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HPLC–MS/MS methods for the quantitative analysis of 5-oxoproline (pyroglutamate) in rat plasma and hepatic cell line culture medium

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ABSTRACT

5-Oxoproline (5-OP; pyroglutamate) is an intermediate in the biosynthesis of the endogenous tripeptide glutathione and has been seen to be elevated in the biofluids and tissues of rats following the administration of glutathione-depleting hepatotoxic xenobiotics such as acetaminophen (paracetamol), bromobenzene and ethionine. As 5-OP is a potential biomarker for hepatotoxicity HPLC–MS/MS methods have been developed for its quantification in *in vitro* cell culture media and rat plasma. For the cell culture media the lower limit of quantification (LLOQ), defined as the lowest concentration on the calibration curve, was 10 ng/ml. Minimal carry over was observed for cell culture media between injections (less than 5% at all concentrations examined), precision and accuracy were generally better than 20% for within and between day analyses. For rat plasma a LLOQ of 50 ng/ml was obtained. Carry over for plasma was less than 5% for all concentrations, within and between batch accuracy and precision were generally better than 20%. The methods were linear for both sample types from the LLOQ up to 1 μg/ml. For samples obtained from rats subjected to chronic administration of the hepatotoxin methapyrilene, concentrations of 5-OP were not observed to increase significantly at any time point compared to controls. 5-OP was also determined in the culture media of human liver epithelial (THLE) cells transfected with cytochrome P450 2E1 (THLE-2E1). Following exposure of THLE-2E1 cells to acetaminophen, large increases in the concentrations of 5-OP were observed, which correlated with reduced cellular glutathione content and with cell toxicity. These results show that LC–MS/MS can be used to perform rapid, sensitive, and quantitative determination of 5-OP *in vivo* and *in vitro* and will enable additional investigations into the utility of 5-OP as a biomarker of liver drug-induced liver injury.

1. Introduction

Drug toxicity is an important cause of human ill health, withdrawal of marketed drugs, failure of regulatory approval, and termination of compounds during drug development. One key mechanism implicated in the toxicity of numerous drugs is *via* the formation of chemically reactive metabolites. In the case of acetaminophen (paracetamol, APAP) toxicity is overtly dose dependent [1–4]. Therapeutic doses of APAP (0.6–4 g/day) are generally well tolerated and, under such circumstances, metabolism of the drug is predominantly *via* chemically stable, unreactive and non-toxic, sulfate and glucuronide conjugates. Following overdose these pathways of metabolism are saturated, resulting in excess APAP being metabolized by cytochrome (CYP) P450 isoforms, in particular CYP 2E1, forming a highly reactive quinoneimine (NAPQI) metabolite. At therapeutic doses, the minimal amount of NAPQI generated is rapidly neutralized by reaction with cellular glutathione to form glutathione conjugates. However, at higher doses, glutathione depletion occurs, which is accompanied by oxidative stress, covalent binding of the metabolite to cellular macromolecules, and organellar injury. These events collectively trigger a complex series of intra- and inter-cellular events that result in cell death, and potentially fatal liver failure [3].

Many other drugs can also cause life threatening drug induced liver injury (DILI), and in numerous instances metabolism to reac-

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tive intermediates which form glutathione conjugates has been demonstrated [5]. However, for DILI inducing drugs other than paracetamol, hepatotoxicity occurs infrequently or very rarely, is not overtly dose dependent, and the relationship between bioactivation, glutathione reactivity and toxicity is poorly defined. The discovery of biomarkers that are indicative of early adverse biological processes caused by drug bioactivation, such as perturbation of glutathione homeostasis, could be of particular value since these might enable early identification of patients who are at high risk of catastrophic organ failure. Such biomarkers might also afford insight into the underlying mechanisms of adverse drug reactions (ADRs) and indicate factors that reflect the susceptibility of individual human subjects. More accurate biomarkers of early toxicity may allow drug administration to be ceased before the development of ADRs such as DILI in susceptible individuals, whilst permitting safe use of the drug in non-susceptible individuals, with clear benefit to both patient and society.

Biomarkers that detect effects on hepatic glutathione depletion are of particular interest given the importance of this molecule in cell defense against reactive intermediates. Recently, ophthalmic acid has been proposed as a potential biomarker that may reflect enhanced glutathione biosynthesis and oxidative stress [6]. However, complexities in the relationship between the biosynthesis of glutathione and ophthalmic acid, which arise due to the involvement of multiple biosynthetic pathways, may complicate interpretation of data obtained from *e.g.*, monitoring circulating concentrations of ophthalmic acid under conditions where only "mild" DILI is seen [7]. Alternatively, metabolites which are more directly involved in glutathione biosynthesis than ophthalmic acid may have greater potential in this area. Glutathione biosynthesis occurs *via* the gamma-glutamyl cycle and a number of studies have shown increases in the concentration of 5-oxoproline (5-OP, pyroglutamate), an intermediate in this pathway, in various biofluids/tissues, following the administration of hepatic toxicants such as acetaminophen [8], bromobenzene [9] and ethionine [10]. The initial association of 5-OP with drug-induced depletion of glutathione followed its detection in rat urine by ^{1}H NMR spectroscopy after dosing rats with APAP [8]. More recent metabonomic studies [9,10] also detected 5-OP using ^{1}H NMR spectroscopy. Thus, at several time points after administration of 1.5 g/kg bromobenzene, 5-OP was increased in blood plasma, urine and liver samples [9]. 5-Oxoprolinuria has also been observed in humans [11–16] with inborn errors of metabolism that resulted in a deficiency in enzymes of the glutamyl cycle (5-oxoprolinase and glutathione synthetase) [11–14] and in other clinical conditions such as metabolic acidocis [15,16] and chronic APAP use [16].

Whilst, as indicated above, NMR spectroscopy-based methods have proved useful in the past for qualitative mechanistic investigations, the evaluation of 5-OP as a biomarker for oxidative stress requires the availability of a rapid, specific and sensitive means of analysis of the type typically offered by chromatographic methods. Gas chromatography (GC) is typically used for clinical investigations [11,15] (though capillary GC has also been described [17]). An LC–UV method has been described for the analysis of protein hydrolysates [18] and more recently LC–MS/MS methods have been applied in studies of the metabolism of 5-OP during pregnancy (based on normal phase LC) [19] and in analysis of cerebrospinal fluid (CSF) in search of neurotransmitter biomarkers (using ion-pair reversed-phase LC) [20] and for the analysis of fermentation media [21]. In order to evaluate the utility of 5-OP as a potential biomarker of glutathione depletion and oxidative stress *in vitro* and *in vivo*, we have therefore developed a quantitative, validated and specific HPLC–MS/MS method that can be applied in toxicity investigations for evaluation of this molecules as a biomarker.

2. Experimental

2.1. Solvents and reagents

The HPLC grade methanol, and formic acid of analytical grade used for LC/MS analysis were purchased from Sigma–Aldrich (Dorset, UK). Ultrapure water (18 MΩ) was obtained from a Purelab Ultra system from Elga (Bucks, UK). 5-OP was obtained from Sigma–Aldrich (Dorset, UK). For cell culture, PMFR P-004 medium was purchased from Gibco (Paisley, UK). Glutamax and fetal bovine serum were purchased from Invitrogen (Paisley, UK), and other media supplements were purchased from Sigma–Aldrich. Human liver epithelial cells immortalised with SV40 large T antigen and transfected with individual cytochrome P450 enzymes (THLE-CYP) were obtained under license from Nestec Ltd., Switzerland.

2.2. Samples

Cell line culture medium samples. The cell culture medium comprised PMFR P-004 supplemented with 7 mM glucose, 2 mM Glutamax, 1.75 μM insulin/transferrin/selenium (ITS), 1 nM hydrocortisone, 0.5 ng/ml epidermal growth factor, 35 μg/ml bovine pituitary extract, 0.33 nM retinoic acid, 3% fetal bovine serum and 150 μg/ml G418. Media was collected from culture flasks containing SV40 large T antigen immortalised human liver epithelial cells transfected with individual cytochrome P450 enzymes (THLE-CYP). The media analysed was taken from THLE-CYP cell lines transfected with either CYP 1A2, 2C9, 2C19, 2D6, 2E1 or 3A4 or an empty construct [22]. For the assessment of the stability, precision, and accuracy of 5-OP a pool was created from the media collected from all of the different THLE-CYP cell lines after incubation for 48 h.

For toxicity studies in cell culture, THLE-2E1 cells were seeded into in a BiocoatTM 96-well plate format (at 15,000 cells/well) for cytotoxicity analysis. These cells were treated with increasing concentrations of either paracetamol (0–25 mM), chlorpromazine (0–300 μM) or streptomycin (0–300 μM) prepared in protein minus media for 24 h. All concentrations were plated out in triplicate. Chlorpromazine and streptomycin were run in every plate as positive and negative controls for cell cytotoxicity. After 24 h cell media were collected from the incubations and analysed for 5-OP concentrations. Cellular effects of paracetamol exposure were assessed via the mitochondrial activity assay (MTS), glutathione content determination, using the protocol described below, and analysis for protein content.

Rat plasma and serum samples. Control rat plasma for method development and validation was obtained from the AstraZeneca animal breeding unit (Alderley Park). Study samples were obtained from three groups of 25 male Crl:CD(SD) rats (supplied by Covance, Charles River Laboratories, Portage, Michigan, USA) that were dosed orally by gavage with methapyrilene at daily doses of 0 mg/kg/day (control), 10 mg/kg/day (low dose) and 50 mg/kg/day (high dose). Methapyrilene was dissolved in reverse osmosis (RO) water and the dose volume was 10 ml/kg. Drug administration continued through the day prior to sacrifice or through day 14 of the dosing phase (for animals designated for recovery). The study was performed in strict compliance with national standards for the conduct of animal studies.

Blood was collected on the day of sacrifice, *via* the *vena cava,* after the animal had been anaesthetized (carbon dioxide) prior to euthanasia. There were five samples from each of the three dose groups for each day (days 3, 7, 11, 15 and 29), apart from day 7 where $n = 6$.

The blood samples were allowed to clot at room temperature for at least 30 min and were centrifuged at 2200 × *g* within 60 min of collection. The serum was divided into aliquots of approximately 0.25 ml each that were flash frozen in liquid nitrogen and stored in

a freezer set to maintain −60 to −80 °C, until packed on dry ice and shipped for analysis.

2.3. Routine sample preparation for LC–MS

Aliquots (50 μl) of both ten-fold diluted plasma/serum samples and the cell culture medium samples were prepared by protein precipitation with 150 μl of cold (−20 °C) methanol. Precipitated proteins were removed by centrifugation (Centrifuge 5417C, Eppendorf, Hamburg, Germany) at 20,800 × *g* for 10 min. Subsequently 180 μl of supernatant was removed and dried using a stream of dry nitrogen at 1 bar and 40 °C (Micro DS96, Porvair Sciences Ltd., Leatherhead, UK) before redissolution in 50 μl of water (with vortexing).

For precision and accuracy, storage, and freeze–thaw stability studies, 5 μl of pooled plasma or cell culture medium (from media incubated with cells for 48 h) were diluted with 45 μl of ultrapure water. Protein precipitation, drying and 5-OP recovering was achieved following the protocol described above.

2.4. Calibration curve and quality controls for cell culture media

Each run included both fresh quality control (QC) samples and calibration samples. 5-OP stock solutions were prepared in water at concentrations of 200 ng/ml to 100 μg/ml. When preparing the cell media calibration curves, samples and biological QC samples were made by adding 2.5 μl of these 5-OP solutions to 47.5 μl of matrix. To reduce matrix effects, we chose a matrix which most resembled the matrix of the cell media samples. For the analysis of 5-OP concentrations following paracetamol exposure the "blank" matrix was media taken from cells incubated in the absence of paracetamol incubated over the same time course as the experiment. For the calibration curves for the precision, accuracy and stability determinations, the media was taken from un-incubated cells. This media was diluted ten-fold in water prior to allow for high 5-OP concentrations in the precision, accuracy and stability samples (see Section 3) before it could be used for spiking the calibration curve.

Calibration curves for 5-OP in cell culture medium were prepared at concentrations of 10 ng/ml, 50 ng/ml, 100 ng/ml, 400 ng/ml, 500 ng/ml, 800 ng/ml and 1 μg/ml. Validation QCs for the culture media were prepared at concentrations of 15, 400 and 800 ng/ml.

2.5. Calibration curve and quality controls for rat plasma and serum samples

Due to the high concentrations of endogenous 5-OP (5000 ng/ml) in plasma samples (see Section 3), and the need for full quantification, calibration curves and QCs were prepared by spiking 5-OP into water. Calibration curves for 5-OP were prepared at concentrations of 50 ng/ml, 100 ng/ml, 400 ng/ml, 500 ng/ml, 800 ng/ml and 1000 ng/ml. Validation QCs for rat plasma were 150, 400 and 800 ng/ml and were also prepared in water.

Samples from the methapyrilene study were analysed in a random order to eliminate effects that might result from run order.

2.6. HPLC–MS/MS conditions

For HPLC–MS, 10 μl of each processed sample was chromatographed using a Perkin-Elmer series 200 high pressure LC micro solvent delivery system (Perkin-Elmer Life Sciences, Cambridge, UK) and a HTC PAL autosampler (CTC Analytics, Switzerland). The autosampler was maintained at 4 °C. Separations were performed on a Symmetry C18 3.5 μm (2.1 × 150 mm) column (Waters Ltd., Elstree, UK) at 40 ± 0.2 °C using a 7990R-1 heater controller (Grace, Deerfield, USA). Between samples the injection system was cleaned using 2 cycles of strong solvent (0.1% formic acid in methanol/water 90:10) and 2 cycles of weak solvent (0.1% formic acid in water).

Reversed-phase gradient chromatography was performed using a binary solvent system composed of formic acid 0.1% (v/v) in methanol (solvent B) and formic acid 0.1% (v/v) in water (solvent A). The solvents were mixed using a Perkin Elmer series 200 mixer (Cambridge, UK) at 400 μl/min as follows: From 0 to 4.2 min the composition of A–B was held at 100% A, from 4.2 to 6 min the composition of the solvent was changed, *via* a linear gradient to 100% B and then held at this solvent composition for the next 2.3 min to wash the column before returning to the initial setting of 100%A. The solvent was then held at 100%A for 3 min for column re-equilibration prior to the next injection.

All of the MS data were acquired on a SCIEX 4000 QTRAP hybrid triple quadrupole linear ion trap mass spectrometer (Applied Biosystems/MDS Analytical Technologies, Warrington, UK) operated in positive electrospray ionisation (ESI) mode with a TurboIonSpray source. Multiple reaction monitoring was used, with Q1 set to *m*/*z* 130.0 ($M+H^+$) and Q3 to *m*/*z* 84.0 ($M+H^+$). The optimized mass spectrometer parameter operating conditions were: curtain gas (CUR) 20 (arbitrary units); ion source gas 1 (GS1) 32 (arbitrary units); ion source gas 2 (GS2) 70 (arbitrary units); source temperature (TEM) 500 °C; declustering potential (DP) 28.5 V; collision energy (CE) 21.3 eV; collision cell entrance potential (EP) 6.4 V; and collision cell exit potential (CXP) 14.0 eV.

The mass spectrometer and the HPLC system were controlled by Analyst 1.4.2 software (Applied Biosystems/MDS SCIEX instruments/Carlsbad/USA).

2.7. Determination of precision, accuracy, lower limit of quantification (LLOQ) and matrix effects

Within-batch precision for endogenous 5-OP was assessed by measuring 6 samples of ten-fold diluted cell media or rat plasma in one batch. Between-batch precision for endogenous 5-OP was assessed by measuring those 6 samples on three different days. Because of the high background concentrations of endogenous 5-OP accuracy could not be determined in this way and therefore accuracy and precision were assessed using the QC samples (5-OP spiked into either un-incubated cell medium or water, see above).

Matrix effects/selectivity were investigated in order to determine the potential for sample components, including endogenous 5-OP, to act as interferences or affect the LLOQ. This was done by examining blank/control matrices for cell culture medium and rat plasma for the presence of 5-OP or other interfering compounds. The percentage of the peak area of any endogenous material was then compared to the peak area of the bottom standard for each of the respective calibration curves to establish the LLOQ.

2.8. Determination of between-injection carry over

Carry over was determined by measuring the peak area for 5-OP following injection of a blank sample immediately after the analysis of the highest concentration of the calibration curve (1 μg/ml). The percentage peak area of the carry over compared to the LLOQ was calculated.

2.9. Stability

The short term stability of the samples was tested by maintaining them at 21 °C for 4 h and at 4 °C for 24 h and 1 week. Longer term freezer storage stability was tested by analyzing samples stored for 24 h, 1 week and 1 month at −20 °C. Freeze-thaw stability was

assessed by determining the concentrations of 5-OP in acquired samples after one, two and three cycles of freezing and defrosting.

2.10. Mitochondrial activity determination (MTS assay)

Mitochondrial activity was measured as an indicator of cell viability using a Promega CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay™ (Promega, Madison, WI). MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) solution was diluted 1 in 5 with media. All treatment media in the incubation plates were removed and replaced with 120 μl of MTS solution. The reaction was allowed to take place in a humidified chamber at 37 °C under 5% CO_2 for 1 h. Then 100 μl of the MTS solution was removed from the incubation plate and transferred into a 96-well plate where the absorbance was immediately read using a Wallac™ EnVision (PerkinElmer, Beaconsfield, UK) at 492 nm.

2.11. Protein content determination

Protein content was determined on cell lysates using the pierce BCA protein assay (Thermo, Rockford, USA). Standards of bovine serum albumin (BSA) were made up between 0 and 2 g/l. Aliquots of 25 μl of standards and samples were treated with 200 μl of working solution, incubated at 37 °C for 30 min and read on a Wallac™ EnVision Spectrophotometer at 580 nm.

2.12. Cell glutathione content determination

The glutathione content of the cell lysate was determined using the luminescence-based GSH-Glo Assay (Promega, Southampton, UK). Glutathione standards were made up for a calibration curve ranging from 0 to 5 μM of glutathione. 10 μl of glutathione standard was added to a white luminometer 96 well plate. The standards and all cell samples were incubated with 100 μl of the assay reagent at room temperature for 30 min. 100 μl Luciferin detection agent was then added with incubation for a further 15 min. The contents of the wells were then transferred to the luminometer 96 well plate and read using a microbeta (Perkin Elmer Envision 2104) multilabel plate reader for glutathione determination.

3. Results and discussion

3.1. Analytical conditions

Several recently reported HPLC–MS/MS methods for 5-OP have employed ion-pairing systems to obtain suitable retention. The first of these methods [21] used an ion-pairing system based on pentadecafluorooctanoic acid (PDFOA) and trifluoracetic acid (TFA) to analyse the compound in fermentation media. More recently a method, utilizing heptafluorobutyric acid (HFBA) and formic acid, has been developed for the analysis of a range of neurotransmitters and related compounds, including 5-OP, in cerebrospinal fluid (CSF) [19]. In the CSF method 5-OP eluted with a retention time of 2.85 min. As a result of the source contamination issues that are often associated with the use of ion-pair systems in LC–MS/MS, we chose to investigate the use of a simpler system based on modifying the organic and aqueous mobile phases with formic acid alone, thereby omitting the use of HFBA, or similar ion pairs. Under these chromatographic conditions 5-OP eluted with a retention time of 2.8 min. Inclusion of a methanol wash step to elute contaminants from the column, followed by a period for column re-equilibration with the starting mobile phase, resulted in a total analytical cycle of 11.3 min per sample.

Sensitive detection of 5-OP by LC–MS/MS following the chromatographic separation was achieved by first selecting the ion at m/z 130.0, corresponding to the parent molecule, using Q1 and then searching for the product ion of the highest intensity in Q3, which corresponded to m/z 84.0 (formed *via* the loss of the –COOH group). This ion was also selected for use in the reversed-phase ion-pair-based LC–MS/MS method developed for 5-OP determination in cerebrospinal fluid (CSF) [19]. Preliminary experiments showed that the MS response under these conditions was linear up to 2 μg/ml.

3.2. Method characteristics for cell culture media

The analysis of cell culture medium before incubation with the THLE cells showed that no detectable concentrations of 5-OP were present. However, when media from control cells that had not been exposed to paracetamol was examined there was clear evidence of the presence of increasing amounts of 5-OP with time. By 48 h after incubation with the cells these concentrations had risen to approximately 4 μg/ml. In the absence of a suitable isotopically labelled internal standard concentrations of 5-OP were determined against a standard curve, generated by spiking ten-fold diluted control medium with 5-OP, at concentrations ranging from 10 ng/ml to 1 μg/ml (the linear equation for the calibration curve over this range in cell culture media was typically $13535x + 556863$ (concentration (ng/ml) against peak area (counts)). All regression coefficients for the calibration curves using the m/z 130.0/84.0 transition were above 0.99 showing that, within this concentration range, the method was linear. Given the absence of interfering endogenous peaks in the calibration curve, for diluted, pre-incubation media the LLOQ was determined as 10 ng/ml (the lowest concentration used in the calibration curve). The percentage carry over was found to be less than 5% at the LLOQ. Whilst linear over the range 0–1 μg/ml we found practically that for the determination of low concentrations of 5-OP accuracy was improved by using a more restricted calibration curve (0–100 ng/ml). Within and between batch precision for culture medium taken from cell incubations, and containing approximately 4.4 μg/ml of 5-OP was better than 15% (Table 1a and b). Within and between batch accuracy and precision for un-incubated culture media spiked with 5-OP were generally better than 20% (Table 2a and b).

3.3. Method characteristics for rat plasma

Examination of control rat plasma samples revealed significant endogenous concentrations of 5-OP (up to 5 μg/ml, see below). Clearly it is possible that plasma concentrations of 5-OP could fall as well as rise during toxicological investigations and for this reason standard curves were prepared in water. To ensure that the plasma samples used for the stability and freeze thaw studies were on the linear portion of the standard curve they were diluted 1:10 (v:v) with water for analysis. Similarly incurred samples were also diluted 1:10 for analysis. The calibration curve was prepared over the concentration range 50–1000 ng/ml. Water blanks showed no evidence of a signal for 5-OP and the LLOQ was taken as 50 ng/ml (0.5 ng on column) which was the lowest concentration of the calibration curve. The calibration curve was linear between 50 and 1000 ng/ml (corresponding to undiluted plasma concentrations of 0.5–10 μg/ml of 5-OP), and typically gave an equation for the line (ng/ml vs. peak area (counts)) of $38966.8x + 931866.6$, with linear correlation regression coefficients above 0.99. The percentage carry over was less than 5% of the LLOQ. Within and between batch precision for plasma containing approximately 4 μg/ml of endogenous 5-OP was better than 15% (Table 3a and b) whilst the within and between batch accuracy and precision for water-spiked 5-OP samples were generally better than 20% (Table 4a and b). These results

Table 1
Within (a) and between (b) run precision for endogenous 5-oxoproline determination in 48 h incubated cell culture medium (corrected for the ten-fold dilution).

(a)

Sample no.	1	2	3	4	5	6	Average concentration (ng/ml ± SD)	Precision (%)
Run 1	4929	4922	3656	4333	3881	4675	4399 ± 539.5	± 12.3
Run 2	3882	3428	3833	3743	4099	3622	3768 ± 230.0	± 6.1
Run 3	4363	4276	4344	4315	3986	4286	4262 ± 139.1	± 3.3

(b)

Average concentration (ng/ml ± SD)	Precision (%)
4143 ± 439.4	± 10.4

Table 2
Within (a) and between (b) run accuracy and precision for 5-oxoproline determination spiked into un-incubated cell culture medium (corrected for the ten-fold dilution).

(a)

Concentration (ng/ml)	1	2	3	Average concentration (ng/ml ± SD)	Accuracy (%)	Precision (RSD %)
Run 1						
15[a]	11.4	14.6	N/R	13 ± 2.2	87	–
400	330	369	327	345 ± 23.6	86	6.9
800	816	794	705	772 ± 59.0	97	7.6
Run 2						
15[a]	20.8	19.1	19.1	19.7 ± 0.99	131	5.1
400	474	394	419	429 ± 40.7	107	9.5
800	968	745	632	785 ± 169.8	98	21.6
Run 3						
15[a]	20.4	22.6.	17.7	20.2 ± 2.4	135	12.0
400	462	451	448	455 ± 7.4	113	1.6
800	686	650	637	658 ± 25.5	82	3.9

(b)

Concentration (ng/ml)	Average concentration (ng/ml)	Accuracy (%)	Precision (RSD %)
15	17.6 ± 4.0	117	22.9
400	408 ± 58.8	102	14.4
800	738 ± 70.2	92	9.5

NR = no result.
[a] Data calculated using a truncated standard curve.

indicate that between 5-OP concentrations of 50 ng/ml (LLOQ) and 1000 ng/ml, the method was linear, precise and accurate.

3.4. Sample stability in cell culture medium and rat plasma

The stability analysis of 5-OP in cell culture media showed that the compound was stable at the concentrations measured and no trends indicating declining/increasing concentrations with time were observed under any of the conditions examined (−20 °C for 1 day, 1 week and 1 month, 21 °C for 4 h and 4 °C for 1 day and 1 week, data not shown) (see also [19]). In contrast to cell media 5-OP concentrations in rat plasma samples were not stable with increasing amounts of 5-OP seen after both 24 h and 1 week storage at 4 °C. The reason(s) for this increase in OP concentration at 4 °C are unclear but, irrespective of the cause, suggest that plasma samples must not be kept for any length of time at this temperature but should be stored frozen at −20 °C or colder, where the compound appeared stable for at least 1 month (data not shown).

Analyses of 5-OP in either plasma or culture medium showed no changes in concentration following up to three freeze thaw cycles (data not shown).

The assay characteristics for both cell culture media and rat plasma were consistent with the requirements of the current FDA guidance for biomarker determination [23].

3.5. Application to the analysis of culture media from THLE-2E1 cells exposed to paracetamol

To examine the utility of the assay for observing perturbations in the gamma glutamyl cycle, we used the assay to determine the

Table 3
Within (a) and between (b) run precision for endogenous 5-oxoproline determination in rat plasma (corrected for the ten-fold dilution).

(a)

Sample no.	1	2	3	4	5	6	Average concentration (ng/ml ± SD)	Precision (RSD %)
Run 1	3236	3516	4290	4253	4952	3805	4009 ± 617.9	15.4
Run 2	4203	4844	4684	4659	4462	4610	4557 ± 221.0	4.8
Run 3	5323	4493	4209	4786	4224	4283	4553 ± 435.8	9.6

(b)

Average concentration (ng/ml ± SD)	Precision (RSD %)
4380 ± 505.4	11.5

Table 4
Within (a) and between (b) run accuracy and precision for 5-oxoproline determination in aqueous QC samples used for plasma analysis (corrected for the ten-fold dilution).

(a)

Concentration (ng/ml)	1	2	3	Average concentration (ng/ml ± SD)	Accuracy (%)	Precision (RSD %)
Run 1						
150	152	147	145	148 ± 2.9	98.7	2.0
400	368	357	337	354 ± 12.6	88.5	3.6
800	746	829	829	801 ± 38.7	100.1	4.8
Run 2						
150	145	147	129	140 ± 9.9	93.3	7.1
400	460	472	436	456 ± 17.9	112.4	3.9
800	840	857	816	838 ± 20.3	104.5	2.4
Run 3						
150	155	160	150	155 ± 4.9	103.2	3.2
400	334	308	295	313 ± 19.6	78.3	6.3
800	671	653	649	648 ± 12.3	82.2	1.9

(b)

Concentration (ng/ml)	Average concentration (ng/ml)	Accuracy (%)	Precision (RSD %)
150	148 ± 7.4	98.7	5.0
400	374 ± 73.7	93.5	19.7
800	766 ± 95.0	95.8	12.4

5-OP concentration in cell culture medium obtained from THLE-2E1 cells following exposure to paracetamol (ion traces can be seen in Fig. 1). In addition to analysis for extracellular 5-OP, cellular effects of paracetamol exposure were determined using both the MTS assay, for the measurement of cell viability, and *via* the determination of intracellular glutathione concentration.

The MTS assay, which measures effects on mitochondrial membrane potential, and is often interpreted as an assay of cell viability, showed a decreased viability at paracetamol concentrations above 5 mM in CYP 2E1-containing THLE cells. The maximum observed

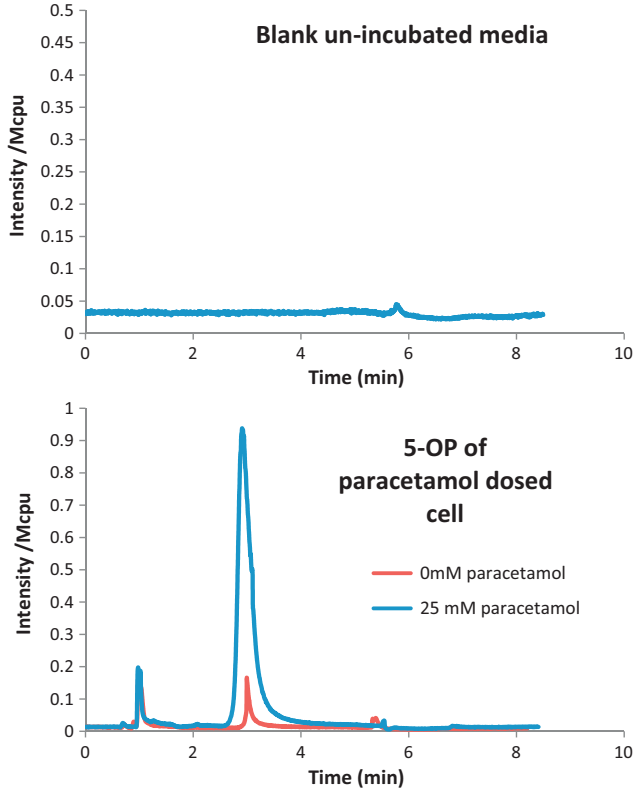

Fig. 1. The LC–MS/MS selected ion current traces for 5-OP in blank, un-incubated media (upper trace) and in 24 h incubated media without paracetamol and with 25 mM paracetamol (lower).

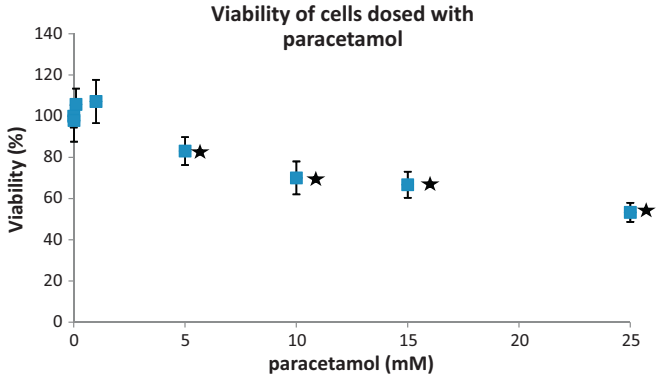

Fig. 2. Mitochondrial activity of cells dosed with paracetamol: the percentage activity as assessed by MTS assay of THLEs after 24 h of exposure to paracetamol. Starred (⋆) points are significantly different ($p < 0.01$ with independent t-test) from results at zero paracetamol.

effect was a 46% loss of MTS activity after 24 h following incubation with 25 mM paracetamol, when compared with control cells (Fig. 2). Chlorpromazine and streptomycin, the positive and nega-

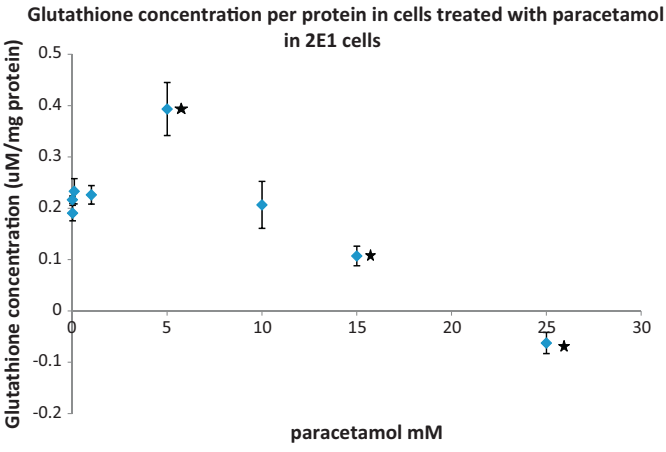

Fig. 3. The concentration of glutathione in cell extracts, corrected for protein content, after 24 h of exposure to paracetamol (error bars are standard error of the mean). Starred (⋆) points are significantly different from the mean at 0 mM paracetamol ($p < 0.05$).

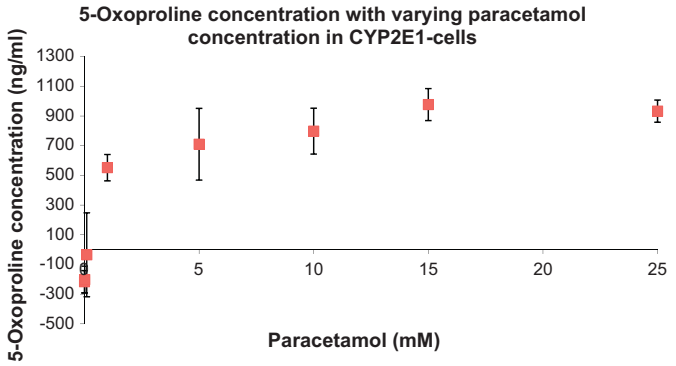

Fig. 4. The concentration of 5-oxoproline in cell media after 24 h of exposure to paracetamol; mean ± SEM; $n = 3$.

tive cytotoxicity control compounds, showed the expected toxicity and absence of toxicity responses, respectively (data not shown). Cellular glutathione analysis (Fig. 3) showed that exposure to paracetamol resulted in an initial increase at 5 mM concentrations of the drug (statistically significant $p = 0.05$ by Student's t-test) with a subsequent decrease in the amount of glutathione detected at concentrations above 15 mM paracetamol (statistically significant $p < 0.05$ by Student's t-test). The analysis of the cell media from CYP2E1-containing THLE cells showed dose-dependent increases in 5-OP, which leveled off after exposure to paracetamol at concentrations of 5 mM and above (Fig. 4). The concentration of 5-OP was markedly elevated at paracetamol concentrations which did yet not cause significant depletion cellular glutathione content (≤10 mM paracetamol). The increase in cellular glutathione concentration seen at paracetamol concentrations of 5 mM could reflect a protective response of the cell to NAPQI, and has been observed in previous studies (as summarised in [24]) and attributed to an adaptive increased glutathione synthesis. We hypothesize that, as the glutathione synthesis increases, the flux through the gamma glutamyl cycle (Fig. 5) also increases, thus increasing the cellular concentration of 5-OP, resulting in elevated concentrations in the medium.

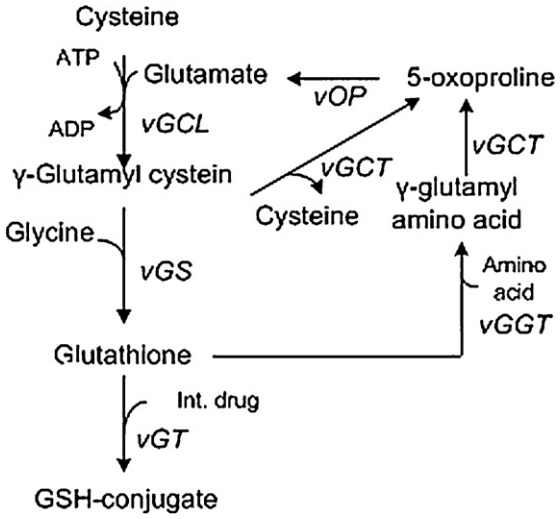

Fig. 5. Metabolism of 5-oxoproline to glutathione. Abbreviations: glutamate-cysteine ligase (GCL), glutathione synthetase (GS), oxoprolinase (OP), glutamylcyclotransferase (GCT), glutamyl transpeptidase (GGT), glutathione transferase (GT).

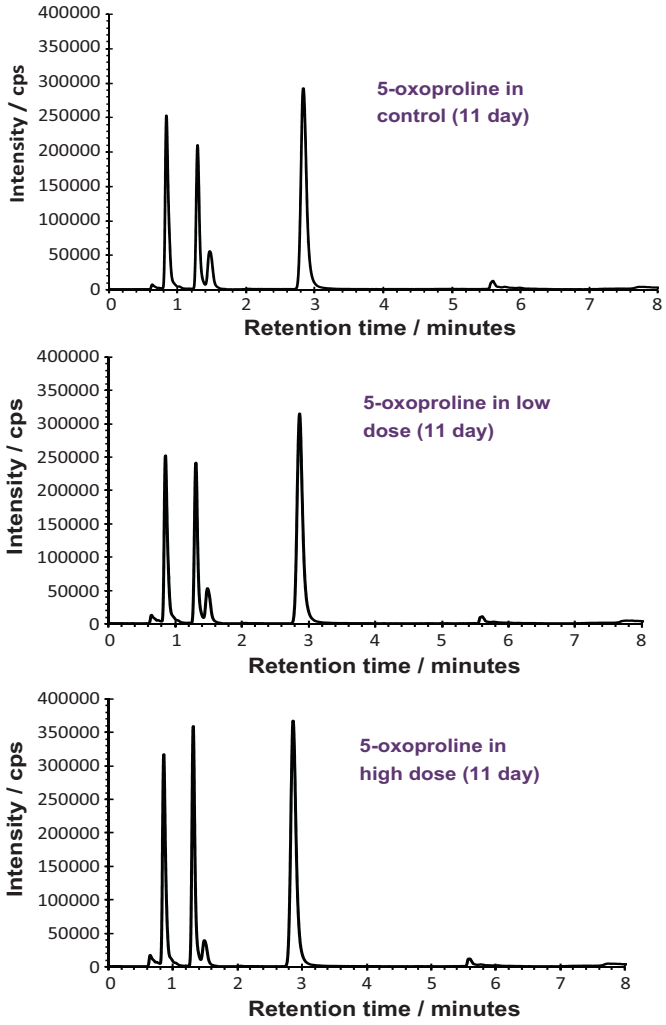

Fig. 6. Mass chromatograms for 5-OP detected in rat samples after 11 days of administration of methapyrilene at 0, 10 or 50 mg/kg/day.

3.6. Application to the analysis of samples from methapyrilene-dosed rats

The HPLC–MS/MS method developed for 5-OP determination was applied to serum samples obtained following 14 days exposure of rats to the drug methapyrilene at doses of 0, 10 or 50 mg/kg/day. As described elsewhere [7], the low 10 mg/kg/day dose was intended to be free of hepatotoxicity based on previous studies with this compound [25,26]). The 50 mg/kg/day dose was expected to result in minimal to mild hepatic necrosis after 7 days of repeat dosing, with an increasing severity of the hepatic injury expected by the end of the 14 days of administration. Typical chromatograms showing the presence of 5-OP in samples obtained from control, 10, and 50 mg/kg/day animals, are provided in Fig. 6 for day 11 of the study. 5-OP was observed in all the samples, irrespective of dose. The mean concentrations of 5-OP in samples from control and methapyrilene dosed rats for all sampling times are illustrated in Fig. 7. Although the results obtained for 5-OP in samples from the dosed animals suggested that, compared to the control, 5-OP concentration may have increased on day 11 in the high dose group, this result was not statistically significant despite large rises in the measured AST levels on day 7 (as we have described elsewhere [7]). This could have been due to rapid clearance of 5-oxoproline from the rat plasma into the urine (since blood was collected from the

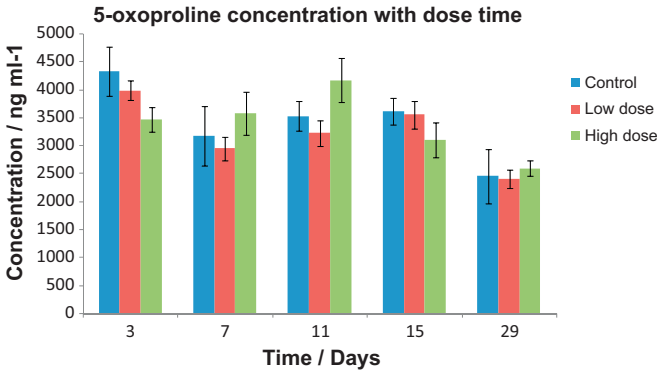

Fig. 7. Variation of the 5-OP concentration with time and dosage of methapyrilene following administration to rats 0, 10 and 50 mg/kg/day for 14 days followed by recovery until day 29. Error bars are SEM; $n = 5$.

animals 24 h after dosing), such that by the time the samples were taken any acute effect of the drug on glutathione metabolism and biosynthesis might have disappeared. As we have noted previously, when discussing our results for the determination of ophthalmic acid in the circulation of these animals [7], these relatively modest effects on circulating 5-OP might, as well as reflecting "pharmacokinetic effects", also result from adaptation of the animals to chronic methapyrilene administration. Effects of methapyrilene on cellular glutathione depletion have previously been noted in isolated rat hepatocytes [27]. In addition, acute dosing of the drug *in vivo* to the rat, over 3 days at 150 mg/kg/day, resulted in clear changes in hepatic GSH levels across the liver that exhibited marked zonality [28]. Thus GSH depletion was evident in periportal hepatocytes coupled to increased GSH concentrations in other hepatocytes. As was the case for ophthalmic acid [7], it seems reasonable to assume that circulating concentrations of 5-OP will be a combination of the effects of methapyrilene across the whole organ. The relationship between the observed zonal changes in hepatic GSH content and in hepatic glutathione cycle homeostasis, and circulating 5-OP concentrations in response to hepatic toxicants will therefore need to be defined. As we have also noted previously [7], in future studies it will be important to investigate both acute and adaptive phases of the liver response to glutathione-depleting compounds in order to establish whether effects on glutathione homeostasis and DILI biomarkers correlate differently in the two phases.

The LC–MS approach we have described appears to be suitable for such analyses, with sufficient sensitivity and acceptable accuracy and precision even in the absence of a stable isotope-labelled internal standard. The described method provides an alternative to the existing RP-ion-pair LC–MS method [20] used for the analysis of 5-OP in CSF, and also the normal phase method used for the plasma of pregnant women and their newborn infants [19].

4. Conclusions

The LC–MS/MS methods described here for culture medium and plasma have enabled rapid, sensitive and quantitative determination of 5-OP concentrations in samples from *in vitro* and *in vivo* studies. Using this methodology, effects on the production of 5-OP have been observed following exposure of CYP2E1-containing THLE cells to paracetamol. These effects correlated with changes in intracellular glutathione concentration and with cell toxicity. LC–MS was also able to determine 5-OP in samples obtained following administration of the hepatotoxin methapyrilene to the rat. However, at least at the time points used to collect samples, 5-OP was less useful than the conventional marker of hepatotoxicity AST. Additional investigations using these methods, applied to both *in vitro* and *in vivo* studies, will be required further to evaluate the utility of 5-OP as a novel biomarker of DILI.

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