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Ophthalmate detection in human plasma with LC–MS–MS

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ABSTRACT

Based on animal experimentations, ophthalmate (OPH) has recently been suggested as a potential plasma biomarker to probe hepatic GSH homeostasis. Up until now, the inability to accurately determine OPH concentrations in human plasma prohibited further studies of OPH metabolism in humans. This study therefore aimed to study the influence of delayed sample preparation on OPH concentrations using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Venous plasma samples from 5 healthy human volunteers were incubated for varying times (5, 30, 60 and 120 min) at temperatures of 4 °C and 37 °C to investigate potential enzymatic degradation. At 37 °C, the decrease in OPH reached significance after 120 min (74.6% (range: 56.2–100.0%; $p < 0.0001$)). At 4 °C, the same trend was observed but did not reach significance. These findings indicate ongoing enzymatic activity, stressing the need for immediate sample deproteinization to obtain reliable plasma concentrations. To investigate the feasibility of the here developed method, baseline arterial plasma values of 21 patients scheduled for partial liver resection were determined to be 0.06 ± 0.03 μmol/l (mean ± s.d.). In addition, in pooled samples from 3 patients, an OPH calibration curve was spiked to arterial plasma, arterial whole blood and liver biopsy material, resulting in a linear calibration curve in all cases. Individual measurements of baseline samples revealed that both arterial whole blood and liver biopsy material contained significant levels of endogenous OPH, namely 16.1 (11.8–16.4) μmol/l and 80.0 (191.8–349.2) μmol/kg, respectively. In conclusion, the present LC–MS/MS assay enables the accurate measurement of OPH in human plasma, whole blood and liver biopsies. Freshly prepared samples and immediate deproteinization are mandatory to block enzymatic degradation.

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1. Introduction

The intracellular content and hepatic synthesis of Glutathione (GSH) is considered to be the main hepatic protection system against redox imbalances and many forms of oxidative stress and intoxications [1–3]. Unfortunately, thus far assessment of hepatic GSH homeostasis in vivo in humans is difficult and the availability of an adequate test to measure hepatic GSH depletion would be of great clinical importance. Estimation of hepatic GSH content would require a liver biopsy, which necessitates an invasive procedure. Although taking a liver biopsy is not an insurmountable issue, measurement of the GSH content of a liver biopsy alone does still not provide information on the capacity of the liver for de novo GSH synthesis.

Recently, ophthalmate (OPH, Fig. 1), an endogenous tripeptide analogue of GSH, has been suggested as a potential biomarker for GSH homeostasis as its production was found to be reciprocally promoted when GSH becomes depleted [4–6]. Kombu et al. [5] suggested that when the hepatic availability of the amino acid cysteine, providing the reducing moiety of GSH, becomes limiting, the amino acid 2-aminobutyrate is incorporated instead, resulting in the formation of OPH [5]. Although the physiological background for this switch is still not elaborated, it has been suggested that OPH makes use of the same transporter system as GSH and in this way OPH would minimize cellular GSH efflux to preserve cell integrity [7]. Since OPH is released into the blood stream following its synthesis in the liver, a reciprocal rise in plasma OPH concentration may thus indicate hepatic GSH depletion.

Although several reports have been published so far describing the analysis of OPH [4,5,8,9], none of them were able to determine the physiological concentrations of OPH in plasma of healthy human subjects. Surprisingly, we obtained plasma OPH levels well above the level of detection (LOD) found before [9]. However, this report focused on the better chemical stability of OPH compared to GSH due to its lack of the thiol moiety, but its resistance

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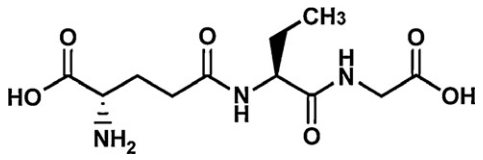

Fig. 1. The structure formula of ophthalmate (OPH), also known as ophthalmic acid.

to enzymatic degradation was not investigated. Hahn et al. [8] suggested that OPH is degraded by the same enzymatic machinery as GSH, which is known to rapidly degrade excreted GSH in the bloodstream. Therefore, we postulated that a rapid enzymatic breakdown might explain the inability to measure human OPH levels. Consequently we aimed to investigate the effects of delayed sample deproteinization and temperature on OPH concentrations. To guarantee optimal selectivity and sensitivity, a liquid chromatography tandem mass spectrometry (LC–MS/MS) method was developed to enable quantitative analysis of OPH in human plasma, whole blood and liver biopsy material.

The here presented protocol meets these requirements, facilitating future studies to investigate the usefulness of OPH as a marker of hepatic GSH depletion in humans.

2. Methods

2.1. Patients and healthy volunteers

Baseline blood samples were obtained from five healthy volunteers. 21 patients, scheduled for liver surgery were also included in the present study. In 3 patients also whole blood and a liver biopsy was collected. This study was approved by the medical ethical committee of the Maastricht University Medical Centre+ and conducted according to the revised version of the declaration of Helsinki (October 2008, Seoul). All subjects and patients gave written informed consent.

2.2. Materials and reagents

OPH was obtained from Bachem (Torrance, CA, USA) and dissolved in Milli-Q water (Waters, Etten-Leur, Netherlands) to a standard of 10.0 mmol/l. The standard was divided into 1.0 ml aliquots and stored at −80 °C, guaranteeing its stability [9]. All reagents and solvents used were of analytical grade or better (Sigma–Aldrich, Zwijndrecht, Netherlands).

2.3. Sample collection and preparation

5 ml blood samples were collected in pre-chilled heparinised vacuum tubes (BD vacutainer, Becton Dickinson Diagnostics, Aalst, Belgium) and kept on ice. In healthy volunteers, blood samples were collected by venepuncture of an antecubital vein. In 21 patients scheduled for liver surgery, a baseline blood sample was taken from an indwelling catheter in the radial artery. Plasma was prepared by centrifugation in a pre-chilled centrifuge at 4 °C at 3000 × *g* for 10 min. and 100 μl aliquots were immediately deproteinized by the addition of 100 μl of 5% solid 5-sulfosalicylic acid solution (5-SSA: British Drug Houses, Amsterdam, The Netherlands) in water and stored at −80 °C until analysis.

From 3 patients, also whole blood and liver biopsy samples were collected. 100 μl aliquots of whole blood were also immediately deproteinized by the addition of 100 μl of 5% 5-SSA and stored at −80 °C until analysis. After collection, liver biopsies were snap-frozen in liquid nitrogen. Next, 10 mg liver biopsies were homogenized in 100 μl 5% ice-cold SSA solution in water, using a mini beat beater (Biospec Products, Applikon, Amsterdam, Netherlands) set to 2 min at full speed. Homogenates were snap frozen in liquid nitrogen and stored at −80 °C until analysis.

Prior to analysis all samples were centrifuged for 10 min at 50,000 × *g* at 4 °C and the clear supernatants were transferred to 200 μl glass inserts spring-loaded in 4 ml WISP style (Waters, Etten-Leur, Netherlands) and stored in the chilled sample compartment of the sample processor systems. The analysis was initiated with the injection of 20 μl sample or standard.

2.4. Calibration and validation

As all biological samples investigated contain endogenous OPH, the limit of detection (LOD) and lower limit of quantification (LOQ) cannot be determined in these samples. Instead, the limit of detection (LOD) and limit of quantification (LOQ) were determined by diluting aqueous standards. LOD was defined as the concentration corresponding to a signal to noise ratio of 3, while LOQ was defined as the concentration corresponding with a signal to noise ratio of 5 according to [13].

For each set of samples and each sample matrix, a calibration curve was prepared. For this, a 10.0 μmol/l aqueous OPH solution was prepared by diluting a freshly thawed 10.0 mmol/l stock solution in Milli-Q water. Calibration curves were prepared by adding 20 μl of pre-diluted OPH-standards to 80 μl of water, plasma supernatant, whole blood supernatant or liver homogenate supernatant. In water and plasma, 9 point calibration curves were constructed to obtain the following concentrations above baseline: 0.0 μmol/l, 0.1 μmol/l, 0.25 μmol/l, 0.5 μmol/l, 0.75 μmol/l, 1.0 μmol/l, 1.5 μmol/l, 2.0 μmol/l and 2.5 μmol/l. Because in whole blood and liver supernatant higher baseline concentrations of OPH were expected, 7 point calibration curves were constructed to obtain the following concentrations above baseline: 0.0 μmol/l, 1.0 μmol/l, 1.5 μmol/l, 2.0 μmol/l, 2.5 μmol/l, 5.0 μmol/l and 10.0 μmol/l. Pearson coefficient was used to determine the linearity of the method. The resulting regression line of these 9 and 7 points standard curves was described as linear equation, and the correlation coefficient (R^2) was calculated.

For each set of samples measured, a control plasma spiked with OPH to raise baseline concentrations with 1 μmol/l was measured in triplicate at the beginning, middle and end of the sample sequence.

The injection carry over was determined by measuring the peak area in 5 blank samples following analysis of samples spiked at a concentration which was the highest concentration of the calibration curve (2.5 μmol/l for plasma and 10.0 μmol/l for whole blood and liver tissue).

We initially aimed at applying an internal standard throughout all experiments, but as the production of the standard ([^{13}C2,^{2}H$_2$]-ophthalmic acid hydrochloride, AlsaChim, Illkirch, France) took too long, we could only apply it for the analysis of the unknown patient plasma samples. In addition, we also constructed a calibration curve in water in which we varied the concentration of unlabelled OPH between 0 and 20 μmol/l, while we kept the concentration of the tracer constant at 10 μmol/l.

2.5. Instrumentation

The HPLC system consisted of a Gilson Model 233 XL sample processor, equipped with Peltier-chilled sample and reagent trays (4 °C) and a Rheodyne 6 way high-pressure valve (Meyvis, Bergen op Zoom, The Netherlands) equipped with a 20 μl sample loop. A high-pressure gradient was generated using two Model PU 2085 pumps connected through a T-piece (Jasco Benelux, Maarssen, The Netherlands). The separation was performed on a 150 mm × 3 mm (i.d.) Allsphere ODS-2 3 μm column (Grace, Breda, The Netherlands) set to 20 °C. The bench top ion-trap mass spectrometer used was

a model LTQ XL (Thermo Electron Veenendaal, The Netherlands), equipped with an ion-max electrospray (ESI) probe. The system was operated in positive mode. To obtain maximal analytical sensitivity, the liquid-chromatographic–mass spectrometry approach described by Geenen et al. [9] was modified. Maximal sensitivity was obtained by applying Selective Reactant Monitoring (SRM) to the precursor ion of OPH at *m/z* 290 and the product ion at *m/z* 215, using 35% (arbitrary value) normalized collision energy. The heated capillary was set to 220 °C and sheath and auxiliary gas were set at 99 and 35 arbitrary system units respectively. The tube lens voltage was 20 V and the spray-voltage was 4.5 kV. Solvent A was a 0.3% formic acid (v/v) solution in Milli-Q-water (pH = 2.4). Solvent B was a mixture of acetonitrile/water (95/5; v/v). Flow was 0.3 ml/min throughout the gradient. From $T = 0$ min to $T = 1$ min, gradient was kept at 100% solvent A, after which solvent B was increased to 50% in 6 min. Next, the column was cleaned by flushing at 100% B for 2 min, after which initial conditions were restored to 100% A in 1 min. The column was next re-equilibrated for 10 min prior to the next analysis. Data were processed using Perkin Elmer TotalChrom version 6.2.0.

2.6. Stability determination of OPH in deproteinized plasma

To determine OPH stability in deproteinized plasma and water during analysis, aliquots of deproteinized aqueous standards and deproteinized plasma were stored in the sample compartment of the Gilson sample processor set at 4 °C and analysed consecutively during an 8 h period.

2.7. Enzymatic activity of plasma on OPH

In order to investigate OPH stability and to quantify enzymatic activity in plasma leading to conversion of OPH, plasma aliquots from the same five volunteers were spiked with OPH (to guarantee measurable concentrations). Next, samples were incubated at 4 °C or 37 °C for 5, 30, 60 and 120 mins. Enzymatic activity was terminated by the addition of 5% SSA solution and subsequent thorough mixing before snap freezing in liquid nitrogen.

2.8. Statistics

Mann Whitney *U* test was applied for two group comparison for continuous data. Wilcoxon signed rank test was applied for pairwise comparison for continuous data. All data are expressed as median and range. A p-value < 0.05 was considered statistically significant. Statistical analysis was performed using Prism 5.0 for Windows (Graphpad Software, Inc, San Diego, CA).

3. Results

3.1. Calibration and validation

The limit of detection, determined according to [13] at a signal to noise ratio of 3 was found to be 4.5 nmol/l. The lower limit of quantification, determined at a signal to noise ratio of 5, was 6.5 nmol/l.

Using the present LC–MS/MS method, the retention time of OPH was about 6.27 min, with a total run time of 17.4 min. Representative chromatograms of aqueous OPH standard, baseline plasma, whole blood and liver samples are shown in Fig. 2, panels A–D. Calibration curves in water and plasma, whole blood and liver were linear (Fig. 3), with the following respective equations: $R^2 = 0.99$, $y = 15.4 \times 10^3$ $(\pm 274.8)x + 477.0$ (± 347.5); $R^2 = 0.99$, $y = 7.0 \times 10^3$ $(\pm 620.6)x - 336.3$ (± 784.5); $R^2 = 0.97$, $y = 6.7 \times 10^3$ $(\pm 508.3)x + 6.5 \times 10^4$ $(\pm 2.3 \times 10^3)$ and $R^2 = 0.94$, $y = 3.4 \times 10^3$ $(\pm 387.9)x + 25.0 \times 10^4$ $(\pm 1.7 \times 10^3)$.

A control plasma, prepared from pooled plasma of the healthy volunteers and spiked to raise baseline concentrations with 1.0 μmol/l was analysed in triplicate, namely at the beginning, middle and end of each sample set. Mean spike recoveries, calculated

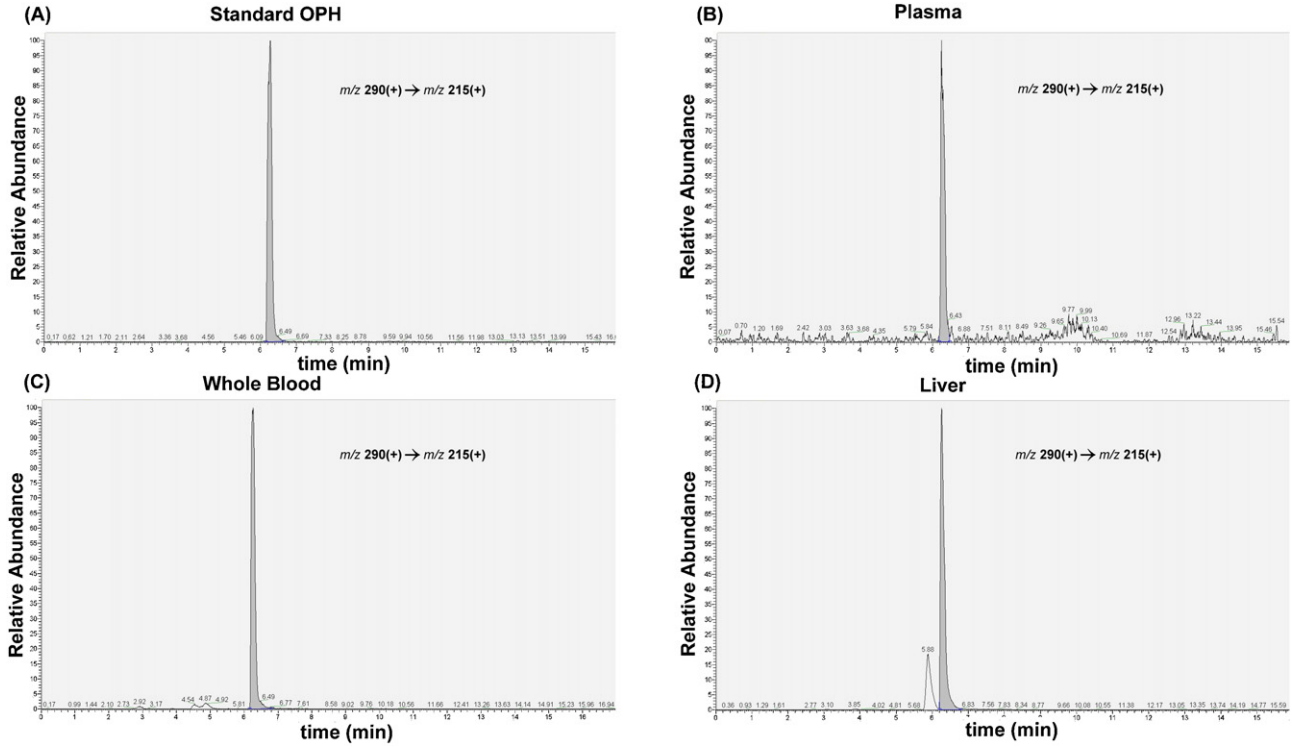

Fig. 2. Chromatographic separation and detection of OPH in the LC–MS/MS chromatogram of (a) spiked 1.5 μM H_2O SSA, (area = 24736), (b) blank human plasma (area = 292), (c) blank human whole blood (area = 106624) and (d) blank human liver (area = 139743).

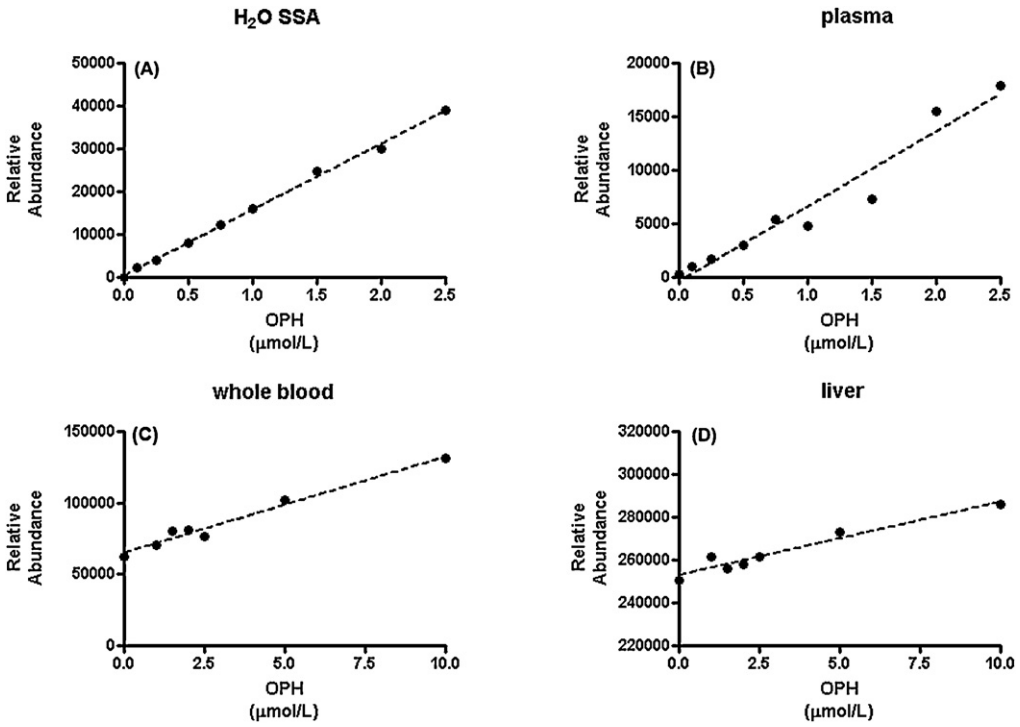

Fig. 3. Calibration curves of OPH in different matrices. Calibration curves of liver and whole blood did not pass through zero because of relatively high endogenous OPH concentration in these matrices.

on 4 consecutive days of analysis varied from 103.4 to 106.2% with a standard deviation, varying between 0.39 and 2.35%.

Injection carry-over for plasma after a sample injection of a 2.5 μmol/l OPH standard was not detectable. Injection carry-over for whole blood and liver tissue after injection of a 10.0 μmol/l OPH standard was negligible with 0.13% and 0.45% respectively.

The internal standard stability was investigated by the construction of a calibration curve in which we varied the unlabelled standard between 0 and 20 μmol/l and kept the labelled internal standard ([$^{13}C_2$,$^{2}H_2$]-ophthalmic acid) constant at 10 μmol/l. In Fig. 4 the results of this experiment are presented, indicating a linear increase in the unlabelled standard, while maintaining a stable area for the internal standard. In addition, we also added 20 μl of a 1 μmol/l internal standard solution to 80 μl of the unknown patient plasma supernatant samples resulting in a final concentration of 0.2 μmol/l. We obtained a mean concentration for the standard of 0.21 μmol, indicating 102.5% recovery. The standard deviation was 0.018 μmol/l, resulting in a coefficient of variation of 8.5% at this low level indicating a good applicability of this approach.

3.2. Baseline OPH levels

The developed method enabled the measurement of endogenous OPH levels in baseline venous control plasma of 5 healthy volunteers at a mean level as low as 0.02 μmol/l (range: 0.01–0.03 μmol/l). Mean OPH levels in arterial blood plasma of 21 patients scheduled for liver surgery were 0.06 μmol/l (with an S.D. of 0.03 μmol/l). Mean OPH levels in whole blood and liver biopsy samples of patients undergoing liver resection were 16.1 μmol/l (range: 11.8–16.4 μmol/l) and 80.0 μmol/l (range: 191.8–349.2 μmol/kg) respectively.

3.3. Stability of OPH

The replicate analysis in time of a spiked deproteinized plasma sample stored in the chilled sample compartment of the sample processor, showed no significant decrease in sample response during an 8 h period, indicating samples can be run safely during this period. The intra-assay precision during this period was 3.5% with an accuracy of 1.9% (Fig. 5).

Stability experiments in spiked human plasma revealed that no significant decrease in exogenous OPH levels occurred at any time

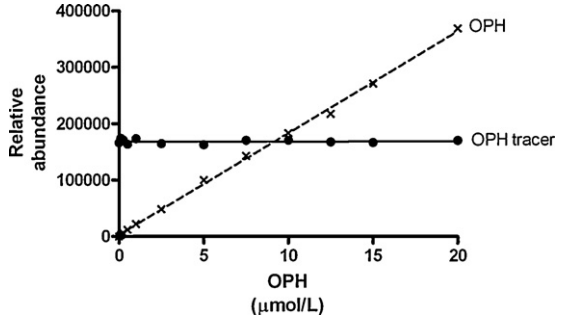

Fig. 4. OPH tracer stability and normal OPH standard.

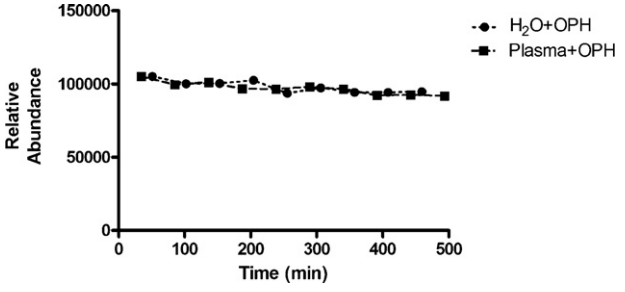

Fig. 5. Stability of OPH in water and deproteinized plasma in the auto sampler for 8 h at 4 °C (n = 5).

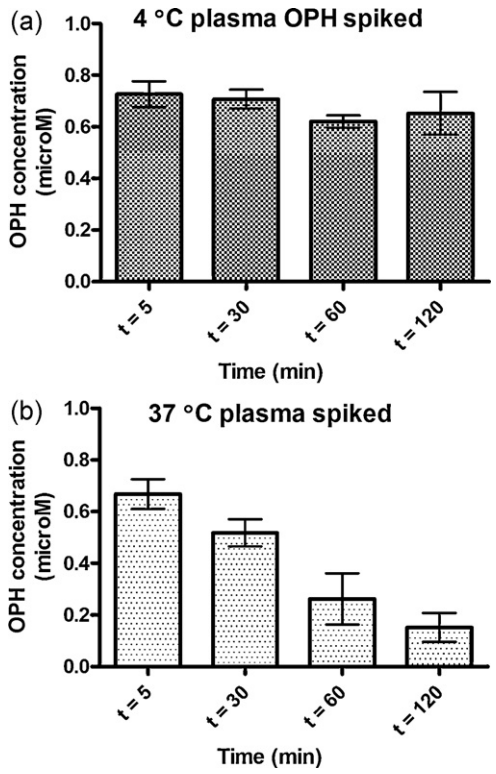

Fig. 6. OPH levels at different time points in untreated plasma that was incubated at (a) 4 °C and (b) 37 °C ($n = 5$), error bars represent standard error of the mean.

point during incubation at 4 °C. However, 120 min incubation at 4 °C resulted in a non-significant tendency towards a decrease of OPH levels in spiked human plasma of 9.7% (6.4–21.0%). Interestingly, when spiked plasma was kept at 37 °C, exogenous OPH levels showed a significant decrease of 74.6% (56.2–100.0%) at 120 min compared to baseline ($p < 0.0001$) (Fig. 6, panels A and B).

4. Discussion

The aim of the present study was to develop and validate a protocol to enable the quantitative analysis of OPH in human plasma, thus enabling future research on the value of OPH as a marker for intra-cellular GSH depletion. The here described protocol indeed enabled this, resulting in baseline OPH levels in venous plasma of healthy human volunteers (10–30 nmol/l) as well as in arterial plasma of patients scheduled for (partial) liver resection (20–140 nmol/l). In human whole blood and liver biopsy homogenates baseline OPH concentrations were markedly higher (μmol/l range) as compared to plasma. It was also shown that immediate deproteinization of plasma with SSA at 4 °C and finally storage at −80 °C was optimal to prevent OPH degradation in collected plasma samples. OPH concentrations in untreated plasma of healthy volunteers did not significantly decrease within 2 h if kept at 4 °C.

An increasing number of studies have suggested that OPH could be a marker of hepatic GSH depletion [4–6,9–11]. However, most of these data are derived from animal experiments and human OPH data are scarce. Kombu et al. [6] investigated the GSH–OPH system by labelling free GSH, bound glutathione (GSSG) and OPH following intravenous infusion of ^{2}H-enriched water and subsequently used an LC–MS–MS assay to determine enrichments. This method provided the opportunity to measure the synthesis of these components in rat plasma, erythrocytes and liver. However, the pool size of OPH in human liver is unknown. Our data show, for the first time, the presence of relatively high levels of OPH in human liver biopsies. From a translational research point of view this observation is crucial support for the value of OPH as a biomarker for hepatic GSH synthesis/depletion. An explanation for these relatively high liver OPH levels may be the fact that the patients enrolled in the present study had colorectal liver metastases. Although liver biopsies were taken remote from the hepatic tumours to be removed, it is known that malignant liver tumour growth, both primary and secondary, can disturb the GSH buffer system and may therefore also be associated with high OPH levels [4,11].

The results of the present study are largely in concordance with results published recently by Geenen et al. [9] who showed that OPH could be measured accurately with LCMS in rat plasma and cell culture media. Since endogenous OPH levels could not be detected in human plasma in their study [9], they speculated on a potential difference between rat OPH metabolism and human OPH metabolism. In contrast to this assumption we postulated that although a chemical degradation had been excluded in this paper, an enzymatic degradation had not been ruled out and this was the key target for this study to investigate. In addition we modified their LC–MS approach, now incorporating an MS–MS transition, as described elsewhere [6] to enhance the level of sensitivity and accuracy even more. This resulted in sensitive and selective method allowing detection of very low endogenous baseline OPH levels in plasma of both healthy volunteers and patients undergoing liver resection.

It is suggested that OPH is subject for the same pathway for enzymatic degradation as GSH, catalysed by γ-glutamyl transpeptidase [7,8]. Therefore, in this study, we focused on optimalization of the sample preparation procedure. Indeed, we were able to show that OPH levels in untreated plasma samples decreased at a temperature and time dependent rate. Especially, when untreated plasma samples spiked with OPH were kept at 37 °C, a significant amount (75%) of the OPH was degraded within 1 h. When kept at 4 °C the degradation process was evidently diminished. These experiments confirmed the necessity to deproteinize collected plasma samples as soon as possible in order to obtain accurate results. Whether OPH follows the same enzymatic breakdown pathway as GSH, *still* needs to be verified in future studies.

As it was clear that even within the period required to prepare plasma still a 5–10% decrease of OPH levels could occur, we tried to further reduce sample preparation time by also measuring whole blood OPH concentrations. As erythrocytes contain a relatively high level of GSH, we found, not unexpectedly, that whole blood contained a 10–20 times higher level of OPH (~12–16 μmol/l) than plasma. This observation could present a potential problem when using OPH as a biomarker of GSH depletion, for instance in case plasma is haemolytic. Although the relation between erythrocyte OPH synthesis and the exchange between plasma/liver OPH and the erythrocytes has not been well studied yet, for GSH it has been investigated thoroughly. Research has previously shown that most of plasma GSH is formed in the liver [12]. Kombu et al. [6] confirmed this by using a stable isotope labelling technique with a ^2H_2O load, followed by measurement of the label incorporation in GSH moieties. The authors showed that there was a rapid labelling of liver GSH, reflected by a rapid turnover of the large GSH/GSSG (the ratio reduced versus oxidized GSH) pool in rats. Interestingly, they also showed that the labelling profile of plasma GSH was comparable to that of liver GSH. The labelling of the GSH/GSSG pool in erythrocytes was shown to be much slower compared to the liver. This is probably due to the low rate of GSH synthesis in erythrocytes and/or due to the low import of plasma GSH into erythrocytes. Since OPH is an analogue of GSH, it is conceivable that OPH follows the same kinetic mechanisms as GSH. This would mean that if haemolysis can be avoided, plasma OPH levels may accurately reflect hepatic OPH synthesis. In order to guarantee accurate measurement of plasma

OPH and to correctly interpret OPH levels in the future it might be necessary to determine the level of haemolysis, for instance by determining free haemoglobin in plasma.

Whether OPH levels can be used reliably as a reciprocal readout of GSH depletion should be investigated in a future proof of principle study, preferably in a human translational research model. If validated thoroughly, OPH could be a candidate dynamic liver function biomarker. This proposed biomarker could for instance be used to preoperatively predict liver function and could also allow early diagnosis of hepatic dysfunction in patients undergoing liver resection. In addition, OPH as a biomarker for GSH depletion could help to titrate the treatment of patients with paracetamol (PCM) intoxication. It could also be used to measure susceptibility to hepatotoxicity to develop individualized pharmacotherapeutic regimens, in order to optimize drug efficacy in the treatment of patients.

5. Conclusion

The developed LC–MS/MS protocol enables the measurement of OPH in plasma of healthy volunteers as well as in patients with colorectal liver metastases undergoing liver resection. Accurate data can only be collected if freshly prepared plasma samples are immediately deproteinized, kept on ice and haemolysis is avoided. With use of this LC–MS/MS assay and the here described pre-storage treatment protocol, it is now possible to validate OPH as a biomarker for hepatic GSH depletion in a future human proof of principle study. Provided the precautions shown in the present study the applicability of OPH as a biomarker for liver function is promising.

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