Comparison of analytical methods for detection of perchloroethylene glutathione conjugates and application in liver fractions

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ABSTRACT
Perchloroethylene (PER) is a degreasing solvent widely used to replace the toxic trichloroethylene. However, PER can also lead to nephrotoxicity via bioactivation by the glutathione (GSH) conjugative pathway. In this study, a postcolumn o-phtaldialdehyde (OPA)/N-acetylcysteine (NAC) derivatisation method has been developed and compared to existing methods for analysis of PER GSH conjugates. Subsequently, the rate of this conjugation has been studied for the first time in human subcellular fractions. The specific activity of PER GSH conjugation in humans shows significant interindividual differences and is 10-fold lower compared to rats, indicating that humans are less susceptible to nephrotoxicity via this pathway.

Keywords
Perchloroethylene, o-phtaldialdehyde (OPA), n-acetylcysteine (NAC), S-(1,2,2-trichlorovinyl)-l-glutathione (TCVG)

INTRODUCTION
Tetrachloroethylene or perchloroethylene (PER) is a volatile and colourless solvent and is widely used as a metal degreasing agent or cleaning solvent in the textile industry[11,13].Occupational exposure has decreased due to technological advances in dry cleaning and degreasing methods in the USA and Europe[5]. However, exposure is still common in developing countries and the widespread use has resulted in environmental contamination of water, soil and food[5,13]. The relatively similar compound trichloroethylene (TRI) has been identified as a Substance of Very High Concern and requires authorisation before it is used since 2016[3]. This regulation has not been enforced for PER, which is a potential alternative to TRI and still widely used by professional workers. Moreover, PER has been found to have an even higher correlation with renal injury compared to TRI[9].

PER nephrotoxicity is the result of metabolism via the glutathione (GSH) conjugative pathway. In this pathway, PER is conjugated to GSH by Glutathione S-Transferases (GSTs) in the liver to form S-(1,2,2-trichlorovinyl)-l-glutathione (TCVG) (Figure 1)[2,9]. TCVG is transported to the kidney, where it is rapidly hydrolysed by γ-glutamyltransferase (GGT) and cysteinylglycine dipeptidase to form S-(1,2,2-trichlorovinyl)-l-cysteine (TCVC)[2,8].

In the kidney, TCVC can be detoxified to urinary excreted products, this reaction can also proceed vice versa. However, TCVC can also be converted in the kidneys by β lyase to dichlorothioketene (DCTK) via a thiol. It is this product which subsequently leads to nephrotoxicity as it can bind to proteins or DNA[2,8,12].

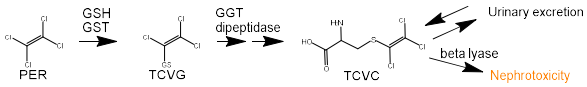

Figure 1 Metabolism of PER via the GSH conjugative pathway. S-(1,2-trichlorovinyl)-l-cysteine (TCVC) can be bioactivated by β lyase, which results in nephrotoxicity.

GSH conjugation kinetics
The rate of metabolism via this pathway, and thus the risk of nephrotoxicity, depends on the activity of GST. Previously, specific activities of GST have been determined in subcellular fractions of F344 rats[9]. The specific activity in male rat liver cytosol after 60 minute incubations with 2 mM PER and 5 mM GSH was 10.6 ± 2.2 nmol/mg protein/60 minutes. However, this rate has not yet been determined for humans. Moreover, it is unknown which GST isoforms play a role in PER metabolism. At least seven GST classes exist, the human variants of these enzymes are described with A, M, P, S, T, O, and Z[7]. The genetically determined isoforms GSTT1, GSTP1 and GSTM1 are most extensively studied and hypothesised to contribute to environment-dependent diseases[4,7]. It is still unknown which isoforms of GST are involved in PER metabolism[1].

Analytical methods for detection of PER GSH conjugates
In order to determine the rate of PER metabolism via this pathway and thus improve public health assessment, a [illegible]. It has already been demonstrated for TRI that the large variation in experimental results is partly due to the difference in analytical methods[14].

In this study, a high performance liquid chromatography (HPLC) method with postcolumn o-phtaldialdehyde/n-acetylcysteine (OPA/NAC) derivatisation technique will be optimised. OPA/NAC is an already established method for quantification of amino acids. In this derivatisation technique, the aldehydes in OPA react with NAC and with the N-terminal amino group of the glutathione conjugates (Figure 2). This product can be detected with fluorometry. Postcolumn OPA/NAC derivatisation has been applied previously and reached limits of detection of respectively 3.88 µM and 9.2 µM [6,10] .

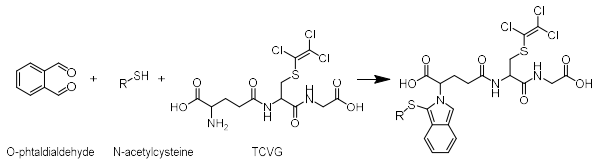

Figure 2 Reaction of O-phtaldialdehyde and N-acetylcysteine (OPA/NAC) with TCVG to the fluorescent derivatisation product which can be detected with fluorometry.

Determination of the rate of PER metabolism via the GSH conjugative pathway in humans requires a sensitive analytical method and is essential for nephrotoxicity risk assessment. Therefore, the aim of this study is twofold. Firstly, sensitive analytical methods for detection of PER GSH conjugated metabolites will be optimised and compared. Secondly, the rate of GSH conjugation and further metabolism of PER will be analysed for the first time in human subcellular fractions and compared to metabolism in rats to determine interindividual and interspecies variability.

METHOD

Development of sensitive analytical method for TCVG analysis

LC-MS analysis

A dilution series of TCVG in 50 mM potassium phosphate buffer pH 7.4 was prepared and analysed with TOF LC-MS 6230 Agilent Technologies, equipped with Luna 5 µm C18 column (150x4 60 mm) in volumes of 300 µL with 30 µL 10% perchloric acid. Eluents A (99% water, 0.2% formic acid, 0.8% acetonitrile) and B (99% acetonitrile, 0.2% formic acid, 0.8% water) were programmed with the following gradient: 0 min: 3% B, 5 min: 3% B, 25 min: 53% B, 28 min: 99%, 30 min: 99%, 31 min: 3% B, 35 min: 3% B. The flow rate was set to 0.5 mL/min and samples were injected at a volume of 50 µL.

HPLC analysis with UV/VIS

A dilution series of TCVG was prepared in 50 mM potassium phosphate buffer pH 7.4. To each 300 µL of sample, 30 µL of 10% perchloric acid was added prior to HPLC analysis. The HPLC system was equipped with a Luna 5µm C18 column (150x4 60 mm), Gilson 234 autoinjector, two Gilson 305 pumps, Shimadzu UV/VIS detector SPD-20A (response 4, lamp 1, aux range 2, range 1). Eluents A (99% water, 0.2% formic acid, 0.8% acetonitrile) and B (99% acetonitrile, 0.2% formic acid, 0.8% water) were run according to the following gradient: 0 min: 3% B, 5 min: 3% B, 25 min: 53% B, 28 min: 99%, 30 min: 99%, 31 min: 3% B, 35 min: 3% B. TCVG was detected with UV/VIS analysis at 265 nm.

HPLC with postcolumn OPA/NAC derivatisation

A dilution series of TCVG was prepared in 50 mM potassium phosphate buffer pH 7.4. To 300 µL of each dilution was added 30 µL 10% perchloric acid in HPLC vials prior to analysis. OPA/NAC reagent was prepared with 100 mg/L OPA (Sigma Aldrich) (0.75 M) and 122 mg/L NAC (Sigma Aldrich) (0.75 M) in 0.1 M Borate buffer pH 10.8. The dilution series was analysed with a HPLC system equipped with Gilson 234 autoinjector, two Gilson 305 pumps, Luna 5 µm C18 column (150x4 60 mm), Solvent delivery system 400 from Applied Biosystems, Shimadzu UV/VIS detector SPD-20A and Shimadzu fluorescence detector RF-10Axl. Eluents A (99.05% water, 0.1% formic acid, 0.85% acetonitrile) and B (99.05% acetonitrile, 0.1% formic acid, 0.85% water) were run according to the following gradient: 0 min: 3% B, 5 min: 3% B, 25 min: 53% B, 28 min: 99%, 30 min: 99%, 31 min: 3% B, 35 min: 3% B. OPA/NAC reagent was added postcolumn at a flow rate of 0.6 mL/min. The reaction was subsequently allowed to take place in a reaction coil (1 mL), heated to 50 °C. The resulting flow was analysed in the fluorophotometer at excitation wavelength 345 nm and emission at 455 nm and with UV/VIS at wavelength 345 nm.

Incubation of human and rat liver factions with PER

Work solution was prepared with 5 mM GSH (Sigma Aldrich) and 2,5 mM PER (Baker Chemicals B.V.) (stock solution in acetonitrile (VWR chemicals), final concentration 1%) in 50 mM potassium phosphate buffer pH 7.4. Stop reagent consisted of 1 mM phenylalanine (Janssen Chimica) in 10% perchloric acid (sample S1329-S1449) or 1 mM 2,2-DCVG in 10% perchloric acid (sample S1336-R1341). All solutions were placed on ice. For each incubation, 250 µL enzyme fraction was added to 750 µL work solution. Samples were incubated at 30 °C for 2 hours. Following incubation, all samples were placed back on ice and 110 µL stop reagent with internal standard was added. Subsequently, samples were vortexed and centrifuged for 15 minutes at 14.000 rpm. Of each supernatant, 300 µL was transferred to vials for LC-MS analysis.

Determination of protein concentration in liver cytosol fractions

A dilution series of Melford Albumine Bovine Fraction (BSA) with concentrations 1, 0.8, 0.6, 0.4, 0.2 and 0 mg/mL was prepared as protein concentration reference. All subcellular fractions were diluted 100x in 50 mM potassium phosphate buffer pH 7.4. For each measurement, 20 µL diluted enzyme fraction or BSA solution was added to 1 mL Bio-Rad Protein Assay Dye Reagent Concentrate, diluted 10x in water. After 3 minutes an extinction spectrum from 500 to 900 nm was measured with UV/VIS Spectrophotometer Ultrospec 2100 pro (Amersham Biosciences).

Incubations of human and rat liver- and kidney fractions with TCVG

Stock solution was prepared with 100 µM TCVG and 1 mM diglycine (cofactor GGT) in 50 mM potassium phosphate buffer pH 7.4. Stop solution was also prepared from 1 mM phenylalanine (internal standard) and 10% perchloric acid in 50 mM potassium phosphate buffer pH 7.4. All solutions were stored on ice. For each incubation, 50 µL enzyme fractions were added to 500 µL stock solution. Samples were incubated for 30 minutes at 37 °C. Subsequently, samples were placed back on ice and 60 µL stop solution was added to each sample. Samples were then vortexed and centrifuged for 15 minutes at 14.000 rpm. Of each supernatant, 300 µL was transferred to vials and analysed with the described LC-MS method.

RESULTS

Comparison of analytical methods and their limits of detection

With LC-MS, TCVG peaks were observed after 23.5 minutes at masses of 436 : 438 : 440 with relative frequency 3 : 3 : 1. The resulting integrated peaks with mass 436 could be measured until concentrations of 90 nM and provide the following calibration curve (Figure 4):

$$AUC=149587[TCVG \text{ in } \mu M]-2900.0 \ (R^2=0.9989)$$

Analysis with the optimised HPLC and UV/VIS detection method shows peaks for TCVG after 21.5 minutes, with detection limit 0.5 µM and the following concentration dependency (Figure 4):

$$AUC=1407[TCVG \text{ in } \mu M]+1208 \ (R^2=0.997)$$

The developed and optimised postcolumn OPA/NAC derivatisation method showed a relatively unstable baseline signal and small, broad peaks at similar TCVG concentrations. The chromatogram obtained for 50 µM TCVG and 50 µM DCVG is shown in Figure 3.

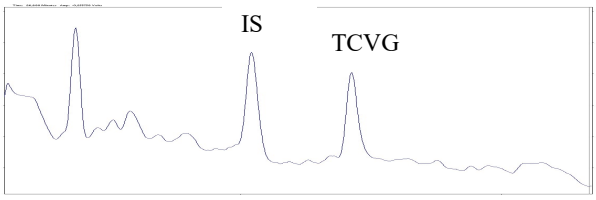

Figure 3 Fluorometric chromatogram section from 10.0 to 25.0 minutes for 100 µM TCVG and 100 µM DCVG as internal standard (IS)

TCVG peaks were observed after 22.3 minutes and could be observed until 4 µM with the following concentration dependency (Figure 4):

$$AUC=698.19[TCVG \text{ in } \mu M]-277.76 \ (R^2=0.9946)$$

Thus, the new postcolumn OPA/NAC derivatisation method developed in this study did not improve the detection limit compared to HPLC analysis without derivatisation. The LC-MS method with a limit of detection of 90 nM was most sensitive and used to analyse incubation samples.

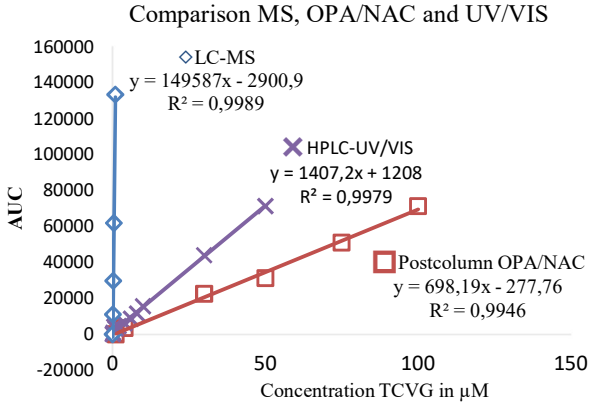

Figure 4 Comparison of LC-MS, HPLC-UV/VIS and postcolumn OPA/NAC methods

Specific activity of TCVG formation in human liver cytosol

After 2 hour incubations with 2.5 mM PER and 5 mM GSH at 30 °C and LC-MS analysis, TCVG peaks were detected. Peaks with mass 436 were integrated and the TCVG concentration was determined with the aid of the calibration line obtained in Figure 4. The protein concentration was determined with the described BSA assay. First, a calibration curve with the relation between absorption at 595 nm and protein concentration in mg/mL was measured for BSA. This standard curve has the following formula:

$$Abs=0.7761 \ [BSA \text{ in mg/ml}]+0.545 \ (R\text{^}2=0.9112)$$

With the aid of this calibration curve, protein concentrations in human liver cytosol fractions were determined. The concentration TCVG in nM formed in 120 minutes, and the protein concentration in mg/ml were used to calculate the specific activity. The specific activity of PER metabolism to TCVG in 20 human liver cytosol fractions is displayed in Figure 5.

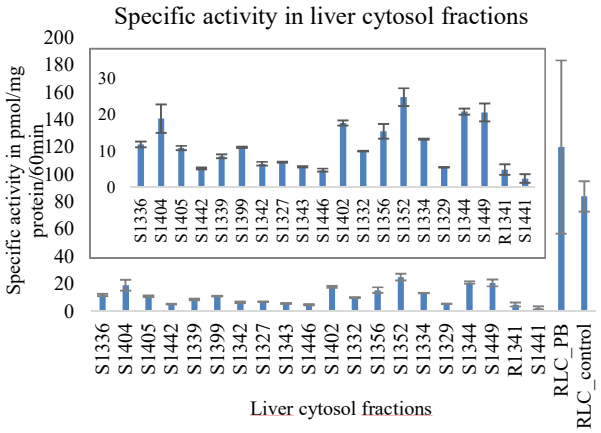

Figure 5 Specific activity of PER metabolism to TCVG in pmol/mg protein/60 minutes in 20 human liver cytosol fractions and two rat liver cytosol fractions (PB-induced (RLC_PB) and control (RLC_control)), obtained by dividing the TCVG concentrations after 120 minute incubations by the protein concentrations. In rat samples, the specific activity was 10-fold higher compared to humans. Moreover, significant interindividual differences were observed between humans.

Secondary metabolism of TCVG

TCVG was incubated with liver and kidney fractions of humans and rats and subsequently analysed with LC-MS. TCVG was detected with mass 436. TCVGC was detected with mass 307, and TCVC with mass 250. Decrease in TCVG and increase in TCVGC and TCVC were expressed as a percentage of the AUC of TCVG in the blank sample (Figure 6). These findings confirm that further metabolism of TCVG takes place primarily in the kidney. Moreover, TCVG was not metabolised further in human liver cytosol or rat liver, indicating that these fractions could be used for incubations with PER to study the specific activity of GST enzymes in the liver responsible for PER metabolism.

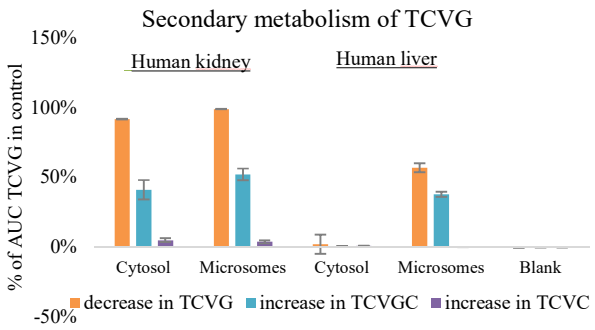

Figure 6 Metabolism of TCVG to TCVGC and TCVC in human liver and kidney samples after incubations of human kidney and liver cytosol- and microsome fractions with 100 µM TCVG during 30 minutes at 37°C. Shown is the decrease in TCVG (orange), increase in TCVGC (blue) and TCVC (purple), expressed as a percentage of AUC of TCVG in the blank sample. TCVG is not further metabolised in the liver cytosol fractions, but is primarily converted in the kidney.

CONCLUSION

A new postcolumn OPA/NAC derivatisation method has been developed for separation of GSH conjugates of PER with a limit of detection of 4 µM TCVG. This is in line with previous postcolumn OPA/NAC methods, which reached limits of detection of 4 µM and 9.2 µM[6,10]. However, the previously developed LC-MS method is still the most sensitive method for TCVG analysis with a detection limit of 90 nM. With the aid of this method, a specific activity of PER GSH conjugation in rat liver cytosol of 83.9 ± 11.1 pmol/mg protein/60 minutes was determined. This is 120-fold lower than the value obtained previously by Lash et al.[9] and could be accounted for by the difference in analytical method applied. In a comparative study, it was found that the HPLC-UV method applied by Lash et al. is not selective for the DCVG analyte[14]. This non-specificity may lead to recoveries of up to 200-fold higher than the actual metabolite levels and could explain the previous overestimation of TCVG formation in rats. Moreover, the specific activity of TCVG formation has been studied for the first time in liver cytosol fractions of 20 different humans. Significant interindividual differences in TCVG formation were observed, indicating that specific GST isoforms may play a role in GSH conjugation of PER. Moreover, GSH conjugation of PER was 10-fold lower in humans compared to rats, which suggests that humans are less susceptible to nephrotoxicity via this pathway. It was also confirmed that further metabolism of TCVG to TCVC takes place primarily in the kidney, and was not observed in human liver cytosol. Determination of the specific GST isoforms which catalyse TCVG formation and their kinetic parameters in future research could improve our understanding of the importance and interindividual variability of this pathway in humans, and thus the risk of nephrotoxicity.

ROLE OF THE STUDENT

Robin Coosen was a Bachelor's student Pharmaceutical Sciences at VU university working under the supervision of dr. J. Commandeur when the research in this report was performed. The topic and study design were proposed by the supervisor. The conduction of the experiments, processing of the results, formulation of the conclusion and writing were performed by the student and discussed with the supervisor.

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