint. Each gray dot represents a single observation. Each grey line represents a single subject.

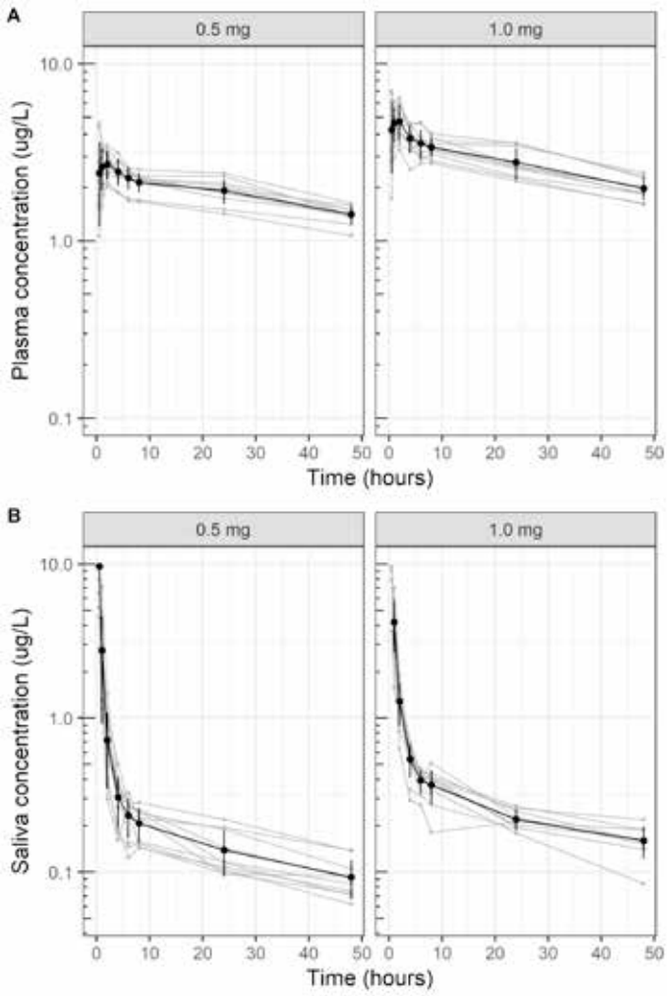

Table 1. Baseline characteristics

Parameter	All subjects (n=20)
Age (years)	22.4 (2.8)
Sex (% male)	45%
Ethnicity (% Caucasian)	100%
Weight (kg)	67.8 (8.3)
Height (cm)	175.1 (7.3)
BMI (kg/m2)	22.2 (2.4)

Data is presented as mean (SD), unless otherwise specified

Population Model

Structural plasma model development resulted in a 2-compartment model which fitted the data best, with a δOFV=-17.34 compared to a 1-compartment model. Inter-individual variability was identified on, in order of inclusion: absorption rate constant (k_A), relative bioavailability, and inter-compartmental clearance. However, the ω^2 on the k_A was high with a value of 0.66 and showed a binominal distribution. This was corrected for by inclusion of a mixture component, in which a fast absorption and a slow absorption population was identified, in which the fast absorption population had a fixed k_A of 100/h. Changing this value to 10/h or 250/h did not change the model fit. This stratification resulted in a significant improvement in model fit and reduced the ω^2 to 0.16, with 75% of subjects in the slow absorption group. No covariates for both subgroups were identified. All parameters were estimated with low RSE's and no changes were made to the proportional residual error structure.

To account for the saliva contamination, a saliva contamination compartment was added, in which a fraction of the full dose (F_{SALIVA}) remained in this compartment. The volume of this compartment was fixed to 1 mL and is represented by the following differential equation:

Equation 1: dxdt Contamination = $-k_{EL}$ * Contamination.

Data exploration showed a nonlinear relationship between the saliva:plasma ratio over the explored concentration range on data > 4 hours post-dose, after which contamination was no contributing factor anymore, in which a steady state ratio was reached at the higher concentrations (*Supplementary Figure S1*). The estimation of a saturable function on the saliva:plasma ratio improved the model fit significantly compared to a constant

saliva:plasma ratio (δOFV = -16.65). As such, the saliva:plasma ratio and saliva concentrations were represented in the model as follows:

Equation 2: Saliva:plasma ratio = $Ratio_{MAX} * C_{PLASMA} / (C_{PLASMA} + Ratio_{KM})$.

Equation 3: $C_{SALIVA} = (Contamination/0.001) + C_{PLASMA} *$ saliva:plasma ratio.

Where 0.001 is the volume of the saliva compartment in liters. Equation 3 therefore accounts for the level of contamination in the initial phase after dosing and for the non-linear saliva:plasma ratio observed in the data.

Inter-individual variability was only identified on the contamination part of the model (F_{SALIVA}, $k_{EL-SALIVA}$) and not on the saliva:plasma ratio. The saliva model gave accurate individual and population model fits over time. The final parameter estimates are displayed in *Table 2* and goodness of fit plots are displayed in *Supplementary Figure S2*. Parameters were estimated with sufficient parameter precision and moderate inter-individual variability and residual error. The plasma residual error was lower than saliva concentrations, indicating that a higher degree of unexplained variability was present in the saliva concentrations over time. The PCVPC show that the model was able to capture the median trend of the data and the level of variability in both matrices correctly.

Simulations

The concentration-time profiles of the simulation cohort are displayed in *Figure 2A*. On average, subjects achieved a median C_{TROUGH} of 2.1 ug/L after dose 1 and 13.7 ug/L at steady state. First, the estimated C_{TROUGH} after dose 1 and at steady state was estimated based on the population PK model parameters and weight of the subject only, which results in a single prediction for each weight, without taking into account any inter-individual variability (equivalent to using '0 samples' for the estimation). This scenario leads to a RMSPE of 1.39 ug/L after dose 1 and 8.8 ug/L at steady state, and an average proportional bias of -3% and -2% (95% LOA -92% - 87%), respectively (*Table 3*), meaning that there was a high level of uncertainty in the predicted individual plasma concentration. In the case of 1 saliva sample, the RMSPE was 3.56 ug/L at steady state, the proportional bias was 4% and the limits of agreement were reduced to -27% - 36% (*Figure 2B*). There was a correlation between true and predicted C_{TROUGH} in this scenario (R 0.93, p < 0.001). Increasing the number of saliva samples improved the accuracy of the prediction, as can be seen by the reduction in the RMSPE and narrowing of the LOA's (*Table 3*). For the simulation scenario with 5 saliva samples, RMSPE was 2.3 ug/L, with a proportional bias of 0% and LOA of -18% - 17%. For all scenario's applying Bayesian optimization, the true and estimated C_{TROUGH} were correlated, with correlation coefficients > 0.93 (p < 0.001, *Supplementary Figure S3 and S4*). There was an eightfold difference in RMSPE between scenario's estimating the C_{TROUGH} after dose 1 compared to scenarios at steady state, which can be explained by the increased concentration after multiple dosing. The proportional bias and LOA's were comparable for the estimation after dose 1 and at steady state.

Figure 2. Population prediction (80% prediction interval) in simulation cohort and visualization of predictive capability based on 1 saliva sample at steady state. A. Median population prediction (solid lines) and 80% prediction interval of the simulation cohort (n = 2000) in plasma (black) and saliva (blue). B. Proportional bias in the prediction (dotted line) and proportional limits of agreement (solid lines) of predicted Ctrough during steady state after Bayesian optimization based on a single saliva sample 11.5h post dose (during steady state). The x-axis displays the mean of the predicted and real Ctrough and the y-axis displayed the proportional difference between the predicted and real Ctrough. C: Pearson correlation between true and predicted plasma Ctrough of the scenario displayed in panel B. Bold black line represents the regression line, thin black line represents the line of unity, and each dot represents a simulated subject. For proportional bias plots and linear correlations of all scenarios, please refer to Supplementary Figures S3 and S4.

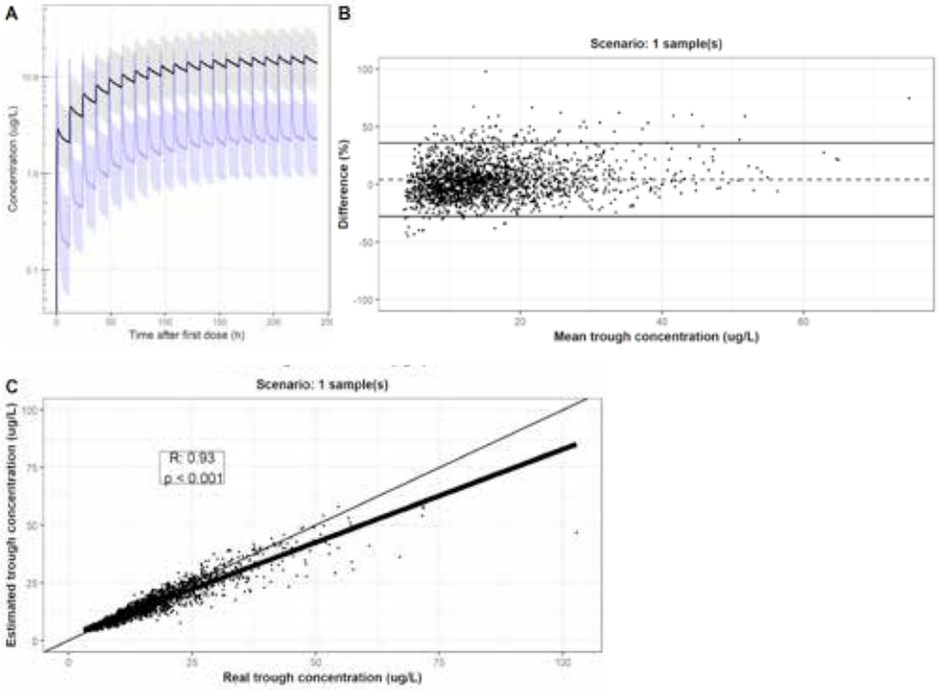

Table 2. Parameter estimates of population pharmacokinetic plasma and saliva model

	Parameter	Estimate [shrinkage %]	SE	RSE (%)
Plasma kinetics	θ k_A - slow group (h-1)	1.106	0.14	12.8
	θ Prob. slow group	0.75	0.10	13.0
	θ VD central (L)	109.5	11.5	10.4
	θ Clearance (L h-1)	2.98	0.16	5.5
	θ VD peripheral (L)	130.6	12.9	9.9
	θ Q (L h-1)	61.37	11.2	18.3
Saliva kinetics	θ F_{SALIVA} (% 1000-1)	0.033	0.005	14.5
	θ K_{EL} saliva (h-1)	1.95	0.12	6.0
	θ $Ratio_{MAX}$	0.195	0.031	16.0
	θ $Ratio_{KM}$ (ug/L)	2.581	0.74	28.5
IIV	ω^2 k_A - slow group	0.16 [2.24%]	0.065	40.8
	ω^2 Q	0.25 [0.33%]	0.12	45.7
	ω^2 F_{PLASMA}	0.026 [3.98%]	0.008	28.9
	ω^2 F_{SALIVA}	0.28 [5.30%]	0.12	42.7
	ω^2 K_{EL} saliva	0.056 [17.2%]	0.025	44.2
Residual error	σ^2 proportional plasma	0.0058 [15.9%]	0.0009	14.9
	σ^2 proportional saliva	0.057 [16.5%]	0.009	15.4

RSE: Relative standard error. IIV: interindividual variability. SE: standard error. / ω^2 and σ^2 are the variances of interindividual variability and residual variability, respectively. / TVCL = θ Clearance * $(WGT/70)^{0.75}$; TVVC = θ VD central * $(WGT/70)^{1}$; TVVP = θVD peripheral * $(WGT/70)^{1}$; TVQ = θ Q * $(WGT/70)^{0.75}$; TVRatio = $Ratio_{MAX}$ * C_{PLASMA} / (C_{PLASMA} + $Ratio_{KM}$)

Table 3. Simulation of predictive capability of using a saliva sample to determine the plasma trough concentration while varying the number of saliva samples used for the analysis.

	C_{TROUGH} after 1st dose				C_{TROUGH} steady state			
Scenario	RMSPE (ug/L)	Bias (%)	LLOA (%)	ULOA (%)	RMSPE (ug/L)	Bias (%)	LLOA (%)	ULOA (%)
Population model	1.39	-2.7	-93	87	8.8	-2.4	-92	87
1 sample, Linear regression[1]	0.57	-9.5	-54	35	4.4	5.2	-40	51
1 sample, Bayesian[1]	0.57	3.7	-28	36	3.6	4.0	-28	36
2 samples, Bayesian[2]	0.44	-0.2	-25	25	2.8	0.1	-25	25
3 samples, Bayesian[3]	0.40	-0.4	-21	21	2.5	-0.2	-21	21
4 samples, Bayesian[4]	0.39	-0.7	-20	19	2.5	-0.4	-20	19
5 samples, Bayesian[5]	0.37	-0.6	-18	17	2.3	-0.3	-18	17

Abbreviations: RMSPE: root mean squared prediction error, LLOA: lower limit of agreement (-2SD), ULOA: upper limit of agreement (+2SD). 1 Sample at 11.5h post-dose. 2 Samples at 5h and 11.5h post-dose. 3 Samples at 5h, 8h, and 11.5h post-dose. 4 Samples at 5h, 6h, 8h, and 10h post-dose. 5 Samples at 5h, 6h, 8h, 10h, and 11.5h post-dose.

Linear regression

The predictive performance of traditional (mixed effects) linear regression was assessed to determine the added value of Bayesian optimization methods. Because of the evident contamination in saliva during the first 4 hours after dosing, the correlation between saliva- and plasma concentrations was not calculated during this time window. The estimated linear regression equation from a linear mixed effects model predicting plasma concentrations from salivary concentrations during this time window was: $C_{PLASMA} = C_{SALIVA} * 5.4172 + 1.1994$ (marginal R^2 0.68). Predicted plasma concentration with the linear regression formula based on the 11.5h post-dose trough sample at steady-state was correlated with the true plasma concentration ($R = 0.91$, $p < 0.001$), but led to a larger RMSPE (4.36 ug/L) compared to Bayesian MAP, with a proportional bias of 4.3% (LOA -41%-50%, *Supplementary Figure S3-4E*).

Discussion

During this study, the pharmacokinetics of saliva- and plasma concentrations of clonazepam was characterized by a nonlinear mixed effects model. Furthermore, the potential use of salivary concentrations for prediction on plasma pharmacokinetics was studied. The developed model was able to predict trough concentrations at steady state with a RMSPE as low as 2.3 ug/L, with an 95% limit of agreement of -18-17%. The model will be used in a future non-invasive study investigating the effects of clonazepam on children with ARID1B-related ID and can be employed in the case of salivary TDM of clonazepam. Additionally, the methodology described in this study can be used to develop and evaluate similar models incorporating both saliva and plasma concentrations to allow for non-invasive pharmacokinetic sampling in future clinical trials in pediatric or other vulnerable populations.

We found high concentrations of clonazepam in saliva samples taken during the first four hours after administration of the oral solution. This indicates significant contamination in the initial saliva samples by clonazepam residue, a finding that has been reported in the past.[18] This precluded the use of rate constants in which there was a slow increase in salivary concentrations driven by plasma pharmacokinetics, because the timepoints earlier than 4h post-dose did not contain any information about the transfer rate from the plasma compartment to the saliva compartment. However, the inclusion of an exponential elimination of the contaminated saliva in combination with a saliva:plasma ratio in the

model led to a good model fit and adequate predictive performance. A consequence of this finding is that saliva samples taken during the first 4 hours do not provide any information regarding the plasma concentrations during that time. However, considering the long half-life and the fact clonazepam therapy is guided via trough concentrations, this has little impact. The estimated model parameters regarding plasma kinetics were comparable to the population model developed by dos Santos *et al.*[19]

Predictive performance was assessed through simulation in a fictional cohort aged 6-30. Simulated salivary samples were obtained that included the residual error component of the model, and the most likely relative bioavailability and the corresponding C_{TROUGH} was estimated via Bayesian MAP optimization. There are several advantages to using Bayesian methodologies for this purpose. First, the prediction error, represented by the RMSPE was lower compared to the prediction error based on linear modelling. Additionally, one can use information obtained from multiple samples to estimate the most likely C_{TROUGH}, reducing the prediction error in the process. We found that, with the current model, predictive performance increases by obtaining additional samples up to 5 samples, and possibly beyond that with even more saliva samples. However, obtaining more than 5 samples in future pediatric PK studies would not be in line with the non-invasive approach taken here. Third, the method allows for convenience sampling at timepoints that are logistically feasible, as long as the chosen timepoints are obtained on timepoints with a valid saliva:plasma correlation, after 4 hours post-dose for the current analysis. Fourth, the optimization process takes residual variability into account, and the prediction shrinks towards the population mean in the case of high residual variability. This prevents that outlier saliva observations are extrapolated to extreme estimated plasma concentrations on which dose adaptions are made. Finally, estimates cannot be outside the constraints provided by the population model, as opposed to linear regression methods that have no such limits. The relevance of several of these advantages are confirmed in our simulations of the predictive capability of Bayesian MAP versus linear regression equations. Predictive capability of the linear regression equation was adequate but inferior to Bayesian MAP based on multiple samples. The simulations confirm that saliva sampling is eminently feasible for monitoring of clonazepam trough concentrations in the context of TDM and for the estimation of individual PK trajectories in the context of clinical trials. Correlations between real and predicted C_{TROUGH} were found, although these showed a slight underprediction at higher concentrations, which can be explained by shrinkage to the population mean during the Bayesian optimization process.

In this study, a saliva:plasma ratio was described which was not constant over time, even after accounting for the initial levels of contamination. The apparent decrease in the ratio driven by decreasing plasma concentrations is a phenomenon that has been reported before[20–22], but the underlying mechanism causing this relationship is unclear. Transport into saliva is partly driven by the free fraction of a drug, but the observed relationship cannot be explained by saturable protein binding. We hypothesize that the observed relationship may be caused by competitive protein binding of clonazepam metabolites, a mechanism previously observed for prednisone[23]. Nevertheless, it remains an important finding in the context of TDM, as this invalidates the use of 'traditional' linear regression equations that do not take this variable saliva:plasma ratio into account. This may be one of the reasons that Bayesian optimization outperformed linear regression, even in the scenario with a single saliva sample.

This study has several limitations. First, this model in this study was based on observations after a single administration of clonazepam. Application of the model for the purpose of TDM or clinical trials will usually occur when subjects have reached steady state, and in that case, it is assumed that the estimates obtained here can be extrapolated to these higher concentrations. As this is the first study systematically exploring the relationship between saliva- and plasma concentrations of clonazepam, this assumption cannot be verified at this time, but can be confirmed in future studies by obtaining paired saliva and plasma samples from subjects who have reached steady state. Using this model in a future pediatric clinical trial is reliant on several other assumptions as well. First, it is assumed that plasma kinetics in children adhere to the allometric scaling employed in this study, which is subject to recurrent discussion.[24,25] However, several studies indicate that this approach is reasonably accurate.[26] The second assumption is that the saliva:plasma ratio is identical in children compared to the young adults included in this study. Little comparative research has been performed on this subject, but Michael *et al.* report highly similar saliva:plasma ratio's in children and adults for voriconazole, and a systematic review regarding saliva:plasma ratio's in infants showed comparability for several compounds.[3,27] Although these assumptions may cause additional variability and less precise results in pediatric patients, we expect the prediction error remains small enough to adequately identify a PKPD relationship based on saliva samples. Furthermore, saliva samples will aid in the identification of ultra-fast metabolizers and subjects that do not adhere to the treatment regimen. The currently developed model can already be applied in adults, where saliva based TDM could be preferable over plasma based TDM.

Future studies may determine whether the underlying assumptions in pediatric populations described above are valid in general and in the case of clonazepam in particular. However, if confirmed, we believe this methodology could be widely implemented to aid clinical trial conduct in pediatrics and apply precision-dosing in pediatric populations.

Conclusion

The developed population pharmacokinetic model describes the salivary- and plasma kinetics of clonazepam well and simulations show that plasma C_{TROUGH} can be predicted from saliva concentrations through Bayesian MAP optimization.

SUPPLEMENTARY DATA

Sup. Figure S1	Nonlinear saliva:plasma ratio
Sup. Figure S2-S5	Goodness of fit plots
Sup. Figure S6	Relative bias, limits of agreement and correlation of predictions in different scenarios after dose 1
Sup. Figure S7	Relative bias, limits of agreement and correlation of predictions in different scenarios at steady state

REFERENCES

1 Patsalos PN, Berry DJ. Therapeutic Drug Monitoring of Antiepileptic Drugs by Use of Saliva. Ther Drug Monit 2013;35(1).
2 Kim HY, Märtson AG, Dreesen E, Spriet I, Wicha SG, McLachlan AJ, Alffenaar JW. Saliva for Precision Dosing of Antifungal Drugs: Saliva Population PK Model for Voriconazole Based on a Systematic Review. Front Pharmacol 2020;11(June).
3 Hutchinson L, Sinclair M, Reid B, Burnett K, Callan B. A descriptive systematic review of salivary therapeutic drug monitoring in neonates and infants. Br J Clin Pharmacol 2018;84(6):1089-1108.
4 Browne TR. Clonazepam. A review of a new anticonvulsant drug. Arch Neurol 1976;33(5):326-332.
5 Cohen LS, Rosenbaum JF. Clonazepam: new uses and potential problems. J Clin Psychiatry 1987;48 Suppl:50-56.
6 Brodie MJ, Chung S, Wade A, Quelen C, Guiraud-Diawara A, François C, Verpillat P, Shen V, Isojarvi J. Clobazam and clonazepam use in epilepsy: Results from a UK database incident user cohort study. Epilepsy Res 2016;123:68-74.
7 Jung EM, Moffat JJ, Liu J, Dravid SM, Gurumurthy CB, Kim WY. ARID1B haploinsufficiency disrupts cortical interneuron development and mouse behavior. Nat Neurosci 2017;20(12):1694-1707.
8 Joseph PD, Craig JC, Caldwell PHY. Clinical trials in children. Br J Clin Pharmacol 2015;79(3):357-369.
9 Gagne JJ, Thompson L, O'Keefe K, Kesselheim AS. Innovative research methods for studying treatments for rare diseases: Methodological review. BMJ 2014;349(November):1-10.
10 Kruizinga MD, Zuiker RGJA, Sali E, de Kam ML, Doll RJ, Groeneveld GJ, Santen GWE, Cohen AF. Finding Suitable Clinical Endpoints for a Potential Treatment of a Rare Genetic Disease: the Case of ARID1B. Neurotherapeutics 2020.
11 Committee for Medicinal Products for Human Use (CHMP). Guideline on bioanalytical method validation. EMEA/CHMP/EWP/192217/2009 Rev 1 Corr 2 2011.
12 Beal SL, Sheiner LB, Boeckmann AJ, *et al.* NONMEM 7.3.0 User Guides. (1989-2013). ICON Dev Solut Hanover, MD.
13 R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.
14 Kyle T. Baron and Marc R. Gastonguay. Simulation from ODE-Based Population PK/PD and Systems Pharmacology Models in R with mrgsolve. J Pharmacokinet Pharmacodyn 2015;42(W-23):S84-S85.
15 National Center for Health Statistics, Data Table of Weight-for-age Charts. [accessed 2020 Nov 20]. https://www.cdc.gov/growthcharts/html_charts/wtage.htm#males
16 Kang D, Bae KS, Houk BE, Savic RM, Karlsson MO. Standard error of empirical bayes estimate in NONMEM® VI. Korean J Physiol Pharmacol 2012;16(2):97-106.
17 Altman DG, Bland JM. Measurement in Medicine: The Analysis of Method Comparison Studies. J R Stat Soc Ser D (The Stat 1983;32(3):307-317.
18 Lins RL, Otoul C, De Smedt F, Coupez R, Stockis A. Comparison of plasma and saliva concentrations of levetiracetam following administration orally as a tablet and as a solution in healthy adult volunteers. Int J Clin Pharmacol Ther 2007;45(1):47-54.
19 Santos FM Dos, Gonçalves JCS, Caminha R, Da Silveira GE, Neves CSDM, Gram KRDS, Ferreira CT, Jacqmin P, Noël F. Pharmacokinetic/pharmacodynamic modeling of psychomotor impairment induced by oral clonazepam in healthy volunteers. Ther Drug Monit 2009;31(5):566-574.
20 Haeckel R. Factors Influencing the Saliva/Plasma Ratio of Drugs. Ann N Y Acad Sci 1993;694(1):128-142.
21 Newton R, Broughton LJ, Lind MJ, Morrison PJ, Rogers HJ, Bradbrook ID. Plasma and salivary pharmacokinetics of caffeine in man. Eur J Clin Pharmacol 1981;21(1):45-52.
22 Malone SA, Eadie MJ, Addison RS, Wright AWE, Dickinson RG. Monitoring salivary lamotrigine concentrations. J Clin Neurosci 2006;13(9):902-907.
23 Unadkat JD, Rowland M. Representation and quantitation of the binding interaction between prednisone, prednisolone and corticosteroid binding globulin. J Pharm Pharmacol 1984;36(9):582-585.
24 Mahmood I. Dosing in children: A critical review of the pharmacokinetic allometric scaling and modelling approaches in paediatric drug development and clinical settings. Clin Pharmacokinet 2014;53(4):327-346.
25 Samant TS, Mangal N, Lukacova V, Schmidt S. Quantitative clinical pharmacology for size and age scaling in pediatric drug development: A systematic review. J Clin Pharmacol 2015;55(11):1207-1217.
26 Momper JD, Mulugeta Y, Green DJ, Karesh A, Krudys KM, Sachs HC, Yao LP, Burckart GJ. Adolescent dosing and labeling since the Food and Drug Administration Amendments Act of 2007. JAMA Pediatr 2013;167(10):926-932.
27 Michael C, Bierbach U, Frenzel K, Lange T, Basara N, Niederwieser D, Mauz-Körholz C, Preiss R. Determination of saliva trough levels for monitoring voriconazole therapy in immunocompromised children and adults. Ther Drug Monit 2010;32(2):194-199.