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A mathematical modelling approach to assessing the reliability of biomarkers of glutathione metabolism

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ABSTRACT

One of the main pathways for the detoxification of reactive metabolites in the liver involves glutathione conjugation. Metabolic profiling studies have shown paradoxical responses in glutathione-related biochemical pathways. One of these is the increase in 5-oxoproline and ophthalmic acid concentrations with increased dosage of paracetamol. Experimental studies have thus far failed to resolve these paradoxes and the robustness of how these proposed biomarkers correlate with liver glutathione levels has been questioned. To better understand how these biomarkers behave in the glutathione system a kinetic model of this pathway was made. By using metabolic control analysis and by simulating biomarker levels under a variety of conditions, we found that 5-oxoproline and ophthalmic acid concentrations may not only depend on the glutathione but also on the methionine status of the cell. We show that neither of the two potential biomarkers are reliable on their own since they need additional information about the methionine status of the system to relate them uniquely to intracellular glutathione concentration. However, when both biomarkers are measured simultaneously a direct inference of the glutathione concentration can be made, irrespective of the methionine concentration in the system.

1. Introduction

Glutathione is often associated with toxicology due to its role in detoxifying electrophilic metabolites leading to the production of *N*-acetyl cysteinyl conjugates (mercapturates) (Meister, 1988). This is particularly true in the case of paracetamol (acetaminophen, APAP). APAP is subject to cytochrome (CYP) P450-based oxidative metabolism via CYP 2E1. This results in the formation of a reactive metabolite that is nucleophilic and at high therapeutic doses is detoxified by reacting with cellular glutathione to form glutathione conjugates. At high concentrations of paracetamol this protective mechanism depletes the system for glutathione leading to high levels of reactive metabolites which in turn can result in oxidative stress and covalent modification of cellular macromolecules (Mitchell et al., 1973).

Many factors can influence the rate of glutathione synthesis and therefore the amount of glutathione available in the cell for drug detoxification (Lu, 2009). These range from the expression level (regulation of enzyme production) to metabolism (effect of surrounding metabolism such as methionine cycle) to environmental (effect of nutrition and uptake of amino acids). Predicting how an individual's glutathione level will react to a xenobiotic attack on the liver is currently difficult. Predictive biomarkers that indicate the level of glutathione metabolism could predict individual responses to drugs and thereby allow prediction of maximum drug dose levels, or aid in the assessment of glutathione levels in clinical trials (Mendrick and Schnackenberg, 2009).

Recently the tripeptide ophthalmic acid, a non-sulphur-containing structural analogue of glutathione, was suggested as a biomarker following APAP administration to mice (Soga et al., 2006). In that study, metabolic profiles were obtained, enabling the determination of global changes in a wide range of metabolites in serum

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and liver extracts of APAP-treated mice. Ophthalmic acid concentrations had increased within one hour after drug administration, remaining above normal for the next 3 h with significant decreases in glutathione concentrations in the same time period.

A number of studies have also shown increases in the concentration of 5-oxoproline following the administration of hepatic toxicants such as acetaminophen and bromobenzene, where CYP dependent bioactivation produces metabolites that require deactivation via glutathione dependent mechanisms, thereby resulting in glutathione depletion. Originally 5-oxoproline was identified in rat urine by NMR spectroscopy after dosing rats with paracetamol (Ghauri et al., 1993). Oxoprolinuria has also been observed in humans (Creer et al., 1989) following deficiency in enzymes of the glutamyl cycle, i.e. 5-oxoprolinase and glutathione synthase. A recent integrated metabonomic study into bromobenzene-induced hepatotoxicity also identified an increase in 5-oxoproline production in rats (Waters et al., 2006) and an LC-MS assay has shown an increase in 5-oxoproline in THLE-2E1 cell system with paracetamol dosing (Geenen et al., in press).

The studies above suggested a link between ophthalmic acid, 5-oxoproline and glutathione depletion. However, it was not investigated why a correlation in changes in their concentrations should exist and without a mechanistic interpretation of the biomarker's functioning it is unknown whether any such correlation is robust. Recent publications did not show an increase in ophthalmic acid (Geenen et al., 2011) concentrations after methapyrilene toxicity in rat plasma samples. In addition measurements where 2E1 THLE-cells (SV40 large T antigen immortalised human liver epithelial cells transfected with individual cytochrome P450 enzymes (Macé et al., 1997)) were dosed with paracetamol, have not shown a significant increase in extracellular ophthalmic acid concentrations (Geenen et al., in press). Here we make use of a mathematical model of what is known about glutathione metabolism. We ask whether one should expect 5-oxoproline and ophthalmic acid to be robust biomarkers for glutathione depletion.

2. Results

2.1. Steady state analysis of the model

For the construction of our mathematical model we adapted an existing model on glutathione metabolism (Reed et al., 2008a). The original model was simplified from 60 to 24 reactions, mostly by removing the folate pathway, and was extended to 41 reactions with relevant parts of drug detoxification metabolism (a schema of the model is shown in Fig. 1). After the adaptations we changed some of the model parameters (V_{max} values and unknown kinetic parameters, a list of which is included in Appendix B) to minimize

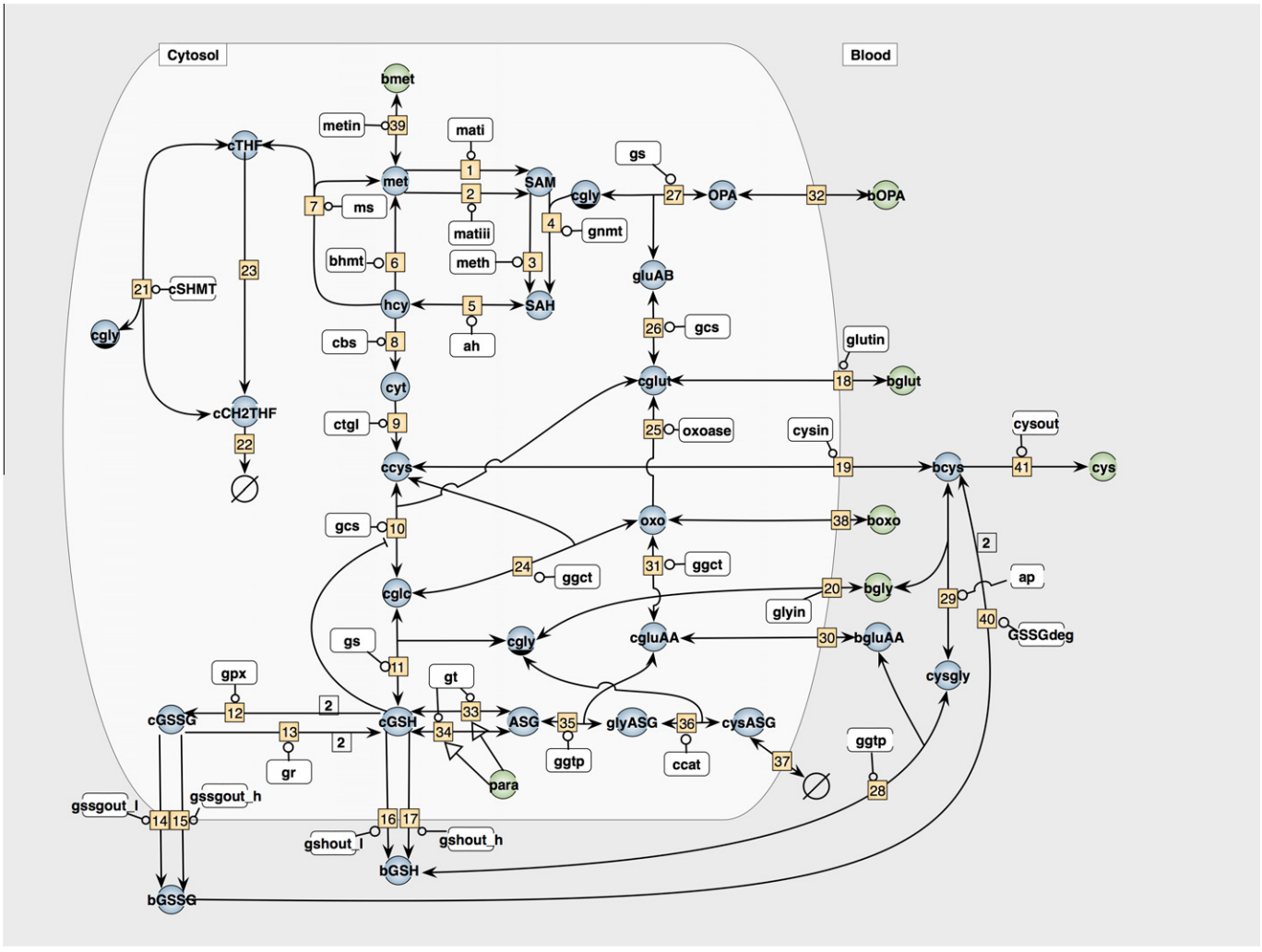

Fig. 1. Schematic representation of the reaction network of the mathematical model. The network contains methionine metabolism, glutathione metabolism, 5-oxoproline and ophthalmic acid metabolism and detoxification pathways.

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Table 1
The steady state metabolite concentrations and flux values at 0, 20 and 500 μM paracetamol. Metabolite concentrations are in μM and fluxes are in μM/h. Fluxes can be related to reactions using Fig. 1. Steady state calculations were performed in Mathematica. All numbers are rounded up to two significant figures, for more precision please solve the steady state at http://jjj.mib.ac.uk/webMathematica/Examples/run.jsp?modelName = geenen.

Paracetamol (μM)	0	20	500
ASG	0.0043	2.8	7.7
bcys	180	120	14
bgluAA	1.1	0.79	0.12
bGSH	8.1	6.0	0.92
bGSSG	0.49	0.30	0.0006
cCH2THF	0.33	0.31	0.27
ccys	210	120	10
cglc	190	140	5.8
cgluAA	4.7	5.2	4.1
cglut	540	670	640
cgly	1800	2100	2900
cGSH	1500	1100	32
cGSSG	69	42	0.084
cTHF	5.3	5.6	6.3
cysASG	5.0	5.7	6.7
cysgly	3.6	2.5	0.35
cyt	33	34	34
gluAB	2.7	5.9	0.86
glyASG	92	120	200
hcy	1.1	1.1	1.1
met	48	47	46
OPA	1.0	1.0	1.0
oxo	2.5	3.2	1.6
SAH	19	20	20
SAM	38	37	36
v[1]	120	120	120
v[2]	66	66	65
v[3]	130	130	130
v[4]	57	59	62
v[5]	190	190	190
v[6]	61	62	63
v[7]	38	37	35
v[8]	90	90	91
v[9]	90	90	91
v[10]	940	960	500
v[11]	110	120	96
v[12]	55	39	0.11
v[13]	50	36	0.1
v[21]	24	23	21
v[22]	38	37	35
v[23]	14	14	14
v[24]	830	840	400
v[25]	930	950	500
v[26]	1.5	4.9	12
v[27]	1.5	4.9	12
v[30]	100	77	12
v[31]	100	110	96
v[33]	0.0	6.7	21
v[34]	0.0	26	64
v[35]	0.0	33	84
v[36]	0.0	33	84
v[40]	5.0	3.1	0.0062
v[28]	100	77	12
v[29]	100	77	12
v[18]	12	12	12
v[19]	25	25	5.1
v[20]	150	120	65
v[14]	3.9	2.4	0.0047
v[15]	1.1	0.7	0.0014
v[16]	42	16	0.00043
v[17]	62	60	12
v[32]	1.5	4.9	12
v[37]	0.0	33	84
v[39]	90	90	91
v[38]	0.57	0.83	0.22
v[41]	90	58	7.0

the differences in steady state metabolite concentrations in the adapted model as compared to the original model.

With fixed external metabolite concentrations, a steady state could be calculated for the model both in the presence and in the absence of paracetamol. In Table 1 (columns 1 and 2) the endogenous metabolite concentrations are shown for the steady state calculated at zero paracetamol. To check whether these calculated steady state metabolite concentrations were within physiologically relevant ranges, we compared the values to available values in the literature (see references in Table 2). It must be noted here that some of these values were also used by Reed et al. when fitting their model and this check can thus not be seen as a validation of the model. When comparing with available values in the literature, most steady state concentrations calculated by the model fell into the expected range (Table 2). The main exception was the ratio of reduced glutathione to glutathione disulphide. We here focus on the glutathione concentrations and we did not go into further detail concerning the ratio of reduced to oxidized glutathione.

Because there is no complete quantitative data set available for paracetamol toxicity or biomarker formation to allow validation of the model in the presence of paracetamol, we cannot presume that this model will be a precise quantitative representation of the biological system. However we assumed that the model was good enough to make an investigation into the qualitative features of glutathione metabolism and paracetamol toxicity worthwhile. In the following, we test the validity of using 5-oxoproline and ophthalmic acid as biomarkers for glutathione depletion, assuming that the mathematical model was correct enough for such an analysis.

2.2. Correlation between biomarkers and glutathione

We analyzed the steady state effects of increasing paracetamol concentrations on the glutathione concentration and the ophthalmic acid and 5-oxoproline production rates. The efflux rates of ophthalmic acid (v32) and 5-oxoproline (v38) can be used to assess their roles as biomarkers; increased export fluxes will lead to increased extracellular concentrations of the molecules. In the model the extracellular concentrations of ophthalmic acid and 5-oxoproline are clamped (i.e. they are kept constant), to be able to do steady state analyses of the system. Fig. 2 shows a negative correlation between ophthalmic acid production rate with increasing glutathione concentrations, caused by varying the paracetamol concentration (an increase in paracetamol leads to a decrease in glutathione concentration, see below). The correlation between 5-oxoproline production rate and glutathione is not monotonic (Fig. 3), there is a maximal 5-oxoproline production rate, and at high glutathione levels (i.e. low paracetamol concentrations) a negative correlation between glutathione and 5-oxoproline production rate is observed. Thus, from these model simulations one would conclude that 5-oxoproline only functions as a biomarker at low paracetamol (high glutathione) concentrations.

To analyse the observed correlations between biomarker production rates and paracetamol levels we determined the steady state metabolite concentrations and flux values at 0, 20 and 500 μM paracetamol (Table 1). As expected, intracellular glutathione and cysteine decreased in concentration with increasing paracetamol concentrations. Few changes occurred in the metabolite concentrations of the methionine pathway (reactions v1 to v10). This may be due to a lack of feedback from cysteine and glutathione metabolism on methionine incorporation.

From the steady state modelling results obtained at 20 and 500 μM paracetamol an increased ophthalmic acid production rate can be observed which correlates well with a decrease in glutathione. We propose that the mechanism underlying this correlation is a competition between the ophthalmic acid pathway and the glutathione synthesis pathway. The synthesis of both ophthalmic acid and glutathione are catalyzed by the same enzymes, glutam-

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Table 2
Comparison of the literature values and model predicted steady state values of metabolite concentrations and fluxes.

	Steady state model value	Literature values	Citation
Cysteine	210 μM	150–250 μM	Wu et al. (2004)
Glutathione (GSH)	1500 μM	500–10000 μM	Inoue et al. (1984) and Wu et al. (2004)
Cystathione	33 μM	40 μM	Tallen et al. (1958)
Blood cysteine	180 μM	110–350 μM	Wu et al. (2004)
Blood glutathione	8.1 μM	2–20 μM	James et al. (2004), Pogribna et al. (2001) and Wu et al. (2004)
Blood glutathione disulphide (GSSG)	0.49 μM	0.14–0.34 μM	James et al. (2004), Pogribna et al. (2001) and Wu et al. (2004)
Methionine cycle flux	190 μM/h	210 μM/h	Finkelstein and Martin (1984)
Remethylation	99 μM/h	100 μM/h	Finkelstein and Martin (1984)
Glutamate	540 μM	250–1400 μM (average 681 μM)	Gressner (1974), Soley and Alemany (1980) and Wu and Bollman (1954)
[GSH]/[GSSG]	22	100	Griffith (1999)

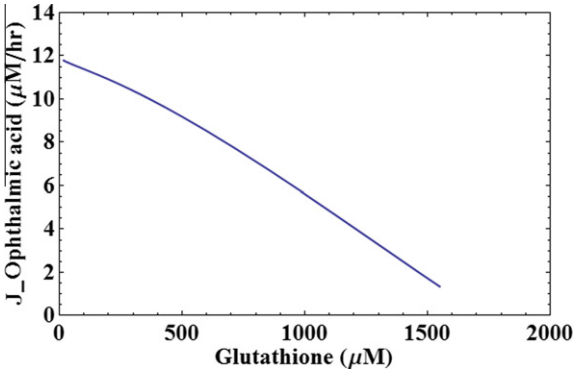

Fig. 2. The relationship between steady state production rate of ophthalmic acid (in this paper J_ symbolises a steady state rate or flux) with intracellular glutathione concentration. Steady state was calculated by scanning paracetamol concentrations (0–1200 μM) at a fixed external methionine concentration of 30 μM.

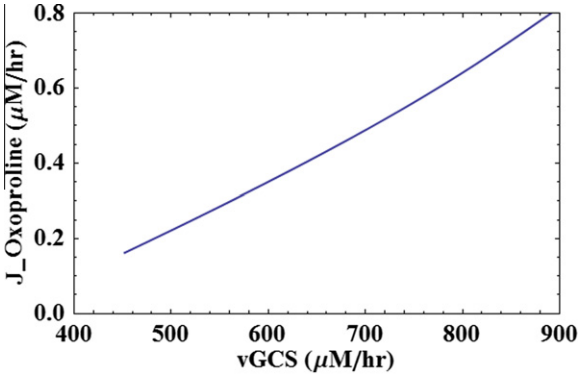

Fig. 4. The relationship between steady state production rate of 5-oxoproline and vGCS (v10). Steady state was scanned at varying paracetamol (0–1200 μM) at a fixed external methionine concentration of 30 μM.

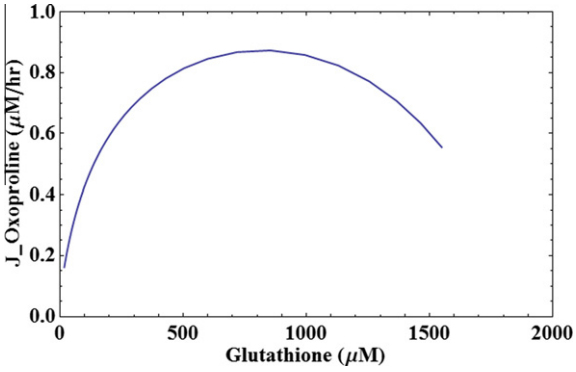

Fig. 3. The relationship between steady state production rate of 5-oxoproline with intracellular glutathione concentration. Steady state was scanned at varying paracetamol (0–1200 μM) at a fixed external methionine concentration of 30 μM.

yl-cysteine synthetase (GCS) and glutathione synthetase (GS). Both these enzymes have a higher affinity for their substrates from the glutathione synthesis pathway (γ-glutamyl cysteine and cysteine) than for their substrates in the ophthalmic acid producing pathway (Meister and Tate, 1976; Waley, 1956). With increasing paracetamol concentrations the intracellular concentrations of cysteine and γ-glutamyl cysteine decrease and thereby make the ophthalmic acid production pathway more competitive, leading to an increased production of this biomarker. To test this we clamped the internal cysteine and internal glutamate concentrations in the model so that they would not decrease even when glutathione was depleted by paracetamol. Under these conditions no ophthalmic acid was produced, showing that the negative correlation between glutathione and ophthalmic acid is dependent on a decrease in cysteine and γ-glutamyl cysteine.

From the steady state results obtained at 20 μM paracetamol an increased 5-oxoproline production rate can be observed, along with an increase in the flux through the entire 5-oxoproline cycle. On the basis of model analyses we propose the following mechanism for the observed correlation between 5-oxoproline and glutathione concentration. At low paracetamol concentrations a decrease in glutathione concentration decreases its inhibition of GCS and GS causing a small increase in v10 and v11. Fig. 4 shows the positive correlation between vGCS (v10) and 5-oxoproline at varying paracetamol concentrations. The increased glutathione production rate causes an increase in the total flux through v35 (recycling of glutamate through paracetamol detoxification pathway) plus v30 (recycling of glutamate through glutathione degradation), thus collectively increasing the rate of formation of 5-oxoproline via v31 (as v30 + v35 = v31). In addition paracetamol addition causes a decrease in the cysteine concentration, a product of reaction v24 and this stimulates the 5-oxoproline production enzyme. The increased production of 5-oxoproline via v31 and v24 leads to an increase in the 5-oxoproline concentration and in its transport across the membrane (v38). As mentioned before (Fig. 3), at higher paracetamol concentrations (i.e. leading to a decrease in glutathione production), a reversal in the correlation between glutathione and 5-oxoproline is observed, i.e. at high paracetamol concentrations a decrease in 5-oxoproline production rate is found. This change in correlation is due to the rapid removal of cysteine from the system via reaction v37 with paracetamol (in-

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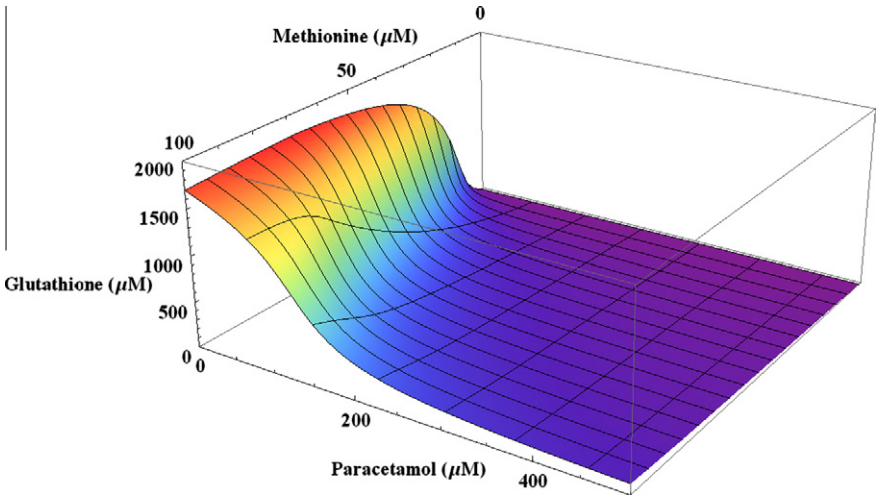

Fig. 5. 3D plot of the relationship between paracetamol, external methionine and intracellular glutathione. Scanning the steady state for varying paracetamol (0–1200 μM) and external methionine concentration (0.5–100 μM).

crease in paracetamol causes an increase in glutathione being removed from the system by v33 and v34. Glutamate is recycled in v35 and glycine in v36 while cysteine is transported out bound to paracetamol by reaction v37). Whereas the normal turnover of glutathione via reactions v16, v17, v28 and v29 leads to a recovery of cysteine via reaction v19, the binding of glutathione to the intracellular drug and its removal from the system via mercapturic acid transport leads to removal of cysteine from the system. Thus, when the influx rate of cysteine via v9 runs at maximal rate, any further decrease in glutathione will no longer enhance v10 and v11 due to the limiting concentration of cysteine. The change from a negative to a positive correlation (shown in Fig. 3) between glutathione concentration and 5-oxoproline production lies at the point where a decrease in inhibition by glutathione (which stimulates v10) does not compensate for the decrease in activity of v10 caused by a decreasing cysteine concentration.

2.3. Control of pathway fluxes

Flux control coefficients of enzymes denote the extent to which the latter limit the flux (Kacser and Burns, 1973; Heinrich and Rapoport, 1974). Metabolic control analysis (MCA) on the model showed that the highest flux control coefficients for ophthalmic acid (v32) and 5-oxoproline (v38) production were in the methionine pathway (transport (v39) and first two reactions methionine adenosyltransferase (vMATi (v1) and vMATiii (v2)) and glutathione transferase (vGT, v33 and v34). Due to the importance of paracetamol detoxification, the control of the glutathione transferases was expected. The high control of methionine transport and methionine cycle entry reactions lead us to believe that the methionine influx rate plays an important role in protecting the cell against paracetamol toxicity, and in the reliability of the biomarkers.

2.4. Steady state analysis at varying external methionine

One of the important properties of a biomarker is that it has to be robust, meaning that under all physiological conditions the correlation between biomarker and glutathione concentration will stay the same. Because of methionine's seeming importance to the glutathione pathway, both in the literature (Ghauri et al., 1993) and in our model, we investigated the relationship of the biomarkers ophthalmic acid and 5-oxoproline with varying external methionine concentrations.

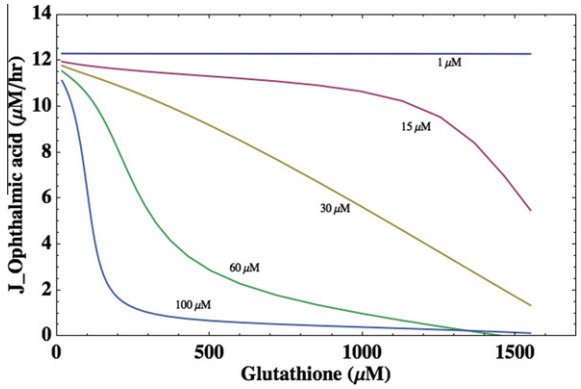

Fig. 6. The relationship between ophthalmic acid production and intracelullar glutathione concentration at 5 external methionine concentrations. Steady state was calculated via scanning the paracetamol (0–1200 μM) and 5 external methionine concentration (1–100 μM).

We first analyzed the steady state relationship between glutathione, methionine and paracetamol (Fig. 5). In the absence of paracetamol an increase in the steady state glutathione concentration was observed, with a maximum of approximately 1.7 mM, with increasing concentrations of methionine (0.5–100 μM). At any methionine concentration paracetamol addition (0–1200 μM) always lead to a decrease in glutathione concentration but the sensitivity for paracetamol was lower at higher methionine concentrations.

We subsequently tested the correlation between biomarker production and glutathione concentration at different external methionine concentrations. We saw earlier (Fig. 2) that ophthalmic acid was a very good biomarker at a methionine concentration of 30 μM, with an almost linear negative correlation of its production rate with glutathione over its full range of concentration variation. However, when tested at various methionine concentrations (Fig. 6), it can be seen that although the relation is always monotonic, the correlation is very dependent on methionine. At low methionine concentrations, ophthalmic acid production reaches maximal rates upon small changes in glutathione concentrations and becomes relatively insensitive for further decreases, while at high methionine concentrations the production rate of ophthalmic acid is low and insensitive at high glutathione concentrations and only increases rapidly at relatively low concentrations of glutathi-

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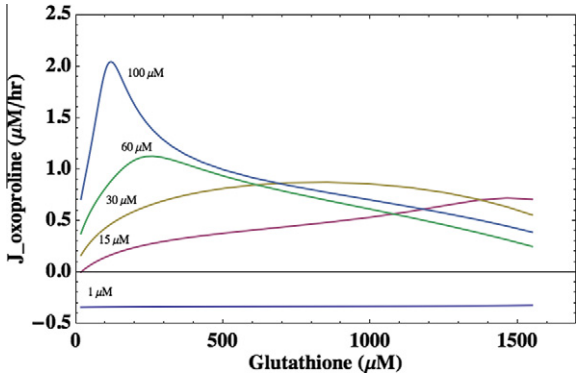

Fig. 7. The relationship between 5-oxoproline production and intracelullar glutathione concentration at 5 external methionine concentrations. Steady state was calculated via scanning the paracetamol (0–1200 μM) and 5 external methionine concentration (1–100 μM). The external concentration of 5-oxoproline is fixed at 1 μM to represent a low endogenous concentration. At low methionine this causes an influx of 5-oxoproline into the cell and therefore a negative value of 5-oxoproline flux.

one. In general, (other than the 1 μM methionine concentration), it can be seen that ophthalmic acid is a better biomarker at high paracetamol (low glutathione) and low methionine concentrations.

Initially (Fig. 3) 5-oxoproline appeared a rather weak biomarker since no unique relation exists between 5-oxoproline production rates and glutathione concentration. However, when tested over a range of methionine concentrations (Fig. 7), it can be seen that at high methionine concentrations, 5-oxoproline is a fairly good biomarker for the glutathione concentration. At high methionine concentration and low paracetamol (high glutathione) there is a negative correlation with glutathione which is expected for the biomarkers. At lower methionine concentrations a positive correlation is observed between 5-oxoproline production rates and glutathione, over the complete physiological range of glutathione concentrations. At the lowest methionine concentration (1 μM) an influx of 5-oxoproline into the cell is observed giving a negative value for 5-oxoproline flux.

From the model analysis at varying external concentrations of methionine it is clear that neither of the two potential biomarkers functions as a good biomarker for the intracellular glutathione concentration. Both metabolites need additional information (e.g. methionine concentration) to relate their production rates to intracellular glutathione levels. The two biomarkers have complementary responses under different methionine concentrations; ophthalmic acid production was a good marker at high paracetamol and low methionine, while 5-oxoproline was better at high methionine and low paracetamol. We tested whether the combined use of both biomarkers would give a good indication of the glutathione concentration. The result of this test is given in Fig. 8, which shows a 3D plot of glutathione concentration, versus 5-oxoproline and ophthalmic acid production rates. The results show the combined steady state effects of varying paracetamol (0–1200 μM) and external methionine concentration (0.5–100 μM). The figure shows a unique relationship between 5-oxoproline and ophthalmic acid production rates and the glutathione concentration. Importantly from these results it can be concluded that if both the rates of 5-oxoproline and ophthalmic acid production are measured, the concentration of intracellular glutathione can be deduced (irrespective of the methionine concentration). For example by looking at the red lines on Fig. 8 it can be seen that with given fluxes of ophthalmic acid production (5 μM/h) and 5-oxoproline (1.5 μM/h) a unique glutathione concentration (1300 μM) can be predicted.

The way in which the biomarkers complement each other can be explained by investigating the mechanism of their correlation with glutathione. Fig. 7 shows that 5-oxoproline is a good biomarker at high methionine and low paracetamol concentrations. The model steady state analysis shows that as described above this is because at this point the flux through glutathione synthesis is increased resulting also in an increase in the flux through the oxoproline cycle. In addition, a decrease in the cysteine concentration is observed, which stimulates 5-oxoproline production reaction v24. At high paracetamol and low methionine concentrations, ophthalmic acid is a good biomarker as, under these conditions, the precursors for glutathione synthesis deplete, reducing the flux through glutathione synthesis and allowing an increase in ophthalmic acid synthesis (which is a competing pathway of glutathione synthesis).

3. Discussion

The effectiveness, toxicity and thereby maximal dosage of many drugs depend on hepatic detoxification pathways. Predictive biomarkers, which would allow assessment of an individual's drug metabolism activity, might indirectly but dramatically enhance individualized therapies by allowing doses to be optimized and dose-related toxicity avoided.

A kinetic model of glutathione metabolism and its detoxification pathway for paracetamol was constructed and used for a tentative investigation of the reliability of two potential biomarkers.

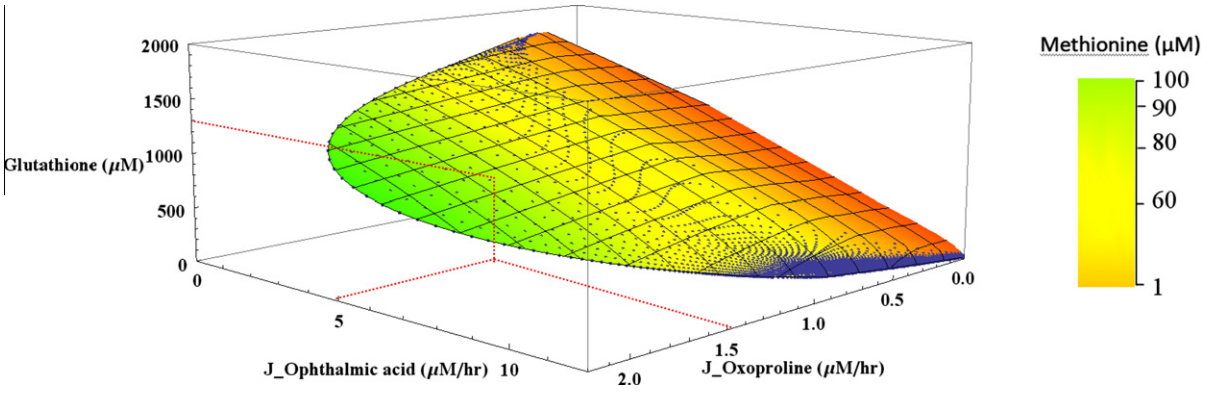

Fig. 8. 3D plot showing the relationship between ophthalmic acid production, 5-oxoproline production and intracelullar glutathione concentration at 5 external methionine concentrations. Steady state was calculated via scanning the paracetamol (0–1200 μM) and external methionine concentration (0.5–100 μM). The simulated points are in blue, while the colouring is representative of the methionine concentration (intensity scale to the right of the figure). The red lines exemplify how if e.g. the flux of ophthalmic acid production is set at 5 μM/hr and the flux of oxoprolineat (1.5 μM/hr it is possible to extrapolate and predict a unique intracellular glutathione concentration (1300 μM).

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Whereas both ophthalmic acid and 5-oxoproline have been suggested individually as biomarkers for the intracellular glutathione concentration, we have shown here that the correlation between either of the two biomarkers and glutathione concentration is highly dependent on the methionine concentration in the system. These results could be used to explain seeming contradictive experimental findings. For example, the fact that it has been reported (Geenen et al., 2011) that ophthalmic acid was not observed under mild liver toxicity of methapyrilene (a drug that also depletes glutathione) might have been due to high methionine concentration present in the liver during this experiment. A striking result of the current study was that a combined use of both biomarkers resulted in a unique relation between their production rates and the glutathione concentration, irrespective of the methionine status of the cell. More than one biomarker may need to be measured to analyse something as complex as liver toxicity.

It should be noted that this study was based on a mathematical model for which many parameter values were uncertain. Consequently, the quantitative predictions made here by the model offer limited certainty. Yet we consider that making these predictions constitutes progress and provides some insight. The progress resides in the fact that new testable predictions have been made for a hypothesis. By testing these, either the existing model can be validated, or an experimental basis for a better model will arise. The insight is useful, because it is based on the best of the molecular and mechanistic knowledge about the relevant pathway that may be available at the moment. Therefore, although the model predictions may not be correct or well-based, they may well be better than any of the intuitive predictions on the basis of which the pharmaceutical industry needed to operate until now. Moreover, this study may have been one of the first approaches of systems toxicology that makes full use of integration with systems biology. Thereby it may serve as a prototype for work towards a systems model of toxicology, contributing to improved understanding, prediction and prevention of adverse drug reactions.

4. Materials and methods

4.1. Modifying the Reed model

The model of glutathione metabolism was based on the model by Reed et al. (Reed et al., 2008b). The Reed model is available from the JWS Online website (Olivier and Snoep, 2004) and can be simulated in a web browser at any of the JWS Online servers (e.g. http://jjj.mib.ac.uk/webMathematica/Examples/run.jsp?modelName=reed). We coded the Reed model in *Mathematica* (http://www.wolfram.com) and the same steady state was obtained as calculated by Reed. The folate pathway, which was modelled very detailed in the Reed model, was strongly reduced for the current study (limited to three reactions), but all reductions were made such that the original steady state was not perturbed. Details of the reactions and kinetics involved can be found in Appendix A.

We needed to extend the Reed model with glutathione detoxifying pathways, and include the formation of ophthalmic acid and 5-oxoproline. Since these reactions were new to the Reed model we treat them in more detail here.

4.2. Glutathione detoxifying pathway

vGT was modelled as previously published by Pabst et al. (1974) and Raucy et al. (1989). vGGTP and vCCAT were added as v35 and v36 as reversible Michaelis Menten reactions in the model. Transport of mercapturic acid out of the cell is modelling by reversible mass action. Parameters can be found in Appendix A.

4.3. Ophthalmic acid synthesis

Ophthalmic acid is synthesised in a two step pathway from aminobutyrate (v26) and glutamate (v27) (Fig. 1). These reactions are catalyzed by the same enzymes that synthesise glutathione (v10 and v11): glutathione synthase (GS) and glutamylcysteine synthetase (GCS). Since each of these enzymes catalyze two different reactions, the substrates (and products) of the individual reactions compete with each other for binding to the active site. This competitive behaviour of the enzymes is reflected in the enzyme kinetics; the denominator of the rate equation is identical for both reactions (and contains substrates and products of both reactions) while the numerator is specific (and contains only the substrates and products for the reaction), see below for details.

Reaction of glutamylcysteine synthetase for glutamylcysteine synthesis (v10):

$$v[10] = \frac{\frac{glu}{K_{glu}} \cdot \frac{cys}{K_{cys}} \cdot V_{max+} \cdot \left(1 - \frac{glc}{glu \cdot cys \cdot K_{eq}}\right)}{\left(1 + \frac{cys}{K_{cys}} + \frac{AB}{K_{AB}}\right) \cdot \left(1 + \frac{glu}{K_{glu}} \frac{glc}{K_{glc}} \frac{gluAB}{K_{gluAB}} \frac{GSH}{K_{GSH}}\right)}$$

Reaction of glutamylcysteine synthetase for glutamylaminobutyrate synthesis (v26):

$$v[26] = \frac{\frac{glu}{K_{glu}} \cdot \frac{AB}{K_{AB}} \cdot V_{max+} \cdot \left(1 - \frac{gluAB}{glu \cdot AB \cdot K_{eq}}\right)}{\left(1 + \frac{cys}{K_{cys}} + \frac{AB}{K_{AB}}\right) \cdot \left(1 + \frac{glu}{K_{glu}} \frac{glc}{K_{glc}} \frac{gluAB}{K_{gluAB}} \frac{GSH}{K_{GSH}}\right)}$$

Reaction of glutathione synthase for glutathione synthesis (v11):

$$v[11] = \frac{\frac{gly}{K_{gly}} \cdot \frac{cglc}{K_{cglc}} \cdot V_{max+} \left(1 - \frac{cGSH}{gly \cdot cglc \cdot K_{eq}}\right)}{\left(1 + \frac{cglc}{K_{cglc}} + \frac{gluAB}{K_{gluAB}}\right) \cdot \left(1 + \frac{OPA}{K_{OPA}} \frac{cgly}{K_{gly}} \frac{GSH}{K_{GSH}}\right)}$$

Reaction of glutathione synthase for ophthalmic acid synthesis (v27):

$$v[27] = \frac{\frac{gly}{K_{gly}} \cdot \frac{gluAB}{K_{cgluAB}} \cdot V_{max+} \left(1 - \frac{OPA}{gly \cdot gluAB \cdot K_{eq}}\right)}{\left(1 + \frac{cglc}{K_{cglc}} + \frac{gluAB}{K_{gluAB}}\right) \cdot \left(1 + \frac{OPA}{K_{OPA}} \frac{cgly}{K_{gly}} \frac{GSH}{K_{GSH}}\right)}$$

Transport of ophthalmic acid was based on mass action kinetics:

$$v[32] = \mathrm{KtrsOPA} \cdot ([\mathrm{OPA}] - [\mathrm{bOPA}]).$$

4.4. Synthesis of 5-oxoproline

5-Oxoproline is synthesized from γ-glutamyl amino acid (v31) or γ-glutamyl cysteine (v24) by Gamma-glutamylcyclotransferase (GCT). The rate equations used for vGCT and for transport of 5-oxoproline (v33) are generic reversible Michaelis Menten equations (see Appendix A). Breakdown of 5-oxoproline via v_{OP} (oxoprolinase) was described with an irreversible Michaelis–Menten equation to reflect the ATP dependency of the reaction.

Transport of 5-oxoproline was based on mass action kinetics:

$$v[38] = \mathrm{Ktrsoxo}([\mathrm{oxo}] - [\mathrm{boxo}]).$$

4.5. Parameter estimation

The parameter values used in the model were obtained from the literature (including values of the original Reed model) see Appendix A. Parameters for which we could not obtain literature values were fitted to steady state metabolite concentrations

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within the physiological range of liver metabolism. For this we used the steady state flux and concentration values of the Reed model at zero paracetamol and the metabolite concentrations as measured by Soga et al. at 12.4 mM paracetamol (Soga et al., 2006). Parameter values were passed to an objective function that fitted the unknown parameter values to minimize the difference with the specified steady state concentrations and fluxes (Appendix B). The fitting procedure worked on a three step principle. Firstly the closeness of fit to the specified steady variables was determined. Secondly, the sensitivity/control of each parameter for the steady state objectives was determined. And thirdly, parameters were changed according to their control values. Thus, parameters with higher control were allowed to change more, thereby reducing the objective value with every run until the best fit is obtained.

4.6. Model dissemination

The model is available for simulation at all three JWS Online (Olivier and Snoep, 2004) servers (e.g. http://jjj.mib.ac.uk/webMathematica/Examples/run.jsp?modelName=geenen), or can be sent in SBML format upon request.

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Appendix A. Abbreviations and kinetic details of glutathione model

A.1. Abbreviations

The complete names of the enzymes and metabolites indicated by acronyms in Fig. 1 are as follows:

Enzyme names and acronyms and EC numbers.

Reaction	Abbreviation	Enzyme name	Ec number
v[1]	mati	Methionine adenosyl transferase I	2.5.1.6
v[2]	matiii	Methionine adenosyl transferase III	2.5.1.6
v[3]	meth	Glycine-*N*-methyltransferase	2.1.1.20
v[4]	gnmt	DNA-methyltransferase	2.1.1.72
v[5]	ah	*S*-adenosylhomocysteine hydrolase	3.3.1.1
v[6]	bhmt	Betaine-homocysteine methyltransferase	2.1.1.5
v[7]	ms	Methionine synthase	2.1.1.13
v[8]	cbs	Cystathionine gamma-synthase	4.2.1.22
v[9]	ctgl	Cystathionase	4.4.1.1
v[10]	gcs	Glutamylcysteine synthetase	6.3.2.2
v[11]	gs	Glutathione synthetase	6.3.2.3
v[12]	gpx	Glutathione peroxidase	1.11.1.9
v[13]	gr	Glutathione reductase	1.8.1.7

Abbreviations (*continued*)

Reaction	Abbreviation	Enzyme name	Ec number
v[24]	ggct	Gamma-glutamylcyclotransferase	2.3.2.4
v[25]	oxoase	5-Oxoprolinase	3.5.2.9
v[26]	gcs	Glutamylcysteine synthetase	6.3.2.2
v[27]	gs	Glutathione synthetase	6.3.2.3
v[28]	ggtp	Gamma-glutamyltranspeptidase	2.3.2.2
v[29]	ap	Aminopeptidase	3.4.11.2
v[31]	ggct	Gamma-glutamylcyclotransferase	2.3.2.4
v[33]	gpx	Glutathione-*S*-transferase	2.5.1.18
v[34]	gpx	Glutathione-*S*-transferase	2.5.1.18
v[35]	ggtp	Gamma-glutamyltranspeptidase	2.3.2.2
v[36]	ccat	Cysteine-*S*-conjugate-*N*-acetyltransferase	2.3.1.80

Names of variables (μM).

THF – tetrahydrofolate
5,20-MTHF – 5-10-methenyltetrahydrofolate
5-MTHF – 5-methyltetrahydrofolate
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

Names and assumed magnitudes of constants (μM) (taken from Reed et al. (2008b) unless specified otherwise).

AB – 10 – 2-aminobutyrate (Soga et al., 2006)
BET – 50 – betaine
bgly – 1300 – blood glycine
bglut – 60 – blood glutamate
bmet – 30 – blood methionine (varies in some experiments)
cNADPH – 50 – nicotinamide adenine dinucleotide phosphate
cser – 540 – cytosolic serine
DUMP – 20 – deoxyuridine monophosphate
GARP – 10 – glycinamide ribonucleotide
H2O2 – 0.01 – cellular hydrogen peroxide
HCHO – 500 – formaldehyde
merc – 5 – mercapturic acid
OPA – 1 – Ophthalmic acid, *N*-[*N*-(γ-glutamyl)-α-aminobutyryl]glycine
Oxo – 1 – Oxoproline, pyroglutamic acid
para – 0 – *N*-acetyl-*p*-aminophenol/paracetamol (varies in some experiments)

Parameter values

All V_{max}s are given in μM/h per cytosolic volume. Concentrations are in μM.

The parameters for the reactions vMATi, vMATiii, vGNMT, vDNMT, vSAH, vCBS vBHM, vMS, vCGL, GSHout, GSSGout were taken from Reed et al. (2008b).

Rate equations used

Reaction of glutamylcysteine synthetase for glutamylcysteine synthesis:

$$v[\mathrm{GCL1}] = \frac{\frac{\mathrm{glu}}{K_{\mathrm{glu}}} \cdot \frac{\mathrm{cys}}{K_{\mathrm{cys}}} \cdot V_{\mathrm{GCLf1}} \cdot \left(1 - \frac{\mathrm{glc}}{\mathrm{glu} \cdot \mathrm{cys} \cdot K_{\mathrm{eqGCL}}}\right)}{\left(1 + \frac{\mathrm{cys}}{K_{\mathrm{cys}}} + \frac{\mathrm{AB}}{K_{\mathrm{AB}}}\right) \cdot \left(1 + \frac{\mathrm{glu}}{K_{\mathrm{glu}}} \frac{\mathrm{glc}}{K_{\mathrm{glc}}} \frac{\mathrm{gluAB}}{K_{\mathrm{gluAB}}} \frac{\mathrm{GSH}}{K_{\mathrm{GSH}}}\right)}$$

Reaction of glutamylcysteine synthetase for glutamylaminobutyrate synthesis:

$$v[\mathrm{GCL2}] = \frac{\frac{\mathrm{glu}}{K_{\mathrm{glu}}} \cdot \frac{\mathrm{AB}}{K_{\mathrm{AB}}} \cdot V_{\mathrm{GCLf1}} \cdot \left(1 - \frac{\mathrm{gluAB}}{\mathrm{glu} \cdot \mathrm{AB} \cdot K_{\mathrm{eqGCL2}}}\right)}{\left(1 + \frac{\mathrm{cys}}{K_{\mathrm{cys}}} + \frac{\mathrm{AB}}{K_{\mathrm{AB}}}\right) \cdot \left(1 + \frac{\mathrm{glu}}{K_{\mathrm{glu}}} \frac{\mathrm{glc}}{K_{\mathrm{glc}}} \frac{\mathrm{gluAB}}{K_{\mathrm{gluAB}}} \frac{\mathrm{GSH}}{K_{\mathrm{GSH}}}\right)}$$

Parameter	Literature value	References	Parameter value used
VGCLf1	36000	Reed et al. (2008b)	36000
KeqGCL			5597
KGCLccys	100	Reed et al. (2008b)	100
KGCLcglut	1900	Reed et al. (2008b)	1900
KGCLcglc	300	Reed et al. (2008b)	300
KGCLAB	2300	Board et al. (1978)	2300
KiGCL	8200	Reed et al. (2008b)	8200
KGCLgluAB	10000	Board et al. (1978)	10000
KeqGCL2			0.00025 (μM)

Reaction of glutathione synthase for glutathione synthesis:

$$v[11] = \frac{\frac{\mathrm{gly}}{K_{\mathrm{gly}}} \cdot \frac{\mathrm{cglc}}{K_{\mathrm{cglc}}} \cdot V_{\mathrm{GSf1}} \left(1 - \frac{\mathrm{cGSH}}{\mathrm{gly} \cdot \mathrm{cglc} \cdot K_{\mathrm{eqGS1}}}\right)}{\left(1 + \frac{\mathrm{cglc}}{K_{\mathrm{cglc}}} + \frac{\mathrm{gluAB}}{K_{\mathrm{gluAB}}}\right) \cdot \left(1 + \frac{\mathrm{OPA}}{K_{\mathrm{OPA}}} \frac{\mathrm{cgly}}{K_{\mathrm{gly}}} \frac{\mathrm{GSH}}{K_{\mathrm{GSH}}}\right)}$$

Reaction of glutathione synthase for ophthalmic acid synthesis:

$$v[27] = \frac{\frac{\mathrm{gly}}{K_{\mathrm{gly}}} \cdot \frac{\mathrm{gluAB}}{K_{\mathrm{cgluAB}}} \cdot V_{\mathrm{GSf1}} \cdot \left(1 - \frac{\mathrm{OPA}}{\mathrm{gly} \cdot \mathrm{gluAB} \cdot K_{\mathrm{eqGS2}}}\right)}{\left(1 + \frac{\mathrm{cglc}}{K_{\mathrm{cglc}}} + \frac{\mathrm{gluAB}}{K_{\mathrm{gluAB}}}\right) \cdot \left(1 + \frac{\mathrm{OPA}}{K_{\mathrm{OPA}}} \frac{\mathrm{cgly}}{K_{\mathrm{gly}}} \frac{\mathrm{GSH}}{K_{\mathrm{GSH}}}\right)}$$

Parameter	Literature value	References	Parameter value used
VGSf1	54000	Reed et al. (2008b)	948.15
KeqGS1			0.22 (μM)
KGScglc	22	Reed et al. (2008b)	22
KGScgly	300	Reed et al. (2008b)	300
KGSgluAB	200	Oppenheimer et al. (1979)	200

equations used (*continued*)

Parameter	Literature value	References	Parameter value used
KGSOPA		Meister and Tate (1976)	100
KeqGS2			0.0022 (μM)
KGScGSH	30		30

Transport of ophthalmic acid was assumed to follow mass action kinetics.

$$v[32] = KtrsOPA.([OPA] - [bOPA])$$

Parameter	Literature value	Reference	Parameter value used
KtrsOPA			900.92

vGCT, vGGT, vDP and transport of γ-glutamyl amino acid were added as reversible Michaelis Menten reactions. v_{OP} was added as a irreversible Michaelis–Menten reaction due to the ATP dependency of the reactions.

vGCT

Parameter	Literature value	References	Parameter value used
VGCTA	51000	Board et al. (1978)	59840.37
KegGCTA			2.80 (μM^{-1})
Kcgctcglc	10		7.98
Kcgctccys	2200	Board et al. (1978)	2177.76
KGCTAoxo			10002.51
KGCTAcgluAA			2200

vGGT

Parameter	Literature value	References	Parameter value used
VGGT	277500	Suzuki et al. (1986)	8745.32
KeqGGT			99915.61 (μM^{-1})
KGGTbGSH	670	Francois et al. (1979)	670
KGGTbgluAA			979.80
KGGTcysgly	1090	Allison (1985)	1090

vDP

Parameter	Literature value	References	Parameter value used
VAP	150000	Kozak and Tate (1982)	145394.4
KeqAP			98.38 (μM^{-1})

(*continued on next page*)

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equations used (*continued*)

Parameter	Literature value	References	Parameter value used
KAPcysgly	2500	Kozak and Tate (1982)	2500
KAPbcys			9988.69
KAPbgly			10000

vOP

Parameter	Literature value	References	Parameter value used
Vop	495000	Van Der Werf et al. (1975)	846929.6
Kopoxo	5	Griffith and Meister (1982)	5
Kopcglut			1.18

vTRS – parameters were estimated to give fast transport and low control in transporter.

Parameter	Literature value	Reference	Parameter value used
VTRS			97881.27
KeqTRS			9999.90
KTRSbgluAA			1000
KTRScgluAA			1000

vGT – the drug to conjugate reactions

Parameter	Literature value	References	Parameter value used
VmP450E1	273150	Raucy et al. (1989)	2731.5
KP450E1para	6500	Raucy et al. (1989)	6500
KP450E1ASG			10000
KP450E1cGSH	270	Pabst et al. (1974)	270
VmP450A2	209850	Raucy et al. (1989)	2098.5
KP450A2cGSH	270	Pabst et al. (1974)	270
KP450A2para	1300	Raucy et al. (1989)	1300
KP450A2ASG			10000

Transport of ASG out of cell: v[37] = KcysASG ∗ (cysASG[t] −merc).

Parameter	Literature value	Reference	Parameter value used
KcysASG	50		50

Transport of 5-oxoproline out of cell: v[38] = Ktrsoxo ∗ (−boxo + oxo[t]).

Parameter	Literature value	Reference	Parameter value used
Ktrsoxo			0.37

Appendix B. Parameters used for fitting model

Table B1. Values optimised to when paracetamol is set to zero. Units are in μM for concentration and μM/h for fluxes.

Model variable	Values
bcys	185.50
bGSSG	0.48
bGSH	12.70
cCH2THF	0.51
cgly	924.43
cTHF	4.62
ccys	194.97
hcy	1.12
SAM	81.17
SAH	19.14
met	49.19
cGSSG	61.32
cGSH	6591.80
cglc	9.81
cyt	36.88
v[1]	124.78
v[2]	80.46
v[3]	143.64
v[4]	61.60
v[5]	205.24
v[6]	61.88
v[7]	40.31
v[8]	103.05
v[9]	103.05

Table B2. Values optimised to when paracetamol is set to 12.4 mM (in μM).

Model variable	Values
cgly	504
ccys	3.02
SAM	13.8
SAH	22.1
met	110.1
cGSSG	21.13
cGSH	385
gluAB	1
OPA	34.32
oxo	20

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Parameters which were allowed to vary in the model:

vmMATi, vmMATiii, Vmmeth, Vmmet, Vmfah, Vmbhmt, vmMS, Vmcbs, vmctgl, vGCLf1, vGSf1, vmGPX, vmgr, vmGSSGl, vmGSSGh, vmgshoutl, vmgshouth, kOPAext, vmglutin, vmcysin, koxoext, vmglyin, VmrcSHMT, VmcMTHFR, vocCH2HF, vop, vGCTA, ktrsOPA, VmP450E1, VmP450A2, vGGT, vAP, kcysASG, ktrsoxo, vSpara, vGpara, vgc, vmmetin, vbGSSGexp, vbcysexp, keqGCL, keqGCL2, keqGS1, keqGS2, kglutin, kccysin, kcgly, kegGCTA, kcgctcglc, kcgctccys, kGCTAoxo, kopcglut, keqGGT, kGGTbgluAA, keqAP, kAPbcys, vTRS, kopoxo, kopcglut, keqTRS.

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