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Title:

Mechanistic and quantitative understanding of pharmacokinetics in zebrafish larvae through nanoscale blood sampling and metabolite modelling of paracetamol

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Abbreviations

dpf	Days post fertilization
$f_{G,e}$	Recovery fraction excreted paracetamol-glucuronide
$f_{P,e}$	Recovery fraction excreted paracetamol
$f_{S,e}$	Recovery fraction excreted paracetamol-sulphate
k_a	Absorption rate constant
$k_{G,e}$	Paracetamol-glucuronide excretion rate
$k_{P,e}$	Paracetamol excretion rate
$k_{PG,f}$	Metabolic formation rate for glucuronidation
$k_{PS,f,0}$	Metabolic formation rate for sulphation at time point 0
$k_{S,e}$	Paracetamol-sulphate excretion rate
LC-MS	Liquid chromatography-mass spectrometry
LLOQ	Lower limit of quantification
PAPS	3'-phosphoadenosine 5'-phosphosulphate

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t_{50}	Time at which time-dependent metabolic formation rate for sulphation is at 50%
V_P	Paracetamol distribution volume
V_G	Paracetamol-glucuronide distribution volume
V_S	Paracetamol-sulphate distribution volume

Keywords

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Abstract

Zebrafish larvae are increasingly used for pharmacological research, but internal drug exposure is often not measured. Understanding pharmacokinetics is necessary for reliable translation of pharmacological results to higher vertebrates, including humans. Quantification of drug clearance and distribution requires measurements of blood concentrations. Additionally, measuring drug metabolites is of importance to understand clearance in this model organism mechanistically. We therefore mechanistically study and quantify pharmacokinetics in zebrafish larvae, and compare this to higher vertebrates, using paracetamol (acetaminophen) as paradigm compound. A method was developed to sample blood from zebrafish larvae five days post fertilization. Blood concentrations of paracetamol and its major metabolites, paracetamol-glucuronide and paracetamol-sulphate, were measured. Blood concentration data were combined with measured amounts in larval homogenates and excreted amounts and simultaneously analysed through non-linear mixed effects modelling, quantifying absolute clearance and distribution volume. Blood sampling from zebrafish larvae was most successful from the posterior cardinal vein with median volume (interquartile range) of 1.12 (0.676-1.66) nL per blood sample. Samples were pooled (n=15-35) to reach measurable levels. Paracetamol blood concentrations at steady state were only 10% of the external paracetamol concentration. Paracetamol-sulphate was the major metabolite and its formation was quantified using a time-dependent metabolic formation rate. Absolute clearance and distribution volume correlated well to reported values in higher vertebrates, including humans. Based on blood concentrations and advanced data analysis, the mechanistic and quantitative understanding of paracetamol pharmacokinetics in zebrafish larvae has been established. This will improve the translational value of this vertebrate model organism in drug discovery and development.

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Significance statement

In early phases of drug development, new compounds are increasingly screened in zebrafish larvae, but the internal drug exposure is often not taken into consideration. We developed innovative experimental and computational methods, including a blood sampling technique, to measure the paradigm drug paracetamol (acetaminophen) and its major metabolites and quantify pharmacokinetics (absorption, distribution, elimination) in zebrafish larvae of 5 days post fertilization with a total volume of only 300 nL. These parameter values were scaled to higher vertebrates, including humans.

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Introduction

In drug discovery and development, the zebrafish (*Danio rerio*) larva is a promising vertebrate model organism (Zon and Peterson, 2005; MacRae and Peterson, 2015). It has many advantages, including 70% genetic homology to humans (Howe *et al.*, 2013), the possibility of high-throughput experimentation, easy genetic modification, and imaging of internal processes due to its transparency in early life (Van Wijk *et al.*, 2017; Schulthess *et al.*, 2018). In experiments with zebrafish larvae it is common practice to expose the larvae to study drugs by waterborne treatment, dissolving the drug of interest in the medium in which the larvae swim. Ignoring the internal exposure as is done in this approach, potentially leads to false positives or negatives (Diekmann and Hill, 2013). More importantly, translation of pharmacological findings to higher vertebrates is very limited without an internal exposure-response relationship (Morgan *et al.*, 2012; Van Wijk *et al.*, 2017). To be able to use zebrafish larvae in pharmacological research to their full potential, a mechanistic understanding of the pharmacokinetic processes in zebrafish larvae is needed together with a framework to quantitatively scale this to higher vertebrates.

Methods to measure internal drug amounts in larval homogenates over time have been established only recently (Kühnert *et al.*, 2013, 2017; Vogs *et al.*, 2015; Kantae *et al.*, 2016). In the case of our paradigm compound paracetamol (acetaminophen), internal paracetamol amounts were subsequently used to quantify the pharmacokinetic processes of absorption and elimination through quantification of the rate of absorption and a clearance relative to total larval volume. Although the clearance derived from relative values in zebrafish larvae scaled quite reasonably to reported absolute clearance values in higher vertebrates (Kantae *et al.*, 2016; Van Wijk *et al.*, 2018), accurate scaling of clearance requires absolute clearance values in zebrafish larvae. However, quantification of absolute clearance requires blood concentrations, which will also allow for the estimation of a distribution volume, but blood sampling methods for

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zebrafish larvae that are only a few hundred microlitres in total volume (Guo *et al.*, 2017) have not yet been described.

In addition, mechanistic characterisation of metabolic clearance in zebrafish larvae is of importance, as the utility of zebrafish larvae in drug development depends on their drug metabolism being similar to higher vertebrates in a qualitative (similar metabolites) and quantitative (similar rate and extent) manner. This is because drug metabolites can be pharmacologically active or toxic. A first step in assessing mechanistic similarities in drug metabolism between vertebrate species is genetic confirmation that similar enzymatic pathways are present in the zebrafish, through DNA sequencing and gene expression of the enzymes and possible co-factors. For example, our paradigm compound paracetamol, is a substrate for two important metabolic pathways, glucuronidation and sulphation (Janus *et al.*, 2003; Neirinckx *et al.*, 2010; Lee *et al.*, 2012; Owens *et al.*, 2012). In zebrafish, the enzyme systems for these pathways have been documented based on genetic homology with humans (Van Den Boom *et al.*, 2012; Van Wijk *et al.*, 2017). However functional and quantitative confirmation that drug metabolites are actually formed *in vivo* and at what rate they are formed and eliminated is crucial. This is currently lacking for most enzymatic pathways that metabolise drugs in zebrafish larvae.

Here the objective is to mechanistically and quantitatively study pharmacokinetics in zebrafish larvae. To that aim, a method is developed to take nanoscale blood samples to measure paracetamol and its major metabolites. A mechanistic metabolite model is developed, to quantify paracetamol absolute clearance and distribution volume, which are compared to those from higher vertebrates, including humans.

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Methods

Experimental design

Experiments were performed in zebrafish (*Danio rerio*) larvae of five days post fertilization (dpf), as this age is within the ethically acceptable time frame for larval experiments, and it offers the largest capacity to metabolise paracetamol within that time frame (Van Wijk *et al.*, 2018). The investigation was divided into two experiments. In the first experiment, larvae were continuously treated with 1 mM waterborne paracetamol concentration in embryo medium for up to 200 minutes. In the second experiment, larvae were treated with 1 mM waterborne paracetamol concentration for 60 minutes, then washed and transferred to clean wash-out embryo medium, and elimination was studied over 240 minutes.

A new method was developed to sample blood from zebrafish larvae. Blood samples were taken during the first experiment. Observations between 10 and 125 minutes of waterborne treatment consisted of minimal 3 replicates of 15-35 pooled blood samples. These data were combined with data from a previous study, which included 6 replicates of measured paracetamol amounts in whole larval homogenates of 5 zebrafish larvae at 10-180 minutes in the first and 60-300 minutes in the second experiment (Van Wijk *et al.*, 2018). Additionally, 6 replicates of wash-out medium of the second experiment were sampled at 60-300 minutes to measure excreted paracetamol and metabolites.

Paracetamol and its two major metabolites, paracetamol-glucuronide and paracetamol-sulphate, were measured in blood, medium, and homogenate samples by LC-MS. Non-linear mixed effects modelling was performed on all data simultaneously to quantify absolute clearance, including metabolic formation and elimination rates, and distribution volume. Finally, obtained parameter values for absolute clearance and distribution volume were compared to published values in higher vertebrates.

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Zebrafish larvae husbandry

Maintenance and handling of zebrafish followed international consensus protocols (Westerfield, 2000), and the planning and execution of all experiments were compliant with European regulation (EU, 2010). Adult wild type AB/TL zebrafish were used to fertilize eggs, and kept in glass aquaria (max 6/L, volume 10L, 120x220x490 mm, Fleuren & Nooijen BV, Nederweert, The Netherlands) with circulating water at $27.7^{\circ}\text{C} \pm 0.1$ on a 14h/10h light/dark cycle (lights on at 08:00) and twice daily feeding with artemia or feed particles (Gemma Micro/Diamond, Skretting, Nutreco NV, Amersfoort, The Netherlands). Water quality was controlled by JUMO Acquis touch S (JUMO GmbH & Co, Weesp, The Netherlands). Fertilised eggs were collected within 20 minutes of fertilization. Eggs and larvae were kept at 28°C in embryo medium which was refreshed daily. Exposure experiments were performed at room temperature.

Blood sampling

To develop a method for blood sampling from zebrafish larvae at five dpf, larvae were washed with embryo medium using Netwell insert filters (Corning Life Sciences B.V., Amsterdam, The Netherlands) and transferred to a microscope slide coated with agarose. Larvae were not anaesthetised to prevent decreased blood flow, instead superficial drying with soft lens paper prevented movement.

Sampling was performed using a needle pulled by a micropipette puller (Sutter Instruments, Novato, California, USA) from a 0.75 mm borosilicate glass capillary (Sutter Instruments, Novato, California, USA) without filament, positioned in a micromanipulator (World Precision Instruments, Berlin, Germany), connected to a manual CellTram Vario oil pump (Eppendorf Nederland B.V., Nijmegen, The Netherlands), under 20x magnification (Leica, Amsterdam, The Netherlands). After decapitation or tail cut were found to result in limited yields, direct sampling from the circulation was explored. To identify the most suitable location, sampling from different

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anatomical locations was tested (Isogai *et al.*, 2001) including the heart, the dorsal aorta, the caudal vein, and the posterior cardinal vein.

An image was taken from each blood sample in the needle to determine the sample volume. The image analysis program Fiji (Schindelin *et al.*, 2012) was used to calculate the volume of the blood sample within the needle with the volume formula of a truncated cone (equation 1).

$$V = \frac{1}{3} \pi \cdot (r_1^2 + r_1 r_2 + r_2^2) \cdot \frac{h}{\cos 45^\circ} \quad (1)$$

where r_1 and r_2 are the upper and lower radii of the blood sample in the pulled needle and h is the length of the blood sample within the needle taking into account the 45° angle of the needle in respect to the microscope. After the blood sample was imaged, it was injected into a 2 μL droplet of heparin solution (5 IE/mL) under microscope both to prevent coagulation and for sample handling. Sample replicates consisted of blood samples from 15-35 larvae to reach measurable levels, of which the total number depended on experimental and time constraints, and were pooled into a 0.5 mL Eppendorf tube and stored at -80°C .

Measurements of paracetamol and its metabolites

On the day of measurement, blood samples were thawed and 200 μL methanol with paracetamol-D4 internal standard (1.4 $\text{pg}/\mu\text{L}$) was added. Samples were centrifuged (16,000g, 10 minutes) and 180 μL supernatant was transferred to a 0.5 mL Eppendorf tube to be evaporated by vacuum centrifuge (Beun-de Ronde, Abcoude, The Netherlands) until dry. Samples were reconstituted in 10 μL 80/20 (v/v) purified water/methanol and transferred to an LC-MS vial for randomised injection of 7 μL into the ultra-performance liquid chromatography system (Acquity, Waters Chromatography B.V., Etten-Leur, The Netherlands) linked to a quadrupole-ion trap MS-MS (QTRAP-6500, AB Sciex B.V., Nieuwekerk aan den IJssel, The Netherlands). An electrospray ionization source in positive (paracetamol) and negative

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(paracetamol-glucuronide and paracetamol-sulphate) mode was used as published before (Kantae *et al.*, 2016; Van Wijk *et al.*, 2018). Method criteria were 90-100% accuracy and a precision corresponding to a relative standard deviation less than 10%. Lower limit of quantification (LLOQ) in blood samples was 0.05 pg/uL for paracetamol and paracetamol-sulphate, and 5 pg/uL for paracetamol-glucuronide.

The homogenate samples were measured as described before (Kantae *et al.*, 2016; Van Wijk *et al.*, 2018). LLOQ in homogenates was 0.09 pg/uL for paracetamol and paracetamol-sulphate, and 9.0 pg/uL for paracetamol-glucuronide. Of the wash-out medium of the second experiment, samples of 1850 μ L were collected and stored at -80°C. On the day of measurement, samples were thawed, 10 μ L 125 pg/ μ L paracetamol-D4 internal standard was added and samples were evaporated by vacuum centrifuge until dry. Samples were reconstituted in 50 μ L purified water, centrifuged (16,000g, 15 minutes), and 25 μ L supernatant was transferred to an LC-MS vial for randomised injection of 5 μ L into the LC-MS system as described above. Background measurement of the wash-out medium at t=60 was subtracted from the sample measurements. A calibration curve ranging from 0.05-100 pg/ μ L was prepared in 50/50 (v/v) methanol/purified water and was used to calculate compound excreted amounts in pmole/larva. LLOQ in wash-out medium was 0.05 pg/uL for paracetamol and paracetamol-sulphate, and 5 pg/uL for paracetamol-glucuronide.

Pharmacokinetic data analysis

A pharmacokinetic model was developed for paracetamol and its metabolites using non-linear mixed effects modelling. NONMEM (version 7.3) (Beal *et al.*, n.d.) was used through interfaces Pirana (version 2.9.6) (Keizer *et al.*, 2011) and PsN (version 4.7.0) (Lindbom *et al.*, 2005), and graphical output was created using R (version 3.4.2) (R Core Team., 2014) through the Rstudio interface (version 1.1.383, RStudio Inc, Boston, Massachusetts, USA).

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The First Order Conditional Estimation algorithm was used. A zero-order absorption rate constant was estimated to quantify paracetamol absorption from the surrounding medium, as we have earlier shown that paracetamol concentrations in the treatment medium remain constant during the experiments (Van Wijk *et al.*, 2018). One and two compartment models were tested for the distribution of each compound. Linear and non-linear metabolic clearance for paracetamol was tested, including one and two substrate Michaelis Menten kinetics (Ingalls, 2012), the latter of which was used to account for possible saturation of the sulphation pathway caused by depletion of the sulphate group donor 3'-phosphoadenosine 5'-phosphosulphate (PAPS) (Reddyhoff *et al.*, 2015). Lastly, time-dependent metabolism using both exponential and sigmoidal relationships were tested (Brochot *et al.*, 2011). First-order rates of excretion into medium with or without recovery fractions were estimated per compound.

Because of the destructive sampling, inter-individual variability could not be distinguished from residual variability, therefore only the latter was estimated, using an additive, proportional, or a combination of additive and proportional per compound per sample type. Level 2 covariance between the residual error for compounds measured in the same sample was tested (Karlsson *et al.*, 1995). All compounds had less than 10% of data points below the limit of quantification, with the sole exception of paracetamol-glucuronide in blood samples (71%), warranting the M2 method of ignoring these values in the analysis (Beal, 2001).

Model selection was based on the likelihood ratio test between nested models, using a drop in objective function value of 6.63 between nested models to indicate statistical significance, corresponding with $p < 0.01$ assuming a χ^2 distribution. Additionally, physiological plausibility of parameter estimates and goodness-of-fit plots were assessed (Nguyen *et al.*, 2017). Structural parameter precision was considered acceptable when relative standard errors were below 50%.

Comparison of absolute clearance and distribution volume of paracetamol to higher vertebrates

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The estimate of absolute paracetamol clearance was compared to values reported in higher vertebrates as previously published (Kantae *et al.*, 2016), as was the estimate of the distribution volume. For the latter, similar to the comparison of clearance values, a literature search was performed in PubMed using "distribution volume OR volume of distribution AND paracetamol OR acetaminophen". Only values published in the last 20 years were included. Values obtained from specific disease models, from combination treatment, or from obese patients, and from models with more than one compartment for paracetamol distribution were excluded. A regression through the log transformed parameter values based on log transformed bodyweight was calculated including 95% confidence interval using R (v.3.4.2) (R Core Team., 2014) through user interface Rstudio (v.1.1.383, RStudio Inc, Boston, Massachusetts, USA). This regression was based on data from mature individuals only, comparable to that of paracetamol clearance. The bodyweight of the larvae was calculated from their previously published volume (Guo *et al.*, 2017) and an assumed density of 0.997 g/mL, corresponding to that of water (Kantae *et al.*, 2016).

Materials

Paracetamol, paracetamol-glucuronide, paracetamol-sulphate, and paracetamol-D4 internal standard were acquired from Sigma (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). Heparin was acquired from Academic Hospital Pharmacy Leiden (LUMC, Leiden, The Netherlands). Embryo medium consisted of demineralised water containing 60 µg/mL Instant Ocean sea salts (Sera, Heinsberg, Germany). Agarose was acquired from Sphaero (Gorinchem, The Netherlands). UPLC/MS grade MeOH was acquired from Biosolve (Biosolve B.V., Valkenswaard, The Netherlands). PURELAB (Veolia Water Technologies B.V., Ede, The Netherlands) was used to purify water.

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Results

Blood sampling

From the different anatomical locations, the posterior cardinal vein proved most efficient for blood sampling and resulted in a median volume of 1.12 nL per larva with an interquartile range of 0.676-1.66 nL. Figure 1 shows an image of this procedure. The sample volume was determined based on a separately captured image of the blood sample within the needle. (Supplementary table 1) lists the number blood samples that were pooled for each sampling time point.

Measurement of paracetamol and metabolites

Paracetamol, paracetamol-glucuronide, and paracetamol-sulphate could be measured in the blood samples, of which representative chromatograms are shown in (Supplementary figure 1). The symbols in Figure 2 show paracetamol and metabolite concentrations and total amounts in blood samples, and in homogenates and wash-out medium, respectively. Although the data are variable, clear trends can be observed.

The paracetamol and metabolite concentrations (top panel) could be measured in the nanoscale blood samples and show that sulphate metabolite is reaching similar concentrations as the parent, while concentrations of the glucuronide metabolite are about tenfold lower. This is similar to the observations in homogenates (middle panel). Paracetamol concentrations reach a steady state value around 0.1 mM or 15 mg/L, which is 10% of the paracetamol concentration in the treatment medium.

The paracetamol amounts in larval homogenate from the wash-out experiment (open symbols) show a mono-exponential decline of paracetamol. For the metabolites, the wash-out experiment shows an increase in internal amounts between 60 and 120 minutes, after which paracetamol-

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glucuronide decreases to 1 pmole/larva and paracetamol-sulphate stabilizes between 10 and 20 pmole/larva at 300 minutes.

The amounts of paracetamol, paracetamol-glucuronide, and paracetamol-sulphate excreted into the medium during the wash-out experiment are shown in Figure 2, bottom panel. These data show low values with high variability, and a minor increase over the wash-out period.

Pharmacokinetic data analysis

Figure 3 shows a schematic representation of the developed parent-metabolite model for paracetamol. A model with zero-order absorption and one compartment per compound best fitted the data. The formation of paracetamol-glucuronide was best described with a first-order metabolic formation rate, while the paracetamol sulphation rate could not be captured with a zero- or first-order metabolic formation rate, as both methods lead to under-predictions at earlier time points and over-prediction at later time points. Conventional Michaelis Menten kinetics depending on parent compound concentrations could not be identified for paracetamol-sulphate, neither could a two-substrate Michaelis Menten kinetic model, which represents the potential rate limiting influence of depletion of the sulphate-group donor as well. Regarding functions based on time-related changes in sulphation rate, a sigmoidal function as described by equation 2 was statistically significantly better than an exponentially declining time-dependent sulphation rate and provided the best fit:

$$k_{PS,f} = k_{PS,f,0} \cdot \left(1 - \frac{t}{t+t_{50}}\right) \quad (2)$$

where $k_{PS,f}$ is the metabolic formation rate for sulphation in min^{-1} , $k_{PS,f,0}$ is the metabolic formation rate for sulphation at time point 0 in min^{-1} , t is the time in min, and t_{50} is the time in min at which the formation rate for the sulphate metabolite is at 50% of its value at time 0.

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The excretion of paracetamol and its metabolites was best captured with a first-order rate constant. A recovery fraction on the excreted paracetamol and metabolite amounts was included to prevent overestimation of the excreted compounds, which resulted in a significantly improved fit both graphically and statistically. Figure 4 shows the paracetamol clearance over time as sum of the constant metabolic formation rate for glucuronidation, the excretion rate, and the time-dependent metabolic formation rate for sulphation according to the model.

Residual variability for homogenate samples was found to be best described with a combination of additive and proportional error for paracetamol and paracetamol-glucuronide and with a proportional error for paracetamol-sulphate, including covariance between the proportional errors of paracetamol and paracetamol-glucuronide, and between the proportional errors of paracetamol-glucuronide and paracetamol-sulphate. Residual variability for blood samples was best described with an additive error for paracetamol, and proportional errors for paracetamol-glucuronide, and for paracetamol-sulphate. The residual variability for medium samples was best described by an additive error for paracetamol, a proportional error for paracetamol-glucuronide, and a combination of additive and proportional for paracetamol-sulphate.

The model predictions (lines) relative to the observed values (symbols) are shown in Figure 2 and show a good description of the data. Table 1 contains the parameter estimates including relative standard error as measure of their precision of the final model. Relative standard errors for structural parameters were acceptable. Additional goodness-of-fit-plots, showing good model accuracy, can be found in (Supplementary figure 2-4).

The final model code and dataset are available through the DDMoRe Repository, Model ID DDMODEL00000300 (<http://repository.ddmore.foundation/model/DDMODEL00000300>).

Comparison of absolute clearance and distribution volume of paracetamol to higher vertebrates

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A correlation between paracetamol clearance and bodyweight in 12 higher vertebrates, including humans, has been reported before (calculated exponent = 0.78, 95% confidence interval: 0.65-0.91, $R^2 = 0.90$) (Kantae *et al.*, 2016; Van Wijk *et al.*, 2018). Paracetamol clearance estimated from homogenate data alone, assuming homogenous distribution of the drug throughout the whole larvae, fell outside of the 95% confidence interval of this correlation (Van Wijk *et al.*, 2018). Here, absolute paracetamol clearance is estimated by simultaneously modelling observed concentrations from blood samples, which could be quantified as the result of the blood sampling method developed here, and observed internal amounts from homogenates. Figure 5 shows the estimated absolute paracetamol clearance of the zebrafish larva in relation to that of 12 higher vertebrates. Because of the time-dependent metabolic formation rate for sulphation, the full range from paracetamol clearance at $t=0$ (solid square) to paracetamol clearance at $t=\infty$ (open square) is shown. This range is partly within the 95% confidence interval. Noticeably, clearance values from higher immature vertebrates are also over-predicted by the correlation based on the values reported for mature organisms.

The literature search resulted in reported values for the distribution volume of paracetamol in 13 higher vertebrates, which are provided with their references in (Supplementary table 2). A correlation between the distribution volume of paracetamol and bodyweight based on data obtained from mature individuals of the higher vertebrates only, is shown in Figure 6 (dashed line including 95% confidence interval). The exponent in this relationship was calculated to be 0.94 (95% confidence interval: 0.67-1.2, $R^2 = 0.68$). Although five orders of magnitude removed from the smallest higher vertebrate, the distribution volume of paracetamol in zebrafish larvae lies well within the 95% confidence interval of the correlation between distribution volume and bodyweight. Even though the values from immature organisms are not taken into account for the correlation between the distribution volume of paracetamol and bodyweight, the data points are in agreement with that correlation.

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Discussion

Mechanistic and quantitative characterisation of pharmacokinetics in zebrafish larvae is crucial for this vertebrate model organism to deliver on its promising role in pharmacological research. To date, a method to take blood samples, which are essential to characterize pharmacokinetics, was lacking. The quantification of absolute clearance and also distribution volume in zebrafish larvae, needed for reliable extrapolation of pharmacokinetics between species, required measurements of these blood concentrations in addition to knowing drug amounts in larval homogenates as determinant of the bioavailable dose. This is analogous to the well-known requirement of knowing both blood concentrations and absolute administered drug amounts by intravenous drug administration, to quantify absolute pharmacokinetic parameters of extravascular administered drugs. Additionally, confirmation of functional drug metabolism is of importance, because drug metabolites can be pharmacologically active or toxic.

We report, for the first time, a method for nanoscale blood sampling from zebrafish larvae at five dpf, in which not only the paradigm compound paracetamol was measured, but also its two major metabolites. Absolute clearance of paracetamol in zebrafish larvae correlated reasonably well with those reported in higher vertebrates. Results from our study suggest that, in addition to the presence of genetic homology for the enzymatic systems established before (Van Wijk *et al.*, 2017), also functionally these metabolic pathways behave similarly to higher vertebrates, as both metabolites are present. Quantitatively, the ratio between the two metabolites was different from those reported in mature humans (Reith *et al.*, 2009). Paracetamol-sulphate proved to be the major metabolite at this larval stage with respect to paracetamol-glucuronide. A similar profile, however, has been shown in human newborns and infants, resulting from limited glucuronidation capacity which increases with increasing age (Anderson *et al.*, 2002; Krekels *et al.*, 2015). This is not unexpected, as zebrafish larvae have also not reached maturity.

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Consequently, the impact of enzymatic maturation needs to be taken into account when translating results from immature zebrafish larva to mature individuals of higher vertebrates.

Metabolic formation rate for sulphation could not be accurately captured using time-constant parameters. A conventional Michaelis-Menten model could not accurately describe the observed pharmacokinetic profiles. Therefore, a two-substrate Michaelis-Menten kinetic model was tested, accounting for both the concentrations of the substrate paracetamol and a hypothetical donor group, representing the sulphate group donor 3'-phosphoadenosine 5'-phosphosulphate (PAPS) which may limit the biotransformation (Slattery *et al.*, 1987; Liu and Klaassen, 1996; Reddyhoff *et al.*, 2015). The data however did not allow for reliable and stable estimation of the relevant model parameters. Therefore, an empiric time-dependent function was tested, with a sigmoidal time-dependent metabolic formation rate resulting in the best fit, but with relatively high RSE values of the estimated parameters. Normalization of the metabolic formation rate for sulphation at time point 0, $k_{PS,t,0}$, at a different time point, reduced its RSE value below 50%, indicating no over-parameterization (Goulooze *et al.*, 2019). (Supplementary figure 5) shows the paracetamol-sulphate fit of the metabolite model, in which the time-dependent metabolic formation rate for sulphation was replaced by a time-constant first-order rate. The parameters of that model are estimated with acceptable precision (RSE < 50%), but a clear misfit of the paracetamol-sulphate data can be observed. The low t_{50} estimate resulted in a steep decline of sulphation in the first minutes of the experiment (Figure 4). We believe this function represents the impact of depletion of the sulphate group donor PAPS on the metabolic formation rate, as the shape of this curve is similar to the shape of simulated PAPS levels from a reported physiological mathematical model at cellular level (Reddyhoff *et al.*, 2015). As time approached infinity, sulphation nears the asymptote of zero metabolic formation rate. This is unlikely, as for example the sulphate group donor PAPS is expected to be produced by the organism at a constant rate, but this production rate could not be estimated with the current data

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and timescale (Reddyhoff *et al.*, 2015). Consequently, interpretation of this model is limited to the time course presented here.

The availability of blood concentrations in addition to absolute internal amounts, allowed for the estimation of absolute values for distribution volume for both the parent and the major metabolites, which is unique. Figure 2 shows variable data yielded from extremely small samples and low concentrations, however, this did not limit accurate estimation of distribution volumes as indicated by RSE values being 25% or lower. The estimated distribution volume of paracetamol in zebrafish larvae correlated well with those reported in higher vertebrates. The estimated distribution volume for the glucuronide and sulphate metabolite is 0.6x and 1.2x the distribution volume of paracetamol, respectively. Reported values show large variability and range from 0.2-1.6x that of paracetamol for paracetamol glucuronide, and 0.09-0.9x that of paracetamol for paracetamol-sulphate in humans (Lowenthal *et al.*, 1976; Owens *et al.*, 2012, 2014; Van Rongen *et al.*, 2016), and 1.2-2.7x for paracetamol glucuronide, and 0.25-1.5 for paracetamol-sulphate in rats (Yamasaki *et al.*, 2011; Lee *et al.*, 2012). These values are in line with our estimates, but care must be taken when interpreting these data. Determining distribution volume of drug metabolites had to be done in patients which all had specific conditions which also impacted normal physiology. In the zebrafish, the total amounts necessary were readily available for that determination.

The developed method of blood sampling results in blood samples at the nanolitre scale. Multiple blood samples have to be pooled into a single analytical replicate to quantify drug and metabolite concentrations by conventional LC-MS. To improve throughput and efficiency, nano-electrospray ionization mass spectrometry could be considered (Fujii *et al.*, 2015). This technique directly injects a sample of sub nanolitre volumes together with ionization solvent into an electron spray ionization source and has for instance been used to sample and quantify the anti-cancer drug tamoxifen concentrations in individual cancer cells (Ali *et al.*, 2019). Currently,

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the high-throughput potential in zebrafish larvae for the purpose of obtaining absolute pharmacokinetic parameter values required for interspecies scaling is limited by the practicalities of the blood sampling and by the need for the development of highly sensitive analytical methods for these samples. We however envision that this step is only performed for promising drug candidates that have been selected for further development. Additionally, microinjection of zebrafish zygotes and embryos has been automated, resulting in higher throughput (Spaink *et al.*, 2013; Cordero-Maldonado *et al.*, 2019). This technique might in the future also be applicable to sampling.

We have confirmed that the paracetamol metabolites are formed by metabolizing enzymes of the zebrafish larvae and not by metabolizing enzymes of the microbiota, by repeating the experiment with germ-free larvae that lack microbiota (data not shown). To establish whether paracetamol-glucuronide and paracetamol-sulphate were the major metabolites in zebrafish, other metabolites were identified and measured by LC-MS. Two minor metabolites, 3-methoxy-paracetamol and n-acetylcysteine-paracetamol, were detected around or below the limit of quantification resulting in negligible impact on the mass balance.

Low fractions of paracetamol and its metabolites excreted according to the model were recovered in the wash-out experiment, for which an empirical recovery fraction was estimated. This might be due to adhesion of these compounds to the wells. Moreover, the accuracy of the measurements of the excreted amounts may be negatively impacted by measurements being close to the limit of quantification. A sensitivity analysis excluding these measurements showed similar parameter estimates, suggesting negligible impact of these data on the final model outcome.

Limitations in estimating distribution volume and thereby absolute clearance values are overcome by having measurements of both drug concentrations in the blood as well as total

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amounts in the larvae. It is however not clear whether this approach will also be applicable to study the pharmacokinetics of lipophilic drugs, as these drugs may be more difficult to wash off the larvae due to skin adhesion, which complicates measurements of total internal amounts. However, alternative methods for oral dosing using nanoparticles might provide a solution for this limitation.

In conclusion, an improved understanding of pharmacokinetics in the vertebrate model organism zebrafish larva is presented. Based on the developed method to sample blood from these larvae, absolute clearance resulting from different metabolic routes, as well as distribution could be quantified for the paradigm compound paracetamol. A comparison of the estimated absolute clearance and distribution volume with those reported for higher vertebrates shows a good correlation. This improved confidence in the translational value of the zebrafish can contribute to the role of this small vertebrate in drug discovery and development.

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Data availability statement

The full dataset and model file are publicly available through the DDMoRe Repository, Model ID DDMODEL00000300 (<http://repository.ddmore.foundation/model/DDMODEL00000300>).

Author contribution

Participated in research design: Van Wijk, Krekels, Spaink, Van der Graaf

Conducted experiments: Van Wijk, Ordas, Kreling

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Contributed new reagent or analytical tools: Kantae, Harms, Hankemeier

Performed data analysis: Van Wijk, Krekels

Wrote or contributed to writing of the manuscript: Van Wijk, Krekels, Kantae, Ordas, Kreling,
Harms, Hankemeier, Spaink, Van der Graaf

Conflict of interest

PHvdG is employee of Certara. All authors declare no conflict of interest.

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Footnotes

- a) This work was supported by the Leiden University Fund (Anita Ordas and Herman P. Spaink).
- b) NA
- c) Piet Hein van der Graaf
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- d) ¹ Mechanistic Biology and Profiling, Discovery Sciences, BioPharmaceuticals R&D, AstraZeneca, Cambridge, UK

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Figure legends

Figure 1. Blood sampling from posterior cardinal vein in a zebrafish larva at five days post fertilization using a pulled needle. Blood sample is shown in needle tip.

Figure 2. Paracetamol (blue circles, left column), paracetamol-glucuronide (magenta triangles, middle column), and paracetamol-sulphate (orange squares, right column) observations in blood samples (top row, concentrations) as well as in homogenates (middle row, amounts), and excreted into medium (bottom row, amounts) after constant waterborne treatment (solid symbols) and wash-out (open symbols) experiments. Blood concentrations are shown in pmole/nL (mM). The model prediction is shown as solid or dotted line for the constant waterborne treatment or wash-out experiment, respectively. The lower number of data points of paracetamol-glucuronide is explained by its lower limit of quantification being higher than that of the other compounds. Paracetamol observations in homogenates have been reported previously (Van Wijk *et al.*, 2018).

Figure 3. Schematic representation of the structural pharmacokinetic model of paracetamol and its metabolites in zebrafish larvae. Compartments depicted with solid lines represent compartments in which both amounts in homogenised larvae or blood concentrations in larvae were available, Compartments indicated with dashed lines represent compartments in which excreted amounts in the medium were available. P = paracetamol, PG = paracetamol-glucuronide, PS = paracetamol-sulphate, k_a = absorption rate constant, $k_{PG,f}$ = metabolic formation rate of glucuronidation, $k_{PS,f}$ = metabolic formation rate for sulphation, $k_{P,e}$ = paracetamol excretion rate, $k_{PG,e}$ = paracetamol-glucuronide excretion rate, $k_{PS,e}$ = paracetamol-sulphate excretion rate.

Figure 4. Paracetamol clearance over time. Total clearance (transparent green solid line) is the sum of the time-dependent metabolic formation rate for sulphation (orange dashed line), the

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metabolic formation rate for glucuronidation (magenta dotted line) and the unchanged excretion rate (blue solid line).

Figure 5. Relationship between paracetamol clearance and bodyweight of 13 vertebrate species. Clearance in zebrafish larvae at five days post fertilization is depicted as the clearance value at $t=0$ (closed square) and $t= \infty$ (open square), with the vertical line depicting the range in clearance over time. Mature individuals shown in blue, immature individuals in red. The correlation (dashed line, shaded area: 95% confidence interval) is based on values from mature individuals of the higher vertebrates only. Adapted with permission from (Kantae *et al.*, 2016).

Figure 6. Relationship between distribution volume of paracetamol and bodyweight of 14 vertebrate species, including zebrafish larvae at five days post fertilization. Mature individuals shown in blue, immature individuals in red. The correlation (dashed line, shaded area: 95% confidence interval) is based on values from mature individuals in higher vertebrates only. Raw data can be found in (Supplementary table 2).

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Tables

Table 1. Parameter estimates for the final model.

	Parameter value	Relative standard error (%)
Structural parameters		
k_a (p mole/min)	0.760	6
$k_{PG,f}$ (min^{-1})	0.00327	10
$k_{PS,f} = k_{PS,f,0} \cdot \left(1 - \frac{t}{t_{50} + t}\right)$		
$k_{PS,f,0}$ (min^{-1})	0.422	57
t_{50} (min)	1.42	70
$k_{P,e}$ (min^{-1})	0.0185	10
$k_{G,e}$ (min^{-1})	0.00743	17
$k_{S,e}$ (min^{-1})	0.000664	66
V_P (nL)	218	24
V_G (nL)	125	25
V_S (nL)	262	20
$f_{P,e}$ (%)	0.113	26
$f_{G,e}$ (%)	0.0900	24
$f_{S,e}$ (%)	3.74	71
Stochastic parameters homogenate		
Variance of paracetamol proportional residual error (-)	0.191	17
Variance of paracetamol-glucuronide proportional residual error (-)	0.279	17

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Variance of paracetamol-sulphate proportional residual error (-)	0.0521	29
Correlation of paracetamol and paracetamol-glucuronide proportional residual error (-)	-0.220	13 ^a
Correlation of paracetamol-glucuronide and p-sulphate proportional residual error (-)	-0.0358	15 ^a
Variance of paracetamol additive residual error (pmole/larva)	0.00461	93
Variance of paracetamol-glucuronide additive residual error (pmole/larva)	0.0131	71
Stochastic parameters blood		
Variance of paracetamol additive residual error (pmole/nL)	0.0148	70
Variance of paracetamol-glucuronide proportional residual error (-)	0.393	50
Variance of paracetamol-sulphate proportional residual error (-)	0.994	33
Stochastic parameters excreted compound		
Variance of paracetamol additive residual error (pmole/larva)	0.000207	41
Variance of paracetamol-glucuronide proportional residual error (-)	0.678	23

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Variance of paracetamol-sulphate proportional residual error (-)	0.941	47
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Variance of paracetamol-sulphate additive residual error (pmole/larva)	0.00113	110
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k_a = absorption rate constant, $k_{PG,f}$ = metabolic formation rate for glucuronidation, $k_{PS,f,0}$ = metabolic formation rate for sulphation at time point 0, $k_{P,e}$ = paracetamol excretion rate, $k_{G,e}$ = paracetamol-glucuronide excretion rate, $k_{S,e}$ = paracetamol-sulphate excretion rate, V_P = paracetamol distribution volume, V_G = paracetamol-glucuronide distribution volume, V_S = paracetamol-sulphate distribution volume, t_{50} = time at which time-dependent metabolic formation rate for sulphation is at 50%, $f_{P,e}$ = recovery fraction excreted paracetamol, $f_{G,e}$ = recovery fraction excreted paracetamol-glucuronide, $f_{S,e}$ = recovery fraction excreted paracetamol-sulphate, ^a relative standard error of the estimate for covariance.

Figures

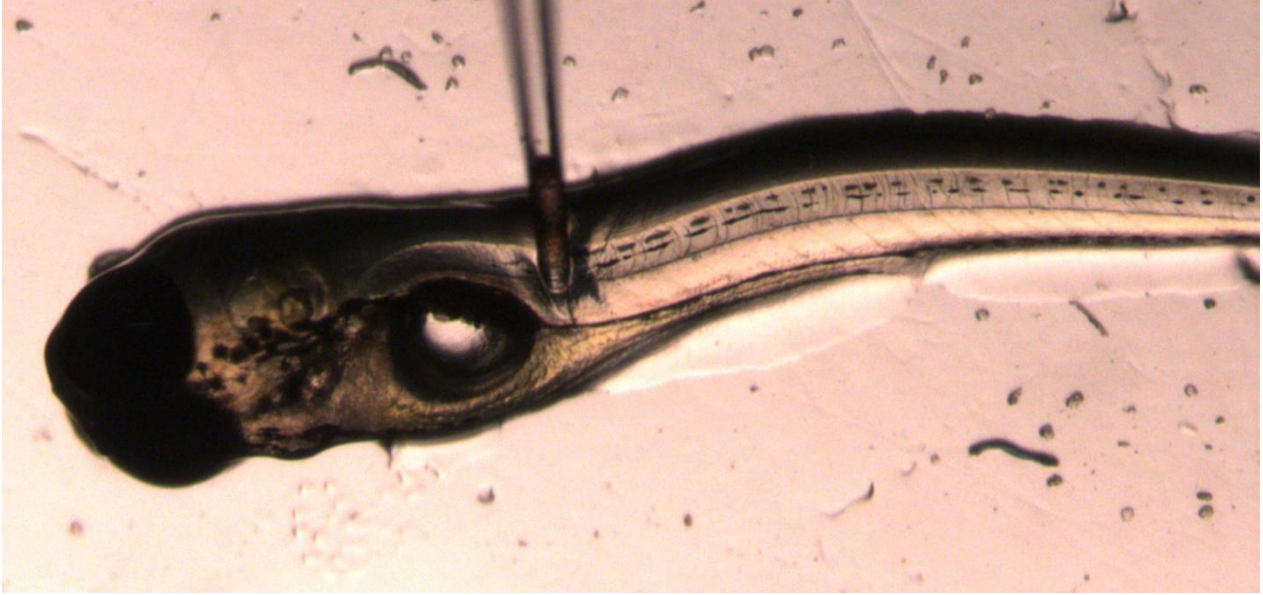


Figure 1.

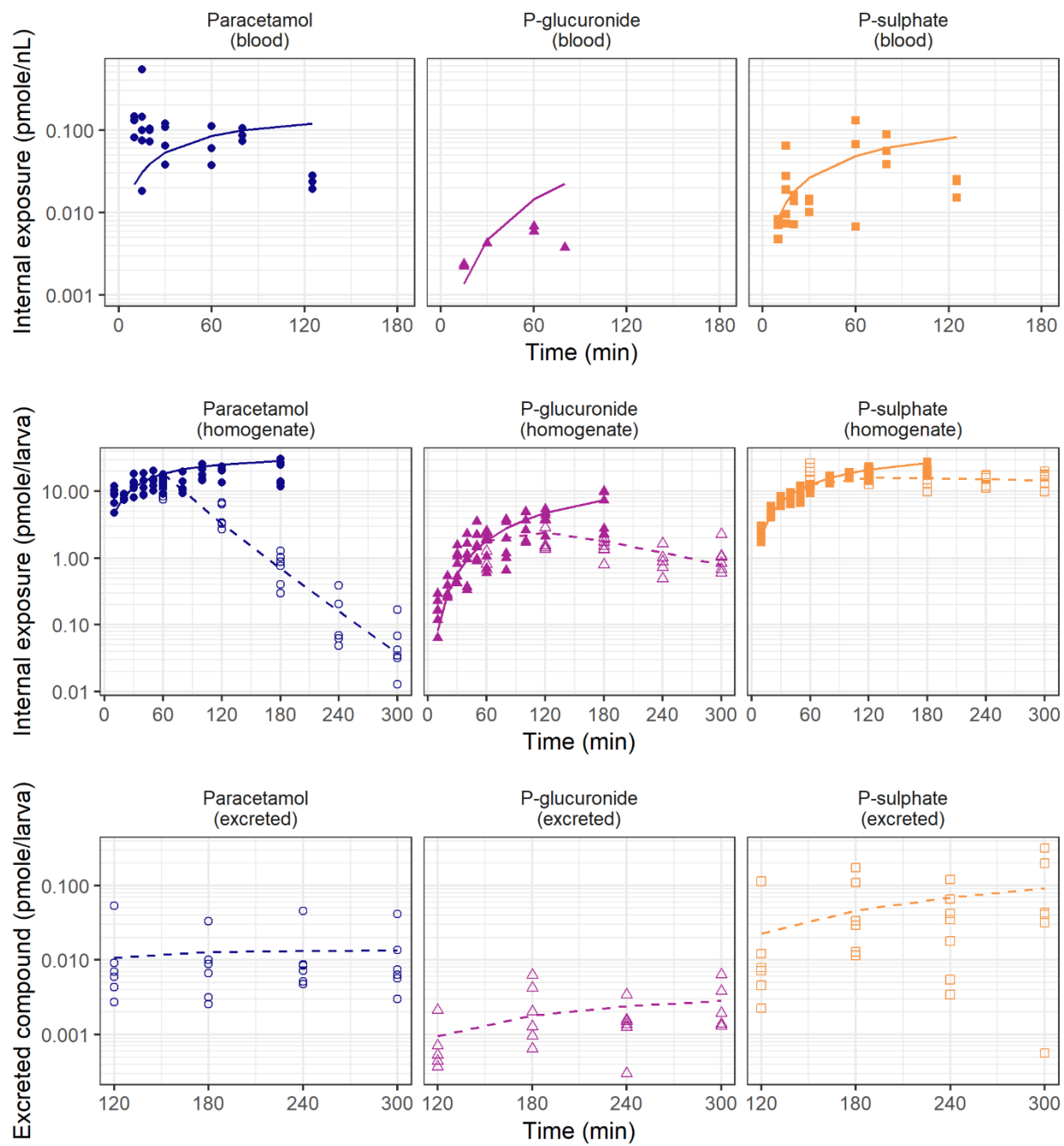


Figure 2.

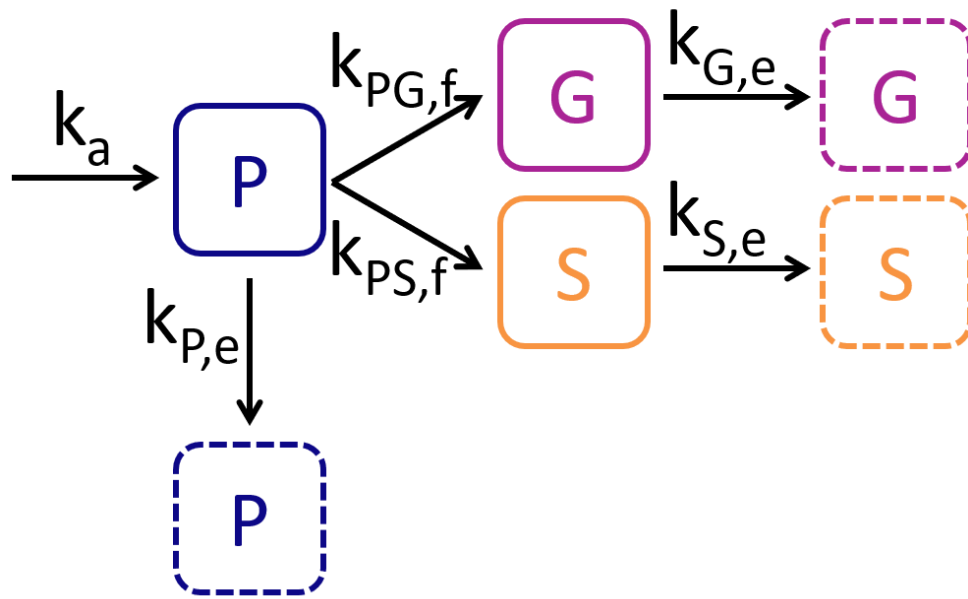


Figure 3.

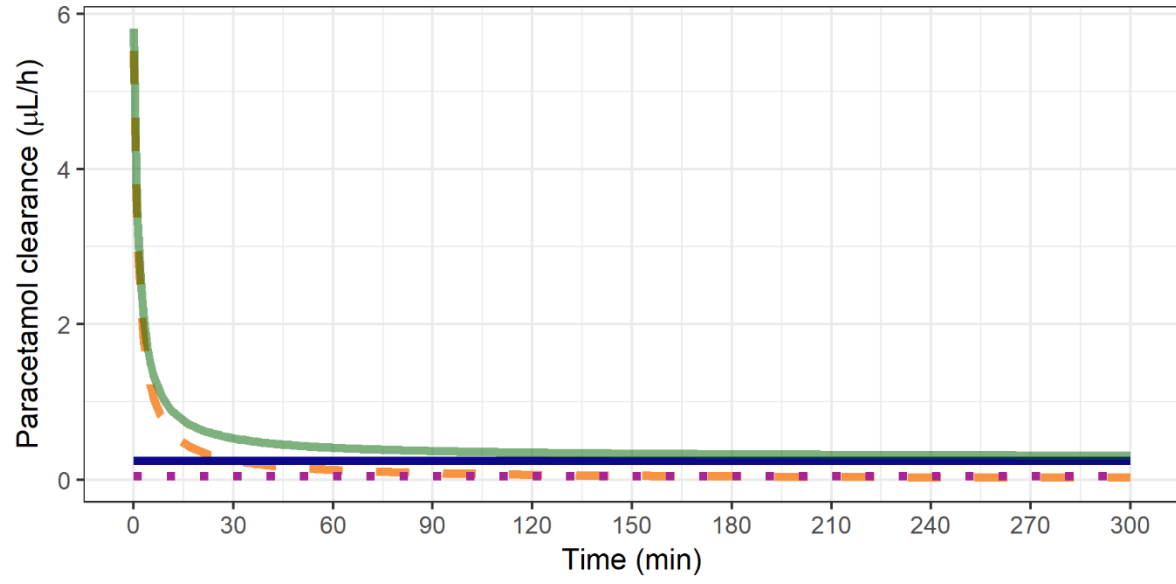


Figure 4.

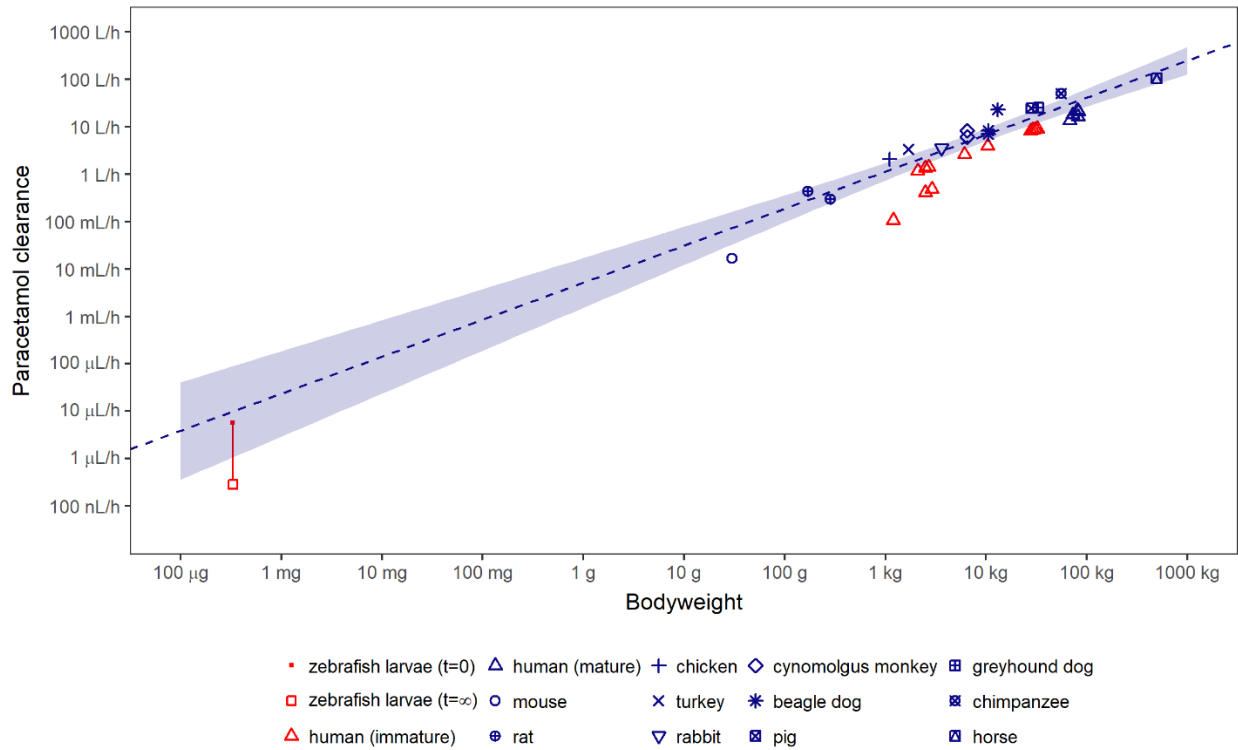


Figure 5.

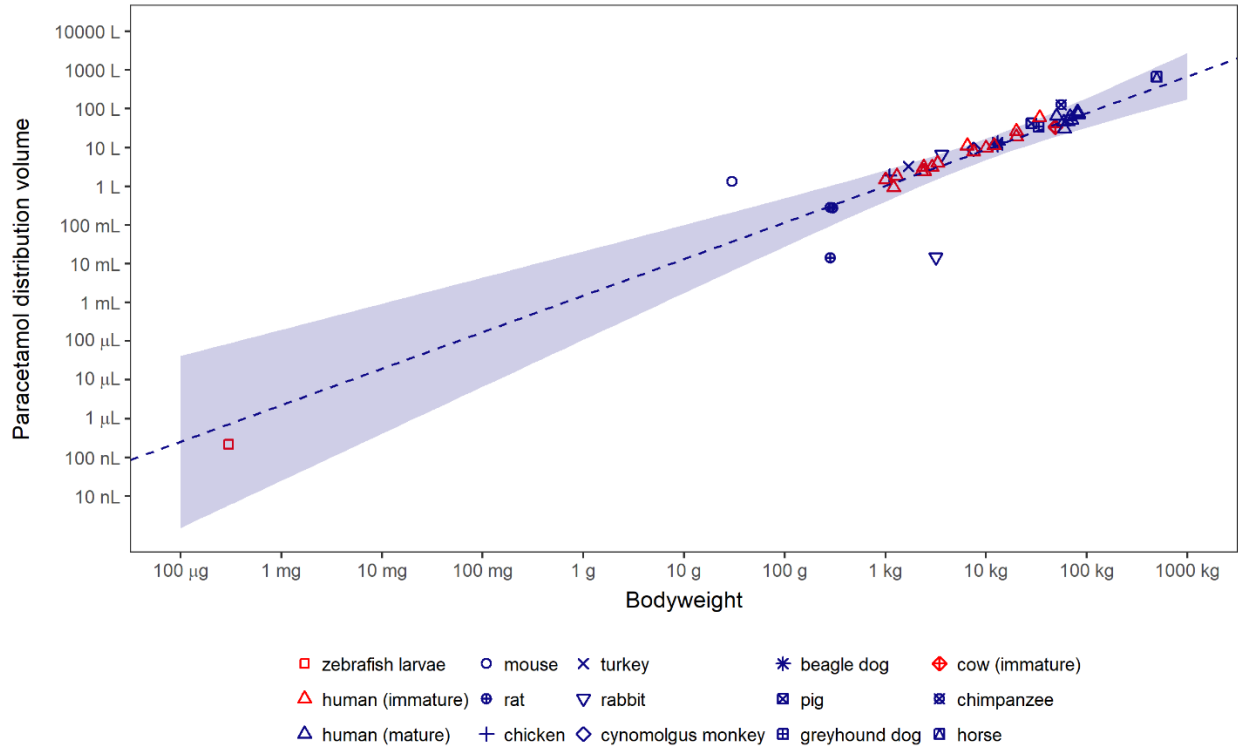


Figure 6.

Supplementary files

Title:

Mechanistic and quantitative understanding of pharmacokinetics in zebrafish larvae through nanoscale blood sampling and metabolite modelling of paracetamol

Authors:

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Affiliations:

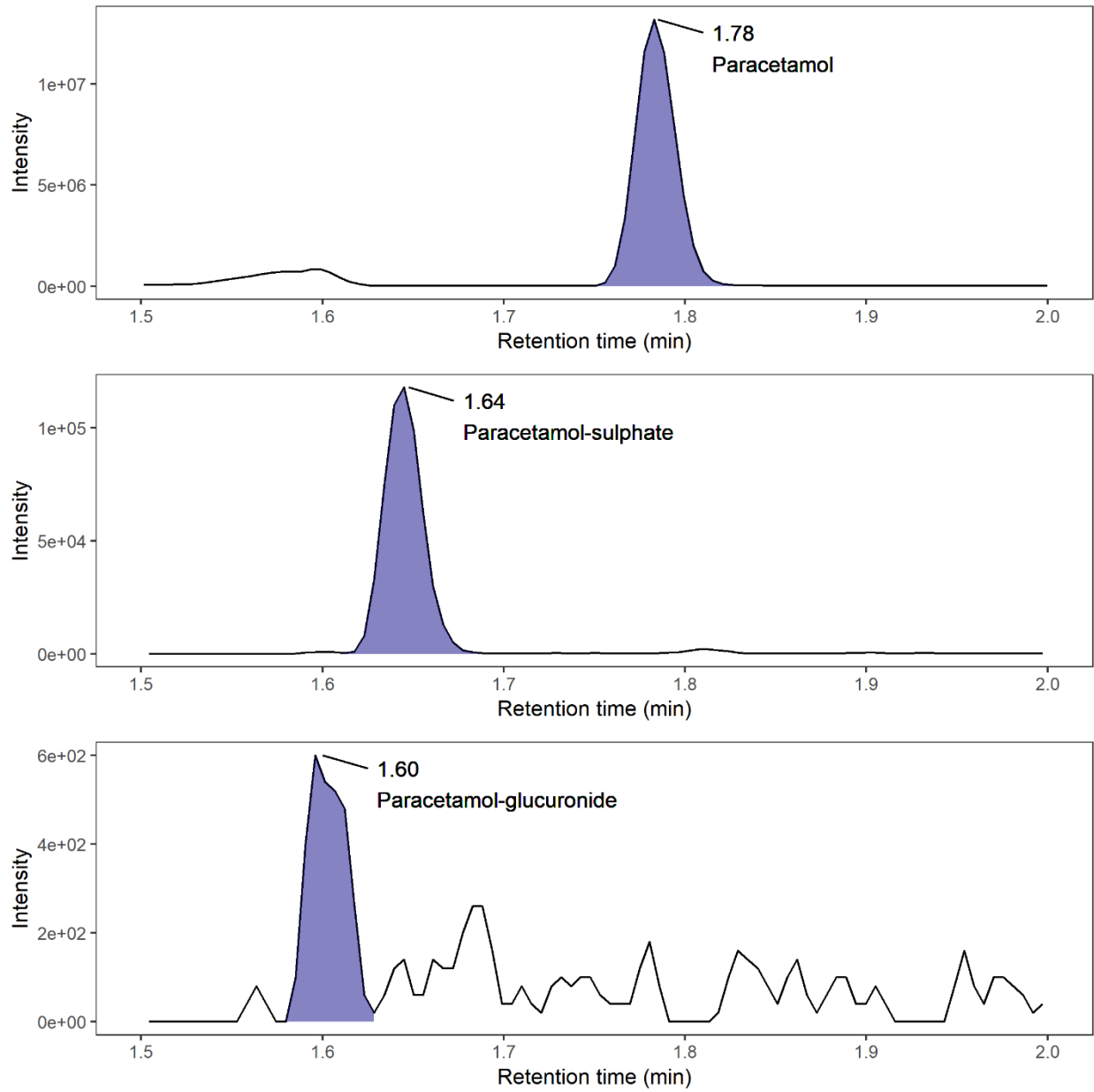
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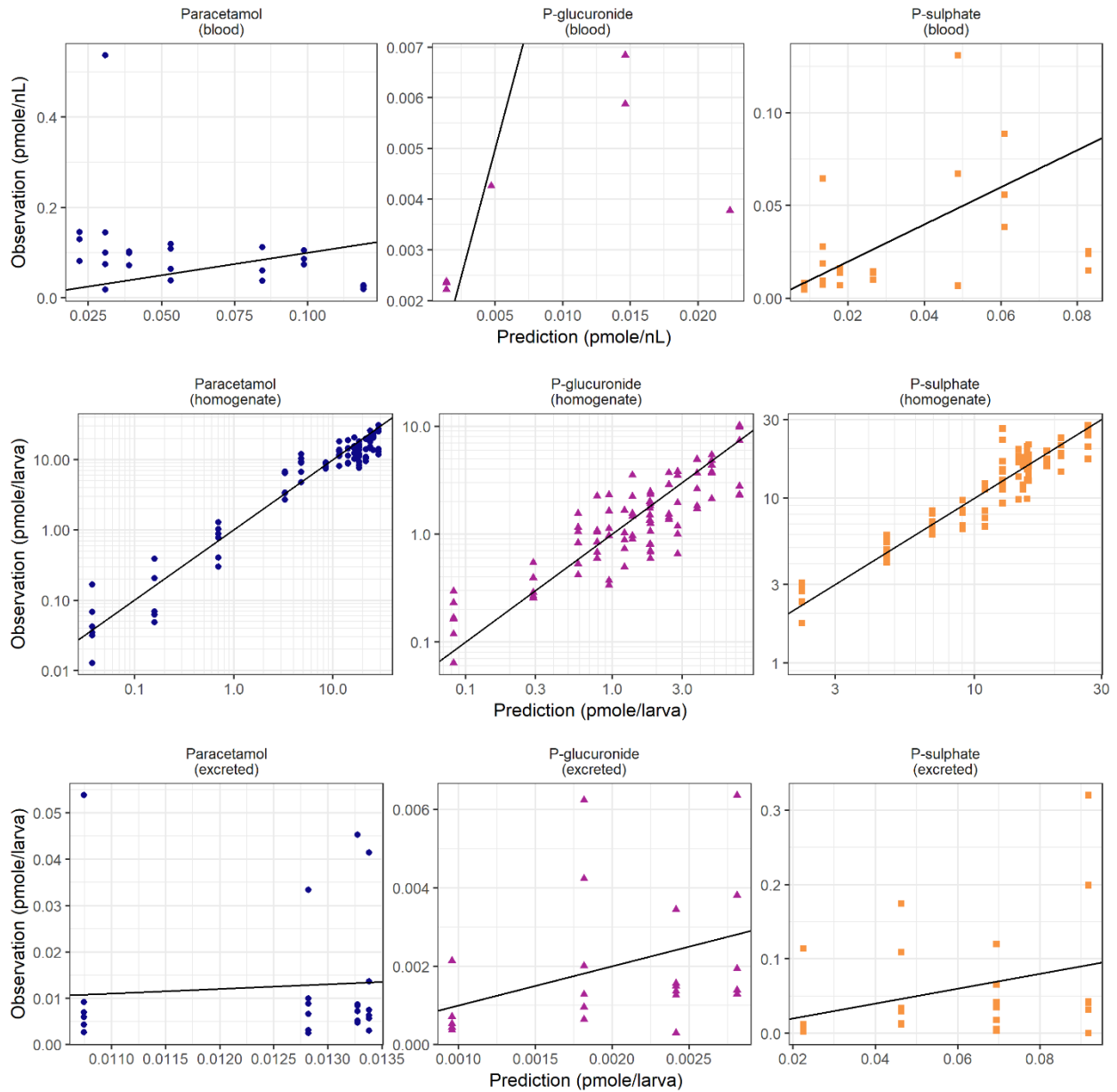
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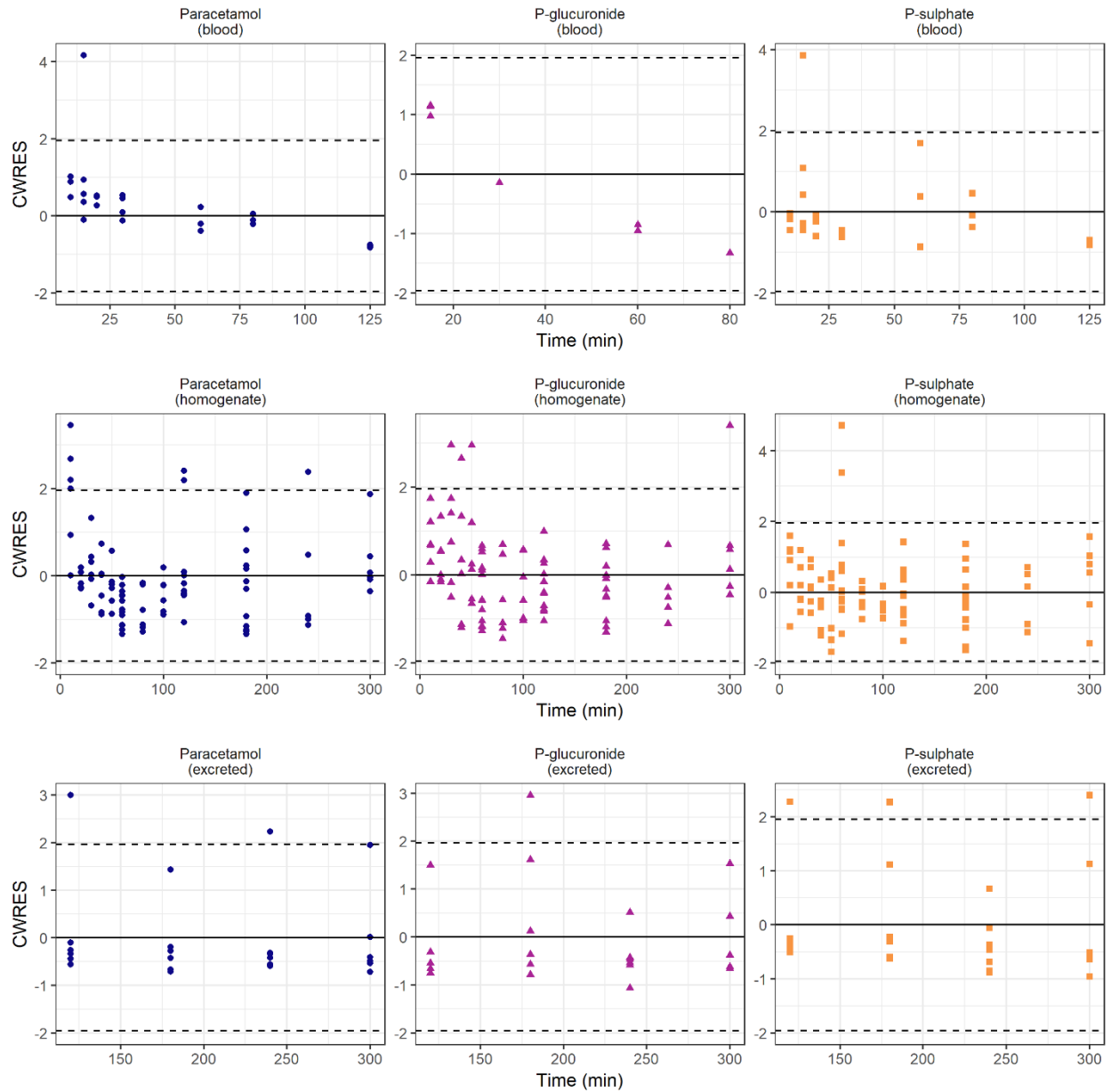
The Journal of Pharmacology and Experimental Therapeutics



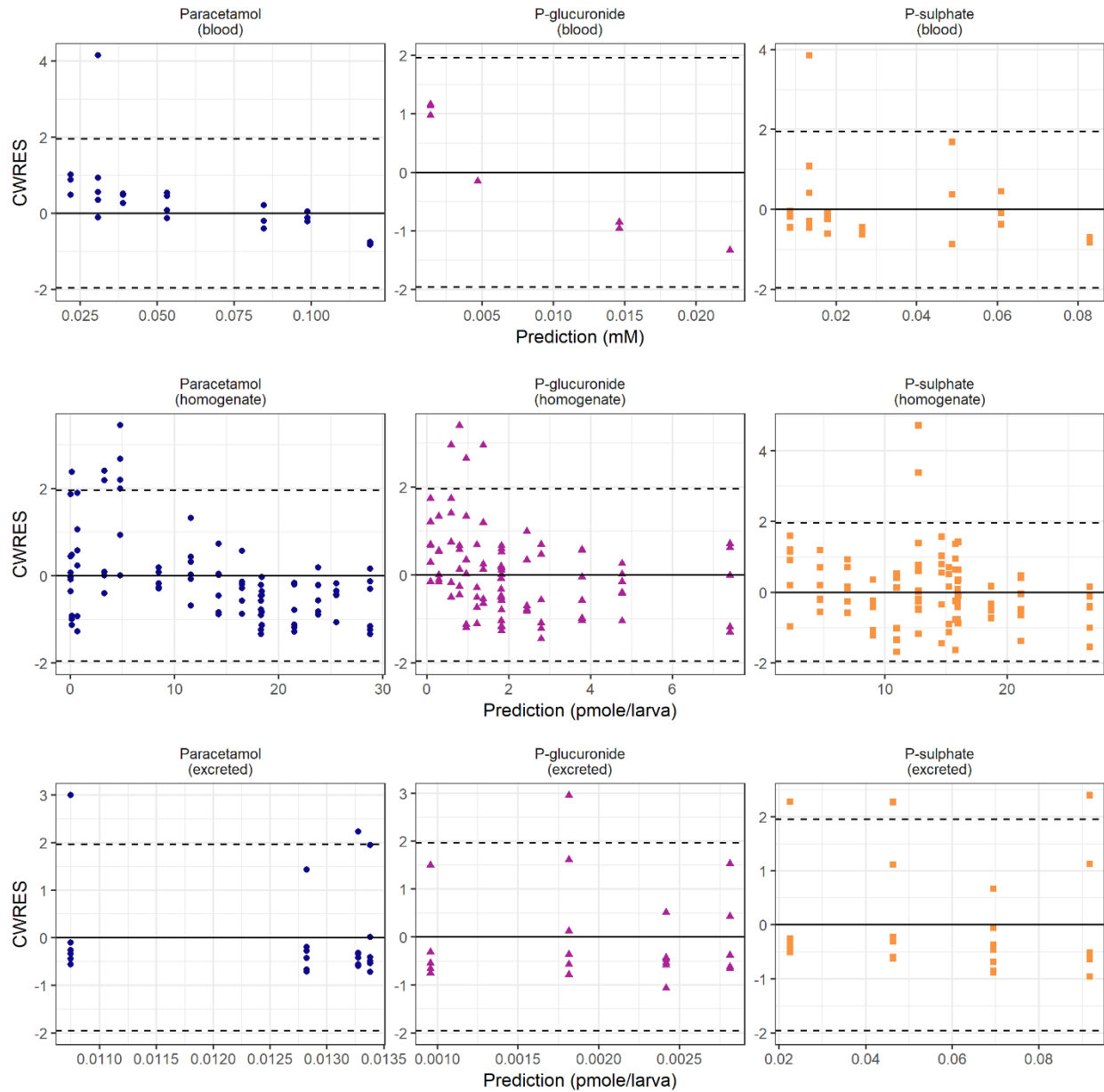
Supplementary figure 1. Representative chromatograms of paracetamol (top), paracetamol-sulphate (middle), and paracetamol-glucuronide (bottom) peaks of a blood sample taken at 80 min. LC-MS separates overlapping peaks by mass difference.



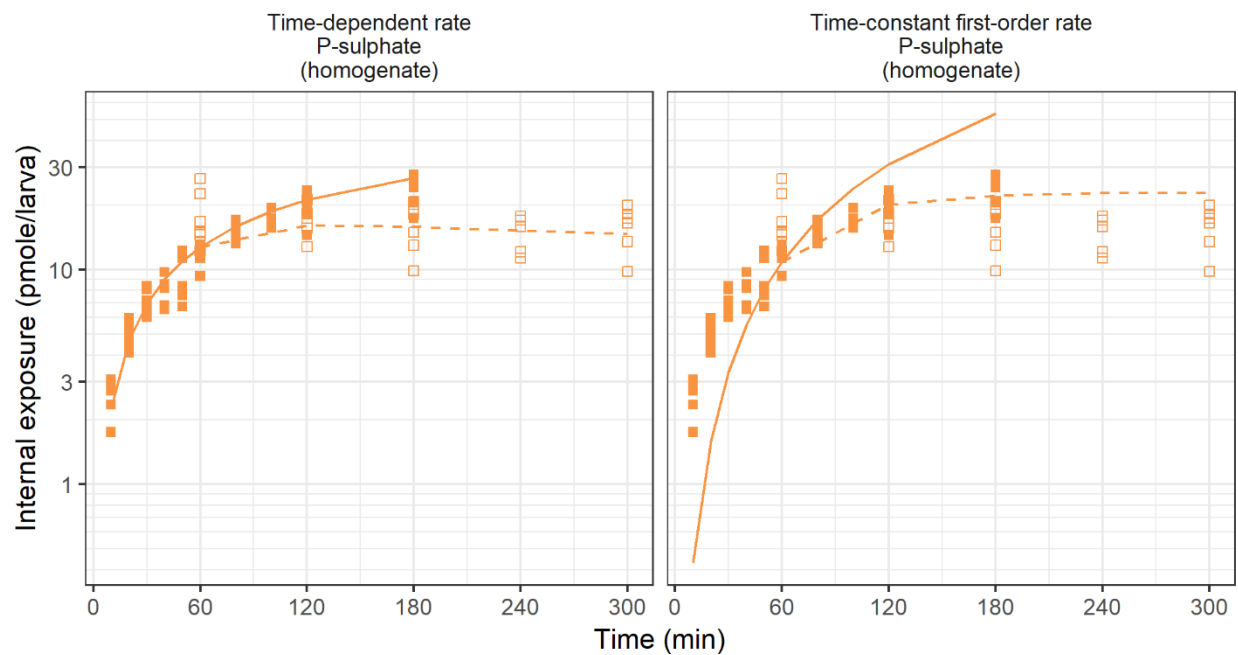
Supplementary figure 2. Observed vs predicted observations for paracetamol (blue circles, left column), paracetamol-glucuronide (magenta triangles, middle column), and paracetamol-sulphate (orange squares, right column). Concentrations in blood samples (top row, concentrations), amounts in homogenates (middle row, amounts), and excreted amounts into medium (bottom row, amounts). The solid black line is line of unity.



Supplementary figure 3. Conditional weighted residuals (CWRES) vs time for paracetamol (blue circles, left column), paracetamol-glucuronide (magenta triangles, middle column), and paracetamol-sulphate (orange squares, right column). Concentrations in blood samples (top row, concentrations), amounts in homogenates (middle row, amounts), and excreted amounts into medium (bottom row, amounts). Solid line is zero with dotted lines for ± 1.96 standard deviations (95% confidence interval).



Supplementary figure 4. Conditional weighted residuals (CWRES) vs prediction for paracetamol (blue circles, left column), paracetamol-glucuronide (magenta triangles, middle column), and paracetamol-sulphate (orange squares, right column). Concentrations in blood samples (top row, concentrations), amounts in homogenates (middle row, amounts), and excreted amounts into medium (bottom row, amounts). Solid line is zero with dotted lines for ± 1.96 standard deviations (95% confidence interval).



Supplementary figure 5. Paracetamol-sulphate for the final model (left) and alternative metabolite model (right) where the time-dependent metabolic formation rate of sulphation is replaced by a first order metabolic formation rate. Paracetamol-sulphate amounts in homogenates after constant waterborne treatment (solid squares) and wash-out (open squares) experiments. The model prediction is shown as solid or dotted line for the constant waterborne treatment or wash-out experiment, respectively.

Supplementary table 1. Overview of the number of replicates and number of blood samples pooled at each time point

Time (min)	Number of replicates	Total number of pooled samples*	Total blood volume (nL)*
10	3	31 (30-34)	45.5 (41.5-49.0)
15	5	34 (18-35)	38.7 (26.2-49.8)
20	3	32 (30-34)	61.9 (48.8-83.4)
30	4	26 (18-34)	18.2 (14.8-30.8)
60	3	31 (30-33)	20.9 (14.2-31.8)
80	3	30 (22-32)	54.3 (35.7-61.6)
125	3	15 (15-16)	11.7 (9.78-12.1)
* median and range			

Supplementary table 2. Literature values of the distribution volume of paracetamol in various vertebrate species.

Species	Weight (kg)	Reported distribution volume (unit)	Distribution volume (L)	Reference
Mouse	0.03	1.34 (L)	1.34	(Shankar <i>et al.</i> , 2003)
Rat	0.285	1.0 (L/kg)	0.285	(Yamasaki <i>et al.</i> , 2011) weight (Lee <i>et al.</i> , 2012)
Rat	0.285	4.96 (ml/kg)	0.014136	(Lee <i>et al.</i> , 2012)
Rat	0.3	0.92 (L/kg)	0.276	(Gandia <i>et al.</i> , 2004)
Chicken	1.1	1.70 (L/kg)	1.87	(Neirinckx <i>et al.</i> , 2010)
Turkey	1.7	1.91 (L/kg)	3.247	(Neirinckx <i>et al.</i> , 2010)
Rabbit	3.17	14.67 (mL)	0.01467	(Karbownik <i>et al.</i> , 2015)
Rabbit	3.61	6.69 (L)	6.69	(Bienert <i>et al.</i> , 2012)
Cynomolgus monkey	7.5	1200 (mL/kg)	9	(Koyanagi <i>et al.</i> , 2014) weight (Rosso <i>et al.</i> , 2016)
Beagle dog	13.1	0.92 (L/kg)	12.052	(Neirinckx <i>et al.</i> , 2010)
Beagle dog	13.1	900 (mL/kg)	11.79	(Koyanagi <i>et al.</i> , 2014) weight (Neirinckx <i>et al.</i> , 2010)
Beagle dog	13.1	1040 (mL/kg)	13.624	(Koyanagi <i>et al.</i> , 2014) weight (Neirinckx <i>et al.</i> , 2010)
Pig	28.1	1.51 (L/kg)	42.431	(Neirinckx <i>et al.</i> , 2010)

Greyhound dog	33.35	1.05 (L/kg)	35.0175	(KuKanich, 2009)
Cow (calf)	48.9	0.684 (L/kg)	33.4476	(Janus <i>et al.</i> , 2003)
Cow (calf)	48.9	0.698 (L/kg)	34.1322	(Janus <i>et al.</i> , 2003)
Chimpanzee	55.6	2.29 (L/kg)	127.324	(Wong <i>et al.</i> , 2006)
Human (preterm)	1	1.47 (L/kg)	1.47	(Anderson <i>et al.</i> , 2002)
Human (preterm)	1.207	0.764 (L/kg)	0.922148	(Van Ganzewinkel <i>et al.</i> , 2014)
Human (preterm)	1.3	1.41 (L/kg)	1.833	(Anderson <i>et al.</i> , 2002)
Human (preterm)	2.3	2.46 (L)	2.46	(Cook <i>et al.</i> , 2016)
Human (preterm)	2.4	1.32 (L/kg)	3.168	(Anderson <i>et al.</i> , 2002)
Human (preterm)	2.44	70.4 (L/70kg)	2.453942	(Allegaert <i>et al.</i> , 2004)
Human (neonate)	2.9	76 (L/70 kg)	3.148571	(Palmer <i>et al.</i> , 2008)
Human (neonate)	3.3	1.21 (L/kg)	3.993	(Anderson <i>et al.</i> , 2002)
Human (infant)	6.5	11.1 (L)	11.1	(Mooij <i>et al.</i> , 2017)
Human (infant)	7.5	1.04 (L/kg)	7.8	(Anderson <i>et al.</i> , 2002)
Human (infant)	10	0.97 (L/kg)	9.7	(Anderson <i>et al.</i> , 2002)
Human (infant)	12	0.95 (L/kg)	11.4	(Anderson <i>et al.</i> , 2002)
Human (child)	20	0.95 (L/kg)	19	(Anderson <i>et al.</i> , 2002)
Human (child)	20	1.32 (L/kg)	26.4	(Hahn <i>et al.</i> , 2000)
Human (child)	34	59.8 (L)	59.8	(Anderson <i>et al.</i> , 1999)
Human (adult)	50	1.3 (L/kg)	65	(Tankanitlert <i>et al.</i> , 2006)
Human (adult)	59	45.9 (L)	45.9	(Kulo <i>et al.</i> , 2014)
Human (adult)	60	30.9 (L)	30.9	(Shinoda <i>et al.</i> , 2007)
Human (adult)	65.3	46.8 (L)	46.8	(Kulo <i>et al.</i> , 2014)
Human (adult)	68	0.92 (L/kg)	62.56	(Liukas <i>et al.</i> , 2011)
Human (adult)	71	51.3 (L)	51.3	(Rincón and Meesters, 2014)

Human (adult)	81	1.04 (L/kg)	84.24	(Liukas <i>et al.</i> , 2011)
Human (adult)	82	0.87 (L/kg)	71.34	(Liukas <i>et al.</i> , 2011)
Human (adult)	83	0.9 (L/kg)	74.7	(Liukas <i>et al.</i> , 2011)
Horse	495	1.35 (L/kg)	668.25	(Neirinckx <i>et al.</i> , 2010)

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Model code (NONMEM format)

The full dataset and model file are publicly available through the DDMoRe Repository, Model ID DDMODEL00000300 (<http://repository.ddmore.foundation/model/DDMODEL00000300>).

```
;; 1. Author: R.C. van Wijk (r.c.van.wijk@lacdr.leidenuniv.nl)
;; 3. Label: Final metabolite model
;; 4. Dataset: Paracetamol, paracetamol-glucuronide, paracetamol-
sulphate in zebrafish larvae of 5 days post fertilization in
homogenates, blood samples, and excreted into medium.

$PROBLEM      PK

$INPUT        ID TIME AMT DV EVID MDV CMT XEXP BQL XEXP2 AGE BLOOD

$DATA         Real_Zebrafish_paracetamol_metabolite.csv IGNORE=@
IGNORE=(BQL.GT.0)

; units

; TIME = min

; DV = pmole / larva or pmole / nL

; V = nL

; kA = pmole / min

$SUBROUTINE ADVAN13 TOL=9

$MODEL        COMP ; CMT 1 dosing compartment
              COMP ; CMT 2 central paracetamol in larva
              COMP ; CMT 3 central paracetamol-glucuronide in larva
              COMP ; CMT 4 central paracetamol-sulphate in larva
              COMP ; CMT 5 excreted paracetamol in medium
              COMP ; CMT 6 excreted paracetamol-glucuronide in medium
```

COMP ; CMT 7 excreted paracetamol-sulphate in medium

\$PK

TVK25 = THETA(1)

TVK12 = THETA(2)

TVK23 = THETA(3)/1000

TVK24 = THETA(4)

TVK36 = THETA(5)/1000

TVK47 = THETA(6)/1000

TVV2 = THETA(7)

TVV3 = THETA(8)

TVV4 = THETA(9)

TVT50 = THETA(10)

TVF5 = THETA(11)

TVF6 = THETA(12)/1000

TVF7 = THETA(13)

K25 = TVK25 * EXP(ETA(1)) ;first order rate of excretion of
paracetamol

K12 = TVK12 ;zero order rate of absorption

K23 = TVK23 ;first order metabolic formation rate for
glucuronidation

K24 = TVK24 ;time-dependent metabolic formation rate for
sulphation

K36 = TVK36 ;first order rate of excretion of
paracetamol-glucuronide

K47 = TVK47 ;first order rate of excretion of
paracetamol-sulphate

V2 = TVV2 ;distribution volume paracetamol

V3 = TVV3 ;distribution volume paracetamol-glucuronide

V4 = TVV4 ;distribution volume paracetamol-sulphate
T50 = TVT50 ;time at which 50% of maximal sulphation is
reached
F5 = TVF5 ;fraction excreted parent retrieved from
medium sample
F6 = TVF6 ;fraction excreted glucuronide-metabolite
retrieved from medium sample
F7 = TVF7 ;fraction excreted sulphate-metabolite
retrieved from medium sample

ET1 = ETA(1)

S2 = V2

S3 = V3

S4 = V4

\$DES

DADT(1) = 0

DADT(2) = K12 * A(1) - K25 * A(2) - K23 * A(2) - K24 * (1 - T/(T50+T))
* A(2)

DADT(3) = K23 * A(2) - K36 * A(3)

DADT(4) = K24 * (1 - T/(T50+T)) * A(2) - K47 * A(4)

DADT(5) = K25 * A(2) * F5

DADT(6) = K36 * A(3) * F6

DADT(7) = K47 * A(4) * F7

\$ERROR

IF (CMT.EQ.2.AND.BLOOD.EQ.0) THEN

IPRED = A(2)


```

Y = IPRED * (1 + EPS(1)) + EPS(7) ; comb error paracetamol in larva
ENDIF

IF(CMT.EQ.3.AND.BLOOD.EQ.0) THEN

IPRED = A(3)

Y = IPRED * (1 + EPS(2)) + EPS(8) ; comb error paracetamol-glucuronide
in larva

ENDIF

IF(CMT.EQ.4.AND.BLOOD.EQ.0) THEN

IPRED = A(4)

Y = IPRED * (1 + EPS(3)) + EPS(9) ; prop error paracetamol-sulphate in
larva

ENDIF

IF(CMT.EQ.2.AND.BLOOD.EQ.1) THEN

IPRED = A(2)/V2

Y = IPRED * (1 + EPS(4)) + EPS(10) ; add error paracetamol in blood

ENDIF

IF(CMT.EQ.3.AND.BLOOD.EQ.1) THEN

IPRED = A(3)/V3

Y = IPRED * (1 + EPS(5)) + EPS(11) ; prop error paracetamol-
glucuronide in blood

ENDIF

IF(CMT.EQ.4.AND.BLOOD.EQ.1) THEN

IPRED = A(4)/V4

Y = IPRED * (1 + EPS(6)) + EPS(12) ; prop error paracetamol-sulphate
in blood

ENDIF

```

```

IF (CMT.EQ.5) THEN

IPRED = A(5)

Y = IPRED * (1 + EPS(13)) + EPS(16) ; add error paracetamol in medium

ENDIF

IF (CMT.EQ.6) THEN

IPRED = A(6)

Y = IPRED * (1 + EPS(14)) + EPS(17) ; prop error paracetamol-
glucuronide in medium

ENDIF

IF (CMT.EQ.7) THEN

IPRED = A(7)

Y = IPRED * (1 + EPS(15)) + EPS(18) ; comb error paracetamol-sulphate
in medium

ENDIF

IRES = DV - IPRED

$THETA (0,0.01142) ; K25
(0,0.3990) ; K12
(0,2.592) ; K23
(0,0.09168) ; K24
(0,5.838) ; K36
(0,0.02447) ; K47
(0,103) ; V2
(0,240) ; V3
(0,262) ; V4
(0,5.253) ; T50

```

(0, 0.01, 1) ; f_Q

(0, 0.01, 1) ; f_G

(0, 0.2, 1) ; f_S

\$OMEGA 0 FIX ; IIV K25

\$SIGMA BLOCK(3)

0.1366 ; prop error paracetamol in larva

-0.1348 0.3150 ; prop error paracetamol-glucuronide in larva

0 0.03749 0.06675 ; prop error paracetamol-sulphate in larva

\$SIGMA

0 FIX ; prop error paracetamol in blood

0.09 ; prop error paracetamol-glucuronide in blood

0.09 ; prop error paracetamol-sulphate in blood

0.5 ; add error paracetamol in larva

0.5 ; add error paracetamol-glucuronide in larva

0 FIX ; add error paracetamol-sulphate in larva

0.05 ; add error paracetamol in blood

0 FIX ; add error paracetamol-glucuronide in blood

0 FIX ; add error paracetamol-sulphate in blood

0 FIX ; prop error paracetamol in medium

0.09 ; prop error paracetamol-glucuronide in medium

0.09 ; prop error paracetamol-sulphate in medium

0.001 ; add error paracetamol in medium
0 FIX ; add error paracetamol-glucuronide in medium
0.05 ; add error paracetamol-sulphate in medium

\$ESTIMATION METHOD=1 MAXEVAL=8000 NOABORT PRINT=10 NSIG=3 SIGL=9
POSTHOC

\$COVARIANCE PRINT=E

\$TABLE ID TIME AMT DV EVID MDV CMT XEXP BQL XEXP2 AGE BLOOD IPRED
CWRES V2 V3 V4 K25 K12 K23 K24 K36 K47 T50 F5 F6 F7 ET1 ONEHEADER
NOPRINT FILE=tab_Zebrafish_paracetamol_metabolite