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HPLC–MS/MS methods for the quantitative analysis of ophthalmic acid in rodent plasma and hepatic cell line culture medium

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ABSTRACT

Ophthalmic acid (OA), an endogenous tripeptide analogue of glutathione, has been suggested as a potential biomarker for paracetamol/acetaminophen hepatotoxicity. Here HPLC–MS/MS methods have been developed for the precise, sensitive and specific detection and quantification of OA in in vitro cell culture medium and plasma. For the cell culture medium the LLOQ was found to be 1 ng/ml, with less than 1% between sample carry over at all concentrations and precision below 15% for within day and below 9% for between day analyses. For rat plasma the presence of endogenous OA resulted in the LLOQ being 25 ng/ml (defined as the lowest concentration on the calibration curve where the base peak was less than 20% of the LLOQ). For the plasma assay the percentage carry over was less than 1% for all concentrations and within and between batch precision was below 21%. The methods were linear for both sample types from the LLOQ up to 5 μg/ml. The method was successfully applied to the determination of OA in samples obtained following the chronic administration of the rat hepatotoxin methapyrilene, where plasma OA concentrations were observed to show a weak negative correlation with those of established liver injury biomarkers such as aspartate aminotransferase (AST).

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1. Introduction

The efficacy and safety of many drugs is influenced by hepatic metabolism, which commonly results in detoxification but can also lead to adverse effects such as hepatotoxicity. For some drugs (e.g. acetaminophen/paracetamol/APAP), drug overdose plays a key role in initiation of toxicity. However, for the vast majority of drugs adverse reactions occur following administration of normal therapeutic doses, and individual susceptibility is considered to result from complex genetic and/or environmental risk factors. Substantial effort has been exerted towards understanding such adverse reactions, which typically occur infrequently in the human population and are idiosyncratic [1,2]. Predictive biomarkers that reflect biological processes, leading to the adverse effects, have the potential to provide insights into underlying mechanisms responsible for adverse reactions. They may also report on the operation of susceptibility factors that differ between human individuals and thereby enable implementation of individualized therapy regimes that optimise drug efficacy whilst minimizing toxicity [3]. More sensitive markers of early toxicity might also provide the opportunity to cease therapy prior to development of clinically significant adverse consequences in susceptible individuals.

Much of our understanding of the role played by glutathione homeostasis in protection against toxicity is derived from studies of the mechanism of hepatotoxicity of APAP. APAP is metabolised by cytochrome (CYP) P450-based oxidative metabolism via CYP 2E1, which results in the formation of a reactive metabolite that is nucleophilic and at normal therapeutic doses is detoxified by reaction with cellular glutathione. Therapeutic doses of acetaminophen are generally metabolically detoxified via formation of sulfate and glucuronide conjugates. However, following overdose both sulphation and glucuronidation are saturated, and high levels of reactive metabolites are formed, which deplete glutathione causing oxidative stress and covalent modification of cellular macromolecules. These initiating mechanisms trigger a complex series of intra- and inter-cellular processes which ultimately result in organellar injury and cell death [4].

A recent series of investigations has implicated analogous mechanisms (i.e. CYP dependent bioactivation, glutathione depletion and covalent modification of macromolecules) in hepatotoxicity

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of the anti-histamine methapyrilene (N-dimethyl-N′-pyridin-2-yl-N′-(2-thienylmethyl)ethane-1,2-diamine) [5,6]. Methapyrilene was withdrawn from use in 1979, due to the production of hepatocellular tumors in Fischer-344 rats. This tumorigenesis has been attributed to non-genotoxic mechanisms that arise secondary to hepatic necrosis [7].

In view of the critical role played by the glutathione system in protection against cell toxicity caused by reactive metabolites of APAP, methapyrilene and numerous other drugs, quantification of components of the glutathione cycle has the potential to provide useful new biomarkers of hepatotoxicity, and of susceptibility to hepatotoxicity. Recently the tripeptide ophthalmic acid (OA), a non-sulphur-containing structural analogue of glutathione, was suggested as a potential biomarker following APAP administration to the mouse [8]. In that study metabolic profiles were analysed by coupling capillary electrophoresis with electrospray ionization time-of-flight mass spectrometry (CE–TOFMS), enabling the determination of global changes in a wide range of metabolites in serum and liver extracts of APAP-treated mice. Data analysis highlighted that 132 metabolites, from the total of 1859 detected in the samples, had changed in concentration as a result of APAP toxicity with the concentrations of OA being observed to have increased within 1 h after drug administration, remaining above normal for the next 3 h. Glutathione concentrations responded in the opposite direction, i.e. there was a significant decrease during the first 3 h after drug administration followed by an increase back up to, or above, normal concentrations for the 24 h observation period. This has lead to an interest in creating methods for the detection of ophthalmic acid in tissue extracts and plasma. Examples include the description of the LC separation of OA, glutathione and glutathione disulphide with MS/MS detection [9] and an LC–MS/MS method for determining H2 incorporation into OA during in vivo studies in healthy control rats administered deuterium oxide [10].

In order to evaluate the utility of OA as a potential biomarker of glutathione depletion and oxidative stress in vitro and in vivo we have developed and validated, specific and quantitative HPLC–MS/MS methods that can be applied in investigative toxicity.

2. Experimental

2.1. Solvents and reagents

Methanol (MeOH) of HPLC grade and formic acid of analytic grade used for LC/MS analysis were purchased from Sigma–Aldrich (Dorset, UK). Water (18.2 MΩ) was obtained from a Purelab Ultra system from Elga (Bucks, UK). Standard ophthalmic acid was purchased from Bachem Holding AG (Budendorf, Switzerland). Methapyrilene for use as an analytical standard was purchased from Sigma–Aldrich (Dorset, UK). For cell culture, PMFR P-004 medium was purchased from Gibco, Glutamax and fetal bovine serum were purchased from Invitrogen, and other media supplements were purchased from Sigma Aldrich. 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. SV40 large T antigen immortalised human liver epithelial cells transfected with individual cytochrome P450 enzymes (THLE–CYP) were obtained under license from Nestec. Ltd., Switzerland.

2.2. Samples

2.2.1. Cell line culture medium samples

Cell culture medium (see above for details) was collected from culture flasks containing SV40 large T antigen immortalised human liver epithelial cells transfected with individual cytochrome P450 enzymes (THLE–CYP) [11]. The medium analysed was taken from THLE–CYP cell lines transfected with the 2C19, 3A4, 1A2, 2C9, 2D6 cytochrome P450s and a mock-transfected cell line (null). For the stability analysis, a pool was created of media collected from the different THLE–CYP cell lines.

2.2.2. Rat plasma and serum samples

Control rat plasma was obtained from the animal breeding unit of AstraZeneca at 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 for control, 10 mg/kg/day for low dose and 50 mg/kg/day for high dose. The drug 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. 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 $n = 6$ as two animals provided samples for both days 7 and 15.

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 250 μl 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.

2.2.3. Human plasma samples

Human plasma was obtained from normal healthy subjects from the AstraZeneca Clinical Pharmacology Unit.

2.3. Routine sample preparation

Aliquots (50 μl) of both the human and the rodent 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 rcf for 10 min. Subsequently the 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).

Ophthalmic acid solutions were made up in water at concentrations of 100 μg/ml to 20 ng/ml. Calibration curve samples, biological quality control (QC) samples and spiked "blank" samples were made by adding a fixed volume of these ophthalmic acid solutions (2.5 μl) to 47.5 μl of matrix. Calibration curves containing 8 concentrations of ophthalmic acid were prepared to concentrations: 1 ng/ml, 5ng/ml, 10 ng/ml, 50 ng/ml, 100 ng/ml, 500 ng/ml, 1 μg/ml and 5 μg/ml for the culture media and for rat plasma. Validation QCs for the culture medium were prepared at concentrations of 2, 200 and 400 ng/ml whilst for rat plasma concentrations of 75, 300, 400 ng/ml were employed. Fresh QCs and calibration curves were prepared for every run.

The rat samples were analysed in a randomized order to eliminate effects that might result from run order. In the event that the matrix in which the calibration curve was made up contained an intrinsic concentration of endogenous ophthalmic acid, which was higher than in the analysed samples, the calibration curve was presumed to be linear and extrapolated in order to obtain the values of the samples. These were then corrected for the intrinsic concen-

tration of ophthalmic acid in the media as analysed compared to a standard curve made up in water.

2.4. HPLC–MS/MS conditions

For HPLC–MS 10 μl of each sample was analysed by HPLC using a Perkin–Elmer series 200 high pressure LC micro solvent delivery system (Perkin–Elmer Life Sciences, Cambridge, UK) and a CTC PAL autosampler (CTC Analytical, Switzerland). The autosampler was maintained at 4 °C. Separations were performed on a Symmetry C18 3.5 μm (2.1 mm × 150 mm) column (Waters Ltd., Elstree, UK). All of the columns used in this study were maintained at 40 ± 0.2 °C during the analysis by using a column heater to ensure temperature stability (Jones Chromatography, Hengoed, U.K.).

Reversed-phase gradient systems, based on binary solvent systems composed of methanol (solvent B) versus water (solvent A) were used. All eluents were acidified with 0.1% formic acid (v/v). For these solvent systems separations were performed using gradient elution with the initial conditions set at 100% A: 0% B for the first 4.2 min post injection, followed by a step to 0% A: 100% B for 1.4 min before returning to the starting conditions and held for 3 min prior to the next injection, making a total cycle time of 8.6 min/sample. A flow rate of 400 μl/min was used.

2.5. Mass spectrometry

All of the MS data were acquired on a 4000 QTRAP hybrid triple quadrupole linear ion trap mass spectrometer (Applied Biosystems/MDS Analytical Technologies, Warrington, U.K.). The TurboIonSpray inlet was operated at 350 °C in positive ESI mode in separate experiments. Multiple reactions monitoring MS was used to separate the target ion of ophthalmic acid. Q1 was set to 290.2 and Q3 to 215.1 and 161.1. The TurboIonSpray voltage was set at +45 V, curtain gas at 20 psi, auxiliary gases at 80 psi and declustering potential at 50 V.

2.6. Determination of precision, accuracy, LLOQ, matrix effects

Precision and accuracy were assessed at spiked concentrations of OA of 70 ng/ml for the cell culture media and 300 ng/ml for rat plasma. Within batch precision and accuracy were assessed by measuring 6 samples in one batch and between batch precision and accuracy were assessed by measuring those 6 samples on three different days. Selectivity was analysed to test for potential interferences in the blank matrix. Six samples of each of the blank matrices were analysed for a response in ophthalmic acid. The percentage of the peak area was then compared to the peak area of the low limit of quantification (LLOQ).

2.7. Determination of between-injection carry over

Carry over was analysed by measuring the peak area in the blank injected after the analysis of the highest concentration of the calibration curve (5 μg/ml). The percentage peak area of the carry over compared to the LLOQ was calculated.

2.8. Stability

For storage stability experiments cell growth medium and rat plasma samples were spiked at an OA concentration of 300 ng/ml. The storage stability samples were tested for bench top stability by keeping samples at 21 °C for 4 h. Freezer stability was tested by analysing precision and accuracy after 24 h, 1 week and 1 month storage at −20 °C. Freeze–thaw stability was assessed by spiking cell growth media samples with 300 ng/ml and rat plasma samples with 400 ng/ml of OA. The concentration of OA was then determined after one, two and three cycles of freezing and defrosting. Stock solution stability was tested by measuring precision and accuracy after 24 h in the autosampler (4 °C).

2.9. Clinical chemistry

Aspartate aminotransferase (AST) values for the methapyrilene study were determined at Covance Laboratories (Wisconsin, USA) using standard clinical chemistry assays.

3. Results and discussion

3.1. Analytical conditions

Detection of OA by LC–MS/MS was achieved by first detecting the parent ion in the Q1 (m/z 290.2) and then searching for the product ions of the highest intensity. These were of m/z 215.1 and 161.1, and arose from the sequential loss of COOH–CH_2–NH_2 and COOH–$CHNH_2$–CH_2–CH_2–C=O, respectively, from ophthalmic acid which have been previously observed by other investigators [8–10]. Due to possible interferences from the rat plasma and media, both the transitions were investigated during method development. The inclusion of formic acid in the HPLC mobile phase was seen to increase the intensity of the signal for ophthalmic acid in positive ESI. Under the chromatographic conditions employed ophthalmic acid eluted with a retention time of ca., 2 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 time of 8.6 min/sample.

3.2. Limit of quantification, linearity, accuracy and precision for cell culture media

In Fig. 1 the LC–MS/MS traces for OA in the blank, bottom standard (1 ng/ml), and 500 ng/ml standard samples of cell culture media are shown. As can be seen from these traces, in comparison to the 1 ng/ml bottom standard, OA was not detected in the THLE cell culture media, whereas addition of OA to the media at concentrations of both 1 ng/ml and 500 ng/ml afforded distinct and readily detected peaks. In the absence of a suitable isotopically labelled internal standard, concentrations of OA were determined against a standard curve generated by spiking control medium with OA at concentrations ranging from 1 ng/ml to 5 μg/ml. The LC–MS peak for OA in control cell culture medium was less than 10% of the lowest point of the calibration curve, and thus the lower limit of quantification (LLOQ), was taken to be 1 ng/ml for the cell culture medium. The linear equation for the calibration curve for ophthalmic acid in the cell culture media was typically $624.56x - 1752$ for the 290/215 transition and $3171.8x + 13{,}000$ for the 290/161 transition. Due to a better signal to noise ratio for the 290/161 transition this transition was chosen for use in routine sample analysis. However, all linear regressions for the calibration curves using either transition were above 0.998. The percentage carry over was found to be less than 1% at both the LLOQ and for the HQC. Within and between batch precision for cell culture media were below 15% (Table 1).

3.3. Limit of quantification, linearity, accuracy and precision for rat plasma

For the rat plasma, a signal for OA was observed in blank plasma, 1 ng/ml and 500 ng/ml samples as shown in Fig. 2. The peak of ophthalmic acid seen in the blank corresponded to an estimated concentration of ca. 5 ng/ml. Thus, the LLOQ, defined as the lowest

Table 1
Within (a) and between (b) run precision and accuracy for ophthalmic acid determination in cell culture medium.

(a)

Sample	1	2	3	4	5	6	Average	SD	Precision	Accuracy
Run 1	84.7	78.7	76.4	79.1	88	73.2	80.0	5.4	6.8	12.5
Run 2	64.9	66.2	76.3	82.3	74.5	87.7	75.3	8.9	11.8	7.1
Run 3	68.7	82.1	71.8	78.5	67.6	75.0	74.0	5.7	7.6	5.3

(b)

Average	SD	Precision	Accuracy
76.4	7	9.1	4.5

Precision was determined in 6 spiked ophthalmic acid samples, in 3 runs on different days in (a) cell culture media within run (spiked at 70 ng/ml) and (b) cell culture media between run (spiked at 70 ng/ml).

Table 2
Within (a) and between (b) run precision and accuracy for ophthalmic acid determination in rat plasma.

(a)

Sample	2	3	4	5	6	Average	SD	Precision	Accuracy
Run1	373.7	317.4	317.4	388.1	315	342.3	35.6	10.4	12.4
Run2	286.1	360.5	264.4	315.1	381.5	321.5	49.2	15.3	6.7
Run3	348.6	369.5	383	213.6	383	339.5	71.8	21.2	11.6

(b)

Average	SD	Precision	Accuracy
334.5	51.2	15.3	10.3

Precision was analysed in 6 spiked ophthalmic acid samples, in 3 runs on different days in (a) rat plasma samples within run (spiked at 300 ng/ml) and (b) rat plasma samples between run (spiked at 300 ng/ml).

concentration of the linear region of the calibration curve where the base peak was less than 20% of the LLOQ, was determined as 25 ng/ml for rat plasma. In the rat plasma samples the calibration curve was around $585x + 43{,}000$ for the 290/215 transition and $3370x + 284{,}000$ for the 290/161 transition. All linear regressions for the calibration curves in both transitions had correlation coefficients in excess of 0.98. As for culture media, the 290/161 transition was used for subsequent quantification. The percentage carry over was less than 1% of both LLOQ and HQC. Within and between batch precision was below 22% for rat plasma (Table 2). These results indicate that between OA concentrations of 25 ng/ml (LLOQ) and 5.0 μg/ml, the method was linear, precise and accurate. Interestingly from a "translational medicines" point of view analysis of control human plasma showed that there were no detectable endogenous concentrations of OA showing a potential difference between human and rat metabolism of this biomarker. This provided a LLOQ of 10 ng/ml (the concentration of the bottom standard of the standard curve). The trace for blank human plasma and for human plasma spiked at a concentration of 500 ng/ml monitored in positive ESI using the 290/161 transition are shown in Fig. 3.

3.4. Sample stability

Results for the stability of OA in cell culture media and rat plasma are shown in Table 3. In culture media the compound appeared to be stable as there was no significant decrease in the concentration measured (precision was better than 30% and accuracy was better than 23%) and no trends indicating declining concentrations with time, under any of the conditions examined. Similarly, in rat plasma the compound appeared stable as there was no significant decrease in concentration measured (precision was better than 18% and accuracy was better than 30%). Analyses of OA in these sample types following up to three freeze thaw cycles showed there was no significant decrease in concentration with increasing number of freeze thaw cycles (precision for cell line and rat plasma were better than 5.5% and accuracy better than 13%).

Table 3
Stability of ophthalmic acid in cell culture media (a) and rat plasma (b).

(a)

	4 h, 21 °C	24 h, 4 °C	24 h, −20 °C	1 w, −20 °C	1 m, −20 °C
Average	319.2	339.5	367.9	306.7	298.9
SD ($n = 3$)	88.0	37.2	48.1	68.5	38.9
Precision	27.5	10.6	13.0	22.3	13.0
Accuracy	6.2	13.7	22.6	2.2	−0.3

(b)

	4 h, 21 °C	24 h, 4 °C	24 h, −20 °C	1 w, −20 °C	1 m, −20 °C
Average	265.1	329.9	387.9	337.6	320.3
SD ($n = 3$)	17.1	59.4	38.3	30.9	37.2
Precision	6.4	18.0	9.8	9.1	11.6
Accuracy	−11.6	9.9	29.3	12.5	6.7

Stability of ophthalmic acid was determined after 4 h at ambient temperature (4 h, 21 °C), 24 h at 4 °C (24 h, 4 °C) and −20 °C (24 h, −20 °C), 1 week at −20 °C (1 w, 20 °C) and 1 month at −20 °C (1 m, −20 °C). These tests were undertaken in (a) cell line culture media (spiked at 300 ng/ml) and (b) in rat plasma (spiked at 300 ng/ml).

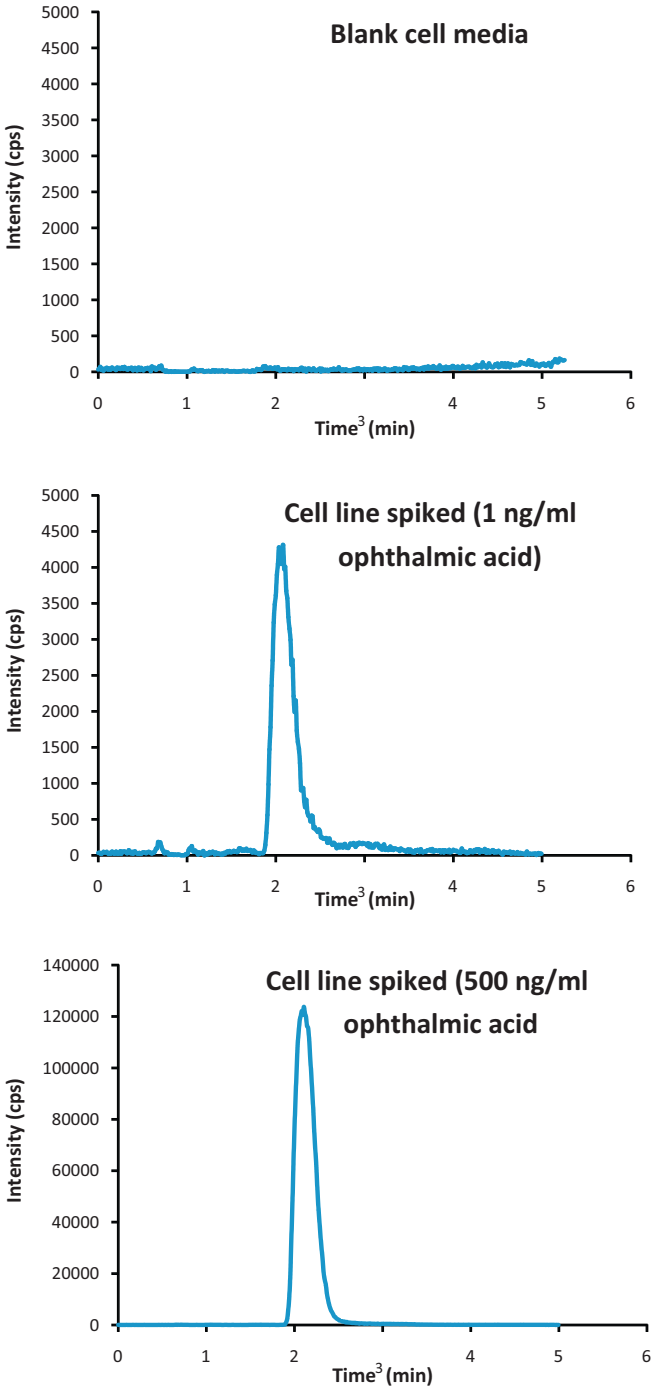

Fig. 1. The LC–MS/MS selected ion current traces for ophthalmic acid in the blank (upper), bottom standard (1 ng/ml) (middle), and 500 ng/ml standard (lower) samples of cell culture media.

The assays for cell culture media and plasma therefore appear to be compatible with the current FDA guidance for biomarker determination [12].

3.5. Analysis of cell culture media

The analysis of cell culture media obtained under normal growth conditions from a range of THLE cells lines after up to 48 h of incubation, with or without transfected cytochrome P450s, did not show detectable concentrations of OA. In future studies, the presence of such low concentrations of this potential biomarker of oxidative stress under normal culture conditions should be of value when exploring the role played by the glutathione system in protection against CYP mediated THLE cell toxicity caused by drugs such as acetaminophen and methapyrilene.

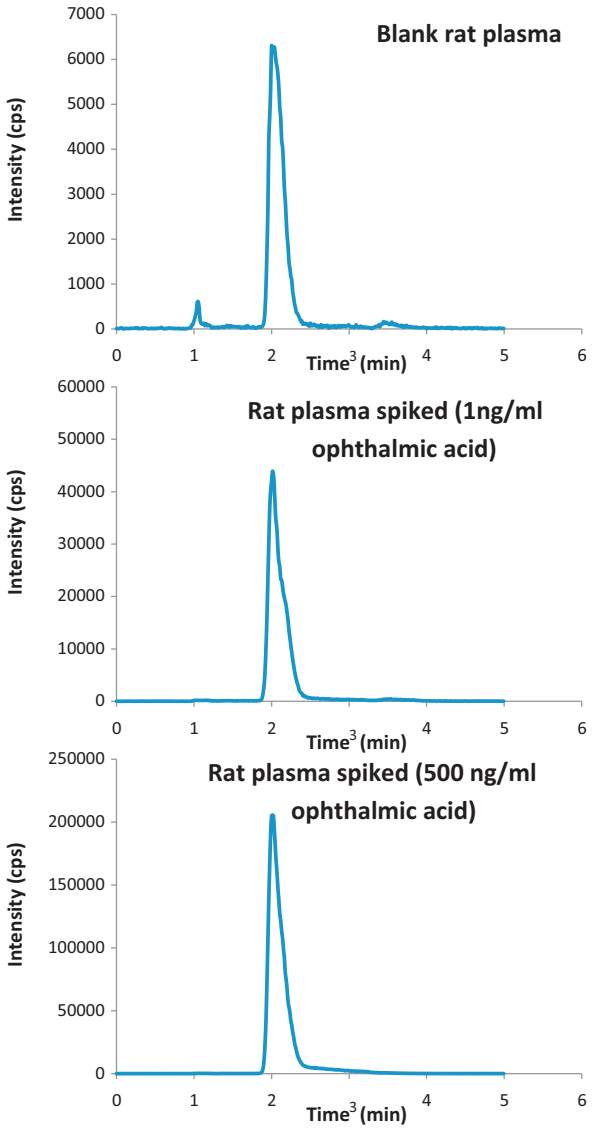

Fig. 2. Chromatographic separation and detection of ophthalmic acid in the LC–mass chromatograms of blank and spiked rat plasma (spiked at 1 and 500 ng/ml).

3.6. Analysis of samples from methapyrilene-dosed rats

The developed HPLC–MS/MS method was applied to serum samples obtained following repeat dose administration of methapyrilene to rats for 14 days at 0, 10 or 50 mg/kg/day. The low dose, which was not expected to result in hepatotoxicity, was based on two previously published studies where the administration of 10 mg/kg/day to male Sprague–Dawley rats was shown to result in no significant changes in known serum markers of hepatotoxicity and no compound-related histopathological changes in the liver itself [13,14]. The high dose of 50 mg/kg/day was chosen in order to induce minimal to mild hepatic necrosis after 7 days of dosing, with increased hepatic injury over the full course of 14 days of treatment. A previous study has shown that methapyrilene at 50 mg/kg/day caused only minimal single cell necrosis without changes in clinical chemistry parameters in a 3 day study [15]. In

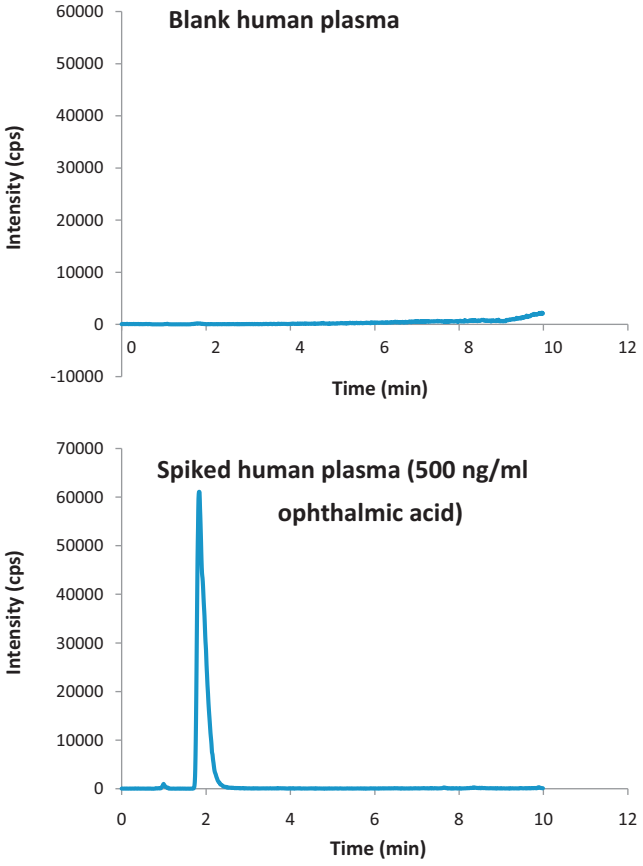

Fig. 3. The LC–MS/MS selected ion current traces for ophthalmic acid in blank and spiked human plasma (spiked at 500 ng/ml).

addition 50 mg/kg/day was chosen to minimize the risk of systemic toxicity due to CNS depression, which has been observed after repeat dosing at 150 mg/kg/day [13,14].

The study design yielded samples obtained at necropsy, which was undertaken on separate groups of animals at days 3, 5, 7, 11, 15 or 29. Examination of the ophthalmic acid concentration data from the control group at day 3 showed some variation between individual animals, with concentrations of OA detected ranging between 40 and 89 ng/ml (mean = 65, SD = 20). Interestingly, when concentrations of OA in the samples obtained from control animals were examined over the time course of the study a degree of fluctuation in the observed OA concentrations (SD = 25) was seen (see Fig. 4).

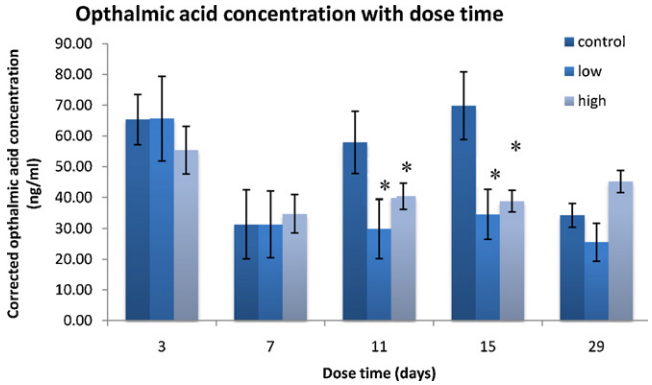

Fig. 4. Analysis of rat plasma from methapyrilene dosed rats with time at control dose (0 mg/kg/day), low dose (10 mg/kg/day) and high dose (50 mg/kg/day). Error bars are standard errors of the mean (SEM). Starred bars are results which are significantly different to control (p value under 0.05 with independent t-test).

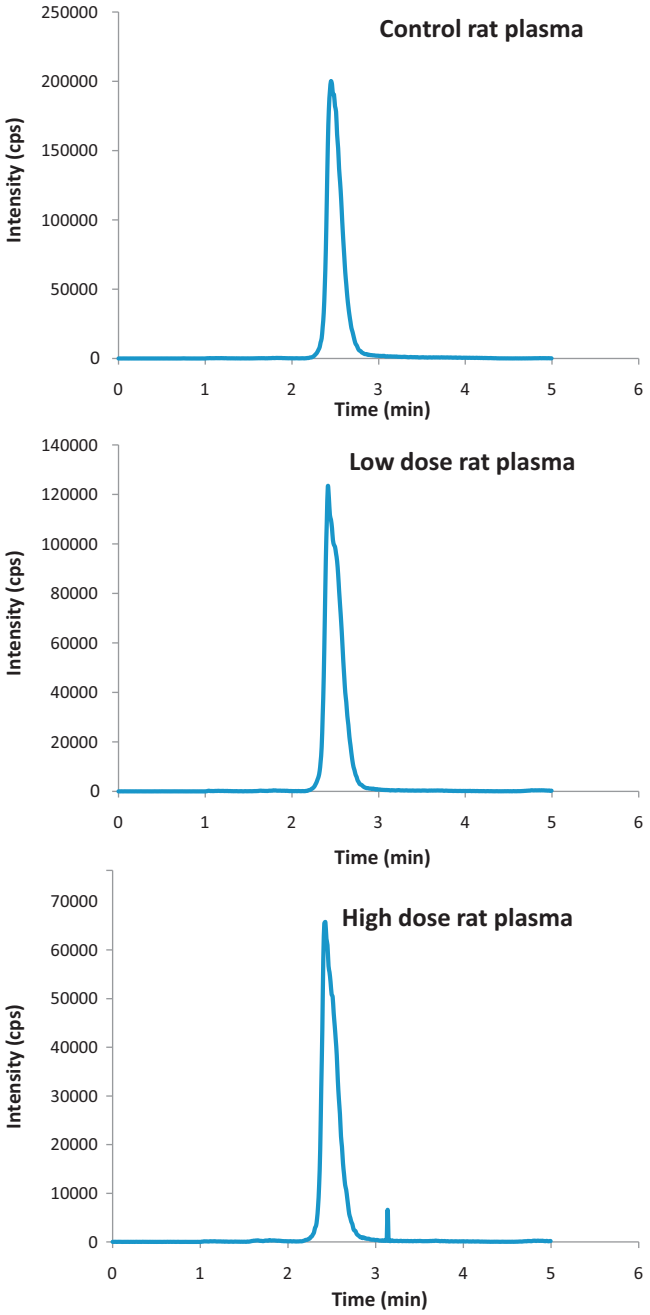

Fig. 5. Typical mass chromatograms for ophthalmic acid detected in rat samples after 11 days of administration of methapyrilene at different doses: 0, 10 or 50 mg/kg/day.

Whilst the reason for these fluctuations in OA concentrations is not clear, it is worth noting that biomarkers of toxicity such as AST also showed similar fluctuations in the samples of the control animals through the time course of the study with similar standard deviation (SD = 27). This leads us to believe that what was observed represents biological variation between animals rather than imprecision of the method.

Typical chromatograms for control, low and high dose rats for day 11 of the study are shown in Fig. 5. These mass chromatograms show the presence of ophthalmic acid in samples from all dose groups, including controls. The concentrations of ophthalmic acid detected in both control and dosed rats for all of the days on which they were sampled are shown in Fig. 4. The results obtained for OA in samples from the dosed animals did not show an increase in

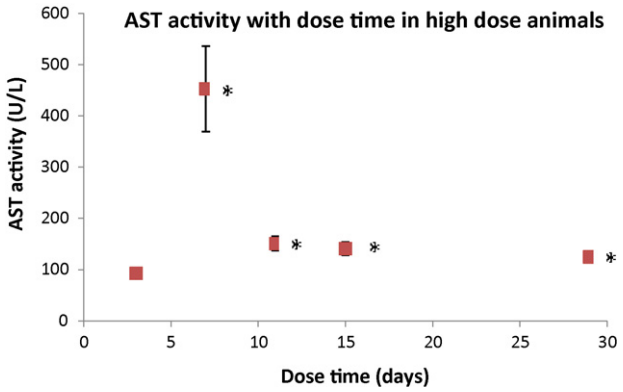

Fig. 6. Observed AST activity for animals dosed with methapyrilene at 50 mg/kg/day. Error bars are SEM ($n = 5$). Starred points are significantly different (p value lower than 0.03 with independent t-test) to results at day 3.

concentrations at any of the sampling points despite large rises in the measured AST levels on day 7 (see Fig. 6) indicative of hepatotoxic effects of the drug. If anything, for days 11 and 15 of the study, ophthalmic acid concentrations were significantly lower than the corresponding controls (p values below 0.05 with the independent t-test). Indeed OA, at this dose, shows a weak negative correlation with AST activity (data not shown).

The correlation between drug metabolism to reactive metabolites, that are detoxified by conjugation with glutathione, and OA concentrations could indeed be complex. In their study of short term effects of APAP, Soga et al. [8] observed a rapid and transient decrease in glutathione concentration accompanied by a similarly transient increase in OA concentration in both mouse liver and mouse serum. In terms of the inter-relationship between glutathione and ophthalmic acid biosynthesis, of which a schematic is shown in Fig. 7, this can be understood by competition between the glutathione and ophthalmic acid synthesis reactions. The synthesis of both ophthalmic acid and glutathione are catalyzed by the same enzymes, glutamyl-cysteine synthetase (GCS) and glutathione synthase (GS). GS and GCS have a higher affinity for γ-glutamyl cysteine and cysteine, respectively, than for the ophthalmic acid producing precursors [16,17]. During this short term

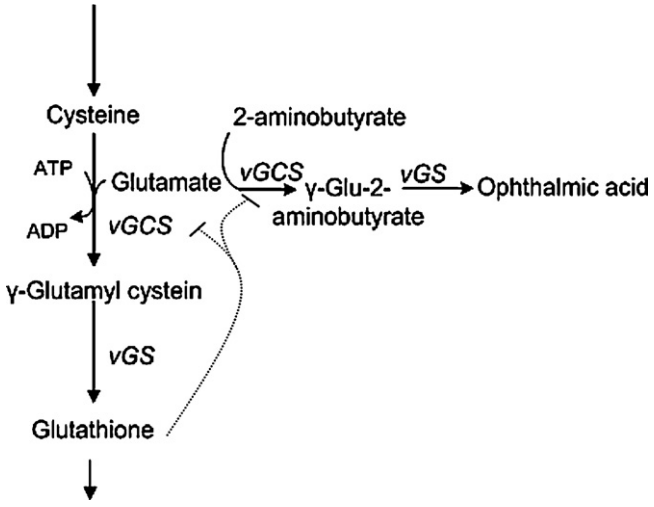

Fig. 7. Schematic of the gamma-glutamyl pathway showing ophthalmic acid and glutathione synthesis. Glutathione is synthesized by glutathione synthase (GS) from γ-glutamyl cysteine which in turn is made by glutamyl-cysteine synthetase (GCS) from cysteine and glutamate. Glutamate can also react with 2-aminobutyrate by GCS to form γ-glu-2-aminobutyrate which can be converted by GS to ophthalmic acid [16,17].

APAP toxicity a decrease in the glutathione precursor cysteine was also seen [8], correlated with the decrease in the production of glutathione. This decrease in the concentration of precursors for glutathione meant that enzymes could react with OA precursors thus forming the biomarker. Such acute drug effects however, might not represent the situation seen in chronic studies such as the methapyrilene study examined here where adaptation of the liver may have occurred in response to drug administration. In the current study animals were also sampled 24 h after drug administration so that any acute effects on hepatic glutathione may have recovered. Indeed, because of adaptive effects at such time scales hepatic glutathione may actually have been increased. In fact increased glutathione concentrations have been observed in livers from methapyrilene treated rats, which could be attributed to transcriptional induction of enzymes of the glutathione cycle [14,15]. For example, in one study administration of methapyrilene to rats at 150 mg/kg/day increased levels of hepatic glutathione levels to 140% of the control values [18], whilst in another similar study the elevation in total glutathione was 1.9-fold [5]. Glutathione S-transferase levels have also been observed to increase to 220% of control values on methapyrilene administration at 150 mg/kg/day [19]. An increase in glutathione would be correlated with a decrease in the enzymatically less favourable and competing pathway of ophthalmic acid production. The decrease in OA levels might thereby reflect such a longer term increase in glutathione levels, particularly reflecting adaptation, because the acute effects would have relaxed in this experimental set up.

It has also been shown [6], over 3 days of administration at 150 mg/kg/day, that changes in hepatic GSH levels across the liver exhibited marked zonality in response to methapyrilene toxicity, with GSH depletion in periportal hepatocytes and increased GSH levels in other hepatocytes which would not sustain damage. Clearly OA concentrations in plasma compartment will reflect the mean change across all hepatocytes.

In future studies it will be important to explore and compare the acute and the adaptive phases of the response to drugs causing hepatotoxicity, as indeed both glutathione and biomarkers may correlate differently with drug dose in the two phases. In addition, the relationship between zonal changes in hepatic GSH content and in hepatic glutathione cycle homeostasis, and plasma ophthalmic acid concentrations will need to be defined.

4. Conclusions

The LC–MS/MS method described here provides a rapid and sensitive method for the quantitative determination of ophthalmic acid concentrations in biologically relevant matrices. The method has been validated in both rat plasma and cell culture media, and has been used to explore ophthalmic acid as a potential novel biomarker for liver effects caused by methapyrilene in the rat in vivo. Additional applications of the developed methodology to in vitro and in vivo studies are planned to further evaluate the utility of ophthalmic acid as a biomarker of liver toxicology caused by this and other drugs.

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