Integrative Biology

RSC Publishing

PAPER

View Article Online
View Journal | View Issue

Cite this: *Integr. Biol.*, 2013, **5**, 877

Multiscale modelling approach combining a kinetic model of glutathione metabolism with PBPK models of paracetamol and the potential glutathione-depletion biomarkers ophthalmic acid and 5-oxoproline in humans and rats†

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Received 11th October 2012,
Accepted 18th April 2013

DOI: 10.1039/c3ib20245c

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A key role of the antioxidant glutathione is detoxification of chemically reactive electrophilic drug metabolites within the liver. Therefore glutathione depletion can have severe toxic consequences. Ophthalmic acid and 5-oxoproline are metabolites involved in glutathione metabolism, which can be measured readily in the blood and urine and have been proposed as candidate biomarkers of hepatic glutathione content. However, currently it is unclear whether their concentrations in plasma exhibit a robust correlation with hepatic glutathione content. To explore this important question, we have developed a novel approach which combines a physiologically based pharmacokinetic (PBPK) model of metabolism and disposition of paracetamol (acetaminophen) with a previously developed mathematical systems model of hepatic glutathione homeostasis. Paracetamol is metabolised to reactive intermediates which deplete glutathione and cause toxicity when given at high doses. Our model correctly predicted that hepatic glutathione depletion following paracetamol administration resulted in elevated concentrations of 5-oxoproline and ophthalmic acid in blood and of 5-oxoproline in urine. However, we also found from the model that concentrations of both of the compounds were likely to be influenced by prolonged administration of paracetamol and by the concentrations of intracellular metabolites such as methionine. We conclude that care must be taken when extrapolating from concentrations of these biomarkers to hepatic glutathione status.

Insight, innovation, integration

Drug induced liver injury (DILI) is a serious issue and a major cause of post marketing drug withdrawal. Non-invasive biomarkers for reactive metabolite induced DILI would contribute to the development of safer therapies. Here a cellular model for glutathione depletion in hepatocytes is integrated with a physiologically based pharmacokinetic model. This novel model incorporates not only cellular processes but also the distribution of a drug and biomarkers of DILI around the body. This integrated model is then used to predict the changes on two biomarkers, ophthalmic acid and 5-oxoproline, after dosing paracetamol to humans. The model predicts a rise in these biomarkers over a short time scale, consistent with medical case histories, and suggests these are useful biomarkers of DILI.

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c3ib20245c

Introduction

Many drugs and other xenobiotics are metabolised within the liver to electrophilic species which interact with cellular macromolecules and thereby cause cell injury that may result in liver toxicity.[1] One of the major roles of the antioxidant tripeptide glutathione is to detoxify such chemically reactive metabolites and prevent toxicity. Consequently, maintenance of high intrahepatic concentrations of glutathione is a critical cellular function and is mediated by enzymes of the glutathione cycle, whose levels of expression are regulated at a transcriptional level *via* the redox sensitive Nrf2 signalling system.[2] Nevertheless, exposure of cells to large amounts of reactive species can cause the depletion of glutathione concentrations within cells of the liver, which in turn provides an early indication of the propensity of a compound to cause hepatotoxicity. The interplay between formation of reactive intermediates, glutathione homeostasis, Nrf2-dependent cell signalling and cell toxicity is especially well characterised for the analgesic paracetamol (acetaminophen, *p*-acetamidophenol, APAP), which causes severe liver injury in humans and in animals when administered at high doses.[3] Liver toxicity caused by paracetamol requires its cytochrome P4502E1-dependent metabolism to the electrophilic intermediate *N*-acetyl-*p*-benzoquinone imine (NAPQI). When given at normal therapeutic doses, paracetamol is metabolised primarily by conjugation (sulfation and glucuronidation) and the relatively small amounts of NAPQI which are formed are neutralised by conjugation with glutathione. However, following administration of high doses of the drug, large amounts of NAPQI are formed which deplete hepatic glutathione concentrations to below 10% of normal values, elicit oxidative stress and covalently modify critical cellular proteins. This in turn initiates a complex downstream cascade of deleterious effects that include activation of the innate immune system and ultimately result in liver toxicity.[3]

Therefore determination of hepatic glutathione status has potential value for prediction of risk of toxicity in patients exposed to paracetamol and other compounds which deplete hepatic glutathione stores. However, since direct determination of hepatic glutathione status would require invasive liver sampling it is not feasible in practice in humans. Circulating biomarkers which are indicative of cellular glutathione depletion within the liver would therefore have considerable value. A number of potential biomarkers of hepatic glutathione status have been suggested in the literature. One of these is 5-oxoproline (pyroglutamate), which has been highlighted as a potential predictive biomarker of susceptibility to liver toxicity caused by bromobenzene[4] or paracetamol (acetaminophen, *p*-acetamidophenol, APAP)[5] induced hepatotoxicity in rats, and also detected in THLE-2E1 cells (SV40 large T antigen immortalized human liver epithelial cells transfected with CYP2E1[6,7]) which were incubated with paracetamol.[7] Ophthalmic acid, which is a non-sulfur-containing analogue of glutathione, has also been suggested as a biomarker of glutathione status following paracetamol administration to mice.[8] A schematic of the glutathione systems model used in this work, showing the relationship between glutathione, 5-oxoproline and ophthalmic acid, is given in Fig. A1.1 (ESI†). Both of these candidate biomarkers can be analysed in blood-derived fluids (serum/plasma) or, in the case of 5-oxoproline, urine samples, and so have the potential to provide a useful indication of hepatic glutathione depletion. The potential utility of 5-oxoproline is supported by a number of published case reports of elevated concentrations of this compound in the plasma and urine of subjects exposed to paracetamol.[9–15] However, it is important to note that the relationship between the elevated 5-oxoproline values and the hepatic glutathione status of the subjects was not explored in these studies.

The metabolism of glutathione is very complex and is influenced by many different factors.[16] Consequently, a mathematical model of the network is required to provide a robust biochemically based understanding of drug-induced glutathione depletion and of its relationship with concentrations of 5-oxoproline, ophthalmic acid and other possible biomarkers in blood-derived fluids. It can be expected that such a model should also aid in design of *in vivo* experiments that can enable testing of the value of predictions arising from the model.

With this in mind, we have constructed a kinetic model of glutathione metabolism and paracetamol toxicity,[16,17] which allowed us to investigate the correlation between the production of 5-oxoproline and ophthalmic acid and the intracellular glutathione concentration in cell systems *in vitro*. This model predicted that the relationship between the concentration of the biomarkers and intracellular glutathione levels was sensitive to both methionine, a precursor involved in glutathione synthesis, and paracetamol concentrations. 5-Oxoproline was a better biomarker at low paracetamol exposure and high methionine concentrations, while ophthalmic acid was the better biomarker at high paracetamol exposure and low methionine concentrations. *In vitro* experimental results obtained following exposure of cultured THLE-2E1 cells which expressed CYP2E1 were consistent with this model.[16] However, a critical limitation of our previous *in vitro* model of glutathione metabolism and homeostasis is that that it did not take account of the pharmacokinetic (PK) aspects of paracetamol exposure *in vivo*, or of the PK properties of the biomarkers. In the previous model, the paracetamol concentration was set to a fixed value and the changes in glutathione concentration and biomarker efflux was assumed to have reached steady state. Whilst these were reasonable assumptions to make when considering *in vitro* cell toxicity data, it is clear that they are insufficient to model effects that can be expected to occur *in vivo*, where the paracetamol concentration will decrease over time as a result of metabolism and excretion from the body and the biomarkers will be cleared from the blood compartment *via* further metabolism or excretion (*e.g.*, *via* the kidneys).

To account for *in vivo* factors, physiologically based pharmacokinetic (PBPK) models can be used which describe the pharmacokinetics of chemical species and have had many applications in drug research.[18] A logical extension of PBPK modeling is its combination with cellular or organ level systems biology models.[19] The combination of systems biology approaches and pharmacology is thought to have a large potential in facilitating drug discovery and development[20–22] and is being

called systems pharmacology.[23] One of the high priority aims of systems pharmacology is therefore to link quantitative systems biology and quantitative pharmacology in order to take the basis for biomarkers from correlative to mechanistic (and probably multivariate) understanding.[21] However, although such multi-scale models have great potential, relatively little progress has been made to date in their development and evaluation. One notable exception is a multi-scale model which predicts the arrhythmogenic potential of new and existing drugs by combining cellular-level descriptions of voltage dependent ion channels with high resolution structural imaging and a probabilistic analysis of inter-individual variability.[24]

The first goal of the work described in this paper was to develop a physiologically based pharmacokinetic (PBPK) model of the disposition of paracetamol which accurately described the concentration of the drug within the livers of humans and rats treated with the drug *in vivo*. The second goal was to combine this model with our previously described systems model of glutathione homeostasis and with PBPK models of 5-oxoproline and ophthalmic acid for the same species, in order to gain novel insights into the relationship between intrahepatic paracetamol and glutathione concentrations and the concentrations of the biomarkers in blood and urine over time.

Materials and methods

PBPK model

A schematic of the PBPK model can be found in Fig. 1. The distribution and elimination kinetics of paracetamol and the two biomarkers are described in terms of first order rates that are functions or organ volume (V), blood flow (Q) and the tissue to blood partition coefficients (K_p). Generically the rate of transfer into a tissue from the arterial side is

$$v_{in} = \frac{Q_{tissue} X_{arterial}}{V_{arterial}},$$

whereas the transfer out of the tissue into the venous blood is

$$v_{out} = \frac{Q_{tissue} C_{tissue}}{V_{tissue} K_{p\ tissue}}.$$

Tissue to blood partition coefficients were predicted in rat based on the molecule's log of the octanol:water partition coefficient ($\log P$), acid dissociation constant (pK_a), log of distribution coefficient ($\log D$) and fraction unbound in plasma (f_{up}), using the Rodgers method.[25] The values we used for $\log P$, pK_a, $\log D$ and f_{up}, and the references from which they were obtained are summarised in Table 1. The partition coefficients obtained and used in the model are given in Table 2.

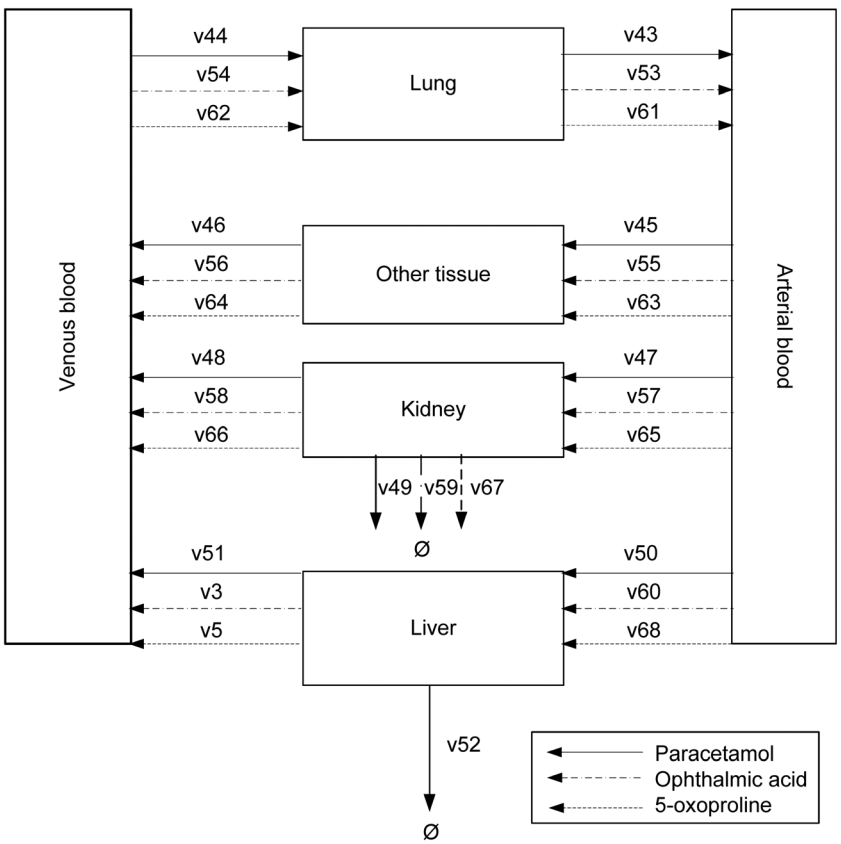

Fig. 1 A schematic of the PBPK model used. Paracetamol, ophthalmic acid and 5-oxoproline distribute around the different compartments according to differential equations whose rate terms, v_i are given on the Fig. 1. The ∅ symbol stands for metabolic or urinary elimination. See text for definitions of rate terms.

Table 1 Log *P*, pK_a, log *D* and f_{up} values used for paracetamol, ophthalmic acid and 5-oxoproline, and the literature from which they were obtained

	Value	Ref.
Paracetamol		
log *P*	0.46	37
pK_a	9.38	37
log *D*	−0.84	37
f_{up} RAT	0.88	37
f_{up} HUMAN	1.00	37
Ophthalmic acid		
log *P*	−3.80	38
pK_a	9.38	39
log *D*	−4.74	40
f_{up} RAT	1.00	41
f_{up} HUMAN	1.00	41
5-Oxoproline		
log *P*	−1.35	42
pK_a	12.76	42
log *D*	−4.88	42
f_{up} RAT	1.00	41
f_{up} HUMAN	1.00	41

Table 2 Tissue to blood partition coefficients for paracetamol, ophthalmic acid and 5-oxoproline

	Human partition coefficients (K_ps)	Rat partition coefficients (K_ps)
Paracetamol		
Other Tissues	0.50	0.29
Kidney	4.00	3.52
Liver	3.68	3.25
Lung	3.33	2.93
Ophthalmic acid		
Other Tissues	0.61	0.62
Kidney	0.77	0.77
Liver	0.75	0.75
Lung	0.79	0.79
5-Oxoproline		
Other Tissues	0.62	0.62
Kidney	0.77	0.77
Liver	0.75	0.75
Lung	0.79	0.79

Fraction of blood flow rate (f_q) and fraction volume (f_v) of the human and rat compartments are given in Table 3. The total body volume (V_{tot}) was set to 82 kg for man and 450 grams for rat. The total cardiac flow rate was calculated using:

$$Q_{\text{Cardiac output}} = 1.33(\text{BW(gr)}^{0.75}) = 387 \text{ L h}^{-1} \quad \text{for humans}$$

$$Q_{\text{Cardiac output}} = 1.15(\text{BW(gr)}^{0.74}) = 6.34 \text{ L h}^{-1} \quad \text{for rats}$$

We know from experimental data that 5-oxoproline is readily secreted into the urine in humans.[26,27] For this reason we have allowed kidney 5-oxoproline to be eliminated into urine with renal clearance CL_{oxuri} (reaction 67). The value of CL_{oxuri} was fitted using data obtained in adult humans from Eldjarn *et al.*[28] This was done by simulating the decrease in venous 5-oxoproline with time after a 116 μmol injection into the venous compartment (see Results). Hahn *et al.*[29] found no evidence for secretion of ophthalmic acid into urine in rats. However, in rats, as well as in mouse, ophthalmic acid is metabolised in the kidneys to the original amino acids.[30,31] For that reason ophthalmic acid in the model is degraded in the kidney with metabolic clearance CL_{opuri} (reaction 59). CL_{opuri} was also fitted using data from Hahn *et al.*[29] This was done by simulating the increase and decrease in kidney ophthalmic acid concentrations after a 0.5 μmol kg^{-1} injection of radioactive ophthalmic acid into the venous compartment (see Results).

This is an early attempt at integrating a systems model with a PBPK-based one. The predictions made in this model will need to be tested experimentally *in vivo* and at this stage it may also be possible to experimentally measure these values to improve the model further. It is clear that model predictions of the biomarkers will be sensitive to the rate of clearance, both the untreated steady state and the rate of response after a paracetamol challenge. In view of the analyses we have undertaken and described here we believe that the parameters we have obtained provide a physiologically relevant and useful model.

Clearances were converted from man to rat and *vice versa* using the allometric equation: clearance rate = $a \times$ body weight (kg)$^{0.75}$.

Intravenous (IV) administration of paracetamol

In humans, a 2 gram IV infusion administration was modelled as a constant infusion of paracetamol at a rate of 53 mmol h^{-1} for 15 minutes.[32] This was added as an event using the open-source mathematical modelling COmplex PAthway SImulator software COPASI.[35]

The same approach was taken for the rat model with a rate of 219 mmol h^{-1} for 30 seconds to compare to data from a 15 mg kg^{-1} IV bolus administration.[33]

Changes to the glutathione model

A few changes were made to our previous model of glutathione metabolism[16] in order to make it more representative of human metabolism. The transport rates of ophthalmic acid *v*3 and 5-oxoproline *v*5 were not PBPK equations in the Geenen *et al.* model[16] and were therefore changed to:

$$v[3] = \frac{F_{Q_{\text{liv}}} Q_{\text{card}} \text{OPA}[t] v_{\text{Liv}}}{f_{v_{\text{Liv}}} V_{\text{tot}} K_{\text{opliv}}}$$

$$v[5] = \frac{F_{Q_{\text{liv}}} Q_{\text{card}} \text{oxo}[t] v_{\text{Liv}}}{K_{\text{oxliv}} f_{v_{\text{Liv}}} V_{\text{tot}}}$$

Fraction of flow rate for liver = $F_{Q_{liv}}$, cardiac flow rate = Q_{card}, volume of liver = v_{Liv}, fraction volume of liver = $f_{v_{Liv}}$, total body volume = V_{tot}, concentration of hepatic ophthalmic acid = OPA[*t*], blood:liver partition coefficient for ophthalmic acid = Kopliv and concentration of hepatic 5-oxoproline = oxo[*t*].

In addition, the V_{max} of p450 enzymes (reaction 34 and 35) were changed. Reactions 34 and 35 describe how the paracetamol is activated by P450 and is neutralized by glutathione (Appendix 1, Fig. A1.1, ESI†). The V_{max} for these reactions in the previous model[14] was fitted to data obtained from an *in vitro* cell

Table 3 Fraction of cardiac output flow rate (F_Q) and fraction of total body volume (f_v) for each compartment in the PBPK models for human and rat[43,44]

	Human		Rat	
	Fraction flow rate	Fraction volume	Fraction flow rate	Fraction volume
Arterial	1.00	0.025	1.00	0.027
Venous	1.00	0.053	1.00	0.054
Tissue	0.41	0.878	0.59	0.869
Kidney	0.17	0.004	0.15	0.007
Liver	0.42	0.026	0.26	0.037
Lung	1	0.008	1	0.005

line. In order to make these more representative of the human values these were changed to values taken from Geenen *et al.* 2011;[16] 2731.5 μM h^{-1} for VmP450E1, and 2098 μM h^{-1} for VmP450A2. Lastly, the cell line which was used to create the Geenen *et al.* 2012[17] model could not form paracetamol sulphate and paracetamol glucuronide conjugates which are found in human metabolism. Therefore, reactions were also added so that the model could describe the cells ability to form paracetamol sulphate and paracetamol glucuronide conjugates. This was done using a previously published equation and Michaelis–Menten kinetics parameters.[34]

The initial concentrations (or quantities) of the joint PBPK and glutathione model were set to their steady state values when paracetamol was not present. Initial urine concentration of 5-oxoproline was set to zero. The units of the PBPK model transport rates are in μmol h^{-1} and those of the GSH model in μM h^{-1}. The stoichiometry of the model therefore had to take this change into account. In order to convert from μmol h^{-1} in the PBPK model to μM h^{-1} in the GSH model the number of moles in the PBPK model was divided by the volume of the liver. The volume of the liver in humans is 2.14 L and in rats is 0.016 L. Therefore in reactions $v3$, $v5$, $v50$, $v51$, $v60$ and $v68$ the stoichiometry was [X]GSHmodel = 2.14 [X]PBPK model in the human model, and [X]GSHmodel = 0.016 [X]PBPK model in the rat model.

The initial concentrations of the model can be found in Appendix 2 (ESI†). The model was compiled and run in COPASI.

Results and discussion

Paracetamol in venous blood

The decrease of paracetamol concentration in the venous compartment over time after IV dosing has been reported in the literature for both humans and rats. We therefore compared experimental data obtained from the literature with values predicted from our PBPK model to assess whether the model was accurate. For humans we simulated a 2 gram paracetamol acetaminophen IV infusion, as described in the literature.[32] There was a good correspondence between the PBPK model predictions and the experimental results (Fig. 2A), although the model slightly under-predicted at the earlier time-points. The rat model was compared to data obtained following IV dosing of paracetamol to ratsat 15 mg kg^{-1} from the literature.[33] Again a reasonably good correspondence was obtained (Fig. 2B).

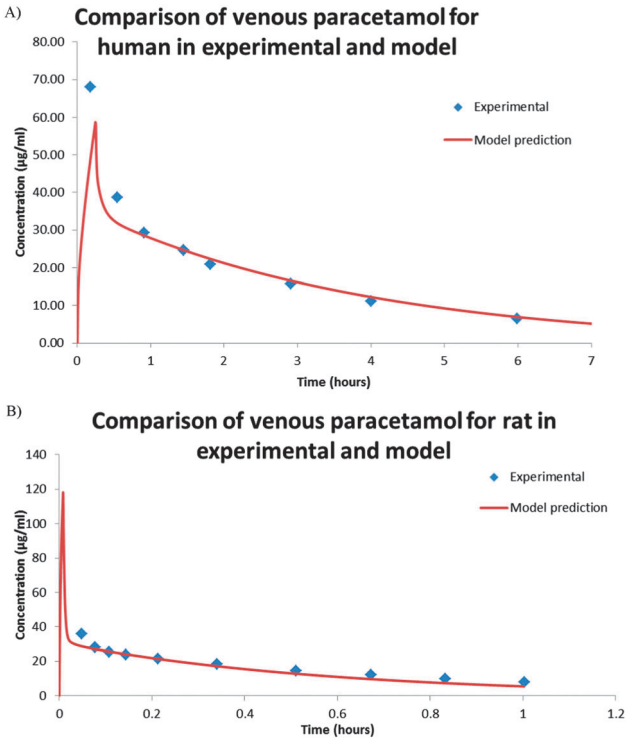

Fig. 2 Comparison between model predictions (lines) and experimentally measured paracetamol venous blood concentration (dots) following IV administration of 2 g paracetamol to human[32] (A) or 15 mg kg^{-1} to rats[33] (B).

Paracetamol in the liver

One of the aims of the PBPK modelling was to improve understanding of the time course of paracetamol concentrations in the liver. Fig. 3 shows the model predictions for hepatic paracetamol in humans after IV administration of the 2 gram dose. The peak paracetamol concentration in the human model occurred after about 15 minutes and then decreased steadily over time. A similar pattern was seen in the rat after a 15 mg kg^{-1} dose, although here the clearance occurred over a shorter time frame (Appendix 3, Fig. A3.1, ESI†).

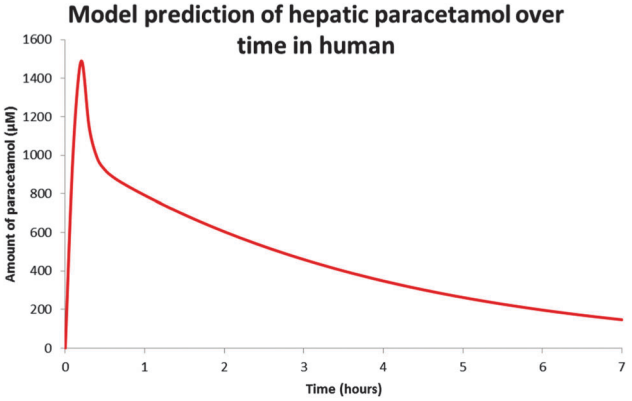

Fig. 3 Model predicted transient concentration of paracetamol in human liver following an IV administration of 2 g paracetamol.

Clearance of 5-oxoproline and ophthalmic acid

When parameterizing the human PBPK component of the model, the 5-oxoproline and ophthalmic acid clearance parameters (CL_{oxuri} and CL_{opuri}) could not be found in the literature. As described in the methods section, this was achieved by empirically adjusting these parameters to enable the model to provide a good fit with experimental data obtained from tracer studies undertaken using radioactive 5-oxoproline in man[28] and radioactive ophthalmic acid in the rat.[29] The best fit obtained is shown in Fig. 4, which shows good agreement between predictions from the model and experimental results.

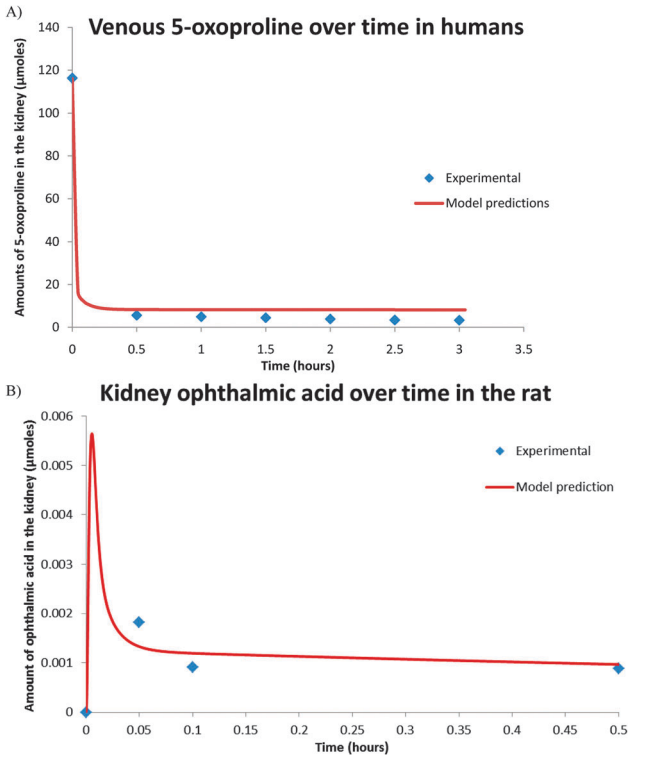

Fig. 4 (A) The experimental (points) and simulated time course (line) in venous 5-oxoproline with time after a 116 µmol injection into the venous compartment.[28] (B) The experimental time course and model prediction of the kidney ophthalmic acid concentrations after a 0.5 µmol kg^{-1} IV injection of ophthalmic acid.[29]

Impact of paracetamol administration on the glutathione pathway

Next, we investigated how changes in the hepatic paracetamol concentration were predicted to affect the intracellular concentrations of hepatic endogenous metabolites of the glutathione pathway in the liver. This is illustrated in Fig. 5, which shows the predicted changes in the concentrations of glutathione, ophthalmic acid and 5-oxoproline in human liver. Administration of paracetamol caused a time dependent decrease in the intrahepatic concentration of glutathione, which is due to conjugation of glutathione with the reactive intermediate NAPQI. The glutathione concentration is predicted to be lowest at 6 hours after dosing and then to recover slowly. As the amount of glutathione decreases, concentrations of 5-oxoproline and ophthalmic acid increase due to increased activity of the glutathione cycle which occurs to replace the glutathione lost by conjugation to NAPQI. Both biomarkers eventually return to their initial values, as both they and paracetamol are cleared from the body. Clearly the rate at which the biomarkers return to their initial value is highly dependent on their clearance rates, which were fitted as explained above. After an initial marked and progressive increase in the intrahepatic concentration of ophthalmic acid over the course of the first 6 hours,

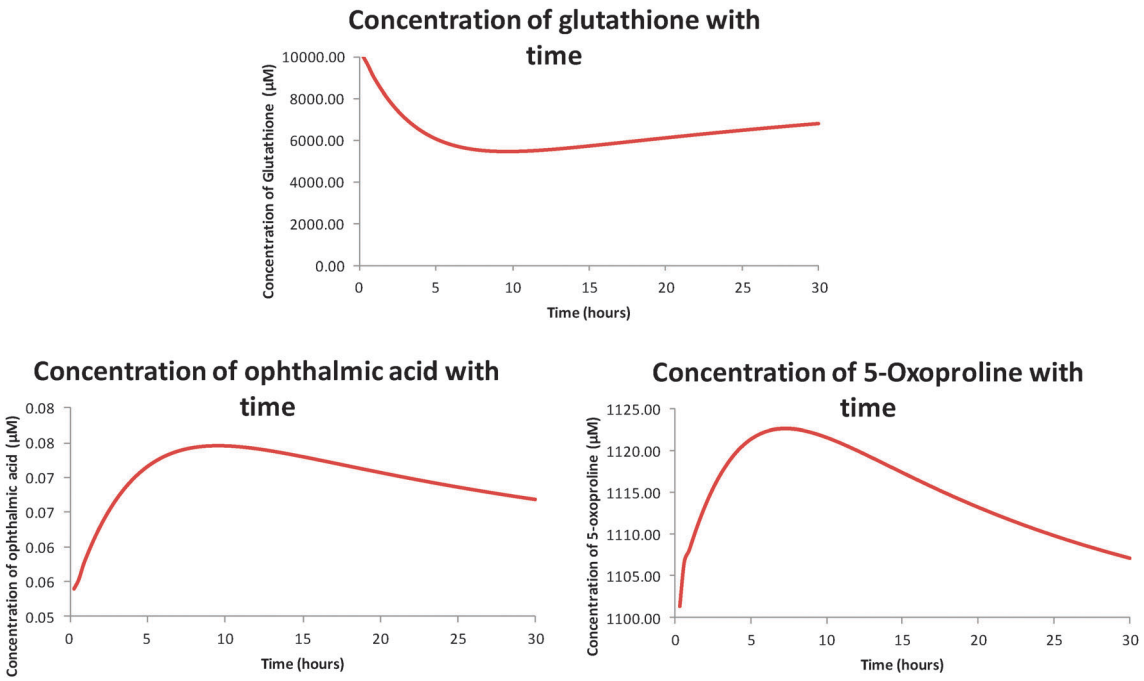

Fig. 5 Predicted time course of glutathione, ophthalmic acid and 5-oxoproline concentrations in the human liver following an IV administration of 2 g paracetamol.

concentrations of this metabolite started decreasing, although these did not return to the initial values until 200 hours post dose. Hepatic 5-oxoproline concentrations returned to initial values after 40 hours and then continued to decrease to values that were below those observed initially. The model predicts that this can be attributed to an initial decrease in the concentration of methionine, which is a key glutathione cycle precursor that is consumed when glutathione is synthesised following its depletion by conjugation to NAPQI after paracetamol dosing. As methionine concentrations recover subsequently, so do the amounts of 5-oxoproline. Both 5-oxoproline and methionine return to their original concentrations after 200 hours (data not shown). A similar pattern of results was seen in the rat model (Appendix 3, Fig. A3.2, ESI†).

PBPK model of 5-oxoproline and ophthalmic acid concentrations in blood and urine

The model predicted increased blood concentrations of both 5-oxoproline and ophthalmic acid with time after paracetamol dose to humans, which closely mirrored the predicted hepatic changes in their concentrations (Fig. 6, paracetamol 13 mmol). Similar time scales were evident for changes in biomarker concentrations in the liver, in the blood and, in the case of 5-oxoproline, in the urine. These results illustrate the importance of timing when sampling blood and urine. Spot sampling of blood only after 48 hours, for example, would not show an increase in 5-oxoproline in either blood or urine. The clearance of ophthalmic acid from the blood was slower than that of 5-oxoprolone, but again a different concentration of ophthalmic acid would be measured depending on when blood is sampled.

Fig. 6 also reveals that the predicted effect of paracetamol on human blood and urine biomarker concentrations was markedly and disproportionately dose dependent. At doses up to and including 7.8 mmol of paracetamol, no effects on the biomarker concentrations were predicted. Increased values were evident after administration of 10.4 mmol paracetamol, while markedly greater increases were seen at the highest modeled dose of 13 mmol paracetamol. Interestingly, similar time scales of effects were seen at 10.4 and 13 mmol paracetamol and at both doses the peak values occurred at about 6 hours. Again, similar trends are seen in the rat model (Appendix 3, Fig. A3.3, ESI†).

Evaluation of concentrations of 5-oxoproline in blood from 11 human subjects, all of whom had ingested paracetamol, revealed values that varied between 2.3 to 11.3 mmol L^{-1}.[15] The present model (Fig. 6) predicted human venous concentration of 5-oxoproline of 2.4 mmol L^{-1} (9620 μmol divided by blood

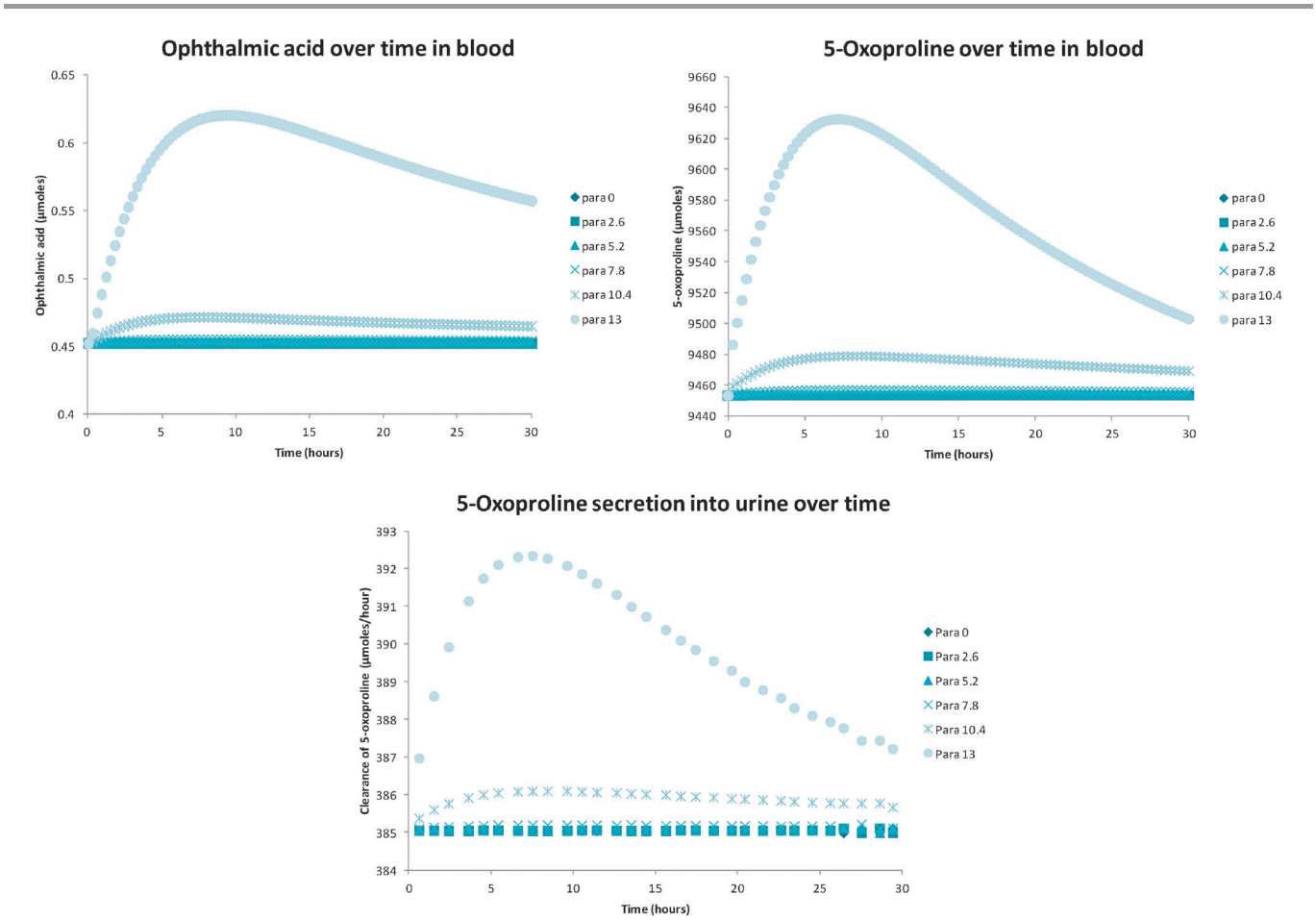

Fig. 6 Model predictions of the concentration of biomarkers in blood (top) and the rate of excrete into the urine (bottom) over time with doses of paracetamol varying from 0 to 13 mmol.

volume 4 L), which falls within that range. The rat model prediction of the endogenous concentration of 5-oxoproline (490 μmol L^{-1}) however, was markedly greater than the value detected in rat plasma of 31 μmol L^{-1}.[7] In addition, the rate of 5-oxoproline excretion in control human subjects has been measured as 50 μmol h^{-1} in one study[27] and in another study was in the range of 11–20 μmol h^{-1}.[26] Compared to these literature values, the clearance rate for 5-oxoproline used in our model was much higher, at 385 μmol h^{-1} (as shown in Fig. 6, bottom pane).

Ophthalmic acid has been measured in serum in the rat and ranged from 0.07 to 0.24 μM[36] whilst the model's prediction of 0.03 μM ophthalmic acid lies just under that range.

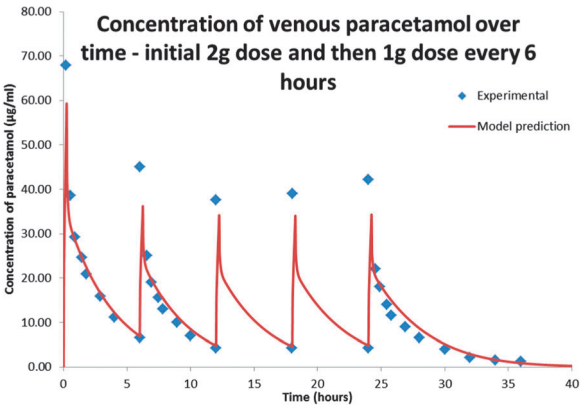

Fig. 7 Multiple paracetamol doses over time in human (initial 2 g dose and then 1 g dose every 6 hours), as measured by Gregoire *et al.*[32] Comparison of experimental values (dots) and model predictions (line) of paracetamol concentration in venous blood as a function of time.

Multiple doses of paracetamol

In a study reported by Gregoire *et al.*[32] multiple doses of paracetamol were given to a patient over time: an initial dose of 2 g and then 1 g every 6 hours. We simulated the same dosing using events in COPASI[35] and predicted the corresponding venous blood concentration of paracetamol. Fig. 7 shows an excellent concordance between experimental observations and model predictions. Fig. 8 shows the corresponding simulations of hepatic concentrations of glutathione, which were predicted to be markedly and progressively depleted, while simulations of ophthalmic acid and 5-oxoproline concentrations became markedly elevated. The model also predicted more modest effects on hepatic concentrations of γ-glutamyl cysteine and methionine.

Discussion

We have developed a joint PBPK model which simulated the pharmacokinetics of paracetamol and two metabolites of the glutathione cycle which have been proposed as candidate biomarkers of glutathione depletion, 5-oxoproline and ophthalmic acid, in humans and rats and combined it with an existing mathematical systems model of glutathione homeostasis.[16] This has allowed us to simulate dynamic changes in the concentration of paracetamol in the liver over time following its IV administration *in vivo* and to predict the subsequent decrease in glutathione concentration which results from detoxification of the reactive metabolite of the drug (NAPQI). We have also shown how this decrease in hepatic glutathione concentration correlates with an increase in the concentrations of 5-oxoproline and ophthalmic acid in the liver, blood and, in the case, of 5-oxoproline the urine. The predicted time scales of

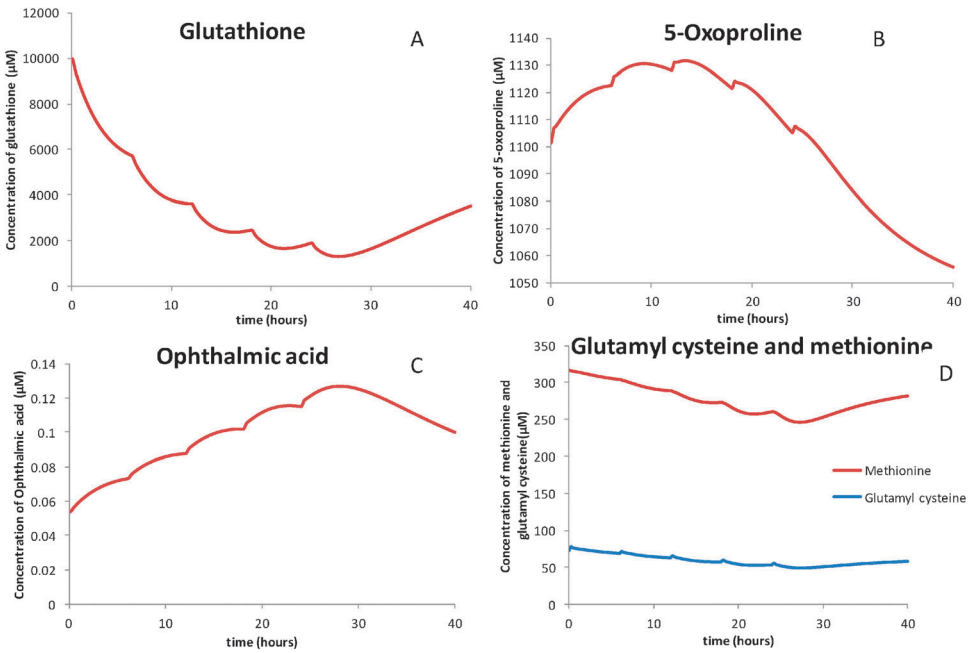

Fig. 8 Multiple paracetamol doses over time in human (initial 2 g dose and then 1 g dose every 6 hours). Changes in endogenous glutathione metabolites in the liver: (A) glutathione, (B) 5-oxoproline, (C) ophthalmic acid, glutamyl cysteine and methionine.

elevated concentrations of the biomarkers, and of their return to normal concentrations, will help with the design in the future of *in vivo* experiments which will be required to refine and test the model. Finally, use of our integrated model to analyse multiple dosing data reported by Gregoire *et al.*[32] has provided further evidence that ophthalmic acid and 5-oxoproline are sensitive biomarkers of hepatic glutathione depletion which may aid in safety testing of drugs such as paracetamol that are metabolised to glutathione reactive metabolites.

The good correlation between experimental measurements and model predictions of paracetamol concentration in venous blood over time provides confidence in the accuracy of the PBPK component of the drug in our model. Currently we cannot be as confident of the precision of the ophthalmic acid and 5-oxoproline components of the PBPK model, since we were unable to find values for hepatic clearance of 5-oxoproline and ophthalmic acid in the literature. These two parameters therefore had to be derived by empirical fitting of experimental data, which was in itself limited. We were reassured that the simulated concentrations of blood 5-oxoproline in humans were within the literature reported ranges. However, the simulated values in the model for concentrations of 5-oxoproline in rat blood, and for clearance from the blood, were much higher than values obtained from the literature.

It can be expected that future experimental determination of 5-oxoproline and ophthalmic acid clearance values will improve the model further. It should also be noted that our predictions assume that the biochemistry of the liver remains constant in response to sustained paracetamol exposure. In particular, other than allowing for increased activity of γ-glutamyl cysteine synthetase (the rate limiting enzyme of the glutathione cycle) following paracetamol administration, which are elicited by glutathione depletion and activation of Nrf2 mediated gene transcription, the current model does not include adaptive changes that might arise in response to chemical insult to liver cells. Given the high likelihood of adaptive changes *in vivo*, experiments are clearly required to verify and/or modify the model input parameters following exposure to paracetamol and other glutathione-depleting hepatotoxic drugs.

The potential utility of the model is exemplified by our simulation of the experiment performed by Gregoire *et al.*[32] After multiple IV administrations of paracetamol to humans, these investigators observed no biochemical signs of hepatotoxicity related to the drug administration and the physical examinations and electrocardiogram (ECG) recordings were normal for all subjects in the study.[32] However, when we used the model to simulate effects on hepatic glutathione during this dosing schedule we predicted a large and sustained decrease in glutathione concentration, which only started to recover following administration of the last dose of paracetamol (Fig. 8A) and was accompanied by predicted increases in hepatic concentrations of both 5-oxoproline and ophthalmic acid (Fig. 8B and C). This finding raises the possibility that ophthalmic acid and 5-oxoproline might be more informative biomarkers of subclinical but mechanistically relevant deleterious effects on liver biochemistry than the classical biomarkers of liver toxicity, such as ALAT, ASAT and prothrombin time, γ-GT, bilirubin and creatinine, measured by Gregoire *et al.*[32]

A closer look at simulated changes in concentrations of 5-oxoproline and ophthalmic acid following repeated paracetamol administration (Fig. 8C) shows that the predicted concentration of ophthalmic acid increased with decreasing amounts of glutathione over the whole time scale. However, the predicted hepatic 5-oxoproline concentrations increased initially at low paracetamol doses but then progressively dropped when subsequent doses of paracetamol were given (Fig. 8B). A likely explanation for this discrepancy is provided by the predicted reductions in hepatic concentrations of the key glutathione cycle precursors methionine and γ-glutamyl cysteine (Fig. 8D). Decreased concentrations of these metabolites will result in a reduction in the rate of glutathione synthesis. As 5-oxoproline and glutathione synthesis rates correlate,[17] this means that the rate of 5-oxoproline synthesis also decreases. Therefore in cases of sustained glutathione depletion, where decreases in endogenous metabolites occur, a decrease in 5-oxoproline concentration can be anticipated. Our previous *in vitro* data have shown that 5-oxoproline is not as useful a biomarker of cellular glutathione depletion as ophthalmic acid at higher paracetamol exposures, where endogenous metabolites are depleted due to severe glutathione lowering.[16,17] In addition to this, our *in vivo* simulations further predict that 5-oxoproline may be less useful as a biomarker of glutathione depletion when the paracetamol administration occurs at sustained therapeutically relevant doses. Furthermore, in instances where determination of both metabolites shows low 5-oxoproline plus high ophthalmic acid concentrations in the blood, reduced hepatic glutathione status can be inferred.

There is some uncertainty as to the exact values of some of the input parameters into the model. We have not evaluated the impact of altering input values on the simulated output from the model. Nevertheless, we consider that the approach we have taken constitutes significant progress. Although there are many examples of PBPK models in the literature, there are relatively few instances where such models have been combined with kinetic models of metabolism and of toxicologically relevant outcomes. The coupling of the PBPK model to a mathematically rigorous systems model of glutathione metabolism which we have undertaken has resulted in a novel approach which has provided useful insights into the interplay between metabolism of paracetamol and the dynamics of intrahepatic glutathione depletion *in vivo*, following administration of single and multiple doses of the drug. It has also highlighted the apparent value and limitations of 5-oxoproline and ophthalmic acid as biomarkers of hepatic glutathione status. In clinical practice, non-invasive monitoring of these biomarkers has the potential to enhance understanding of the relationship between treatment with *N*-acetyl cysteine to replenish depleted glutathione stores, *in vivo* glutathione status and clinical outcome in patients who sustain sever liver injury due to accidental or deliberate paracetamol overdose. In view of the limitations of the current model that we have identified, further refinement will be required before it can be considered suitable for clinical

application. Extension of the modelling approach to other drugs which form glutathione conjugates and cause hepatotoxicity is also warranted, since this can be expected to provide novel insights into the role of glutathione depletion in drug toxicity and the value of 5-oxoproline and ophthalmic acid as mechanistically relevant safety biomarkers.

Abbreviations

Abbreviations for enzymes monitored in classical liver toxicity monitoring

ALAT	alanine transaminase
ASAT	aspartate transaminase
γ-GT	gamma-glutamyl transpeptidase

Parameters for partition coefficient calculations

log *P*	log of the partition octanol:water coefficient
pK_a	acid dissociation constant
log *D*	log of distribution coefficient
f_{up}	fraction unbound in plasma

Compartments of the PBPK model

art	arteriol compartment
ven	venous compartment
lung	lung compartment
liv	liver compartment
tiss	tissue compartment
kid	kidney compartment

Parameters of PBPK model

K_ps	partition coefficients
f_q	fraction of flow rate
f_v	fraction volume
V_{tot}	total volume
Q_{card}	cardiac flow rate
CL_{opuri}	clearance rate of ophthalmic acid
CL_{oxuri}	clearance rate of 5-oxoproline

Enzymes and the reaction rates (v) they catalyse

v[34]	gpx-glutathione *S*-transferase-2.5.1.18
v[35]	gpx-glutathione *S*-transferase-2.5.1.18

Metabolites present at variable concentrations

met	methionine
SAM	*S*-adenosylmethionine
SAH	*S*-adenosylhomocysteine
hcy	homocysteine
cyt	cystathionine
ccys	cytosolic cysteine
bcys	blood cysteine
glc	glutamyl-cysteine
cGSH	cytosolic glutathione
bGSH	blood glutathione
cGSSG	cytosolic glutathione disulfide
bGSSG	blood glutathione disulfide
cgly	cytosolic glycine
cglut	cytosolic glutamate
opa	ophthalmic acid, *N*-[*N*-(γ-glutamyl)-α-aminobutyryl]glycine
oxo	oxoproline, pyroglutamic acid
ASG	acetaminophen glutathione adduct
gluAB	glutamyl aminobutyrate
bgluAA	blood glutamyl amino acid
gln	glutamine
prot	protein

Metabolites assumed to be present at fixed concentrations

AB	2-aminobutyrate
BET	betaine
bgly	blood glycine
bglut	blood glutamate
bmet	blood methionine
cNADPH	nicotinamide adenine dinucleotide phosphate
cser	cytosolic serine
H_2O_2	cellular hydrogen peroxide
bOPA	blood ophthalmic acid, *N*-[*N*-(γ-glutamyl)-α-aminobutyryl]glycine
boxo	blood oxoproline, pyroglutamic acid
bASG	blood acetaminophen glutathione adduct
para	paracetamol (acetaminophen)

Acknowledgements

This work was partly supported by the EPSRC/BBSRC Doctoral Training centre grants (*e.g.* BB/G530225/1) to HVW, as well as by other grants supporting his and this work from the EPSRC (EP/D508053/1), BBSRC (BB/C008219/1, BB/5302251, BB/I004688/1, BB/I017186/1, BB/I004696/1, BB/I00470X/1, BB/J003883/1, BB/J020060/1, BB/J500422/1), NWO (various), and EU-FP7 (EC-MOAN [NO043235], BioSim, NucSys [MRTN-CT-019496], ITFoM, ISBE, SYNPOL).

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