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RESEARCH ARTICLE

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Ethylene glycol: Evidence of glucuronidation *in vivo* shown by analysis of clinical toxicology samples

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Abstract

In the search for improved laboratory methods for the diagnosis of ethylene glycol poisoning, the *in vivo* formation of a glucuronide metabolite of ethylene glycol was hypothesized. Chemically pure standards of the β-*O*-glucuronide of ethylene glycol (EG-GLUC) and a deuterated analog (d_4-EG-GLUC) were synthesized. A high-performance liquid chromatography and tandem mass spectrometry method for determination of EG-GLUC in serum after ultrafiltration was validated. Inter-assay precision (%RSD) was 3.9% to 15.1% and inter-assay %bias was −2.8% to 12.2%. The measuring range was 2–100 μmol/L (0.48–24 mg/L). Specificity testing showed no endogenous amounts in routine clinical samples (n = 40). The method was used to analyze authentic, clinical serum samples (n = 31) from patients intoxicated with ethylene glycol. EG-GLUC was quantified in 15 of these samples, with a mean concentration of 6.5 μmol/L (1.6 mg/L), ranging from 2.3 to 15.6 μmol/L (0.55 to 3.7 mg/L). In five samples, EG-GLUC was detected below the limit of quantification (2 μmol/L) and it was below the limit of detection in 11 samples (1 μmol/L). Compared to the millimolar concentrations of ethylene glycol present in blood after intoxications and potentially available for conjugation, the concentrations of EG-GLUC found in clinical serum samples are very low, but comparable to concentrations of ethyl glucuronide after medium dose ethanol intake. In theory, EG-GLUC has a potential value as a biomarker for ethylene glycol intake, but the pharmacokinetic properties, *in vivo/vitro* stability and the biosynthetic pathways of EG-GLUC must be further studied in a larger number of patients and other biological matrices.

KEYWORDS

biomarker, clinical toxicology, ethylene glycol, glucuronide, poisoning

1 | INTRODUCTION

Ethylene glycol (EG) or monoethylene glycol (ethane-1,2-diol) is an odorless, colorless, viscous, sweet-tasting liquid. It is primarily used for industrial production of plastic polymers, polyester fibers, and films. Other uses include anti-freeze products for automobiles; coolant, and heat transfer agents for air-conditioning systems; and de-icing fluids along with other niche industrial applications. The world

production of ethylene glycols, of which EG accounts for about 90% of the total market, was estimated at approximately 35 million tons in 2016 and its consumption rate is increasing by 5%–6% per year.[1]

Toxicity after ingestion of EG remains a major concern worldwide in human clinical toxicology with unintentional exposures in adolescents, children, and the elderly or exposure due to self-poisonings or suicide attempts.[2] EG poisonings are also described in veterinary toxicology (livestock, pets).[3] To limit poisoning incidents, the bitter-tasting compound denatonium benzoate has been added to antifreeze products in many countries. However, in the United States (USA) implementation of this procedure caused no reduction of oral EG ingestion cases in humans.[4]

EG in itself is moderately toxic in humans with an estimated oral lethal dose of 1.0 to 2.0 g/kg body mass.[5] EG is metabolized *in vivo* by hepatic enzymatic pathways to glycolic acid and glyoxal, which are further oxidized into glyoxylic acid and oxalic acid.[6] These metabolites cause severe metabolic acidosis, central nervous system depression, and cardio-pulmonary toxicity. Moreover, calcium oxalate can precipitate in organs and cause kidney injury.[2,6]

Treatment can include supportive care, inhibition with either ethanol or fomepizole, and in some cases hemodialysis. The classic treatment with ethanol has largely shifted to the use of fomepizole, which is a competitive inhibitor of alcohol dehydrogenase.[7] However, while the difference in total outcome between the two antidotes has been debated, ethanol is associated with more frequent adverse reactions.[8]

In the USA from 2000 to 2013, 85 891 poisonings with EG were registered (> 94% acute intoxications), of which 480 (0.6%) had a fatal outcome.[9] In a study from the United Kingdom (UK), covering two years (2010 and 2012), 1.3% of the total reported telephone enquiries to the National Poison Information Centre related specifically to suspected exposure to ethylene glycol or methanol.[10] Although ethylene poisoning is uncommon in the UK, and assays for ethylene glycol are available on a 24-hour basis, it was reported that antidote treatment was often provided before analytical results were available.[10]

Recognition of clinical symptoms of EG intoxication is often difficult, leaving clinicians − in countries where clinical analysis of EG is not available (eg, Denmark with 5 million inhabitants) − to rely on surrogate biochemical parameters with insufficient specificity and sensitivity, such as lactate gap,[11-13] anion gap[14-18] or osmolar gap.[19] Generally, well-organized, fast analytical services and antidote supplies are needed, since a delay in testing may also cause delays in proper treatment.[20]

Poisonings with EG can be diagnosed by quantitative analysis of EG with gas chromatography (GC), in serum, plasma, post-mortem blood and urine.[21-35] This may involve different pre-treatment techniques, like liquid-liquid extraction (LLE), ultrafiltration, and/or derivatization followed by head-space sampling, direct thermal desorption, or injection of extracts on a GC apparatus coupled to flame ionization (GC–FID) or mass spectrometry (GC–MS). Some methods include determination of glycolic acid, which correlates well with the degree of metabolic acidosis.[29] Liquid chromatography (LC) has been used to analyze EG in animals[36] or human serum[37-40] with various derivatization techniques. The use of automated, enzymatic screening assays for EG or glycolic acid has also been implemented.[41-45] Such methods may be used for screening tests to rule out EG poisoning. However, both GC–FID and enzymatic methods may suffer from interference with other alcohols or glycols, for example 2,3-butanediol and propylene glycol.[45,46] Thus, to exclude these rare false positive screening results GC–MS may be used as a confirmatory reference method. Recently, an approach that employs liquid chromatography–tandem mass spectrometry (LC–MS/MS) with detection of ethylene glycol as a cluster ion was published; however, this method has not been evaluated in a clinical study.[47]

Following the implementation of high-performance LC–MS/MS as a routine apparatus in bioanalytical laboratories, analysis of phase II metabolites of medical drugs and illicit substances has gained importance in clinical and forensic toxicology.[48] This includes the glucuronides and sulfates of small polar molecules, for example ethanol and γ-hydroxybutanoic acid in blood, urine and hair.[49-52] Ethyl glucuronide (EtG), formed by glucuronidation of ethanol by uridine diphosphate glucuronosyltransferases (UGTs), has proved suitable as a direct biomarker for ethanol consumption.[53]

However, so far the glucuronides of two of the most important alcohols in clinical toxicology, methanol and EG, have not been investigated. In the search of improved laboratory methods for diagnosis of EG poisoning, the *in vivo* formation of a glucuronide metabolite of EG was hypothesized. Chemically pure standards of the β-*O*-glucuronide of EG (EG-GLUC) and a deuterated analog for use as internal standard (IS) (d_4-EG-GLUC) were synthesized. An LC–MS/MS method for quantitative determination of EG-GLUC in serum after ultrafiltration was developed and validated. For a proof-of-concept of *in vivo* formation of EG-GLUC, authentic clinical samples from confirmed toxicology cases were analyzed for EG-GLUC.

2 | METHODS

2.1 | Materials and reagents

The β-*O*-glucuronide of ethylene glycol (EG-GLUC) and a corresponding deuterium-labeled analog (d_4-EG-GLUC) was synthesized at the Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen by an approach similar to that reported previously (Figure 1).[54] The Supporting

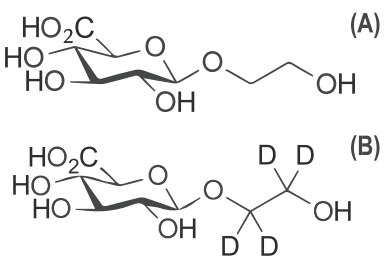

FIGURE 1 Structure of A, ethylene glycol glucuronide (EG-GLUC); B, d_4-ethylene glycol glucuronide (d_4-EG-GLUC)

Information details the method of synthesis and full characterization with high-resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR) spectroscopy. The EG-GLUC contained an impurity of 8 mol% 3-hydroxy-propanoic acid; the d_4-EG-GLUC substance contained 10 mol% 3-hydroxy-propanoic acid. During preparation of the stock solutions described in the next paragraph, no correction factor was used for this impurity.

Stock solutions (10 mmol/L) of EG-GLUC and d_4-EG-GLUC (10 mmol/L) were prepared in purified water and stored at −20°C. The EG-GLUC stock solution was used to prepare quality control samples (QC) in serum. The IS aqueous working solution of d_4-EG-GLUC was 200 µmol/L. Methanol was LiChrosolv LC–MS hypergrade (Merck, Darmstadt, Germany). Formic acid (99%) was HiPerSolv LC–MS grade (VWR International, Søborg, Denmark). Purified water (18 MΩ) was generated in an ELGA LabWater CENTRA® RDS system (High Wycombe, UK). Other reagents were of analytical grade.

2.2 | Liquid chromatography–tandem mass spectrometry

The LC–MS/MS system used was a 6460 triple-quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) with a Jetstream electrospray ion source, operated at unit resolution. The chromatographic system consisted of an Agilent 1200 binary pump, a 1200 SL well-plate autosampler and a 1200 column department. Separation was performed on a Kinetex Biphenyl 100A column (100 × 3 mm), particle size 2.6 µm (Phenomenex, Macclesfield, UK). Flow rate was 550 µL/min using the mobile phases: (A) 0.1% formic acid in purified water; and (B) 0.1% formic acid in methanol. Gradient elution was as follows: 2% B for 0.5 minutes, then up to 95% B in 4.5 minutes, 95% B for 3 minutes followed by equilibration at initial conditions for 2 minutes. Total run time was 9 minutes and the column temperature was maintained at 40°C. Autosampler injection volume was 1 µL and the sample tray was kept at 20°C.

The electrospray source parameters were as follows: capillary voltage: 3500 V; declustering potential 120V; nitrogen gas flow: 8 L/min; nitrogen gas temperature: 350°C; sheath gas flow: 8.5 L/min; sheath gas temperature: 375°C; nozzle voltage: 400 V; nebulizer pressure: 25 p.s.i.

Declustering potential and individual collision energies for EG-GLUC and d_4-EG-GLUC were optimized manually in flow injection mode. Multiple reaction monitoring (MRM) was performed in negative ionization mode (−ES) using the deprotonated precursor ions $[M-H]^-$ for both EG-GLUC (*m/z* 237.1) and d_4-EG-GLUC (*m/z* 241.1) (Table 1). The MRM transitions for EG-GLUC, *m/z* 237.1 →m/z 85, and for d_4-EG-GLUC, *m/z* 241.1 → *m/z* 75, was used for quantification. MRM dwell time was fixed at 40 ms. Sample concentrations were determined from peak-area ratios of EG-GLUC to d_4-EG-GLUC by reference to a calibration curve. Data acquisition and post-analysis was performed with MassHunter™ software, version B.04.01 (Agilent Technologies, Palo Alto, CA, USA). Figures 2 and 3 were directly exported to PDF-format from MassHunter software and annotated using Adobe InDesign. Figure 4 was constructed using mass spectral data export to SigmaPlot 12.0 and annotated using Adobe InDesign.

The identification of EG-GLUC in clinical samples was approved in case of correct assigned retention time (tolerance ±2.5%) and from MRM data, when the relative ratios for seven qualifying ions, expressed as a percentage of the intensity of the most intense transition, matched with the mean values determined in calibrators in the same batch. Acceptable tolerances are shown in Table 1. Performance criteria for qualifying ions described in an EU directive[55] as well as in technical documents used by laboratories accredited by the World Anti-Doping Agency (WADA),[56] were used. There is a general consensus in endogenous compound identification (by environmental and food analysis, doping control, etc.) for using different acceptance tolerances (range: 20%–50%) for qualifying ions depending on the

TABLE 1 Multiple reaction monitoring (MRM) parameters and tolerances for qualifying ion ratios

Compound	Precursor Ion *m/z*	Fragment Ion *m/z*	Collision Energy (V)	Qualifying Ion Ratio (%) *	Qualifying Ion Ratio Tolerance (%)
EG-GLUC	237.1	85	10	Used for quantification	-
		113	10	60.5	± 20
		99	10	13.6	± 30
		75.3	10	65.9	± 20
		57	20	31.2	± 25
		55	30	23.9	± 25
		45	30	8.7	± 50
		43	25	6.4	± 50
d_4-EG-GLUC	241.1	75	10	Used for quantification	-
		113.2	10	63.4	± 20
		94.8	15	11.3	± 30
		85	15	109	± 20
		71	20	20.4	± 25
		57	20	35.1	± 25
		55.2	30	28.5	± 25
		43	20	6.4	± 50

*values show inter-batch variability.

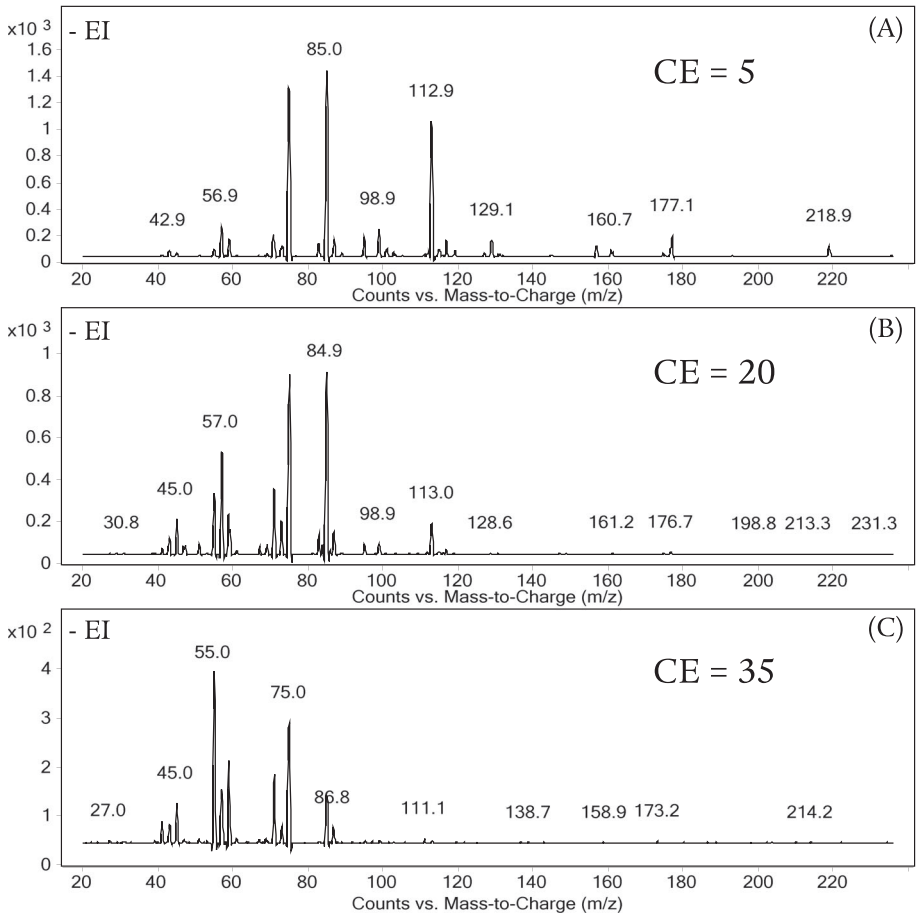

FIGURE 2 Negative ionization electrospray product ion spectra of ethylene glycol glucuronide (EG-GLUC), $[M-H]^-$ = *m/z* 237.1, at different collision energies (CE): A, 5 V; B, 20 V; C, 35 V. Declustering potential: 120 V

relative ion abundances (% of base peak). These criteria were also evaluated and found applicable for confirmatory drug analysis in plasma and urine by LC–MS/MS.[57]

2.3 | Evaluation of internal standard purity

Prior to method development, initial testing of a stock solution of d_4-EG-GLUC (1 mmol/L), using the LC–MS/MS method described in section 2.2, showed less than 0.63% content EG-GLUC calculated from the peak area ratios. This trace amount of EG-GLUC is acceptable for use of d_4-EG-GLUC as IS.

2.4 | Calibration standards and quality controls

2.4.1 | Serum calibrators

Serum calibrators (*n* = 6) were prepared by spiking aqueous working solutions of EG-GLUC into serum obtained from a large blood donor pool (*n* = 1000), prepared for quality control materials at the Department of Biochemistry, North Denmark Regional Hospital. The serum was kept at −80°C until use. The final calibrator concentrations used for method validation were 100, 50, 25, 10, 5, and 2 μmol/L.

2.4.2 | Calibration curve and batch setup

Calibration curves were based on least-squares linear regression (Y = aX + b), not forced through zero, with no weighting factor. Every batch also contained a zero sample (matrix processed with IS) and a blank sample (matrix processed without IS). For the batches of clinical serum samples (with unknown concentrations), each sample was separated by a blank sample.

2.4.3 | Spiked quality control samples (QC)

Spiked serum samples were prepared as described for serum calibrators (section 2.4.1), including a QC sample at 250 μmol/L for dilution integrity testing. The QC samples were used during method validation and as QC samples during analysis of clinical samples. They were kept at −20°C until use.

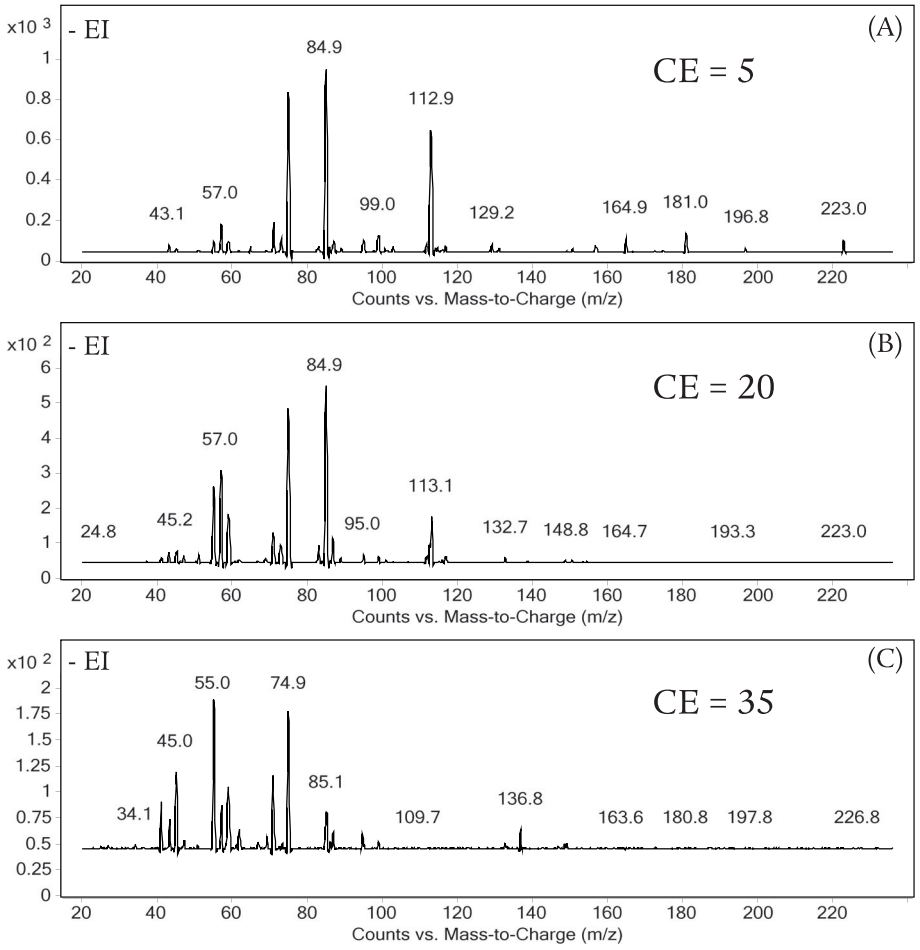

FIGURE 3 Negative ionization electrospray product ion spectra of the deuterated internal standard d_4-ethylene glycol glucuronide (d_4-EG-GLUC), $[M-H]^-$ = *m/z* 241.1, at different collision energies (CE): A, 5 V;B, 20 V; C, 35 V. Declustering potential: 120 V

2.4.4 | Aqueous calibrators

A series of aqueous calibrators (*n* = 9) were prepared by spiking aqueous working solutions of EG-GLUC into purified water. The concentrations were 1000, 500, 250, 200, 100, 50, 25, 10, 5, 2, and 1 μmol/L.

2.5 | Clinical toxicology samples

Anonymized, authentic clinical samples (*n* = 31) from patients with confirmed EG intoxications were provided by CHU Ste-Justine, Montreal (Quebec, Canada). The samples were serum aliquots from routine blood sampling taken at the admission of patients suspected of ethylene glycol intoxication. No extra blood samples were retrieved for the present project. It was not registered if the patients had received treatment with fomepizole. For samples #15 to #31, the concentrations of EG are available. Due to the anonymization, age, gender, and dates of sampling are not available for this study. However, no samples were stored more than a year from the time of sampling to date of analysis. The samples were shipped frozen (on dry ice) to the Department of Clinical Biochemistry, North Denmark Regional Hospital, and stored at −80°C until analysis. According to Danish Law on Scientific Research # 593 of June14, 2011, § 14,[3] anonymized clinical samples can be studied without approval of the Human Ethics Committee. The EG-GLUC results had no influence on the handling and clinical care of patients.

2.6 | Sample preparation by ultrafiltration

Sample preparation was identical for calibrators, spiked quality controls, and clinical samples. IS working solution (10 μL) and serum sample (250 μL) was added directly into an Amicon Ultra centrifugal filter device (0.5 mL) with a 30 kDa nominal molecular weight limit cut-off (Millipore/Merck, Darmstadt, Germany). After vortex mixing for 2 seconds, the tube was centrifuged for 30 minutes at 12 000 × *g*. Then 100 μL of ultrafiltrate was transferred to a 300 μL fixed insert vial (Chromacol, Langenwehe, Germany), capped, and placed in the thermostatted autosampler at 20°C until analysis.

2.7 | Sample preparation by protein precipitation

To evaluate method specificity and the final sample preparation technique by ultrafiltration, protein precipitation was performed ad hoc by

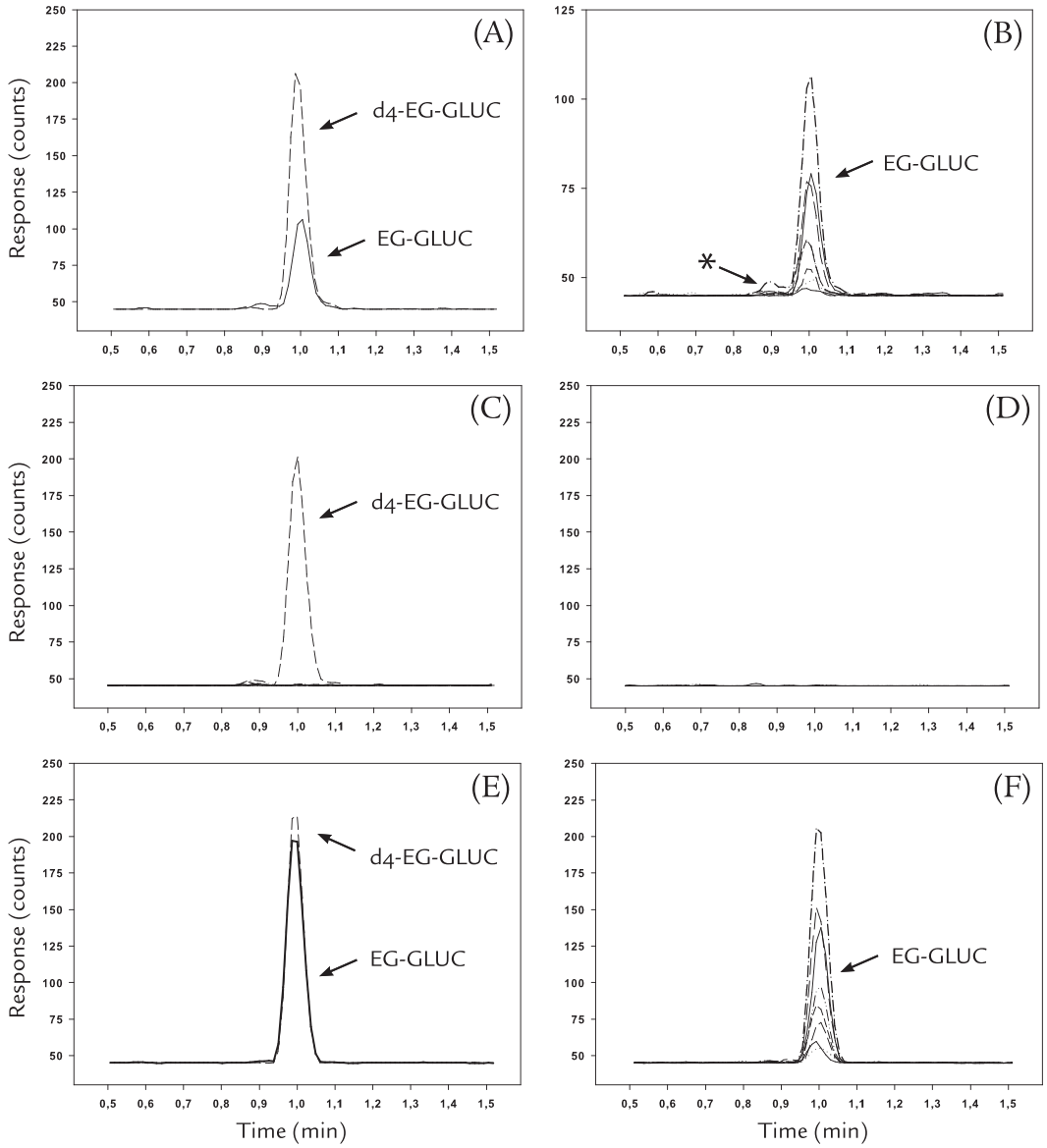

FIGURE 4 Extracted multiple-reaction monitoring (MRM) ion chromatograms for LC–MS/MS analysis of EG-GLUC in serum. A, Target ion *m/z* 237.1 → *m/z* 85 (EG-GLUC) and *m/z* 241.1 → *m/z* 75 (d_4-EG-GLUC) in a serum calibrator (2 μmol/L); B, target and qualifying ions for EG-GLUC (overlaid) in a serum calibrator at LOQ (2 μmol/L). The asterisk (*) shows closely eluting, unknown endogenous compounds detected in most transitions; C, zero sample with IS and target/qualifying ions for EG-GLUC (overlaid); D, blank serum sample without IS and target/qualifying ions for EG-GLUC overlaid; E, target ion *m/z* 237.1 → *m/z* 85 (EG-GLUC) and *m/z* 241.1 → *m/z* 75 (d_4-EG-GLUC) in a clinical sample (#12) at 5.67 μmol/L; (F) target and qualifying ions for EG-GLUC (overlaid) in a clinical sample (#12). Plots A, C, D, E, and F are in the same y-axis scale. Plot B is in half y-axis scale

the following procedure: Serum (100 μL) was added to an Eppendorf tube (1.5 mL) with 400 μL cold acetonitrile, mixed for 30 seconds and centrifuged 10 minutes at 12000 × *g*.

3 | METHOD VALIDATION

Method performance was validated according to the key principles in *Guidance for Industry*, *Bioanalytical Method Validation*.[58] This guideline sets out specific method validation criteria for LC–MS/MS analysis of drugs or metabolites in biological matrices. It was supplemented by (a) characterization of the custom synthesized standard of EG-GLUC (Supporting Information), (b) evaluation of the IS purity, and (c) extended acceptance criteria for using qualifying ions for unambiguous identification of EG-GLUC in clinical samples (Table 1).

3.1 | Selectivity/specificity

Method selectivity was assessed by analyses of (a) anonymized, blood donor serum aliquots (*n* = 20), obtained within one week after sampling from the local blood bank; (b) anonymized serum samples

(n = 20), randomly selected among routine clinical samples at the Department of Clinical Biochemistry, North Denmark Regional Hospital. The sampling was performed in polypropylene collection tubes without additives. All samples were kept at 4–8°C for up to 2 weeks prior to analysis. During testing of specificity, samples were analyzed for EG-GLUC and isobaric interferences after both protein precipitation and ultrafiltration. Standards of the ethanol conjugates – EtG and ethyl sulfate (EtS) – at 2000 ng/mL in ultrafiltrate were injected to evaluate interfering effects.

3.2 | Evaluation of sample preparation method

The sample preparation by ultrafiltration was evaluated at three concentration levels (in both aqueous and serum samples) with spiking of the IS to the final ultrafiltrate. The recovery of EG-GLUC was quantified using aqueous calibrators (because ultrafiltrated serum calibrators would mask the effect of protein binding). Chromatograms were evaluated for samples using ultrafiltration versus protein precipitation.

3.3 | Linearity and calibration

Linearity was evaluated by calibration curve data from different batches (n = 6). Linearity was expressed by the correlation coefficient (r), with the acceptance criterion $r > 0.999$. The repeatability of calibration was assessed by the relative standard deviation (%RSD) of the calibration curve slopes and the Y-axis intercept values.

3.4 | Precision and accuracy

Accuracy and precision was evaluated with at least four QC concentration levels. Intra-assay data was obtained by five determinations per concentration level within one batch. Intermediate bias and precision data were obtained from batches on five different working days. Precision was expressed as the relative standard deviation (%RSD). Accuracy was expressed as %bias (recovery% – 100), defined as recovery% = [measured concentration/spiked concentration × 100%]. The acceptance criteria for precision and accuracy was %RSD < 15 and %bias ±15, except at the limit of quantification (LOQ).

3.5 | Limit of quantification/limit of detection

The LOQ was the lowest concentration that could be analyzed within %RSD < 20 and %bias ±20, and using all qualifying ions within the acceptance criteria shown in Table 1. The limit of detection (LOD) was calculated as mean ± 3 × standard deviations (SD) in zero samples. Signal-to-noise was also calculated for LOQ and LOD.

3.6 | Carry-over

The effect from carry-over was assessed by analyzing a QC sample (250 μmol/L) followed by a blank (with no IS), and was reported in peak area-%. Carry-over was also evaluated in zero samples during method validation batches and clinical samples.

3.7 | Dilution integrity

To test dilution integrity, a QC sample (250 μmol/L) was diluted 1:5, 1:10, 1:25, and 1:50 with blank serum prior to analysis. The average %bias and %RSD were reported. The acceptance criterion was 15%.

3.8 | Evaluation of matrix effects

Quantitative matrix effects (ion suppression of signals) were calculated by the ratio of quantification ion intensities (m/z 237.1 → m/z 85) in equimolar aqueous calibrators relative to serum calibrators, and expressed in percentages.

Qualitative matrix effects were evaluated by a post-column infusion experiment using a single-syringe infusion pump (Cole Palmer, Vernon Hills, IL, USA) delivering a fixed amount of EG-GLUC per time. Concentration in the final eluent was 25 μmol/L. Blank serum ultrafiltrate (1 μL) was injected and the total ion chromatogram of the LC–MS/MS method was monitored.

3.9 | Stability

Stability studies were limited by the relatively small amount of substance available for the study (5.37 mg), where preparation of new stock solutions for each stability batch was not possible. Thus, all stability testing results were calculated relative to the serum calibrators prepared from a single stock solution which was kept at −20°C. The solid standard substance of EG-GLUC was assumed to be fully stable at −80°C. In all stability experiments , EG-GLUC was considered stable if the deviations were within ±15%, which is the method validation criterion for bias.

3.9.1 | Stability toward heating and forced acidic and alkaline hydrolysis

Clinical toxicology laboratories may use chemical hydrolysis during pre-treatment of samples. Hence, the stability toward simple heating and acidic/alkaline hydrolysis was tested by the following procedure: Aqueous calibrators (100 μmol/L) were incubated at (a) 60°C or 100°C without hydrolyzing reagents; (b) 60°C in 4 M HCl; or 100°C in 8 M HCl; (c) 60°C in 4 M NaOH; or 100°C in 8 M NaOH;. The incubation time was 15 minutes. After cooling, the acidic or alkaline solutions were neutralized with NaOH or HCl, respectively. All samples were adjusted to the equal final volumes, spiked with IS, and analyzed with LC–MS/MS. Incubation at 100°C was performed in a pressure-cooking device. Degradation was calculated relative to an aqueous calibrator, kept at room temperature during the experiments.

3.9.2 | In-sampler stability

A series of QC serum samples was left in the thermostatted autosampler at 20°C for four days, reanalyzed and quantified with a freshly prepared calibration curve. The difference was calculated in percentage of the initial result.

3.9.3 | Long-term stability

The stability of EG-GLUC in serum at 4°C was assessed at 4, 14, 27, and 155 days. The influence of storage at −20°C for two months including freeze–thaw cycles (n = 3) were evaluated in a single QC sample (100 μmol/L). Results within ±15% from spiked concentrations were considered acceptable.

4 | RESULTS AND DISCUSSION

4.1 | Mass spectrometry

EG-GLUC detection was performed in negative electrospray ionization mode (−ES). No molecular ion $[M+H]^+$ was observed in full scanning +ES mode, probably due to high sodium ion affinity of the glucuronide as only this adduct ion $[M+ Na]^+$ was observed. The −ES product ion mass spectra of deprotonated EG-GLUC, m/z 237.1 and deprotonated d_4-EG-GLUC, m/z 241.1 are shown in Figures 2 and 3. Due to the absence of fragment ions with +4 (originating from the deuterium-labeled aglycone), it was concluded that most peaks originate from the electrophilic glycosyl moiety. The ions m/z 75, 85, 95 and 113 have been reported in the product ion spectra of other glucuronides[59,60] and are part of a general fragmentation pattern observed for *O*-glucuronides.[61]

4.2 | Liquid chromatography

LC was performed in gradient mode using a biphenyl column that can separate the positional isomers of, for example, morphine glucuronides. With a low pH (approximately 2.6) in the mobile phase, EG-GLUC eluted as a symmetric peak with the retention time (RT) 1 minute, as seen in the chromatograms of a calibrator and a clinical sample (Figure 4). RSD% for RT in samples with measured EG-GLUC (above LOQ) was below 1%. EG-GLUC and EtG co-elute during analysis, but there is no interference in the measurements of the two glucuronides. Injection of serum ultrafiltrate versus the analyte dissolved in the mobile phase had a significant matrix effect on retention time, as EtG elutes approximately 0.5 minutes later in aqueous solution or in urine compared to ultrafiltrate using identical LC parameters. Given the minor differences in chemical structure between EG-GLUC and EtG, separation by reversed-phase chromatography may be challenging. The retention (capacity) factor for EG-GLUC was estimated to 0.25, which shows poor retention.

4.3 | Evaluation and choice of sample preparation method

Although plasma protein binding of some drug glucuronides have been reported,[62] little is known about the protein binding of small, hydrophilic compounds and their glucuronides. Thus, there are no reports on plasma protein binding of EtG, which is close to EG-GLUC in structure.[63] Generally, ether *O*-glucuronides are more stable and show higher stability than the electrophilic acryl-glucuronides, which may undergo intra-molecular rearrangement and irreversible, covalent binding to plasma proteins.[64] For small polar compounds with no protein binding, like methylmalonic acid, ultrafiltration can be used for sample pre-treatment prior to analysis by LC.[65] Ultrafiltration is a relative simple technique, which only depends on access to a high-speed centrifuge, normally available in a clinical laboratory. No disadvantages were observed for ultrafiltration as a pre-treatment tool during the present study: It is a solvent-free isolation technique with a low dilution factor only due to spiking of the internal standard with little or no effect on chromatography column lifetime. Protein precipitation with acetonitrile has the disadvantage of a high dilution factor prior to analysis. Chromatograms in this study were characterized by more noise and interfering peaks for protein precipitation compared with ultrafiltration.

4.4 | Method validation results

Method precision and accuracy results are shown in Table 2 and other validation parameters and results are summarized in Table 3. In section 4.4.1 to 4.4.6 the critical parameters are discussed in detail.

4.4.1 | Selectivity/specificity

Specificity testing showed no endogenous amounts above LOD in routine clinical samples used for specificity testing (n = 40). Furthermore, EG-GLUC was not detected in the serum pool used to prepare calibrators and QCs and blank samples. At low concentrations, unknown endogenous peaks eluting prior to EG-GLUC was observed in all samples (Figure 4). However, the chromatographic resolution was suitable and did not introduce bias on the determination of concentrations in the final measuring range. Furthermore, EtG/EtS did not cause interference during detection of EG-GLUC.

4.4.2 | Linearity and calibration

Standard curves of both aqueous and serum calibrators showed linearity up to at least 100 μmol/L with correlation coefficients (r^2) above 0.99. The average calibration curve slopes in these two matrices were 0.159 (n = 3) and 0.175 (n = 8), for aqueous and serum calibrators respectively. The serum calibration curves were reproducible (slope %RSD = 4.0) over a time period of eight months.

TABLE 2 Method validation data (n = 5) for quantification of EG-GLUC in serum

	Concentration		Intra-assay Precision		Intermediate Precision	
Sample	µmol/L	µg/mL	RSD%	Bias (%)	RSD%	Bias (%)
Spiked serum	100	23.8	4.0	+1.6	4.2	+1.8
	50	11.9	3.1	− 0.8	4.4	− 2.8
	10	2.38	8.5	+2.5	3.9	− 1.8
	2	0.48	7.5	+1.7	15.1	+ 12.2
	1	0.24	7.3	− 35.4	Not tested	Not tested

4.4.3 | Precision, bias, LOQ, and LOD

The intra-assay precision (%RSD) was 3.9 to 15.1% and %bias in the range − 2.8 to 12.2%, which is considered acceptable for bioanalytical methods.[58] LOQ was 2 µmol/L, and LOD was calculated to 0.1 µmol/L. The signal-to-noise at LOQ (n = 5) was in the range 35–53 and for LOD (n = 5) in the range 11–21. In theory, the LOD also depends on the minor impurity of EG-GLUC in the IS. However, the IS amount used during sample preparation was not a limiting factor for LOD in this study. Above LOQ, all qualifying ion ratios for EG-GLUC in clinical samples were within acceptance criteria (Table 1). In a few single injections, the response ratios of two qualifying ions for IS (m/z 57 and m/z 94.8) were outside the range. This was of no importance for the results of the present study. The qualifying ions with ratios below 10% and tolerances of ±50% could be excluded in further research methods, but all qualifying ions were used herein to maximize the identification power in this first report on EG-GLUC in a biological sample.

4.4.4 | Dilution integrity

All samples validated for dilution integrity showed acceptable %bias and RSD% below 15%.

4.4.5 | Matrix effects

Due to the low retention of glucuronides to the biphenyl column, EG-GLUC elutes in the region where matrix effects are expected. Matrix effects were estimated quantitatively by comparing ion intensities of serum calibrators to aqueous calibrators with equimolar concentration. These results showed up to 49.4% ion suppression (Table 3). A post-infusion ion-suppression experiment (Figure 5) showed that EG-GLUC elutes in a zone where ion suppression is evident. However, it is generally assumed that the isotope-labeled, co-eluting internal standard (d_4-EG-GLUC) compensates for the alteration in signal, thereby minimizing matrix and ion-suppression effects on quantification results. However, ion suppression has an impact on the LOD. The variation in IS response for the batches in this study, expressed as RSD%, was below 15, which was considered acceptable. In conclusion, ion-suppression effects were not considered to be a problem for the use of the present method and for the validity of the obtained results.

4.4.6 | Stability

Effect of acidic or alkaline hydrolysis

During forced degradation in 8 M HCl by incubation in a pressure cooker, EG-GLUC was degraded completely, while 87% of EG-GLUC was recovered after autoclaving in 8 M NaOH.

Simple heating at 60°C or in a pressure cooker without hydrolyzing reagents; and with both 4 M HCl and 4 M NaOH did not affect the recovery of EG-GLUC (Table 3).

In-sampler stability

Samples were stable for at least 4 days at 20°C in the autosampler as the differences in pre- and post-analysis was comparable to inter-assay bias (Table 3). The maximum difference-% in a sample was 9.3.

Long-term stability

Serum calibrators with EG-GLUC (100, 50, 25 and 10 µmol/L) were stable for a minimum of 155 days at 4–8°C and for a minimum of two months at −20°C including freeze–thaw cycles (n = 3). Based on these data and the forced degradation experiment, EG-GLUC is considered stable in both aqueous solution and serum under standard laboratory working and storage conditions.

4.5 | Clinical sample results

The method was used to analyze authentic clinical serum samples (n = 31) from patients intoxicated with ethylene glycol. EG-GLUC was quantified in 15 of these samples, with a mean concentration of 6.5 µmol/L (1.6 mg/L), ranging from 2.3 to 15.6 µmol/L (0.55 to 3.7 mg/L). In 5 samples, EG-GLUC was detected below LOQ and in 11 samples EG-GLUC concentrations were below LOD (Table 4). There was no meaningful correlation between EG and EG-GLUC concentrations and statistical analysis on these data were not pursued.

4.6 | Potential use of EG-GLUC in bioanalysis

The glucuronidation in vivo of small aliphatic alcohols and carboxylic acids is complex and not yet fully understood. The ether glucuronide of an alcohol differs in terms of stability from the ester (acyl) glucuronide of a carboxylic acid, which can undergo rearrangement processes into isomers.[64]

TABLE 3 Summary of method validation data

Validation of the 7-point calibration curve (inter-assay, *n* = 7)	
Slope (mean ± S.D.)	0.173256 ± 0.004187
C.V. (%) Slope	2.42
r (mean ± S.D.)	0.999645 ± 0.000326
Intercept (mean ± S.D.)	0.050863 ± 0.033657
Limits of quantification and detection	
Upper limit of quantification (μmol/L)	100
Lower limit of quantification (μmol/L)	2
Limit of detection (μmol/L)	1
Carry-over	
Calibrator 250 μmol/L to blank sample (no IS), peak area-%	0,026%
In blanks in batches with clinical samples	< LOD
Stability toward heating and acid/alkaline hydrolysis (15 min)	
Recovery after heating at 60°C; or incubation in a pressure cooker	104%,;102%
Recovery after heating at 60°C with 4 M HCl; or 4 M NaOH	113%; 111%
Recovery after incubation in a pressure cooker with 8 M HCl	0%
Recovery after incubation in a pressure cooker with 8 M NaOH	87%
In-sampler stability (4 days at 20°C)	
Calibrators (kept at 18 days in a fridge prior to the experiment), mean difference-% and range	1.6 (−2.6 to 9.3)
Calibrators (kept at 7 days in a freezer prior to the experiment), mean difference-% and range	3.9 (−3.5 to 7.9)
Long-term stability	
Serum calibrators (100, 50, 25, and 10 μmol/L), 155 days at 4–8 °Crecovery-% mean and range	102 (98.3–108)
Serum calibrator (100 μmol/L) at −20°C, freeze–thaw cycles (*n* = 3) for two months, final recovery	96.4%
Ion suppression (matrix effect)	
Serum calibrators/aqueous calibrators, mean response-% and range	40.9 (30.3 to 49.4)
Recovery-% after ultrafiltration *	
Aqueous calibrators (12.5; 25 and 100 μmol/L), mean and range	97.5 (94.8 to 100)
Serum calibrators (12.5; 25, 50 and 100 μmol/L), mean and range	110.5 (103.5 to 118.8)

*quantified with calibration curve from aqueous calibrators (see text for explanation).

Consequently, in the present study only the ether β-*O*-glucuronide was synthesized, as this is the sole product formed in vivo by UDP-glucuronosyltransferase.

The most important prospect of EG-GLUC is the potential to use the metabolite as a biomarker for EG intoxication, in analogy with EtG, which is used as a routine biomarker for ethanol consumption.[53] This would require a comprehensive clinical evaluation of patients involving analysis of various biological matrices. Compared to the millimolar concentrations of EG present in blood after intoxications and potentially available as substrate for conjugation, the concentrations of EG-GLUC found in clinical samples were very low (Table 4).

4.6.1 | Comparison with EtG and EtS

Here, a comparison with ingested amounts of alcohol versus serum concentrations of EtG and EtS is relevant. In a study of healthy volunteers, after intake of a single ethanol dose (44–90 g), EtG did not exceed 16.6 μmol/L (3.7 mg/L) and EtG in serum peaked 2–3.5 hours later than ethanol and could still be determined up to 8 hours after complete ethanol elimination.[66] There was an exponential decline in EtG with a half-life of 2–3 hours.

It is important that concentrations of EG and EtG do not peak at the same which was also shown in a kinetic model of the pharmacokinetics of EtG in humans based on data from drinking experiments.[67]

In a kinetic study of EtG in heavy drinkers (*n* = 14) during detoxification, EtG in serum was in the range 0.45 to 27 μmol/L (0.1 to 5.9 mg/L) and EtS in serum was in the range 0.79 to 8.6 μmol/L (0.1 to 1.9 mg/L), except for one subject with outlying EtG and EtS concentrations caused by serious renal and hepatic disease. The last ethanol intake was 122 g median (range 25 to 376 g).[68]

In a pharmacokinetic study by Halter et al of EtG formation after medium doses of ethanol (0.5 to 0.78 g ethanol/kg body mass), for

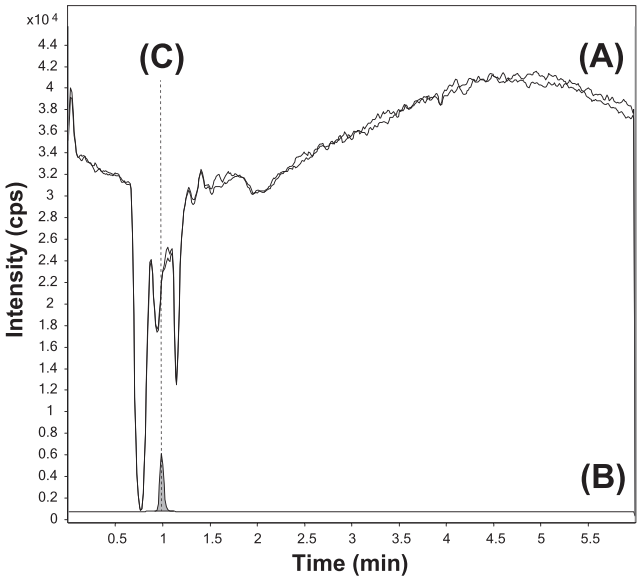

FIGURE 5 Post-column infusion experiment to determine matrix effects (ion suppression/enhancement). Concentration of EG-GLUC in eluent after infusion: 25 µmol/L. A, Total ion chromatogram (TIC) in counts-per-second (cps) after injection of blank serum ultrafiltrate (two injections overlaid). B, Total ion chromatogram (TIC) of EG-GLUC standard (25 µmol/L). C, Dotted line shows the retention time of EG-GLUC

which equimolar ingestions of EG could be life threatening, serum concentrations of EtG peaked in the range 1.2 to 4.9 µmol/L, and decreased to 1–2 µmol/L after 10–11 hours.[69] The LC–MS/MS method used by Halter et al has an LOQ of 0.45 µmol/L for EtG, with a 10-fold higher injection volume as used in the present study.

Thus, the serum concentrations of EG-GLUC found in the present study are comparable to the peak and ranges of EtG or EtS in serum expected after intake of moderate to high ethanol doses. The low or missing EG-GLUC concentrations could partly be explained by early sampling, also taken into consideration that concentrations below 1 µmol/L are not detected by the applied method.

4.6.2 | The prospects of urine analysis

Studies on EtG, EtS, and γ-hydroxybutanoic acid glucuronide (GHB-GLUC) have shown at least 100 times higher peak concentrations of glucuronides in urine versus plasma.[70,71] For qualitative analysis of EG-GLUC to report whether the patient has ingested EG, or for determination of chronic exposure, urine may be a more suitable sample matrix, although serum/plasma or whole blood is normally preferred for clinical toxicology analysis of EG. For urine analysis, a suitable cut-off concentration would be needed, for example based on EG-GLUC levels related to occupational exposure to EG. In a study of Canadian aviation workers exposed to de-icing fluid, EG was measured in next-morning urine samples up to 129 mmol/mol creatinine without indication of important health effects from the exposure.[72]

TABLE 4 Analysis of EG-GLUC in clinical serum/plasma samples from patients with confirmed ethylene glycol intoxications (n = 31)

Sample #	EG		EG-GLUC	
	mmol/L*	g/L	µmol/L	mg/L
1	n.a.	n.a.	3.4	0.81
2	n.a.	n.a.	7.2	1.7
3	n.a.	n.a.	2.3	0.55
4	n.a.	n.a.	4.9	1.2
5	n.a.	n.a.	3.0	0.72
6	n.a.	n.a.	< 2	< 0.48
7	n.a.	n.a.	15.6	3.7
8	n.a.	n.a.	13.4	3.2
9	n.a.	n.a.	12.6	3.0
10	n.a.	n.a.	14.8	3.5
11	n.a.	n.a.	< LOD	< LOD
12	n.a.	n.a.	5.7	1.4
13	n.a.	n.a.	< 2	< 0.48
14	n.a.	n.a.	< 2	< 0.48
15	75.9	4.7	< LOD	< LOD
16	18.2	1.1	2.8	0.67
17	12.2	0.76	2.5	0.60
18	4.0	0.25	< 2	< 0.48
19	1.2	0.074	< LOD	< LOD
20	3.3	0.21	< LOD	< LOD
21	8.7	0.54	< 2	< 0.48
22	17.9	1.1	< LOD	< LOD
23	3.7	0.23	< LOD	< LOD
24	2.0	0.12	< LOD	< LOD
25	1.2	0.075	< LOD	< LOD
26	2.3	0.14	2.2	0.52
27	3.1	0.19	< LOD	< LOD
28	3.8	0.24	< LOD	< LOD
29	22.4	1.4	< LOD	< LOD
30	5.6	0.35	2.4	0.57
31	14.5	0.90	4.3	1.0

*EG analysis by GC-FID available, LOQ: 1 mmol/L (0.062 g/L).

n.a. = not available.

Unfortunately, urine samples from clinical toxicology or working places were not available for the present study.

4.6.3 | Other conjugates of EG

The role of sulfate conjugation in the metabolism of EG has not been investigated and a reference standard of this compound (4-(sulfooxy) ethylene glycol, EGS) is not commercially available. Therefore, at present it is unknown whether a sulfate of EG is present in clinical

toxicology samples. The synthesis of EGS should in principle be possible by a method previously reported by Mehling et al by treating EG with sulfurtrioxide pyridine complex.[73] However, due to the presence of two hydroxy groups in EG, o- and di-sulfonated as well as unreacted EG is likely to be obtained. Moreover, the stability of EGS is likely to be poor because the sulfonated alcohol represents a good leaving group, which by intramolecular ring closure would expel sulfate to form ethyleneoxide.

Formation of double conjugates in humans by UDP glucuronosyltransferase of both the acyl- and aryl types have been reported for, for example morphine (morphine 3,6-diglucuronide),[74] bilirubin (bilirubin diglucuronide)[75] and bisphenol-A (mono- and di-glucuronides, mono- and di-sulfates).[76] Polyphenols may contain mixed sulfates/glucuronide conjugates,[77] and this may theoretically also be the case for glycols.

It may be possible to qualitatively search for the presence of disulfates, diglucuronides, or mixed sulfate/glucuronide metabolites of ethylene glycol in biological samples by non-targeted analysis with high resolution mass spectrometers. However, it was outside the scope of the present study, where the clinical sample amount was limited and none of these metabolites are available as chemical standards.

4.7 | Limitations of the study

The present study was solely aimed at the proof-of-concept for the in vivo formation of EG-GLUC and the authors do not make any claims about the suitability of EG-GLUC as a biomarker in serum for ingested EG. In fact, this aspect does not look promising for serum as a sample matrix, based on the data obtained by the LC–MS/MS method used in this study. Further research of EG-GLUC in serum should optimize the LC–MS/MS method sensitivity in order to detect sub-micromolar concentrations.

The choice of analytical column was based on our experience with high robustness and reproducibility of the biphenyl phase in routine determination of other glucuronides, including direct injection of biological specimen (urine, serum ultrafiltrate). However, the retention of these very polar compounds is poor, and matrix effects can cause bias and impression if co-eluting, isotope-labeled ISs are not used. More polar column materials or hydrophilic interaction chromatography (HILIC) could theoretically be promising regarding matrix effects, but this approach was not pursued in the present study. In addition, we believe that HILIC would be better suited for protein precipitation with acetonitrile rather than for direct injection of serum ultrafiltrate.

The present study shows 11 clinical samples >(Table 4) where EG-GLUC could not be detected. Although the stability testing showed EG-GLUC to be stable for at least 155 days at 4–8°C and a minimum of 2 months at −20°C (including 3 freeze-thaw cycles), degradation by enzymes or other hydrolysis mechanisms in serum cannot be excluded during the total storage period for up to one year in the present study. Furthermore, it is uncertain whether clinical and medical treatment of patients may have influenced the formation of EG-GLUC in vivo.

Stock solution and pure solid substance stability was not studied due to the limited amount of EG-GLUC synthesized.

5 | CONCLUSIONS

We have shown that EG-GLUC is a hitherto unrecognized metabolite of EG with a prospective use in analytical toxicology. Methods for chemical synthesis of reference standards of EG-GLUC and an isotope-labeled IS d_4-EG-GLUC are now available for continued research in the subject. The LC–MS/MS method − which was developed and validated specifically for the present study − can be adapted using fewer qualifying ions and a higher injection volume for routine determination of EG-GLUC in serum or other matrices.

To address the potential value of including EG-GLUC in bioanalytical methods for diagnosis of EG ingestion, the pharmacokinetic properties and the biosynthetic pathways of EG-GLUC must be characterized by in vitro system studies, analyses in a larger number of intoxicated patients, and analyses in other biological matrices, including a complete profile of all relevant EG metabolites.

If analytical procedures for the determination of EG in blood are not available for patient-near testing − and EG-GLUC is proven useful as biomarker for EG intoxication − the growing number of clinical biochemical laboratories with access to routine LC–MS/MS apparatus could analyze EG-GLUC and ensure faster diagnosis. This could prospectively benefit treatment outcome.

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REFERENCES

1. Plastic Insight. Mono-Ethylene Glycol (MEG): Production, Market, Price and its Properties. Available at https://www.plasticsinsight.com/resin-intelligence/resin-prices/mono-ethylene-glycol-meg. Assessed on November 6, 2018
2. Porter WH. Ethylene glycol poisoning: quintessential clinical toxicology; analytical conundrum. *Clin Chim Acta*. 2012;413(3–4):365-377.
3. Khan SA, Schell MM, Trammel HL, Hansen SR, Knight MW. Ethylene glycol exposures managed by the ASPCA National Animal Poison Control Center from July 1995 to December 1997. *Vet Hum Toxicol*. 1999;41(6):403-406.
4. Jobson MA, Hogan SL, Maxwell CS, et al. Clinical features of reported ethylene glycol exposures in the United States. *PLoS ONE*. 2015;10(11):e0143044.
5. U.S. National Library of Medicine (NKM), U.S. Department of Health & Human Services (DHHS). Available at https://toxnet.nlm.nih.gov/cgi-

bin/sis/search/a?dbs+hsdb:@term+@DOCNO+69. Accessed on August 3, 2018

6. Kruse JA. Methanol and ethylene glycol intoxication. *Crit Care Clin*. 2012;28(4):661-711.
7. Rietjens SJ, de Lange DW, Meulenbelt J. Ethylene glycol or methanol intoxication: which antidote should be used, fomepizole or ethanol? *Neth J Med*. 2014;72(2):73-79.
8. Thanacoody RH, Gilfillan C, Bradberry SM, et al. Management of poisoning with ethylene glycol and methanol in the UK: a prospective study conducted by the National Poisons Information Service (NPIS). *Clin Toxicol (Phila)*. 2016;54(2):134-140.
9. Ghannoum M, Hoffman RS, Mowry JB, Lavergne V. Trends in toxic alcohol exposures in the United States from 2000 to 2013: a focus on the use of antidotes and extracorporeal treatments. *Semin Dial*. 2014;27(4):395-401.
10. Ford LT, Berg JD. Five-year review of a UK 24 hour testing service for plasma ethylene glycol and diethylene glycol. *Ann Clin Biochem*. 2016;53(Pt 4(4):459-465.
11. Jorens PG. Ethylene glycol poisoning and lactate concentrations. *J Anal Toxicol*. 2009;33(7):395. author reply 6
12. Manini AF, Hoffman RS, McMartin KE, Nelson LS. Relationship between serum glycolate and falsely elevated lactate in severe ethylene glycol poisoning. *J Anal Toxicol*. 2009;33(3):174-176.
13. Verelst S, Vermeersch P, Desmet K. Ethylene glycol poisoning presenting with a falsely elevated lactate level. *Clin Toxicol (Phila)*. 2009;47(3):236-238.
14. Ammar KA, Heckerling PS. Ethylene glycol poisoning with a normal anion gap caused by concurrent ethanol ingestion: importance of the osmolal gap. *Am J Kidney Dis*. 1996;27(1):130-133.
15. Giner T, Ojinaga V, Neu N, Koessler M, Cortina G. Ethylene glycol intoxication presenting with high anion gap metabolic acidosis, acute kidney injury and elevated lactate. *Pediatr Int*. 2018;60(2):194-195.
16. Krasowski MD, Wilcoxon RM, Miron J. A retrospective analysis of glycol and toxic alcohol ingestion: utility of anion and osmolal gaps. *BMC Clin Pathol*. 2012;12:1-10. https://doi.org/10.1186/1472-6890-12-1 Assessed on November 6, 2018.
17. Leon M, Graeber C. Absence of high anion gap metabolic acidosis in severe ethylene glycol poisoning: a potential effect of simultaneous lithium carbonate ingestion. *Am J Kidney Dis*. 1994;23(2):313-316.
18. Moossavi S, Wadhwa NK, Nord EP. Recurrent severe anion gap metabolic acidosis secondary to episodic ethylene glycol intoxication. *Clin Nephrol*. 2003;60(3):205-210.
19. Purssell RA, Lynd LD, Koga Y. The use of the osmole gap as a screening test for the presence of exogenous substances. *Toxicol Rev*. 2004;23(3):189-202.
20. McQuade DJ, Dargan PI, Wood DM. Challenges in the diagnosis of ethylene glycol poisoning. *Ann Clin Biochem*. 2014;51(Pt 2):167-178.
21. Bost RO, Sunshine I. Ethylene glycol analysis by gas chromatography. *J Anal Toxicol*. 1980;4(2):102-103.
22. Cummings KC, Jatlow PI. Sample preparation by ultrafiltration for direct gas chromatographic analysis of ethylene glycol in plasma. *J Anal Toxicol*. 1982;6(6):324-326.
23. Jonsson JA, Eklund A, Molin L. Determination of ethylene glycol in postmortem blood by capillary gas chromatography. *J Anal Toxicol*. 1989;13(1):25-26.
24. Fraser AD, MacNeil W. Colorimetric and gas chromatographic procedures for glycolic acid in serum: the major toxic metabolite of ethylene glycol. *J Toxicol Clin Toxicol*. 1993;31(3):397-405.
25. Houze P, Chaussard J, Harry P, Pays M. Simultaneous determination of ethylene glycol, propylene glycol, 1,3-butylene glycol and 2,3-butylene glycol in human serum and urine by wide-bore column gas chromatography. *J Chromatogr*. 1993;619(2):251-257.
26. Aarstad K, Dale O, Aakervik O, Ovrebo S, Zahlsen K. A rapid gas chromatographic method for determination of ethylene glycol in serum and urine. *J Anal Toxicol*. 1993;17(4):218-221.
27. Dasgupta A, Blackwell W, Griego J, Malik S. Gas chromatographic-mass spectrometric identification and quantitation of ethylene glycol in serum after derivatization with perfluorooctanoyl chloride: a novel derivative. *J Chromatogr B Biomed Appl*. 1995;666(1):63-70.
28. Dasgupta A, Macaulay R. A novel derivatization of ethylene glycol from human serum using 4-carbethoxyhexafluorobutyryl chloride for unambiguous gas chromatography-chemical ionization mass spectrometric identification and quantification. *Am J Clin Pathol*. 1995;104(3):283-288.
29. Porter WH, Rutter PW, Yao HH. Simultaneous determination of ethylene glycol and glycolic acid in serum by gas chromatography-mass spectrometry. *J Anal Toxicol*. 1999;23(7):591-597.
30. Maurer HH, Peters FT, Paul LD, Kraemer T. Validated gas chromatographic-mass spectrometric assay for determination of the antifreezes ethylene glycol and diethylene glycol in human plasma after microwave-assisted pivalylation. *J Chromatogr B Biomed Sci Appl*. 2001;754(2):401-409.
31. Van Hee P, Neels H, De Doncker M, et al. Analysis of gamma-hydroxybutyric acid, DL-lactic acid, glycolic acid, ethylene glycol and other glycols in body fluids by a direct injection gas chromatography-mass spectrometry assay for wide use. *Clin Chem Lab Med*. 2004;42(11):1341-1345.
32. Meyer MR, Weber AA, Maurer HH. A validated GC-MS procedure for fast, simple, and cost-effective quantification of glycols and GHB in human plasma and their identification in urine and plasma developed for emergency toxicology. *Anal Bioanal Chem*. 2011;400(2):411-414.
33. Ehlers A, Morris C, Krasowski MD. A rapid analysis of plasma/serum ethylene and propylene glycol by headspace gas chromatography. *Springerplus*. 2013;2(1):203.
34. Hlozek T, Bursova M, Cabala R. Simultaneous and cost-effective determination of ethylene glycol and glycolic acid in human serum and urine for emergency toxicology by GC-MS. *Clin Biochem*. 2015;48(3):189-191.
35. Robson J, Townsend S, Bowdler P, Honeychurch KC. Direct thermal desorption gas chromatographic determination of toxicologically relevant concentrations of ethylene glycol in whole blood. *Analyst*. 2018;143(4):963-969.
36. Smith RA, Lang DG. Rapid determination of ethylene glycol and glycolic acid in biological fluids. *Vet Hum Toxicol*. 2000;42(6):358-360.
37. Gupta RN, Eng F, Gupta ML. Liquid-chromatographic determination of ethylene glycol in plasma. *Clin Chem*. 1982;28(1):32-33.
38. Wu NM, Malinin TI. High performance liquid chromatography determination of ethylene glycol and ethylene chlorohydrin in tissues. *J Anal Toxicol*. 1987;11(2):63-66.
39. Vollmer PA, Harty DC, Erickson NB, Balhon AC, Dean RA. Serum ethylene glycol by high-performance liquid chromatography. *J Chromatogr B Biomed Appl*. 1996;685(2):370-374.
40. Imbert L, Saussereau E, Lacroix C. Analysis of eight glycols in serum using LC-ESI-MS-MS. *J Anal Toxicol*. 2014;38(9):676-680.
41. Hansson P, Masson P. Simple enzymatic screening assay for ethylene glycol (ethane-1,2-diol) in serum. *Clin Chim Acta*. 1989;182(1):95-101.
42. Juenke JM, Hardy L, McMillin GA, Horowitz GL. Rapid and specific quantification of ethylene glycol levels: adaptation of a commercial

enzymatic assay to automated chemistry analyzers. *Am J Clin Pathol*. 2011;136(2):318-324.

43. Hanton SL, Watson ID. An enzymatic assay for the detection of glycolic acid in serum as a marker of ethylene glycol poisoning. *Ther Drug Monit*. 2013;35(6):836-843.
44. Rooney SL, Ehlers A, Morris C, et al. Use of a rapid ethylene glycol assay: a 4-year retrospective study at an Academic Medical Center. *J Med Toxicol*. 2016;12(2):172-179.
45. Robson AF, Lawson AJ, Lewis L, Jones A, George S. Validation of a rapid, automated method for the measurement of ethylene glycol in human plasma. *Ann Clin Biochem*. 2017;54(4):481-489.
46. Gomolka E, Cudzich-Czop S, Sulka A. Determination of ethylene glycol in biological fluids--propylene glycol interferences. *Przegl Lek*. 2013;70(8):511-513.
47. Dziadosz M. Direct analysis of ethylene glycol in human serum on the basis of analyte adduct formation and liquid chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2018;1072:100-104.
48. Kaeferstein H. Forensic relevance of glucuronidation in phase-II-metabolism of alcohols and drugs. *Leg Med (Tokyo)*. 2009;11(Suppl 1): S22-S26.
49. McDonell MG, Skalisky J, Leickly E, et al. Using ethyl glucuronide in urine to detect light and heavy drinking in alcohol dependent outpatients. *Drug Alcohol Depend*. 2015;157:184-187.
50. Arndt T, Beyreiss R, Hartmann W, Schrofel S, Stemmerich K. Excessive urinary excretion of isopropyl glucuronide after isopropanol abuse. *Forensic Sci Int*. 2016;266:250-253.
51. Dias AS, Castro AL, Melo P, et al. A fast method for GHB-GLUC quantitation in whole blood by GC-MS/MS (TQD) for forensic purposes. *J Pharm Biomed Anal*. 2017;150:107-111.
52. Verbeek J, Crunelle CL, Leurquin-Sterk G, et al. Ethyl glucuronide in hair is an accurate biomarker of chronic excessive alcohol use in patients with alcoholic cirrhosis. *Clin Gastroenterol Hepatol*. 2017;16:454-456.
53. Walsham NE, Sherwood RA. Ethyl glucuronide. *Ann Clin Biochem*. 2012;49(Pt 2):110-117.
54. Nymann Petersen I, Langgaard Kristensen J, Tortzen C, Breindahl T, Sejer Pedersen D. Synthesis and stability study of a new major metabolite of gamma-hydroxybutyric acid. *Beilstein J Org Chem*. 2013;9:641-646.
55. 2002/657/EC: Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Available at https://publications.europa.eu/en/publication-detail/-/publication/ed928116-a955-4a84-b10a-cf7a82bad858/language-en. Assessed on November 6, 2018.
56. World Anti-Doping Agency: WADA Technical Document – TD2010IDCR: Identification criteria for qualitative assays incorporating column chromatography and mass spectrometry. 2010. Available at https://www.wada-ama.org/sites/default/files/resources/files/WADA_TD2010IDCRv1.0_Identification%20Criteria%20for%20Qualitative%20Assays_May%2008%202010_EN.doc.pdf. Assessed on November 6, 2018.
57. Maralikova B, Weinmann W. Confirmatory analysis for drugs of abuse in plasma and urine by high-performance liquid chromatography-tandem mass spectrometry with respect to criteria for compound identification. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2004;811(1):21-30.
58. Guidance for Industry, Bioanalytical Method Validation (2001). U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM). Available at https://www.fda.gov/downloads/drugs/guidances/ucm070107.Pdf. Accessed on November 6, 2018.
59. Janda I, Weinmann W, Kuehnle T, Lahode M, Alt A. Determination of ethyl glucuronide in human hair by SPE and LC-MS/MS. *Forensic Sci Int*. 2002;128(1–2):59-65.
60. Petersen IN, Tortzen C, Kristensen JL, Pedersen DS, Breindahl T. Identification of a new metabolite of GHB: gamma-hydroxybutyric acid glucuronide. *J Anal Toxicol*. 2013;37(5):291-297.
61. Gu J, Zhong D, Chen X. Analysis of O-glucuronide conjugates in urine by electrospray ion trap mass spectrometry. *Fresen J Anal Chem*. 1999;365(6):553-558.
62. Boudinot FD, Homon CA, Jusko WJ, Ruelius HW. Protein binding of oxazepam and its glucuronide conjugates to human albumin. *Biochem Pharmacol*. 1985;34(12):2115-2121.
63. Hoiseth G, Morini L, Polettini A, et al. Serum/whole blood concentration ratio for ethylglucuronide and ethyl sulfate. *J Anal Toxicol*. 2009;33(4):208-211.
64. Skonberg C, Olsen J, Madsen KG, Hansen SH, Grillo MP. Metabolic activation of carboxylic acids. *Expert Opin Drug Metab Toxicol*. 2008;4(4):425-438.
65. Blom HJ, van Rooij A, Hogeveen M. A simple high-throughput method for the determination of plasma methylmalonic acid by liquid chromatography-tandem mass spectrometry. *Clin Chem Lab Med*. 2007;45(5):645-650.
66. Schmitt G, Droenner P, Skopp G, Aderjan R. Ethyl glucuronide concentration in serum of human volunteers, teetotalers, and suspected drinking drivers. *J Forensic Sci*. 1997;42(6):1099-1102.
67. Droenner P, Schmitt G, Aderjan R, Zimmer H. A kinetic model describing the pharmacokinetics of ethyl glucuronide in humans. *Forensic Sci Int*. 2002;126(1):24-29.
68. Hoiseth G, Morini L, Polettini A, Christophersen A, Morland J. Blood kinetics of ethyl glucuronide and ethyl sulphate in heavy drinkers during alcohol detoxification. *Forensic Sci Int*. 2009;188(1–3):52-56.
69. Halter CC, Dresen S, Auwaerter V, Wurst FM, Weinmann W. Kinetics in serum and urinary excretion of ethyl sulfate and ethyl glucuronide after medium dose ethanol intake. *Int J Leg Med*. 2008;122(2):123-128.
70. Lostia AM, Vicente JL, Cowan DA. Measurement of ethyl glucuronide, ethyl sulphate and their ratio in the urine and serum of healthy volunteers after two doses of alcohol. *Alcohol Alcohol*. 2013;48(1):74-82.
71. Mehling L-M, Piper T, Spottke A, et al. GHB-O-β-glucuronide in blood and urine is not a suitable tool for the extension of the detection window after GHB intake. *Forensic Toxicol*. 2017;35(2):263-274.
72. Gerin M, Patrice S, Begin D, et al. A study of ethylene glycol exposure and kidney function of aircraft de-icing workers. *Int Arch Occup Environ Health*. 1997;69(4):255-265.
73. Mehling L-M, Piper T, Dib J, et al. Development and validation of a HPLC-QTOF-MS method for the determination of GHB-β-O-glucuronide and GHB-4-sulfate in plasma and urine. *Forensic Toxicol*. 2017;35(1):77-85.
74. Yeh SY, Gorodetzky CW, Krebs HA. Isolation and identification of morphine 3- and 6-glucuronides, morphine 3,6-diglucuronide, morphine 3-ethereal sulfate, normorphine, and normorphine 6-glucuronide as morphine metabolites in humans. *J Pharm Sci*. 1977;66(9):1288-1293.
75. Burchell B, Blanckaert N. Bilirubin mono- and di-glucuronide formation by purified rat liver microsomal bilirubin UDP-glucuronyltransferase. *Biochem J*. 1984;223(2):461-465.
76. Ho KL, Yuen KK, Yau MS, et al. Glucuronide and sulfate conjugates of bisphenol A: Chemical synthesis and correlation between their urinary levels and plasma bisphenol A content in voluntary human donors. *Arch Environ Contam Toxicol*. 2017;73(3):410-420.

77. van der Woude H, Boersma MG, Vervoort J, Rietjens IM. Identification of 14 quercetin phase II mono- and mixed conjugates and their formation by rat and human phase II in vitro model systems. *Chem Res Toxicol*. 2004;17(11):1520-1530.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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