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# Generic method for the absolute quantification of glutathione S-conjugates: Application to the conjugates of acetaminophen, clozapine and diclofenac

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## ABSTRACT

Modification of cellular macromolecules by reactive drug metabolites is considered to play an important role in the initiation of tissue injury by many drugs. Detection and identification of reactive intermediates is often performed by analyzing the conjugates formed after trapping by glutathione (GSH). Although sensitivity of modern mass spectrometrical methods is extremely high, absolute quantification of GSH-conjugates is critically dependent on the availability of authentic references. Although <sup>1</sup>H NMR is currently the method of choice for quantification of metabolites formed biosynthetically, its intrinsically low sensitivity can be a limiting factor in quantification of GSH-conjugates which generally are formed at low levels. In the present study, a simple but sensitive and generic method for absolute quantification of GSH-conjugates is presented. The method is based on quantitative alkaline hydrolysis of GSH-conjugates and subsequent quantification of glutamic acid and glycine by HPLC after precolumn derivatization with o-phthalaldehyde/N-acetylcysteine (OPA/NAC). Because of the lower stability of the glycine OPA/NAC-derivate, quantification of the glutamic acid OPA/NAC-derivate appeared most suitable for quantification of GSH-conjugates. The novel method was used to quantify the concentrations of GSH-conjugates of diclofenac, clozapine and acetaminophen and quantification was consistent with <sup>1</sup>H NMR, but with a more than 100-fold lower detection limit for absolute quantification.

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## 1. Introduction

Although extensive preclinical animal studies are performed to test the safety of novel drug candidates, the occurrence of severe adverse drug reactions during the clinical development stage and postmarketing remains a problem for pharmaceutical industry. For many drugs causing drug-induced liver injury (DILI) in humans,

the formation of highly reactive metabolites that can react with cellular macromolecules, such as proteins and DNA, is believed to play a role in the onset of liver injury [1–4]. The liver is a frequent target organ because of its relatively high exposure to orally administered drugs and its high metabolic capacity. Except for acetaminophen (N-acetyl-p-aminophenol; APAP), which shows predictable and dose-dependent DILI, most clinically used drugs causing DILI affect only a very small subgroup of the patients, explaining why these reactions usually escape detection during clinical development. The exact mechanisms underlying these so-called idiosyncratic reactions still remain to be established. Due to the safety concerns related to bioactivation, minimizing the formation of reactive metabolites has become an integral part of the drug optimization strategies [1,2,4]. Therefore, to analyze and quantify reactive metabolites many analytical strategies have been developed.

One of the most widely used methods to detect reactive metabolites is incubation of drugs with human liver microsomes (HLM) in presence of glutathione (GSH;  $\gamma$ -glutamylcysteinylglycine) as a trapping agent. GSH is a physiologically relevant nucleophile,

**Abbreviations:** APAP, acetaminophen; CLZ, clozapine; DCF, diclofenac; GSH, glutathione; GST, glutathione S-transferases; HLM, human liver microsomes; KPi, potassium phosphate; NMR, nuclear magnetic resonance; NAC, N-acetylcysteine; OPA, o-phthalaldehyde; TMSP, trimethylsilyl propionic acid-d<sub>4</sub>; APAP-SG, acetaminophen-glutathione; S-propyl-GSH, S-propyl-glutathione; S-hexyl-GSH, S-hexyl-glutathione; S-nonyl-GSH, S-nonyl-glutathione; CG-1, C-6 glutathionyl-clozapine; CG-3, C-9 glutathionyl-clozapine; CG-4, C-7 glutathionyl-clozapine; CG-6, C-8 glutathionyl deschloroclozapine; CG-5, C-2/3 glutathionyl-clozapine; M1, 4-glutathion-S-yl-5-hydroxy-diclofenac; M2, 3'-glutathion-S-yl-4-hydroxy-diclofenac; M3, 6-glutathion-S-yl-5-hydroxy-diclofenac; M5, 2'-glutathion-S-yl-4'-hydroxy-deschlorodiclofenac.

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present at millimolar concentrations in cells, which allows detection of reactive metabolites in both cellular models and *in vivo* by measuring excretion of GSH-conjugates in bile [5,6]. Many different LC-MS methods have been developed for the detection and characterization of GSH-conjugates [7]. With the advance in sensitivity and resolution of modern mass spectrometers extremely low levels of GSH-conjugates can be detected in biological samples, aided by the characteristic fragmentation pattern that GSH-conjugates have in common. However, absolute quantification and structural characterization of detected GSH-conjugates by LC-MS methodologies is often hampered by the inavailability of reference compounds. Although absolute quantification of GSH-conjugates can be achieved by using radiolabeled GSH ( $[^3\text{H}]\text{-GSH}$ ) or ( $[^{35}\text{S}]\text{-GSH}$ ) in microsomal incubations and radiometric detection of GSH-conjugates, extensive sample clean-up and relatively low sensitivity are limitations of this methodology [7,8]. Several semi-quantitative methods using non-radiolabeled GSH-analogues, such as dansylated GSH [9] and quaternary-ammonium-derivatised GSH [10] have been developed. However, methods using radiolabeled GSH or GSH-analogues cannot be applied to demonstrate and quantify reactive intermediates in cellular models and *in vivo*. In addition, these methods cannot be used to quantify enzymatic GSH conjugation by glutathione S-transferases (GSTs) since the GSH-analogues cannot substitute GSH in enzymatic GSH conjugation. Because in recent years formation of several GSH-conjugates of drugs, such as clozapine (CLZ), diclofenac (DCF), duloxetine and nevirapine even appeared to be fully dependent on GSTs [11–15], quantitative methods for the detection of electrophiles to unmodified GSH are required.

In absence of authentic synthetical standards, several alternative approaches for the absolute quantification of drug metabolites have been proposed such as  $^1\text{H}$  NMR and UV-detection [16–18].  $^1\text{H}$  NMR is considered the method of choice, since it allows both structure determination, check of purity and quantitative measurement by using calibration curves of structurally related chemicals, such as the parent drug itself [16–18]. A drawback of  $^1\text{H}$  NMR, however, is its intrinsic low sensitivity compared to other detection methods. Because GSH-conjugates of drugs formed in incubations with HLM usually represent only minor metabolites, quantification of GSH-conjugates by  $^1\text{H}$  NMR could only be achieved recently by generating sufficient amounts using highly active drug metabolizing mutants of CYP102A1 [19–21]. To enable quantification of GSH-conjugates in incubations with HLM more sensitive analytical methods are required. As an alternative approach, GSH-conjugates have also been quantified by HPLC with UV-detection (HPLC-UV), using calibration curves of the parent drug and by assuming that the extinction coefficients of GSH-conjugates and parent drug is unchanged [11,12,22]. Although being more than 100-fold more sensitive than quantitative  $^1\text{H}$  NMR, quantification by UV-detection is only valid in case the chromophore responsible for UV-absorbance is unaltered by the metabolism [17,18]. However, even if the absorption spectrum of a metabolite is similar to that of the parent drug, the molar absorptivity can be changed by up to 30% [18].

The aim of the present study was to develop a sensitive and generic methodology for the absolute quantification of GSH-conjugates, which can be performed with low-cost lab facilities. Because all GSH-conjugates have the glutathionyl-moiety in common, of which only the cysteinyl-group is chemically modified by the intercepted electrophile, quantitative hydrolysis of the peptide bonds of GSH-conjugates will yield equimolar amounts of glutamic acid and glycine, next to the modified cysteine-residue. Because many sensitive methods are available for the accurate quantification of amino acids, as reviewed in [23], this approach would enable quantification of low concentrations of GSH-conjugates provided that hydrolysis to glutamic acid and glycine is

quantitative and that these amino acids are not further degraded under the conditions used. Hydrolysis of GSH-conjugates can be done non-enzymatically by acidic or alkaline hydrolysis [23] or using  $\gamma$ -glutamyltranspeptidase and dipeptidases [24,25]. In the present study alkaline hydrolysis was applied followed by subsequently derivatization by o-phthaldialdehyde/N-acetylcytisteine (OPA/NAC), a well-established and highly sensitive method for fluorimetric quantification of amino acids [23,26]. Calibration curves were constructed using stock solutions of authentic glutamic acid and glycine. The methodology of alkaline hydrolysis and OPA/NAC-derivatization was first validated using gravimetrically prepared solutions of commercially available S-propyl-glutathione (S-propyl-GSH), S-hexyl-glutathione (S-hexyl-GSH) and S-nonyl-glutathione (S-nonyl GSH). Subsequently, the method was applied to the regioisomeric GSH conjugates of DCF, CLZ and the GSH-conjugate of APAP (APAP-SG), for structures see Fig. 1. The novel methodology was compared with quantification by  $^1\text{H}$  NMR and with the HPLC-UV-methods described previously [11,12,22,27].

## 2. Materials and methods

### 2.1. Chemicals

L-Glutamic acid, glycine, L-phenylalanine, o-phthaldialdehyde (OPA), N-acetyl-L-cysteine (NAC), GSH, S-propyl-GSH, S-hexyl-GSH, S-nonyl-GSH, DCF and APAP were from Sigma-Aldrich (Zwijndrecht, The Netherlands). CLZ was obtained from Duchefa (Haarlem, The Netherlands). Acetaminophen-glutathione conjugate (APAP-SG) was from Toronto Research Chemicals (North York, Canada). Trimethylsilyl propionic acid-d<sub>4</sub> sodium salt (TMSP) was obtained from Merck (Amsterdam, The Netherlands). All other chemicals and reagents were of analytical grade and obtained from standard suppliers.

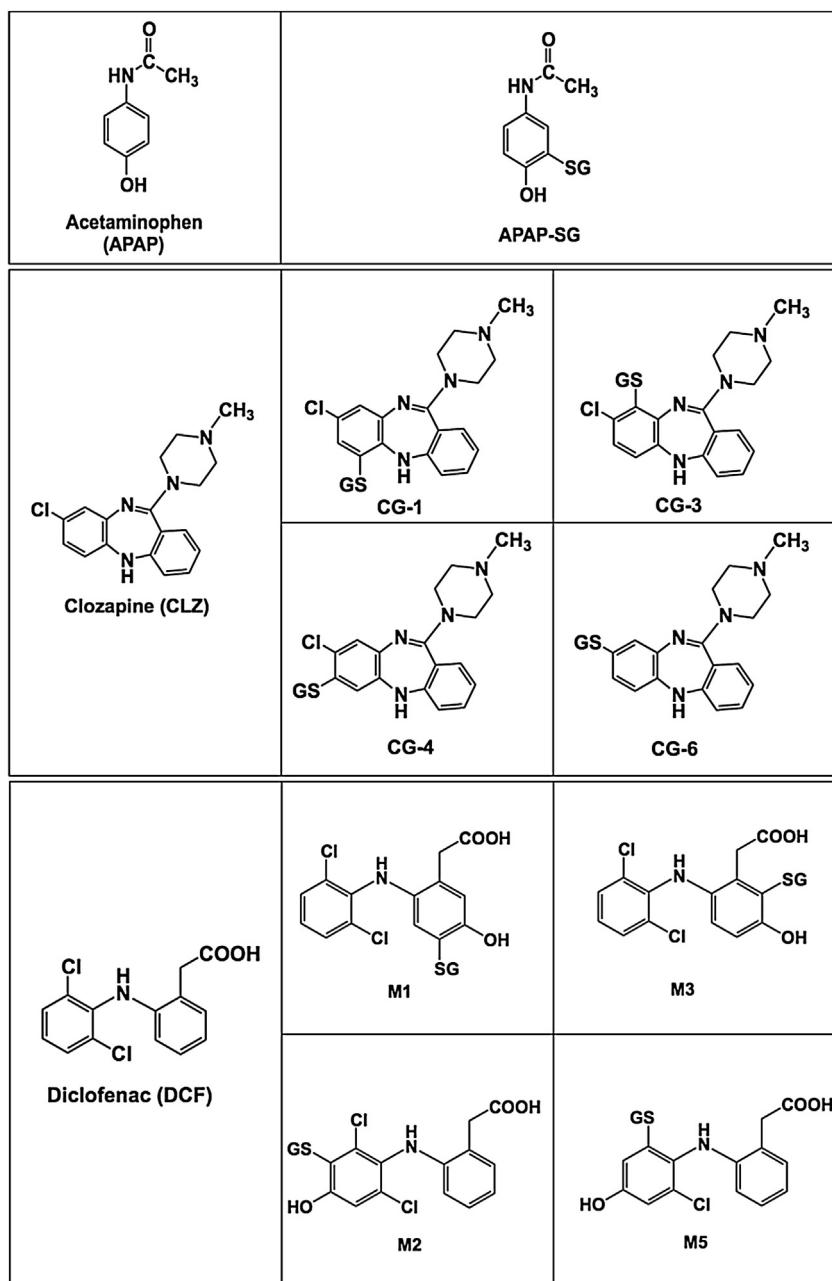
Regiosomeric GSH-conjugates of CLZ (**CG1**, **CG3**, **CG4** and **CG6**) and DCF (**M1**, **M2**, **M3** and **M5**), were prepared biosynthetically using CYP102A1 mutants and recombinant glutathione S-transferase P1-1 and isolated by preparative HPLC as described previously [20,22] with minor modifications, as detailed in the Supplemental Materials.

### 2.2. Alkaline hydrolysis of GSH-conjugates and OPA/NAC-derivatization

Peptide bonds of proteins and peptides can be hydrolysed completely by heating in strongly acidic or alkaline conditions for 20–24 h [23]. In the present study alkaline hydrolysis at 4 M sodium hydroxide was selected because pilot experiments showed that quantitative hydrolysis of GSH-conjugates could be achieved within 30 min, whereas acidic hydrolysis in 6 M hydrochloric acid took several hours to reach completion [data not shown]. Furthermore, acid hydrolysis of GSH-conjugates leads to formation of corresponding cysteine S-conjugates which also will appear as a peak after OPA/NAC-derivatization. The fact that these peaks may interfere with those of glutamic acid and glycine would make the method less generic. In case of alkaline hydrolysis cysteine conjugates are degraded by beta-elimination [29,30], so that they can not interfere with glutamic acid and glycine analysis.

Parent GSH-conjugates and amino acids were analyzed by HPLC after OPA/NAC-derivatization. OPA/NAC-derivatization was performed after complete or partial neutralization of the alkaline samples. The OPA/NAC-reagent used was prepared daily by mixing 1 mL stock solution of 100 mg OPA and 50 mg NAC in 5 mL methanol with 9 mL 150 mM sodium borate buffer pH 9.5.

To study the stability of the amino acids under the conditions of alkaline hydrolysis, standard curves were prepared of mixtures of



**Fig. 1.** Structures of acetaminophen (APAP), clozapine (CLZ), diclofenac (DCF) and corresponding GSH-conjugates used in this study.

glycine and glutamic acid in 4 M sodium hydroxide, ranging from 12.5 to 100  $\mu$ M, and kept on ice or heated in a boiling water bath. To all mixtures 100  $\mu$ M phenylalanine was added as internal standard because this amino acid appeared resistant to the used alkaline conditions and because the retention time of the OPA/NAC-labeled phenylalanine did not interfere with the peaks of derivatized glutamic acid, glycine and GSH-conjugates.

One standard curve was prepared by mixing 100  $\mu$ L of the amino acid mixtures in water with ice-cold 100  $\mu$ L 8 M sodium hydroxide, and kept on ice for 30 min and subsequently partially neutralized by addition of 200  $\mu$ L ice-cold 2 M hydrochloric acid. Next, 100  $\mu$ L of each sample was mixed with 200  $\mu$ L of OPA/NAC-reagent, derivatized for 15 min at room temperature and diluted 10-fold using HPLC-eluent A. The samples were transferred to HPLC-vials and analyzed by the HPLC-method described in Section 2.3.

The second standard curve was prepared by mixing 100  $\mu$ L of the amino acid mixtures in water with 100  $\mu$ L ice-cold 8 M sodium hydroxide, and subsequently heated for 30 min in a boiling water bath. The samples were allowed to cool and were partially neutralized by addition of 200  $\mu$ L 2 M hydrochloric acid. 100  $\mu$ L of each sample was mixed with 200  $\mu$ L of OPA/NAC-reagent, derivatized for 15 min at room temperature and diluted 10-fold using HPLC-eluent A, and analyzed by the HPLC-method described in Section 2.3.

To study the stability of the OPA/NAC-derivatized amino acids, to a 100  $\mu$ L mixture of glutamic acid, glycine and phenylalanine (each 100  $\mu$ M) subsequently was added 100  $\mu$ L 8 M sodium hydroxide, 200  $\mu$ L 2 M hydrochloric acid and 200  $\mu$ L OPA/NAC-reagent. After 15 min at room temperature, 3.6 mL of HPLC eluent A was added. This sample was subsequently analyzed over a period of 16 h by injection of 50  $\mu$ L sample every 50 min.

To study the time-course of alkaline degradation of GSH-conjugates, 1 mL of 200  $\mu$ M S-hexyl-GSH in water was mixed with 1 mL of 8 M sodium hydroxide on ice after which 100  $\mu$ L 1 mM phenylalanine was added as internal standard. After placing the mixture in a boiling water bath, at several timepoints a sample of 100  $\mu$ L was taken and added to cups containing 100  $\mu$ L 2 M hydrogen chloride on ice. After sampling the last timepoint (120 min), 100  $\mu$ L of each sample was mixed with 200  $\mu$ L of OPA/NAC-reagent and derivatized for 15 min at room temperature. The samples were subsequently diluted 10 times with HPLC eluent A and analyzed by the HPLC-method described in Section 2.3.

To confirm the quantitative hydrolysis of GSH-conjugates after 30 min of the alkaline hydrolysis, 5 mM stock solutions of S-propyl-GSH, S-hexyl-GSH and S-nonyl-GSH were prepared gravimetrically and subsequently diluted to 25, 50 and 75  $\mu$ M concentrations. Samples of 100  $\mu$ L were mixed with 100  $\mu$ L 8 M sodium hydroxide, after which 10  $\mu$ L 1 mM phenylalanine was added as internal standard. The samples were subsequently heated for 30 min in a boiling water bath. After cooling the samples to room temperature, 200  $\mu$ L 2 M hydrogen chloride was added. A 100  $\mu$ L sample was taken and added to 200  $\mu$ L OPA/NAC-reagent. After 15 min at room temperature, the samples were diluted 10 times with HPLC eluent A and transferred to HPLC-vials. The amount of glycine and glutamic acid formed by hydrolysis was quantified using calibration curves of these amino acids ranging from 10 to 100  $\mu$ M.

Finally, the method described above was applied to solutions of GSH-conjugates of CLZ and DCF which were quantified by  $^1$ H NMR, and gravimetrically prepared APAP-SG, see Section 2.4.

### 2.3. HPLC-analysis of OPA/NAC-derivatized amino acids and GSH-conjugates

The OPA/NAC-derivatized amino acids and GSH-conjugates were analyzed by reversed phase liquid chromatography using a Shimadzu HPLC-system equipped with a SIL20AC autosampler (cooled at 4 °C), two LC-20AD pumps and RF-10AxL fluorescence detector. A Chrompack ChromSpher 5 C18 column (4.6 × 150 mm) protected by a Phenomenex SecurityGuard Cartridge system (C18; 4.0 × 3.0 mm) was used as the stationary phase for the separation of the derivatized amino acids and GSH-conjugates. A binary gradient composed of solvent A (5% methanol in 50 mM potassium phosphate (KPi) buffer pH 6.5) and solvent B (60% methanol in 50 mM KPi buffer pH 6.5) was used. The gradient was programmed as follows: 0–15 min, linear increase from 17 to 25% solvent B; 15–25 min, linear increase from 25 to 85% solvent B; 25–30 min, isocratic at 85% solvent B; 30–31 min, linear decrease from 85% to 17% solvent B; 32–50 min, re-equilibration at 17% solvent B. The flow rate was set at 0.6 mL/min and fluorescence detection was performed with excitation at 344 nm and emission at 433 nm. Samples were injected at a volume of 50  $\mu$ L.

### 2.4. Quantitative analysis of drugs and GSH-conjugates by $^1$ H NMR

$^1$ H NMR spectra of CLZ, DF, APAP and their corresponding GSH-conjugates were recorded on a Bruker Avans 250 MHz spectrometer operating at the basic frequency of 250,18 MHz. The spectrometer was controlled using TopSpin (Bruker BioSpin). Each sample contained 0.7 mL of deuterium oxide as solvent containing 0.45 mM TMSP as internal standard, and was frequency locked using the deuterium signal of deuterium oxide. Shimming was performed on each sample prior to data acquisition. Data were collected using the ICON-NMR software (Bruker BioSpin). 128 scans were collected, with an acquisition time of 4.5 s per scan. The data were Fourier transformed with a 1 Hz line broadening function and the chemical shifts were referenced to the inter-

nal standard TMSP. After phase correction, the signals of the aromatic hydrogen atoms of the drugs and corresponding GSH-conjugates were integrated manually using TopSpin and referenced to the integral of the methyl-groups of the internal standard TMSP (0.45 mM).

The accuracy of the  $^1$ H NMR quantification was first validated by recording spectra of gravimetrically prepared solutions of APAP, DCF and CLZ in deuterium oxide at concentrations ranging from 250  $\mu$ M to 10 mM. All samples contained 0.45 mM TMSP as internal standard for quantification. The concentrations of the drugs were quantified by  $^1$ H NMR by integrating the signals of their aromatic protons and that of the methyl-groups of the internal standard TMSP.

The concentrations of GSH-conjugates of APAP, DCF and CLZ were subsequently quantified in the same manner by integration the signals of their aromatic protons and the methyl-groups of TMSP.

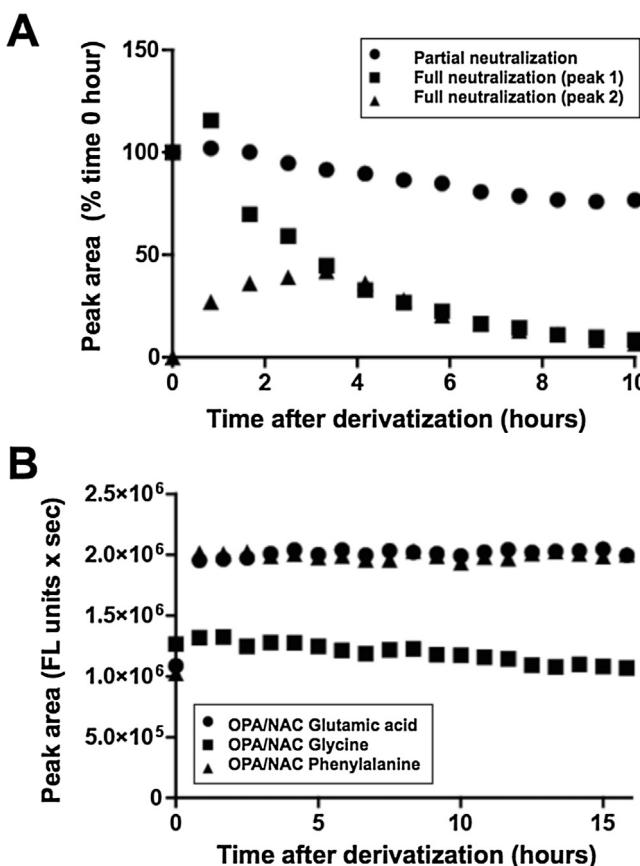
### 2.5. Quantification of GSH-conjugates by HPLC-UV detection

In earlier studies, concentrations of DCF, CLZ and APAP conjugates were determined by HPLC with UV-detection, using standard curves of parent drugs assuming that extinction coefficients of GSH-conjugates are identical to that of the parent drugs [11,12,22,27]. To compare the quantification of GSH-conjugates by UV-detection with the quantification by the alkaline hydrolysis method, the solutions of the GSH-conjugates of CLZ, DF and APAP were also quantified by HPLC-UV as described previously [11,12,22,27]. UV absorption spectra of parent drugs and GSH-conjugates were recorded on-line using an UV-vis diode-array detector (Agilent 1200 Series Rapid resolution LC system).

## 3. Results and discussion

### 3.1. Derivatization of glutamic acid, glycine and phenylalanine by OPA/NAC-reagent

First the OPA/NAC-derivatization procedure was validated using solutions of glycine, glutamic acid and phenylalanine dissolved in 4 M sodium hydroxide. Phenylalanine was selected as internal standard to correct for evaporation effects during hydrolysis and was added prior to heating of the samples. Initially, the alkaline samples were neutralized by addition of an equal volume of 4 M hydrochloric acid prior to the derivatization with OPA/NAC. However under these conditions derivatization of glycine resulted in two peaks on HPLC in poorly reproducible ratio, consistent with previous studies [28]. In addition, repeated injection of these samples showed a low stability of OPA/NAC-derivatized glycine because its peaks decreased by more than 90% after 10 h, Fig. 2A. When 50% neutralization was applied, by addition of an equal volume of 2 M hydrochloric acid prior to OPA/NAC-derivatization, one major peak of derivatized glycine was eluting at 12.5 min after OPA/NAC-derivatization. A minor peak was eluting at 22.5 min, but the peak area was less than 5% of the major peak (see Fig. 4 for a representative chromatogram). Repeated injection of these samples also showed a much slower decrease in peak area of derivatized glycine, Fig. 2A. Both glutamic acid and the internal standard phenylalanine showed a single peak after complete or 50% neutralization prior to OPA/NAC-derivatization. When a mixture of 50  $\mu$ M glycine, glutamic acid and phenylalanine was derivatized after 50% neutralization, and injected repeatedly over a period of 16 h, no significant decrease of the peaks of glutamic acid and phenylalanine was observed (Fig. 2B). Derivatized glycine gave a 35% lower peak area, and decreased by about 20% over 16 h.

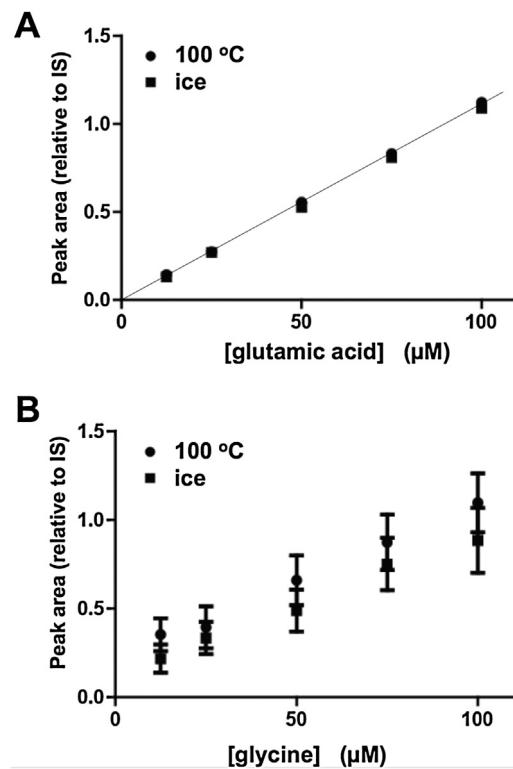


**Fig. 2.** Stability of OPA/NAC derivatized amino acids. **A.** Effect of full or partial neutralization on stability of OPA/NAC-derivatized glycine; **B.** Stability of OPA/NAC-derivatized glutamic acid, glycine and phenylalanine after partial neutralization. After derivatization of amino acids samples were analyzed with intervals of 50 min by HPLC with fluorescence detection.

To investigate the stability of glutamic acid and glycine under the alkaline hydrolysis conditions, standard curves of mixtures of glutamic acid and glycine, containing 100  $\mu$ M phenylalanine as internal standard, were prepared in 4 M sodium hydroxide and either were kept on ice or heated for 30 min in a boiling water-bath before partial neutralization and OPA/NAC-derivatization. As shown in Fig. 3A, the standard curves of glutamic acid showed high linearity, whereas no significant difference was found between the standard curve kept on ice and heated in a boiling water bath. For glycine, however, duplicate measurements showed 15–20% variability, Fig. 3B, which may result from the lower stability of the OPA/NAC-derivate, as described above. Because of the higher stability of derivatized glutamic acid and higher accuracy of quantification, analysis of glutamic acid appears the preferred way for the accurate quantification of GSH-conjugates after alkaline hydrolysis. Simultaneous detection of glycine can be considered as confirmation of analysis of a GSH-conjugate.

### 3.2. Alkaline hydrolysis of GSH-conjugates

Fig. 4 shows the HPLC-chromatograms of 100  $\mu$ M S-hexyl-GSH before and after alkaline hydrolysis by heating for 30 min in 4 M sodium hydroxide. After the heat treatment the peak of S-hexyl-GSH was completely disappeared and two major peaks appeared at 4.25 and 12.5 min, corresponding to OPA/NAC-derivatized glutamic acid and glycine, respectively. When studying the time course of alkaline hydrolysis, already after 5 min of heating no intact S-hexyl-GSH was observed anymore, Supplemental Fig. S1A. Alkaline

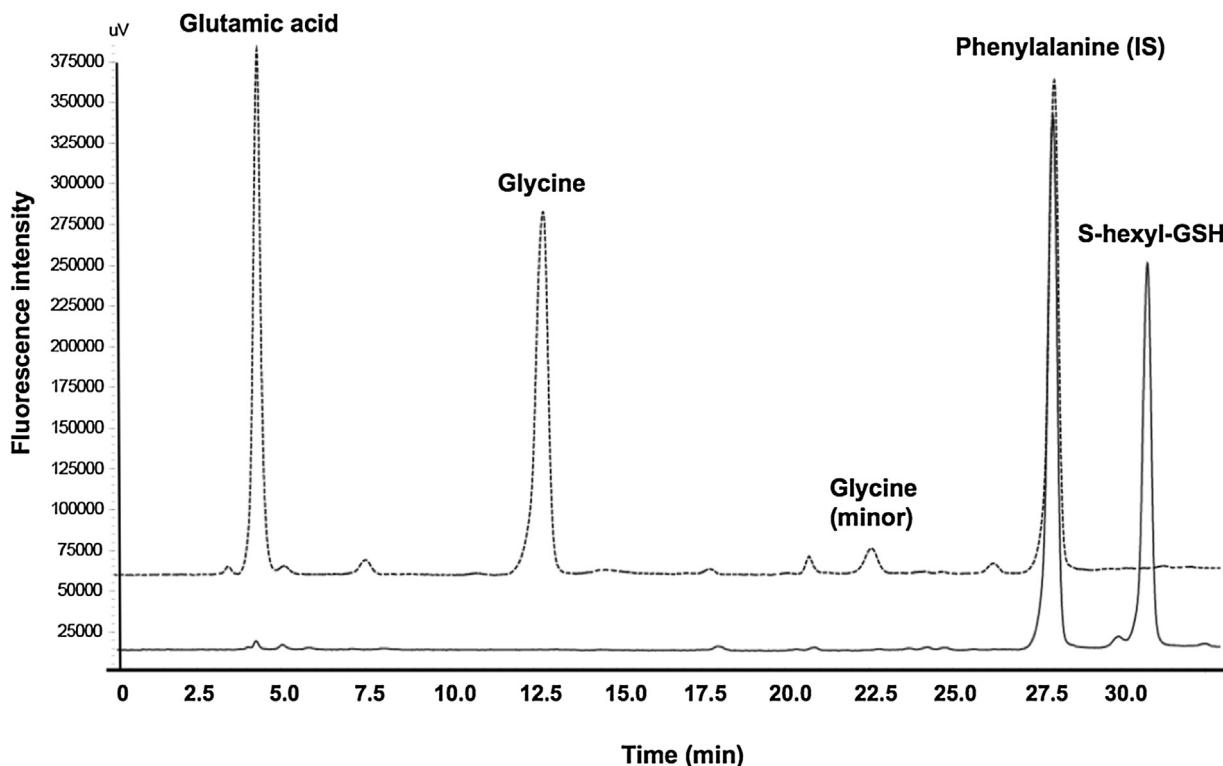


**Fig. 3.** The effect of heat treatment of amino acids in alkaline conditions on the calibration curves of glutamic acid (A) and glycine (B) using phenylalanine as internal standard (IS). After partial neutralization, samples were derivatized by OPA/NAC and analyzed by HPLC with fluorescence detection. As control, calibration curves were constructed of alkaline samples which were kept on ice until partial neutralization and derivatization. Each data point represents the mean of duplicates (+/– range). The line in panel A represents the linear regression of the calibration curve of glutamic acid ranging from 0 to 100  $\mu$ M ( $r^2 = 0.99$ ,  $y = 0.011x - 0.005$ ).

hydrolysis of S-hexyl GSH appeared to be complete after 30 min of heating since no further increase of glutamic acid was observed Supplemental Fig. S1B. The amount of glutamic acid detected was still unchanged after 120 min of heat treatment in 4 M sodium hydroxide, indicative for its stability under the condition of alkaline hydrolysis. Alkaline hydrolysis of all other GSH-conjugates tested also showed quantitative hydrolysis after 30 min heating in a boiling water bath. After this period, intermediates as cysteine-glutamic acid- and cysteine-glycine S-conjugates were not detected. As expected no cysteine S-conjugates were found, due to the facile beta-elimination of cysteine conjugates in strong alkaline conditions [29,30].

### 3.3. Validation of the alkaline hydrolysis method using gravimetrically prepared GSH-conjugates

To validate the novel method for quantification of GSH-conjugates, first standard curves of commercially available S-propyl-, S-hexyl- and S-nonyl GSH were prepared from gravimetrically prepared stock solutions. First the method was applied to three different concentrations (25, 50 and 75  $\mu$ M) of GSH-conjugate. Calibration curves of glutamic acid (ranging from 1.5 to 100  $\mu$ M) were used to quantify the amount of glutamic acid formed by alkaline hydrolysis of the S-alkyl GSH-conjugates. In Table 1 the concentrations of glutamic acid quantified after alkaline hydrolysis of the different concentrations of the S-alkyl GSH-conjugates is shown. Concentrations of glutamic acid formed corresponded well with the gravimetrically defined concentrations of GSH-conjugates used. The assay precision is reflected as the standard deviation (SD) between triplicates which was below 10%, which is within



**Fig. 4.** Representative chromatograms of 100  $\mu\text{M}$  S-hexyl GSH hydrolysis before (solid line) and after (dashed line) basic hydrolysis. Samples were derivatized by OPA/NAC and analyzed by HPLC with fluorescence detection.

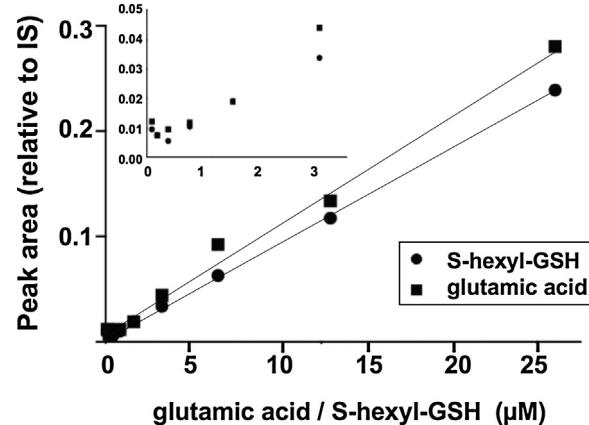
**Table 1**

Quantification of glutamic acid formed after alkaline hydrolysis of gravimetrically prepared solutions of S-alkyl-glutathione conjugates.

Conjugate	Concentration $\mu\text{M}$	Glutamic acid	
		$\mu\text{M}$	% theoretical
S-propyl GSH	25	$22.5 \pm 0.3$	$90 \pm 1.2$
	50	$45.2 \pm 0.6$	$90 \pm 1.0$
	75	$69.0 \pm 1.0$	$92 \pm 1.5$
S-hexyl GSH	25	$26.4 \pm 2.7$	$106 \pm 10$
	50	$49.0 \pm 3.0$	$98 \pm 6.1$
	75	$74.7 \pm 0.8$	$100 \pm 10$
S-nonyl GSH	25	$21.9 \pm 1.6$	$88 \pm 7.3$
	50	$42.7 \pm 3.3$	$85 \pm 6.9$
	75	$72.2 \pm 4.9$	$96 \pm 6.8$

Values are represented as means ( $n=3$ )  $\pm$  SD.

the precision required for bioanalytical analysis of drug metabolites [31]. Method precision and accuracy were not significantly influenced by the nature of the S-alkyl group and concentration of the GSH-conjugate. To determine the lower limit of quantification, standard curves of glutamic acid and S-hexyl-GSH were prepared ranging from 0.1 to 25  $\mu\text{M}$  and both were analyzed by the alkaline hydrolysis assay. As shown in Fig. 5, the standard curve of glutamic acid quantified after hydrolysis of S-hexyl-GSH closely matched that of the standard curve of glutamic acid when quantified directly. Trendlines obtained by linear regression were: hydrolysed S-hexyl-GSH:  $y = 0.0094 * x + 0.0035$ ,  $r^2 = 0.999$ ; glutamic acid:  $y = 0.0105 * x + 0.0147$ ,  $r^2 = 0.990$ . A lower limit of quantification was estimated to be approximately 1  $\mu\text{M}$  of glutamic acid and S-hexyl-GSH. Below this concentration, the signal-to-noise ratio of the glutamic acid peak did not allow accurate quantification.



**Fig. 5.** Calibration curves of glutamic acid and S-hexyl-GSH after alkaline hydrolysis treatment and OPA/NAC-derivatization. S-hexyl-GSH was quantified as glutamic acid formed after alkaline hydrolysis. The lines represent the linear regression of the calibration curves of glutamic acid (upper line) and hydrolysed S-hexyl-GSH (lower line) ranging from 0 to 25  $\mu\text{M}$ . The insert represent the calibration curve from 0 to 3  $\mu\text{M}$ , showing a lower limit of quantification of 1  $\mu\text{M}$  due to low signal to noise ratio. IS, internal standard phenylalanine.

### 3.4. Comparison of quantification by alkaline hydrolysis method with $^1\text{H}$ NMR quantification

For the GSH-conjugates of CLZ, DCF and APAP, quantification by the alkaline hydrolysis method was compared with quantification by  $^1\text{H}$  NMR. Previously, Espina and co-workers showed that metabolite solutions of at least 100  $\mu\text{M}$  can be quantified by  $^1\text{H}$  NMR with 10% accuracy [16]. As shown in Supplemental Fig. S2, calibration curves of the parent drugs CLZ, DCF and APAP obtained by using the integrals of their aromatic signals relative to the integral of the methyl-groups of the internal standard TMSP

**Table 2**

Comparison of determined concentrations of drug derived GSH-conjugates by  $^1\text{H}$  NMR with quantification based on glutamic acid concentration formed after alkaline hydrolysis of GSH-conjugates.

Parent drug	GSH-Conjugate	Quantification by $^1\text{H}$ NMR <sup>a</sup> ( $\mu\text{M}$ )	Concentration glutamic acid <sup>b</sup>	
			( $\mu\text{M}$ )	% NMR
Acetaminophen	APAP-SG	7980 $\pm$ 110	8205 $\pm$ 395	103
	<b>CG-1</b>	1120 $\pm$ 30	965 $\pm$ 110	86
	<b>CG-3</b>	715 $\pm$ 30	620 $\pm$ 15	87
	<b>CG-4</b>	N.D.	24 $\pm$ 2	—
	<b>CG-6</b>	140 $\pm$ 10	128 $\pm$ 1	92
	<b>M1</b>	200 $\pm$ 15	250 $\pm$ 10	125
Clozapine	<b>M2</b>	275 $\pm$ 50	206 $\pm$ 2	75
	<b>M3</b>	385 $\pm$ 5	425 $\pm$ 20	110
	<b>M5</b>	120 $\pm$ 20	60 $\pm$ 2	50

Values represent means of triplicate measurements  $\pm$  standard deviations.

<sup>a</sup>  $^1\text{H}$  NMR quantification is based on the integration of signals of aromatic protons, normalized to the number of aromatic protons, and by referencing to the proton-normalized integral of internal standard 0.45 mM TMSP.  $^1\text{H}$  NMR spectra were collected by acquiring 128 scans, except for **M5** and **CG-6** for which 1024 scans were required for good signal to noise ratios of aromatic protons. For **CG-4** no good signal to noise ratio could be obtained, even after collecting 4096 scans.

<sup>b</sup> Prior to the alkaline hydrolysis treatment, GSH-conjugates were first diluted to 50  $\mu\text{M}$ , based on the concentration determined by  $^1\text{H}$  NMR. **CG-4** was quantified without dilution.

**Table 3**

UV Response of GSH conjugates of drugs relative to that of the parent compounds, based on peak areas obtained by HPLC-UV quantification.

Relative UV-response of GSH-conjugate (% of parent drug) <sup>a</sup>				
Parent drug	GSH Conjugate	254 nm	280 nm	245 nm
Acetaminophen	APAP-SG	149	—	141
	<b>CG-1</b>	81	—	—
	<b>CG-3</b>	83	—	—
	<b>CG-4</b>	70	—	—
	<b>CG-6</b>	62	—	—
	<b>M1</b>	115	64	—
Clozapine	<b>M2</b>	272	135	—
	<b>M3</b>	183	103	—
	<b>M5</b>	289	78	—
Diclofenac				

<sup>a</sup> Relative UV-response were calculated from the ratio of the slopes of the calibration curve of the GSH-conjugates and the corresponding drug. Concentrations of the stocks of GSH-conjugates were determined by glutamic acid quantification after alkaline hydrolysis. The wavelengths were used in previous described HPLC-UV methods for quantification of GSH-conjugates of acetaminophen, clozapine and diclofenac [11,12,22,27].

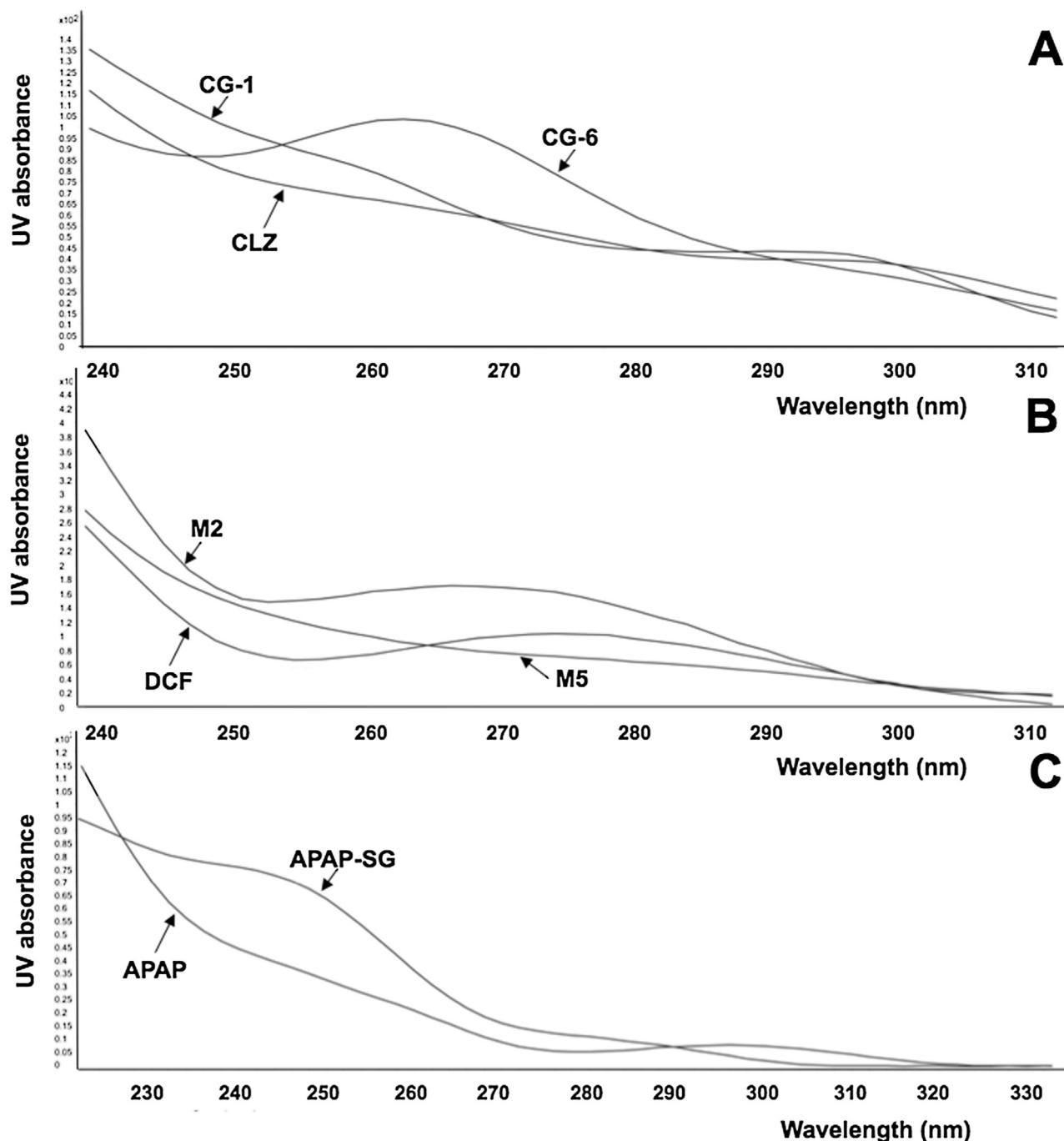
showed linear responses, consistent with the high dynamic range of  $^1\text{H}$  NMR-quantification. Although previous quantitative  $^1\text{H}$  NMR-studies used 600 MHz NMR-equipment with dedicated acquisition parameters [16,18,19], the 250 MHz NMR-equipment used in the present study appeared suitable for quantification of concentrations of APAP, CLZ and DCF in the range of 250  $\mu\text{M}$  to 10 mM. As shown in Table 2, the  $^1\text{H}$  NMR-quantification of the APAP-SG solution correctly corresponds with gravimetrically prepared 8 mM concentration. Using the same procedure, the concentrations of the regiosomeric GSH-conjugates of CLZ and DCF were quantified and ranged from 120 to 1120  $\mu\text{M}$ , Table 3. The concentration of CLZ conjugate **CG-4** was too low to obtain sufficient signal-to-noise ratio for accurate integration.

Based on the concentrations determined by  $^1\text{H}$  NMR, the stock solutions of the GSH-conjugates were first diluted to 50  $\mu\text{M}$  prior to alkaline hydrolysis, to be within the linear range of the standard curve of glutamic acid. Table 2 shows that for seven of the eight GSH-conjugates analyzed, the difference in concentration as determined by  $^1\text{H}$  NMR and as determined by glutamic acid quantification varied between 3 and 25%. The accuracy of glutamic acid quantification was reflected by the small SD (less than 11%) between triplicate measurements. The relatively poor correspondence between the two methods for DCF conjugate **M5**, may be explained by the low signal to noise ratio of the aromatic signals in the  $^1\text{H}$  NMR spectrum.

### 3.5. Comparison of absolute quantification of GSH-conjugates by alkaline hydrolysis method and quantification by HPLC-UV

In contrast to mass spectrometry, for which significant ionization differences are reported between parent and phase I metabolites [32], quantification of metabolites by UV-detection, using a calibration curve of the parent drug, is an accepted method for metabolite quantification, as long as the  $\lambda_{\max}$  of the chromophore of the molecule is unchanged by the metabolism [17,18]. Metabolites with the same  $\lambda_{\max}$  as the parent drug could be quantified using calibration curves of the parent with deviations of maximal 30% [18]. In contrast, in case of the metabolites were  $\lambda_{\max}$  is different from that of the parent drug, differences in relative responses on UV-detection can be more than 400% [18]. In case of GSH-conjugates of reactive metabolites of thiophene compounds major changes in UV-absorption were found [33]. In our previous studies, the GSH-conjugates of DF, CLZ and APAP were quantified by HPLC-UV using standard curves of the parent drugs, assuming that the extinction coefficient of parent and metabolite are equal [11,12,22,27]. As shown in Fig. 6, significant differences are present in the absorption spectra of GSH-conjugates and parent drug, when measured by on-line diode-array-detection. These differences can be explained by the addition of a GSH-moiety to aromatic ring, substitution of a chlorine-atom by GSH and aromatic hydroxylation.

To test whether the previously used quantification of GSH-conjugates by HPLC-UV require significant correction, calibration



**Fig. 6.** UV-spectra of parent drugs and corresponding GSH conjugates measured on-line by diode-array-detection. **A.** UV-spectra of CLZ, CG6 and CG3. GSH-conjugates CG1 and CG4 showed the same UV spectrum as CG-3 [data not shown]; **B** UV-spectra of DCF, M2 and M5. GSH-conjugates M1 and M3 showed the same UV spectra as DCF and M2, respectively [data not shown]; **C**, UV spectra of APAP and APAP-SG.

curves of GSH-conjugates and corresponding parent drugs were constructed by analyzing them by the HPLC-UV methods used previously for quantification. As shown in Supplemental Fig. S3, linear curves were obtained by plotting the peak areas of drugs and their GSH-conjugates against their concentrations. By comparing their slopes, the response of the GSH-conjugates relative to the parent drug was calculated at different wavelengths, see Table 3. In case of APAP and APAP-SG, the slope of the calibration curve of APAP-SG was 49% higher when analyzed at 254 nm and 41% higher when analyzed at 245 nm.

Previously, we demonstrated that incubations of CLZ with HLM in presence of GSH resulted in significant amounts of

GSH-conjugates when quantified by HPLC-UV using a standard curve of CLZ at 254 nm. It was estimated that the total amount of GSH-conjugates of CLZ almost equaled the amounts of clozapine N-oxide and desmethylclozapine, the major metabolites of CLZ [34]. The present study shows that the response of the four GSH-conjugates of CLZ at 254 nm was 19–38% lower than that of CLZ, Supplemental Fig. S3 and Table 3. This implicates that the amounts of GSH-conjugates of CLZ quantified previously were actually underestimated rather than overestimated. The high degree of GSH-conjugation of CLZ in *in vitro* incubations is not reflected by a high excretion of mercapturic acids or other thioethers in urine [35]. This might implicate that reductive inactivation of the

nitrenium ion of CLZ by quinone reductases or other reductive enzymes is more efficient in hepatocytes than GSH-conjugation. Interestingly two association studies suggest that NQO2-polymorphisms, leading to reduced expression of NQO2, increase the risk for CLZ-induced agranulocytosis [36,37]. Whether this quinonereductase is active in inactivation of the nitrenium ion remains to be established experimentally, however.

Calibration curves of GSH-conjugates of DCF and the parent DCF also showed different slopes when measured at 254 nm or 280 nm. The different responses at these wavelengths can be attributed to the different UV-spectra of these analytes, see Fig. 6B, in which the spectra of DCF and GSH-conjugates M2 and M5 are presented. When measured at 254 nm, the peak areas of these GSH-conjugates were more than two-fold than that of DCF when injected at the same concentration. Because both DCF and GSH-conjugate M2 show an absorption maximum around 280 nm, their peak areas were higher than that of GSH-conjugate M5. As shown in Table 3, different correction factors are required when quantifying these GSH-conjugates at different wavelengths.

#### 4. Conclusion

In this paper, we present a simple and generic method applicable for the absolute quantification of low amounts of isolated GSH-conjugates. The method relies on the quantitative analysis of glutamic acid formed after alkaline hydrolysis of the glutathionyl-moiety of GSH-conjugate. Method precision and accuracy were confirmed with three gravimetrically quantified commercial GSH-conjugates and by <sup>1</sup>H NMR quantification. Although quantification of glycine appeared less accurate due to lower stability of its OPA/NAC derivate, the simultaneous appearance of both glutamic acid and glycine can be used to confirm the presence of a GSH-conjugate. The main advantage of the novel methodology over <sup>1</sup>H NMR- quantification is its more than 100-fold higher sensitivity (low μM range) and the lower costs of required equipment. Using this method significant differences in UV-responses between GSH-conjugate and parent drugs were found, implicating that previous quantifications of GSH-conjugates by HPLC-UV require up to 2-fold correction in case standard curves of parent drug were used.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jchromb.2017.02.004>.

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