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Highlights

Procedures for analysis of an isothiocyanate in rat blood including Edman degradation and “total measurement“(free plus sulfhydryl bound)

Analytical Approaches for Quantification of a Nrf2 Pathway Activator: Overcoming Bioanalytical Challenges to Support a Toxicity Study

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Abstract

Activation of the Nrf2 stress pathway is known to play an important role in the defense mechanism against electrophilic and oxidative damage to biological macromolecules (DNA, lipids, and proteins). Chemical inducers of Nrf2 such as sulforaphane, dimethyl fumarate (Tecfidera®), CDDO-Me (bardoxolone-methyl), and 3-(dimethylamino)-4-((3-isothiocyanatopropyl)(methyl)amino)cyclobut-3-ene-1,2-dione (a synthetic sulforaphane analogue; will be referred as **1**) have the ability to react with Keap1 cysteine residues, leading to activation of the Antioxidant Response Element (ARE). Due to their electrophilic nature and poor matrix stability, these compounds represent great challenges when developing bioanalytical methods to evaluate in-vivo exposure. **1** like SFN, reacts rapidly with glutathione (GSH) and nucleophilic groups in proteins to form covalent adducts. In this work, three procedures were developed to estimate the exposure of **1** in a non-GLP 7-day safety study in rats: (1) protein precipitation of blood samples with methanol containing free thiol trapping reagent 4 - fluoro - 7 - aminosulfonylbenzofurazan (ABD-F) to measure GSH- and N-acetyl cysteine conjugated metabolites of **1**; (2) an Edman degradation procedure to cleave and analyze N-terminal adducts of **1** at the valine moiety; and (3) treatment with ammonium hydroxide to measure circulating free- and all sulfhydryl bound **1**.

Keywords: 3-(Dimethylamino)-4-((3-isothiocyanatopropyl)(methyl)amino)cyclobut-3-ene-1,2-dione; sulforaphane; Edman degradation; covalent drugs, isothiocyanates.

1. Introduction

The nuclear factor-E2 related factor 2 (Nrf2) pathway (see Fig. 1) is believed to play an essential role in activating cellular defense mechanisms against oxidative stress by activation of a large set of Phase II metabolizing enzymes, such as glutathione S-transferase and quinone reductase [NAD(P)H: (quinone-acceptor) oxidoreductase 1][1-3]. As a key transcription factor, the levels of Nrf2 are highly regulated in cells[4]. Nrf2 binds to a dimeric protein, Keap1, which targets it for Cullin-3 mediated ubiquitin degradation[3,5]. Under stress conditions, the Keap1/Cul3-dependent degradation of Nrf2 is disrupted leading to accumulation of Nrf2 in the cytoplasm which is followed by translocation to the nucleus where it complexes with other proteins (such as MafK), binds Antioxidant Response Elements (ARE) of DNA and ultimately leads to induction of the Phase II gene machinery[3,5]. There are two approaches for activation of the Nrf2 pathway (see Fig. 1): (1) targeting the direct inhibition of Nrf2 binding to the Kelch domain of Keap1[6-7] and (2) disrupting the Keap1/Cul3/Nrf2 complex by reversible-covalent modification of reactive cysteine residues including Cys151 in the BTB domain of Keap1[8-15]. Alkylation of reactive cysteine residues including Cys151 is considered to be the mechanism of action of many natural and synthetic Nrf2 activators including sulforaphane (SFN)[8-13], CDDO-Me (bardoxolone-methyl)[14], and dimethylfumarate (Tecfidera®)[15]. This mechanism has been evaluated in drug discovery programs to target diseases such as chronic kidney disease, obstructive pulmonary disease (COPD), asthma, multiple sclerosis (MS) and Parkinson's[16].

SFN is a molecule containing an isothiocyanate functional group and is generated by the enzyme myrosinase from the natural product, glucoraphanin, when cruciferous vegetables such as broccoli, brussels sprouts or cabbages [9] are damaged (e.g. by chewing). Animal experimental models suggest that SFN may have anti-cancer and antimicrobial activity[9-11] and several clinical trials are in progress[12-13] including a phase II trial for prostate cancer[13] . SFN is a liquid and therefore its oral formulations are limited. For this reason, SFN has mostly been administered, in the form of its natural product precursor, the glucosinolate glucoraphanin, as extract from three-day old broccoli sprouts[12]. Evgen Pharma has developed a stable synthetic formulation, Sulforadex®, containing SFN as non-cytotoxic agent against tumor proliferation and also designed to target and destroy cancer stem cells, the underlying cause of tumor recurrence and metastasis [17]. The company reported successful completion of a first-in-man clinical trial; and a trial in prostate cancer patients is intended for 2014[17].

3-(Dimethylamino)-4-((3-isothiocyanatopropyl)(methyl)amino)cyclobut-3-ene-1,2-dione (**1,** see Fig 2) is a synthetic isothiocyanate (SFN analog) with *in vitro* potency similar to SFN and as a crystalline solid compound, it provides the potential for improved developability properties compared to SFN itself. Compound **1**, like SFN, readily undergoes conjugation with glutathione (GSH) and nucleophilic groups in other proteins. The GSH conjugated metabolite of **1** (referred as conjugate **2** in this publication), as many other GSH conjugates[18], is expected to be formed in the liver and further metabolized in the kidney by gamma-glutamyltransferase and dipeptidases; enzymes that catalyze the sequential removal of the glutamyl and glycyl moieties;

respectively, to form a cysteine S-conjugate. Cysteine conjugate metabolites of **1** is then transported back to the liver and acetylated by intracellular N-acetyl-transferases, to form a mercapturic acid derivative or N-acetylcysteine (NAC) S-conjugate (referred as conjugate **3** in this publication).

In this work, three procedures were developed to estimate the exposure of **1** in a non-GLP 7-day safety study in rats: (1) protein precipitation of blood samples upon collection (separate aliquot) with methanol containing free thiol trapping reagent 4 - fluoro - 7 - aminosulfonylbenzofurazan (ABD-F) to measure conjugates **2** and **3**; (2) an Edman degradation procedure of blood samples to analyze N-terminal adducts of **1** at the valine moiety in hemoglobin; (3) treatment of blood samples with ammonium hydroxide leading to the formation of a thiourea derivative **4** allowing analysis of "total measurement" (free **1** plus sulfhydryl bound **1**; e.g. bound to GSH (conjugate **2**), N-acetylcysteine (conjugate **3**), cysteine in hemoglobin). These methodologies enabled a successful bioanalytical support of a non-GLP 7-day rat safety study with oral doses of **1** at 3, 30 or 100 mg/kg/day. The study was successfully completed and evaluated. These procedures may be applied to similar compounds containing isothiocyanates functional groups such as SFN.

2. Experimental

2.1. Chemicals and reagents

SFN (≥90% (HPLC), synthetic liquid), ABD-F, ammonium formate, acetonitrile, methanol, dimethylformamide (DMF), 3,4-diethoxycyclobut-3-ene-1,2-dione dichloromethane, triethylamine, glutathione (GSH), N-acetyl-cysteine (NAC), carbon disulfide (CS_2), tosyl chloride (TsCl), hexane, dioxane, isopropanol, nitrophenyl isothiocyanate, trifluoroacetic acid (TFA) and tert-butyl methyl ether (MTBE) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid was purchased from Alfa Aesar (Ward Hill, MA, USA). Compounds **1-4** and the alkylated N-terminal peptide 1-VHLTPEEK were prepared by the Respiratory Stress & Repair DPU at GlaxoSmithKline (King of Prussia, USA). tert-Butyl (3-(methylamino)propyl)carbamate was obtained from Advanced ChemBlocks Inc, (Burlingame, CA, USA) . Ethanol was purchased from Decon labs, Inc.(King of Prussia, PA, USA). VHLTPEEK was prepared by 21 Century Biochemicals (Marlborough, MA, USA). Rat blood was obtained from Bioreclamation Inc. (East Meadow, NY, USA).

2.2. Equipment

An Eppendorf 5810R centrifuge with a rotor capacity for four 96–well plates (Brinkmann Instrument, Westbury, NY, USA) and a Mettler UMX2 balance (Columbus, OH, USA) were used. ArcticWhite LLC 96-well round 2 mL polypropylene plates, ArctiSeal silicone mats with PTFE film (Bethlehem, PA, USA), VWR Economy

Incubator model 1500E (Radnor, PA, USA) and Barnstead Lab Line Titer Plate Shaker (Radnor, PA, USA) were used. Waters one-milliliter plastic plates (Milford, MA, USA) along with Arctiseal mats (Bethlehem, PA, USA) were used for sample introduction to the LC-MS/MS. An ACQUITY™ UPLC integrated system from Waters (Milford, MA, USA) consisting of a sample manager combined with a sample organizer, capable of holding ten 96-deep well plates, and a binary solvent manager were used. A triple quadrupole mass spectrometer API-4000 (Applied Biosystems/MDS–Sciex, Concord, Ontario, Canada) and Waters Xevo-TQS (Waters co, MA, USA) were used. Quadra 4 SPE™ Liquid Handling Workstation from TOMTEC (Connecticut, USA) was used for liquid-liquid extraction (LLE). The Biotage V-10™ solvent evaporation system (Biotage AB, Sweden) was in the synthesis of **1**-VHLTPEEK.

*2.3. Preparation of alkylated N-terminal peptide **1**-VHLTPEEK*

VHLTPEEK (20 mg) was dissolved in 2 mL of pyridine-water (50:50). The solution was warmed to 40°C and the pH was adjusted to 9.0 by adding three drops of 1 M NaOH. Then 11.18 mg **1** was added. The reaction was stirred at 40°C for 5.5 h. The solvent was evaporated using a Biotage V-10™ and purified by Gilson LC using a Sunfire C_{18} (19mm×100mm; 5µm). The mobile phase composition was 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). The separation was performed using a linear gradient from 10% B to 50%B in 15 min and the flow rate was kept at 18 mL/min. The desired product was characterized by LC-MS (m/z 1206.4 $[M+H]^+$) and the overall yield was 9.3 mg (24.5%).

*2.4. Preparation of compounds **1** to **4***

Compound **1** was prepared in four steps in 34% overall yield from the commercially available 3,4-diethoxycyclobut-3-ene-1,2-dione and tert-butyl (3-(methylamino)propyl)carbamate as depicted in Fig 2. Treatment of **1** with GSH and in the presence of sodium hydroxide (pH 7.8) provides conjugate **(2)**. Treatment of compound **1** with NAC in the presence of sodium hydroxide (pH 7.8) provides conjugate **3**. Treatment of **1** with ammonia provides compound **4** as described in Fig 2. These compounds were characterized by ^{1}H NMR and the following characteristic data was recorded. ^{1}H NMR (400 MHz, DMSO-d6) δ 3.60 - 3.95 (m, 4H), 3.27 - 3.41 (m, 3H), 3.21 (s, 6H), 1.75 - 2.17 (m, 2H) for **1**;^{1}H NMR (400 MHz, deuterium oxide) δ 2.02 - 2.14 (m, 4 H), 2.46 (m, 2 H), 3.18 (s, 3 H), 3.22 - 3.27 (m, 6 H), 3.50 (m, 1 H), 3.68 - 3.89 (m, 8 H), for **2**; ^{1}H NMR (400 MHz, deuterium oxide) δ 1.97 (s, 3 H), 2.04 (m, 2 H), 3.18 (s, 3 H), 3.24 (s, 6 H), 3.51 (m, 1 H), 3.70 - 3.89 (m, 5 H) for **3**; and ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.60 (br. s., 1H), 6.97 (br. s., 1H), 3.67 (br. s., 2H), 3.38 (br. s., 2H), 3.21 (s,6H), 3.13 (s, 3H), 1.81 (br. s., 2H) for **4**.

2.5. Internal standard (hemoglobin adduct of SFN) preparation

An aliquot of SFN (100 μL of 1 mg/ml acetonitrile) was added to 1.9 mL fresh rat blood. The above solution was incubated at 37ºC for 24 hours under constant and gentle mixing. After incubation, the solution was diluted 10-fold with water and used as the internal standard.

2.6. *Analysis of conjugates **2** and **3***

Stock solutions of conjugates **2** and **3** were individually prepared in 50/50 (v/v) acetonitrile/water solution at concentration of 0.5 mg/mL. These stock solutions were stored at 4ºC. Separate working solutions (WS) at 0.5 mM were prepared fresh on the day of analysis for **3** (WS-A) and **2** (WS-B), respectively, in 10 mM ammonium acetate (native pH). An aliquot of each of the working solutions (50 µL) were combined and added to 150 µL 10 mM ammonium acetate (native pH) to make working solution (WS1) containing **3** and **2** conjugates at concentration 100 µM. Two additional working solutions (WS2, WS3) at 10 and 1 µM were prepared by diluting WS1 10-and 100- fold, respectively, with 10 mM ammonium acetate (native pH).

These working solutions were used to make calibration standards in rat blood containing both analytes at 10000, 5000, 2500, 1000, 500, 250, 100, 50, 25, and 10 nM **3** and **2** conjugates in rat blood. The quality control (QC) samples were prepared in rat blood at 8000, 400, and 30 nM **3** and **2** conjugates. Due to limited stability of these analytes the samples were processed immediately following preparation as described below.

Aliquots (50 µL) of calibration and QC samples were transferred to uniquely-labeled MATRIX Screwtop Trakmate™ tubes (1.4 mL) with Screwtop Trakmate caps. An aliquot (400 µL) of internal standard solution (100 ng/mL of N-acetyl cysteine conjugate of SFN) in a cold methanol solution containing ABD-F at 1 mg/mL was added to all tubes with the exception of the blanks, which instead received 400 µL of 1 mg/mL ABD-

F solution in methanol. The tubes were capped and vortex-mixed for approximately 3 min. After vortex-mixing, the tubes were centrifuged for approximately 5 min at approximately 1000×g. After centrifugation, 350 µL of the supernatant was transferred to uniquely-labeled 1.4 mL MATRIX Screwtop Trakmate™ tubes with Screwtop Trakmate™ caps, and 20 µL of 10% formic acid in water was added to all tubes and briefly vortex-mixed. The samples were analyzed by LC-MS/MS.

*2.7. LC-MS/MS analysis of **3** and **2** conjugates*

The analytical column used was an Imtakt Cadenza CD-C18 50 mm x 2 mm, with 3 µm particle size. The column temperature was held at 30ºC and the sample compartment was at ambient temperature. Mobile phase A consisted of 0.1% formic acid in water and acetonitrile was used as mobile phase B. Mobile phase B was held at 10% until 0.3 min, followed by a linear gradient from 10% B to 80% B for 2.0 min, after which the system was returned to the initial condition. The total run time, including sample loading was approximately 3.0 min and the flow rate was maintained at 0.7 mL/min throughout the run. A typical injection volume of 4 µL in a 10 µL loop (partial loop injection mode) of a Waters® ACQUITY UPLC® H-Class System (SM-FTN) was used. The UPLC weak wash was a solution of 10/90 acetonitrile/water (v/v) and the strong wash consisted of 0.1% formic acid in a solution of 40:40:20 acetonitrile:isopropanol:water (v/v).

A Sciex API-4000 with a TurboIonspray (TIS) interface was operated in positive ionization mode. The instrument was optimized for conjugates **2**, **3** and the internal standard by infusing a 10 ng/mL solution of the analytes in acetonitrile/water (50/50 v/v)

at 0.5 mL/min through an auxiliary Agilent pump 1100 series (Palo Alto, CA, USA) directly connected to the mass spectrometer. The MRM transitions of m/z 417→254, m/z 561→254, and 341→114 were chosen for conjugates **2**, **3**, and the internal standard, respectively. Dwell times of 125 msec were used for the analytes and the internal standard. The optimized mass spectrometric conditions included the following MS conditions: TIS source temperature, 650ºC; TIS voltage, 5500 V; curtain gas, 40 psi (nitrogen); nebulizer gas (GS1), 80 psi (zero air); turbo gas (GS2), 80 psi (zero air); collision energy, 42 eV for **2** and 25 eV for **3**; declustering potential 60 eV.

2.8. Analysis of valine adducts in hemoglobin

Stock solutions of **1**-VHLTPEEK were prepared in water at a concentration of 1 mM. These stock solutions were stored at 4ºC. Additional working solutions of **1**-VHLTPEEK at 100, 20, and 4 μM were prepared fresh on the day of analysis in rat blood. These solutions were used to make calibration standards in rat blood at 100, 50, 20, 10, 4, 2, 1, 0.4, 0.2 and 0.1 μM **1**-VHLTPEEK in rat blood. The QC samples were prepared in rat blood at 80, 5, and 0.3 μM **1**-VHLTPEEK.

Aliquots of 20 μL of calibration and QC blood samples were transferred to uniquely-labeled MATRIX Screwtop Trakmate™ tubes (1.4 mL) with Screwtop Trakmate caps. A 20 μL aliquot of internal standard solution (alkylated blood with SFN see Section 2.4) was added to all wells with the exception of the blanks, which instead received 20 μL of water only. A 200 μL aliquot of methanol was added to all samples to precipitate blood proteins. The tubes were capped and vortex-mixed for approximately 3 min. After vortex-

mixing, the samples were centrifuged for approximately 5 min at approximately 3000×g to concentrate the samples on the bottom of the tube. The samples (containing the supernatant and precipitate) were dried down using a steady stream of nitrogen at 45°C. A 100 µL aliquot of concentrated TFA was then added to all dried samples followed by incubation at 65°C for 20 min under constant vortex mixing. After incubation, the samples were dried down using a steady stream of nitrogen at 45°C to remove TFA. An aliquot of acetonitrile (400 µL) was added to all tubes and followed by vortex-mixing of samples for approximately 5 min. The samples were then centrifuged for approximately 5 min to remove the solid residue at approximately 3000×g and 300 µL of supernatant was transferred to clean tubes. The samples were analyzed LC−MS/MS.

2.9. LC MS/MS analysis of valine adducts in hemoglobin

The analytical column used was an ACQUITY HSS-T3, 2.1 mm x 50 mm with 1.8 µm particle size from Waters Co. The column temperature was held at 65ºC and the sample compartment at 4 ºC. Mobile phase A consisted of 0.1% formic acid in water and mobile phase B was acetonitrile. Mobile phase B was held at 5% until 0.1 min, followed by a linear gradient from 5% B to 45% B for 1.2 min and then a steep gradient from 45% B to 95% B from 1.2 min to 1.21 min. The system was held at 95% B until 1.70 min to remove late eluting substances from the analytical column, after which the system was returned to the initial condition. The total run time, including sample loading was approximately 2.0 min and the flow rate was maintained at 0.7 mL/min throughout the run. A typical injection volume of 1 µL in a Waters® ACQUITY UPLC® I-Class System (SM-FTN) was used.

Waters Xevo-TQS (Waters co, MA, USA) with TIS interface was operated in the positive ionization mode. The instrument was optimized for Edman derivatives of **1**-valine and sulforaphane-valine by infusing corresponding derivatized solutions at 2 ng/mL in acetonitrile/water (50/50, v/v) using flow rate at 300 µL/min through an Agilent pump 1100 series (Palo Alto, CA, USA. The MRM transitions of m/z 353→254 and m/z 277→178 were used for the Edman derivatives of **1**-valine and sulforaphane-valine, respectively. The optimized mass spectrometric conditions for both analytes were used as follows: desolvation temperature, 600°C; source temperature, 150°C; cone voltage, 5 V; cone gas flow 150 L/h; desolvation gas flow, 900 L/h; nebulizer gas, 7 bar; and collision energy, 20 eV.

*2.10. Total measurement (**1** bound to sulfhydryl moiety and free fraction of **1**)*

Stock solutions of **1** were prepared in water at concentration of 3.95 mM. These stock solutions were stored at 4ºC. Additional working solutions of **1** at 100, 20, and 4 µM were prepared fresh on the day of analysis in rat blood. The WS were used to make calibration standards in rat blood at 100, 50, 20, 10, 4, 2, 1, 0.4, 0.2, and 0.1 µM **1** in rat blood. The QC samples were prepared in rat blood at 80, 5, and 0.3 µM **1**.

MTBE (0.5 mL) was added to each well of the 2 mL ArcticWhite 96-well PTFE coated plate. The plate was sealed with the ArcticSeal PTFE coated mat and vortex-mixed in an inverted position for approximately 3 min. Subsequently, the MTBE was discarded and the plate was left to dry in a chemical hood. This wash step was used to remove any plastic residue from the plates and plate seals. Aliquots (20 µL) of calibration

and QC blood samples were transferred to the washed ArcticWhite 96-well plate. A 120 µL aliquot of internal standard solution (freshly prepared 1 µg/mL of nitrophenyl isothiocyanate in ammonium hydroxide) was added to all wells with the exception of the blanks, which instead received 120 µL of concentrated ammonium hydroxide containing no internal standard solution. The plate was sealed with the ArcticSeal mat and vortex-mixed for approximately 2 min. After vortex-mixing, the plate was incubated for approximately 20 min at approximately 65°C. After incubation, 1 mL ethyl acetate was added to each well and vortex-mixed for 3 min. The plate was then centrifuged for approximately 5 min at approximately 3000×g. An aliquot (50 µL) of DMF was added to a clean silanized glass inserts (into the 2 ml 96-well collection plate). Using a liquid handler (Tomtech), 825 µL of the ethyl acetate supernatant was transferred to silanized glass inserts containing 50 µL of DMF. The ethyl acetate residue was removed using a steady stream of nitrogen at 45°C approximately to the level corresponding to 50 µL DMF. It is recommended to stop the drying process before DMF is completely evaporated. An aliquot of a 100 µL of water/acetonitrile solution (1/1; v/v) was then added to each wells. The plate with glass inserts was capped and vortex-mixed for approximately 1 min and then centrifuged for 2 min at approximately 1000×g. The samples were analyzed LC−MS/MS.

*2.11. LC-MS/MS analysis of compound **4** (**1**-thiourea derivative)*

The analytical column used was an ACQUITY HSS-T3, 2.1 mm x 50 mm with 1.8 µm particle size from Waters Co. The column temperature was held at 65ºC and the sample compartment was at 4ºC. Mobile phase A consisted of 0.1% formic acid in water

and acetonitrile was used as mobile phase B. Mobile phase B was held at 10% until 0.1 min, followed by a linear gradient from 10% B to 45% B for 1.2 min and then a steep gradient from 45% B to 95% B from 1.2 min to 1.21 min. The system was held at 95% B until 1.70 min to remove late eluting substances from the column, after which the system was returned to the initial condition. The total run time, including sample loading was approximately 2.0 min and the flow rate was maintained at 0.7 mL/min throughout the run. A typical injection volume of 1 µL in a Waters® ACQUITY UPLC® I-Class System (SM-FTN) was used. Waters Xevo-TQS (Waters co, MA, USA) with TIS interface was operated in the positive ionization mode. The instrument was optimized for the thiourea derivatives of **1** and nitrophenyl isothiocynate (internal standard), by infusing corresponding derivatized solutions at 2 ng/mL in acetonitrile/water (50/50, v/v) using flow rate of 300 µL/min through an Agilent pump 1100 series (Palo Alto, CA, USA. The MRM transitions of m/z 271→254 and m/z 198→135 were chosen for the thiourea derivatives of **1** and nitrophenyl isothiocynate, respectively. The optimized mass spectrometric conditions for both analytes were used as follows: desolvation temperature, 600°C; source temperature 150°C, cone voltage, 5 V; cone gas flow 150 L/h; desolvation gas flow, 900 L/h; nebulizer gas, 7 bar; collision energy for the thiourea derivatives of **1** and nitro phenyl isothiocynate were 20 eV and 30 eV, respectively.

2.12. Toxicity study

The 7-day oral toxicity study was conducted at GSK Safety Assessment Facility in accordance with the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals and was reviewed by the Institutional Animal Care and Use Committee at GSK.

The objective of the study was to characterize the toxicity and toxicokinetics (TK) of **1** following oral administration to male Crl:WI(Hans) rats [Charles River Laboratories, Inc., Stone Ridge, NY] for 7 days. Rats were group-housed (up to 3 rats/cage) in clear plastic cages with ALPHA-dri^{TM} bedding (Shepherd Specialty Papers, Inc., Kalamazoo, MI). The rats were in the range of 10 to 12 weeks of age and weighed approximately 200 to 400 g at the initiation of dosing. Rats were offered 5002 Certified Rodent Diet (PMI Nutrition International, Richmond, IN) ad libitum. Filtered tap water (supplied by Aqua Pennsylvania, Inc. and periodically analyzed) was available ad libitum from an automatic watering system. Compound **1** was administered orally, once daily for up to 7 days at 3, 30 or 100 mg/kg/day. The vehicle used was 0.5% hydroxpropylmethycellulose (K15M with 0.1% (w/v) polyoxyethylene sorbitan monooleate 80™. A serial sampling scheme was used (3 animals per dose group) at 0.25, 0.5, 1, 4, 8, and 24 h after dosing on Day1 and 7. The same animals provided samples on Day 1 and 7. Approximately 0.2 mL blood was collected from each rat per time point in uniquely labelled tubes containing EDTA and split into two aliquots (Aliquot A and B). For Aliquot-A, a 50 µL sample of whole blood was used for analysis of **2** and **3**. The remaining blood, Aliquot-B was used for analysis of total measurement and hemoglobin valine adducts. Aliquot A was precipitated immediately upon collection with internal standard solution (N-acetyl cysteine conjugate of SFN) in a solution of methanol containing ABD-F and further processed in an identical manner as the calibration and QC samples.

2.13. Data analysis

MS data were acquired and processed (integrated) using the proprietary software application Analyst™ (Version 1.4.2, Applied Biosystems/MDS–Sciex, Canada) for analysis of conjugates **2** and **3** and MassLynx 4.1 (Waters co, MA, USA) for analysis of the Edman- and thiourea- derivatives.

Calibration plots of analyte/internal standard peak area ratio versus analyte concentrations were constructed and a weighted $1/x^2$ linear regression was used. Concentrations of investigated analytes in validation samples were determined from the appropriate calibration line and used to calculate the bias and precision of the method with an in–house LIMS (Study Management System, SMS2000, version 2.3, GlaxoSmithKline).

Toxicokinetic analysis was performed by noncompartmental pharmacokinetic analysis using WinNonlin™ (WNL), Version 6.1(Pharsight Corp., USA)

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3. Results and discussion

3.1. Challenges during method development

The objective was to develop rugged and sensitive LC–MS/MS methods allowing determination of the exposure of **1** in a rat safety assessment study. The data generated was crucial for decision making in the further progression of **1** into development. The major challenge faced during method development was associated with poor stability and low recovery of **1** in rat blood. Approximately 90% loss of **1** was observed when blood was fortified and then immediately extracted using protein precipitation. Compound **1** reacts rapidly with GSH and nucleophilic groups in proteins (adducts), making it challenging to develop a method for determination of free **1** in blood. All attempts to establish a method for quantification of free **1** in rat blood were unsuccessful, with the results showing lack of reproducibility indicating that analysis of free **1** would be unreliable.

Since **1** like SFN contains an isothiocyanate functional group, it was decided to evaluate the method available for determination of SFN in biological matrices. Ye, et al.,[19] described a method for total analysis of isothiocyanates and dithiocarbamates using a reaction with 1,2-benzenedithiol to produce 1,3-benzodithiole-2-thione that can be quantified photodiode array integration. Although this cyclocondensation reaction has been highly useful for analyzing plant material and urine samples, the determination of isothiocyanetes in biological matrices has been limited by assay sensitivity and selectivity. Our efforts developing similar method for analysis of **1** using this reagent and

LC-MS/MS as a detector were also unsuccessful. Due to the importance of having methodologies available to support a rodent non-GLP toxicology study, numerous unconventional bioanalytical approaches were evaluated to estimate the exposure of **1** in rodents.

*3.2. Analysis of conjugates **2** and **3***

Considering the conjugate **2** exists in equilibrium with the free **1**, in order to avoid any potential shift in the equilibrium during sample handling, it was decided to process blood samples immediately upon collection. Conjugate **2** and **3** were analyzed the same day of sample collection by protein precipitation using ice-cold methanol solution. It was observed that the recovery and consistency of the assay was improved by adding ABD-F at 1 mg/mL to the methanol solution. ABD-F is well documented to react with GSH and cysteine in proteins. It is important to clarify that ABD-F is used in this procedure to alkylate free thiols (thiol scavengers) in rat blood to prevent thiol exchange reactions and it does not form any derivative with conjugates **2** and **3.** In addition, formic acid was used to stabilize these conjugates during storage in the auto sampler. Based on QC data analysis, conjugates **2** and **3** are stable in the described process extract solution for at least 24 hours at ambient temperature.

*3.3. Edman degradation procedure to cleave and analyze N-terminal adducts of **1***

Another approach evaluated during method development in the intention to assess exposure of **1** in the safety study utilized the Edman degradation procedure[20] to determine adducts formed on the N-terminal valine. Edman degradation procedure is typically used

to determine the peptide amino acid sequence from the N-terminus (see Fig. 3). This technique employs the reaction of the N-terminal α amino group with phenyl isothiocyanate at slightly basic pH to give a phenylthiocarbamyl (PTC) derivative[20]. The PTC derivative is then treated with a strong acid (e.g., TFA) to cleave the peptide at the last peptide bond leading to the formation of a phenylthiohydantoin (see Fig. 3). Compound **1** is expected to react with N-terminal valine in hemoglobin in a similar way, and if treated with TFA a thiohydantoin derivative is expected to be formed thus allowing determination of the fraction of **1** that reacted with this moiety in hemoglobin. This is in principle the same technique that has been used for determination of exposure of many reactive industrial chemicals[21-23]. In these cases, industrial chemicals and/or their metabolites react with N-terminal valine in hemoglobin and a modified Edman reagent pentafluorophenyl isothiocyanate is used to cleave the valine adduct and subsequently analyzed by LC-MS/MS or GC-MS/MS[23].

The cleavage of adducted valine with **1** was optimized (see section 2.7) for a 20 µL aliquot rat blood. Since the reaction requires an aqueous free environment, methanol was used to precipitate blood proteins facilitating the dry down process. Lower recovery was observed when separating the supernatant from the solid material and therefore the TFA treatment was performed after drying down the whole fraction containing both solid material and the supernatant. A 100 µL aliquot of concentrated TFA was used to dissolve the extract and to cleave the valine adduct. Incubation at 65°C for 20 min was optimum for the cleavage to be performed. After incubation, the samples were dried down to remove TFA and re-dissolved in acetonitrile. A Centrifugation step was employed to

remove some solid residue before LC−MS/MS analysis. An adducted peptide "**1**-VHLTPEEK" with an amino acid sequence the same as for the eight N-terminal residues in hemoglobin was used as reference standard for preparation of calibration standards and QC samples for this assay. This approach has been used before for analysis of acrylamine adducts of hemoglobin[24]. Similar peptide alkylated with labeled **1** (^{13}C- or deuterium labeled) would be an ideal internal standard for this assay; however due to time constraints in supporting safety study this synthesis was not prioritized. Instead, alkylated hemoglobin with SFN was used as internal standard. SFN was left to react with blood proteins to form adducts with all nucleophillic groups in blood including valine adducts. Valine adducts are known to be stable stable and the Edman procedure is reproducible[21-24]; therefore a treated solution may be used as internal standard. The approach has been used in the past for determination of hemoglobin adducts of industrial chemicals due to difficulties with the synthesis of alkylated hemoglobin[25-26]. The Edman procedure described in this work may be applied to similar compounds containing isothiocyanates functional groups such as SFN.

*3.4. Total measurement (bound to sulfhydryl moiety and free fraction of **1**)*

Since **1** is expected to undergo equilibrium reaction with GSH and cysteine in haemoglobin, sought to shift the equilibrium towards the formation of free **1** by changing the solution pH to basic. An aliquot of ammonium hydroxide was added to a neat solution containing equal quantities of **1**, and the two conjugates, **2** and **3**. The solution was injected repeatedly on the LC-MS operated in full scan mode. The levels of free **1** did not increase as expected; however, a new peak was increasing with retention time close to

that for **3** (see Fig. 4). After an hour of treatment, only trace of the investigated analytes were observed and a peak with m/z = 271 corresponding to the thiourea derivative of **1**, compound **4** was detected. This observation led to the idea of using ammonium hydroxide for quantification of **1** in its free form plus the fraction bound to sulfhydryl moiety in hemoglobin (this fraction is referred as total measurement). To enable this, it was necessary to estimate the recovery of thiourea derivative when these analytes are fortified into blood samples. For that reason, it was decided to synthesize the thiourea derivative from **1**. Addition of ammonium hydroxide to blood samples, containing **1**, and the conjugates, **2** and **3**, converted all sulfhydryl conjugates and the free **1** to the thiourea derivative **4** to high extent (see Fig. 5). As mentioned earlier, approximately 90% of **1** is lost when rat blood is fortified with this compound. The treatment with ammonium hydroxide recovers **1** in form of a thiourea derivative to a high extent (in molar quantities). For that reason, **1** is added to the preparation of calibration standards for quantification of total measurement (bound to sulfhydryl moiety and free fraction of **1**). The thiourea derivative **4** was extracted by LLE with ethyl acetate before LC-MS/MS. It is important to mention that this thiourea derivative is also lost during the dry down process. To overcome this issue, an aliquot (50 µL) of DMF was added to collection silanized glass inserts (into the 2 ml 96-well collection plate). The ethyl acetate phase was then evaporated to approximately to the level corresponding to 50 µL DMF to avoid complete dry down. The formation of thiourea derivative from SFN was also observed to high extent, therefore it was initially evaluated as internal standard for the analysis of total **1** assay; however, this derivative had shorter retention time than that for **4**. For that

reason, nitrophenyl isothiocyanate had similar retention time as **4** and was used as the internal standard.

3.5. Summary of TK data from the 7-day rat safety study

The methodologies described above enabled successful bioanalytical support of a non-GLP 7-day rat safety study with doses of 3, 30 or 100 mg/kg/day with **1**. Analysis of free **1** was not performed due to poor stability of the compound and high variability in the assay. Alternatively, the total measurement, hemoglobin valine adduct of **1**, and adducts **2** and **3** were used as surrogate markers of evaluation of the systemic exposure of **1**.

Following oral administration of **1** at doses of 3, 30 or 100 mg/kg/day for 7 days to the rat, total measurement and valine adduct concentrations were quantifiable during the entire 24-hour sampling period after dosing on Days 1 and 7 except the 3 mg/kg Day 1 dose group with total measurement concentrations quantifiable for up to 8 h post dose. Concentrations of conjugates **2** and **3** were also quantifiable for up to 8 h post dose for all dose levels on Days 1 and 7 except the 3 mg/kg Day 7 dose group with concentrations quantifiable for up to 4 h post dose. The maximum blood concentrations after dosing were observed at 0.25 to 1 h for total measurement, 0.5 to 8.0 h for valine adduct, 0.25 to 8.0 h for conjugate **3** and 0.25 to 0.5 h for conjugate **2**. A summary of TK parameters and blood concentration profiles on Day 1 and 7 are presented in Table 1 to Table 4 and shown graphically in Fig. 6-7.

Systemic exposure ($AUC_{0\text{-}t}$ and C_{max} values) based on the total measurement increased dose proportionally from 3 to 100 mg/kg/day. For the 33.3-fold increase in dose, the mean $AUC_{0\text{-}t}$ and C_{max} values increased 43.6- and 35.0-fold, respectively on Day 1, and 24.8- and 9.1-fold, respectively on Day 7. Following 7 days of repeat dosing, there was no marked (>2-fold) change in the systemic exposure of total measurement from Day 1 to Day 7 for any of the dose levels, except for the mean C_{max} values at 100 mg/kg/day, which decreased 3.4-fold from Day 1 to Day 7.

The systemic exposure based on the data from valine adducts in hemoglobin was also pronounced and the $AUC_{0\text{-}t}$ and C_{max} values increased proportionally with dose from 3 to 100 mg/kg/day. For the 33.3-fold increase in dose, the mean $AUC_{0\text{-}t}$ and C_{max} values increased 40.3- and 47.6-fold, respectively on Day 1 and 22.6- and 20.9-fold, respectively on Day 7. On both Days 1 and 7, the concentrations of valine adduct generally were constant throughout the 24-hour sampling period. There was a trend toward higher systemic exposure on Day 7 than that on Day 1. $AUC_{0\text{-}t}$ ratios of total measurement /valine adducts ranged from 0.780 to 1.24 on Day 1 and 0.431 to 0.690 on Day 7. $AUC_{0\text{-}t}$ ratios of total measurement/conjugate **3** and total measurement/conjugate **2** were variable and overlapped between Day 1 and Day 7, ranging from 12.2 to 681 and 13.6 to 56.5, respectively.

4. Conclusion

A multiple component semi-automated sample preparation methodology in 96–well plate format for determination of in vivo exposure of **1** in rat blood was developed. These methodologies enabled a successful bioanalytical support of a non-GLP 7-day rat safety study with oral doses of **1** at 3, 30, or 100 mg/kg/day. Even though all three methodologies described in this publication provided good understanding on the in vivo exposure of 1, the total measurement methodology is advised for assessing exposure of similar compounds bearing an isothiocyanate group due to the following reasons, 1) the simplicity of the methodology and 2) these compounds most likely exist mainly as sulfhydryl conjugates in vivo, which are well recovered with the total measurement methodology.

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Figure Legends:

Fig. 1. Targeting Nrf2 activation

Fig 2. Preparation of compounds 1 to 4

Fig 3. Application of Edman degradation procedure for analysis of 1 in rat blood.

Fig. 4. Formation of thiourea derivative from 1, 2 and 3, after treatment with ammonium hydroxide. LC-MS total ion chromatograms (TIC) at time t=0 h (top chromatogram) and after storage in the autosampler for an hour (bottom chromatogram) under ambient conditions.

Fig. 5. Recovery of the thiourea derivative 4 from blood samples fortified with 1, 2 and 3 after treatment with ammonium hydroxide. Formation conjugate 3 is a result of inter-organ synthesis by gamma-glutamyltransferase and dipeptidases in the kidney and acetylation by intracellular N-acetyl-transferases in the liver.

Fig. 6. Concentration profiles for conjugates 2 and 3 for Day 1 and 7 after oral administration of 1

Fig. 7. Concentration profiles for valine adducts and total measurement for Day 1 and 7 after oral administration of 1

Table Legends:

Table 1. Toxicokinetic parameters for conjugate 2

Table 2. Toxicokinetic parameters for conjugate 3

Table3. Toxicokinetic parameters for hemoglobin valine adduct of 1

Table 4. Toxicokinetic parameters for the total measurement (1 bound to sulfhydryl moiety and free fraction of 1)

Fig. 1. Targeting Nrf2 activation

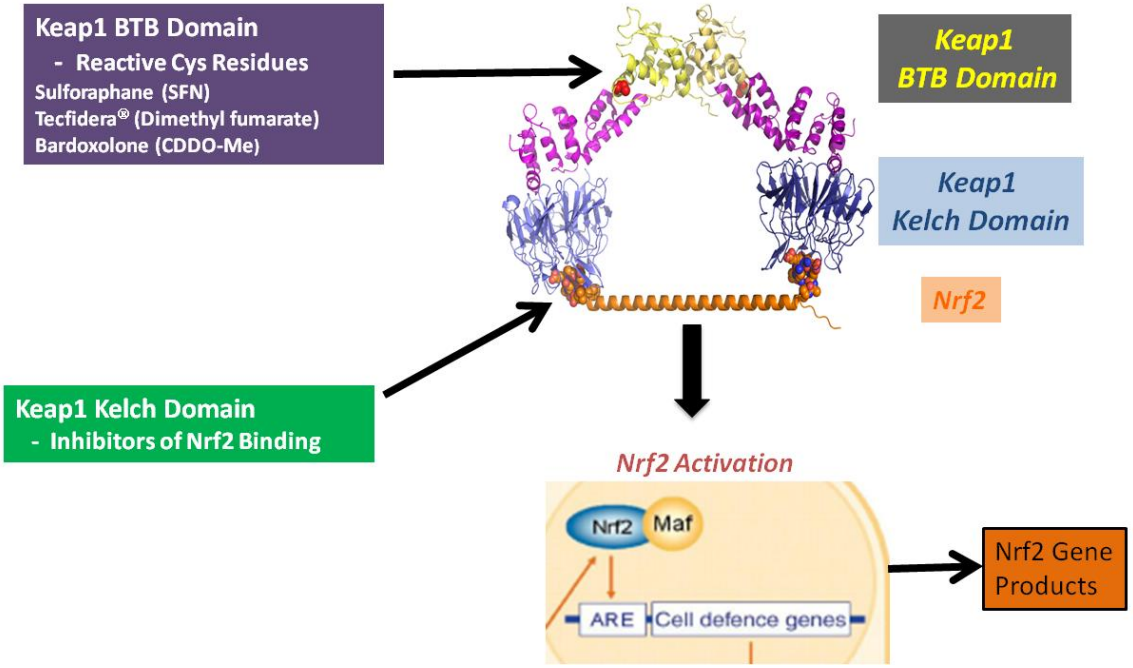


Fig. 2. Preparation of compounds 1 to 4

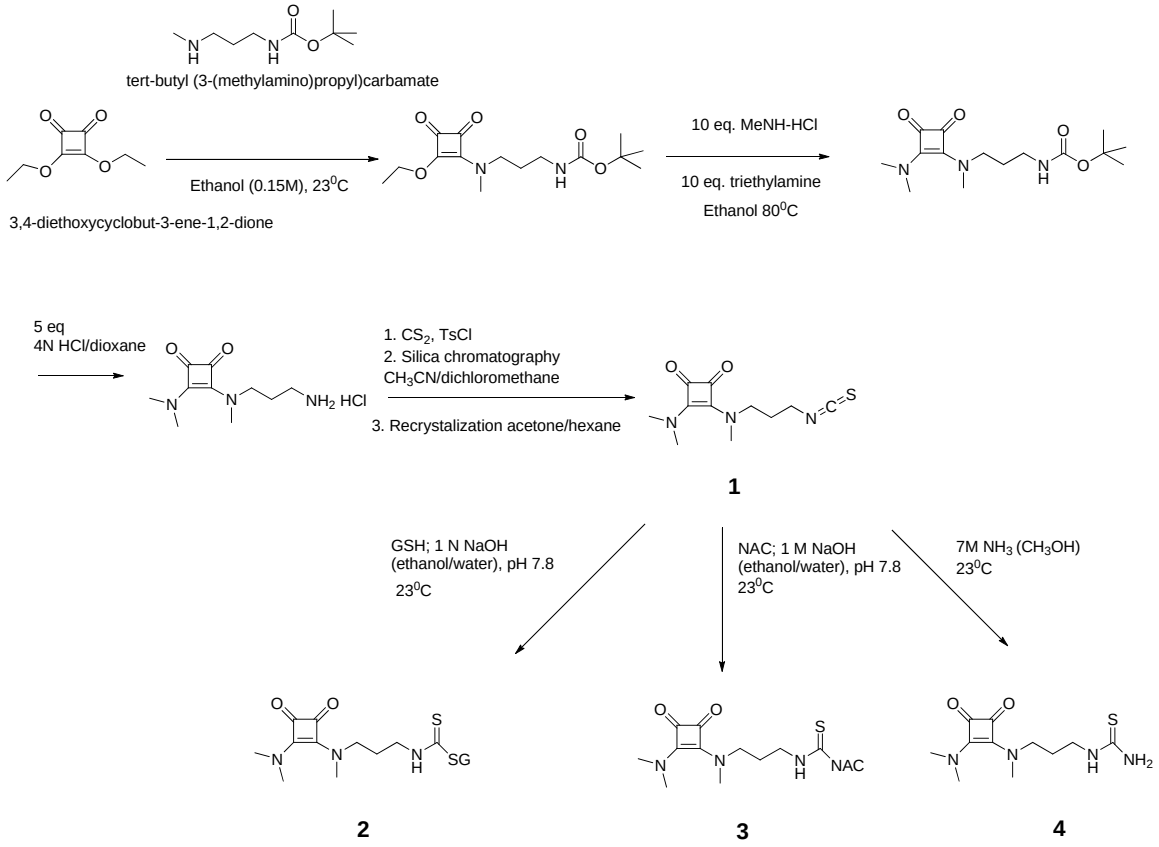
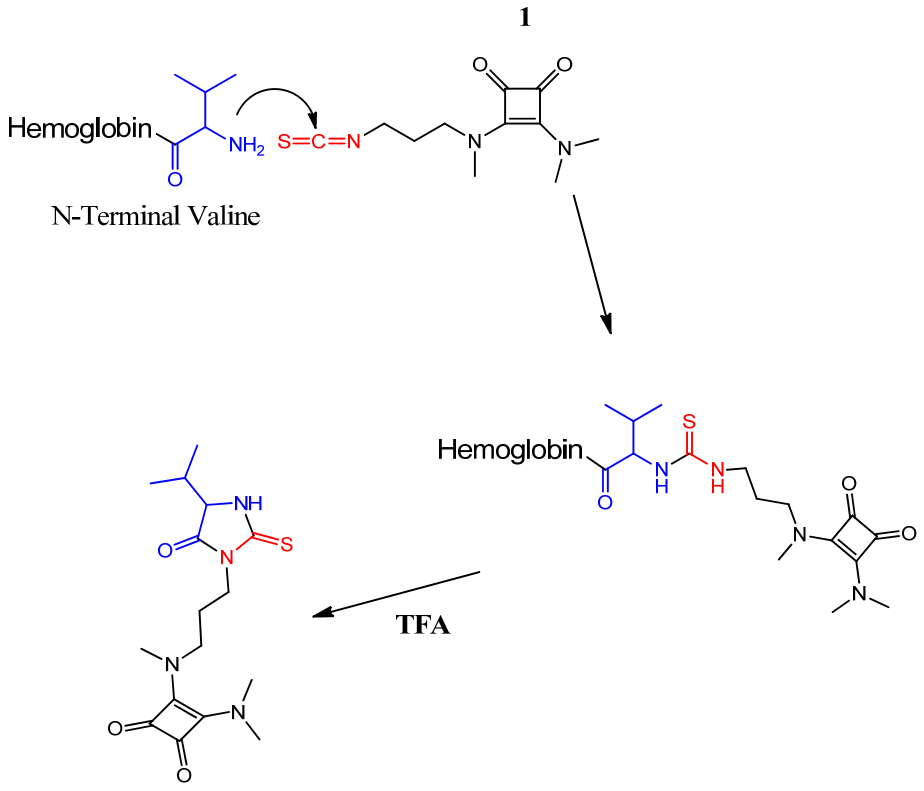


Fig. 3. Application of Edman degradation procedure for analysis of 1 in rat blood.



Thiohydantoin Derivative of **1**

Fig. 4. Formation of thiourea derivative 4 from 1, and conjugates 2 and 3 after treatment with ammonium hydroxide. LC-MS total ion chromatograms (TIC) at time t=0 h (top chromatogram) and after storage in the autosampler for an hour (bottom chromatogram) under ambient conditions.

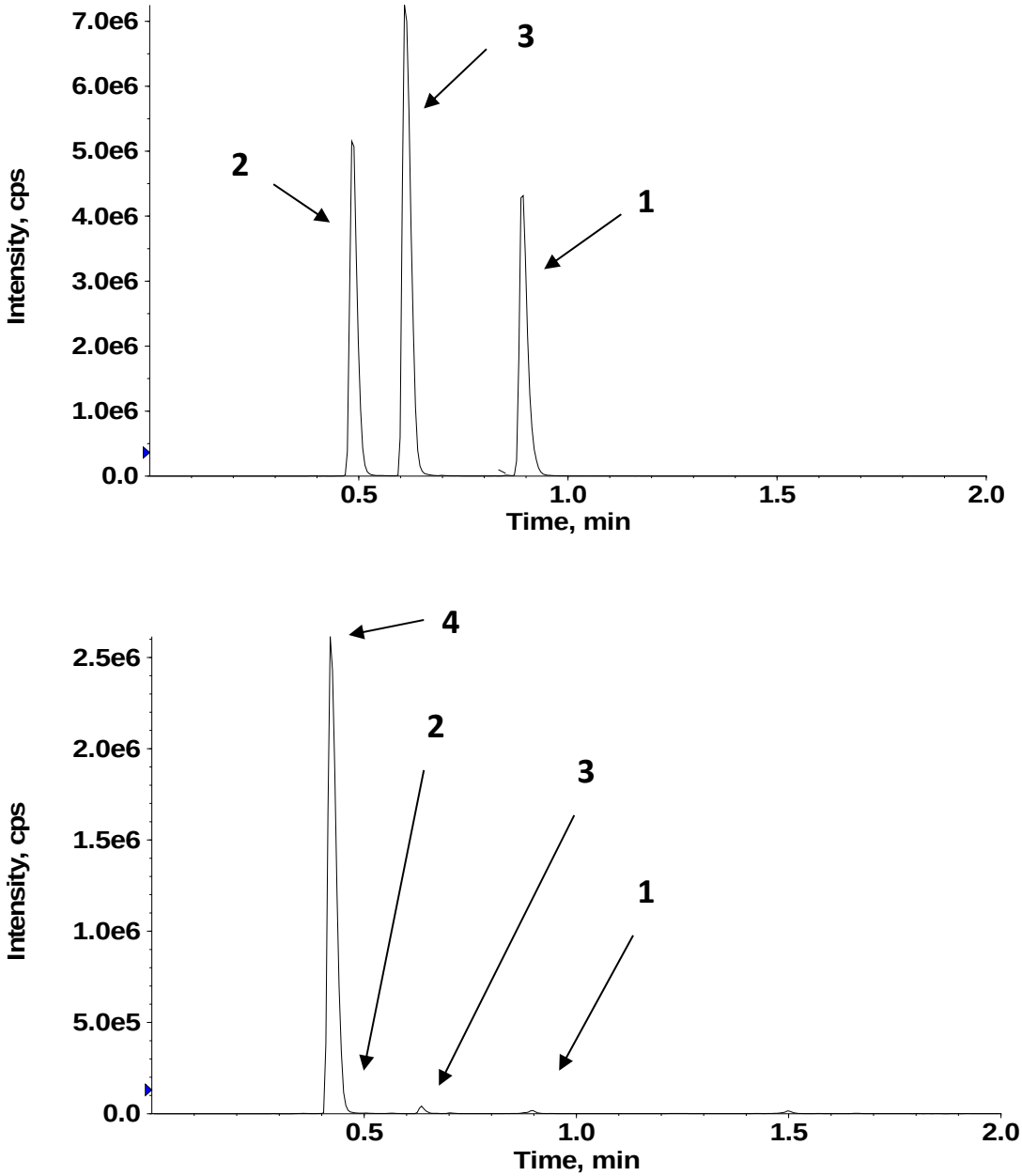


Fig. 5. Recovery of the thiourea derivative 4 from blood samples fortified with 1 and conjugates 2 and 3 after treatment with ammonium hydroxide. Formation conjugate 3 is a result of inter-organ synthesis by gamma-glutamyltransferase and dipeptidases in the kidney and acetylation by intracellular N-acetyl-transferases in the liver.

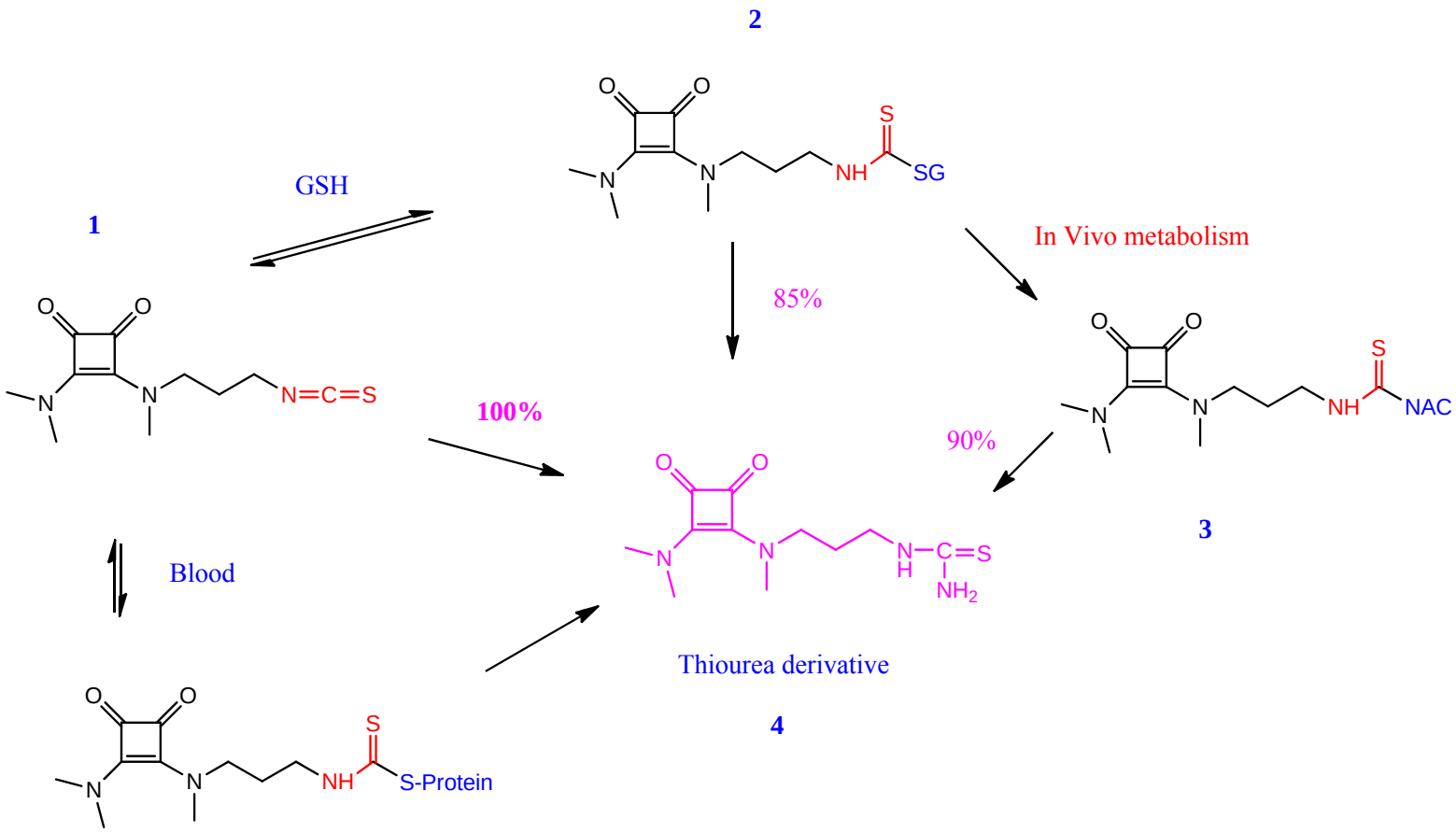


Fig. 6. Concentration profiles for conjugates 2 and 3 for Day 1 and 7 after oral administration of 1

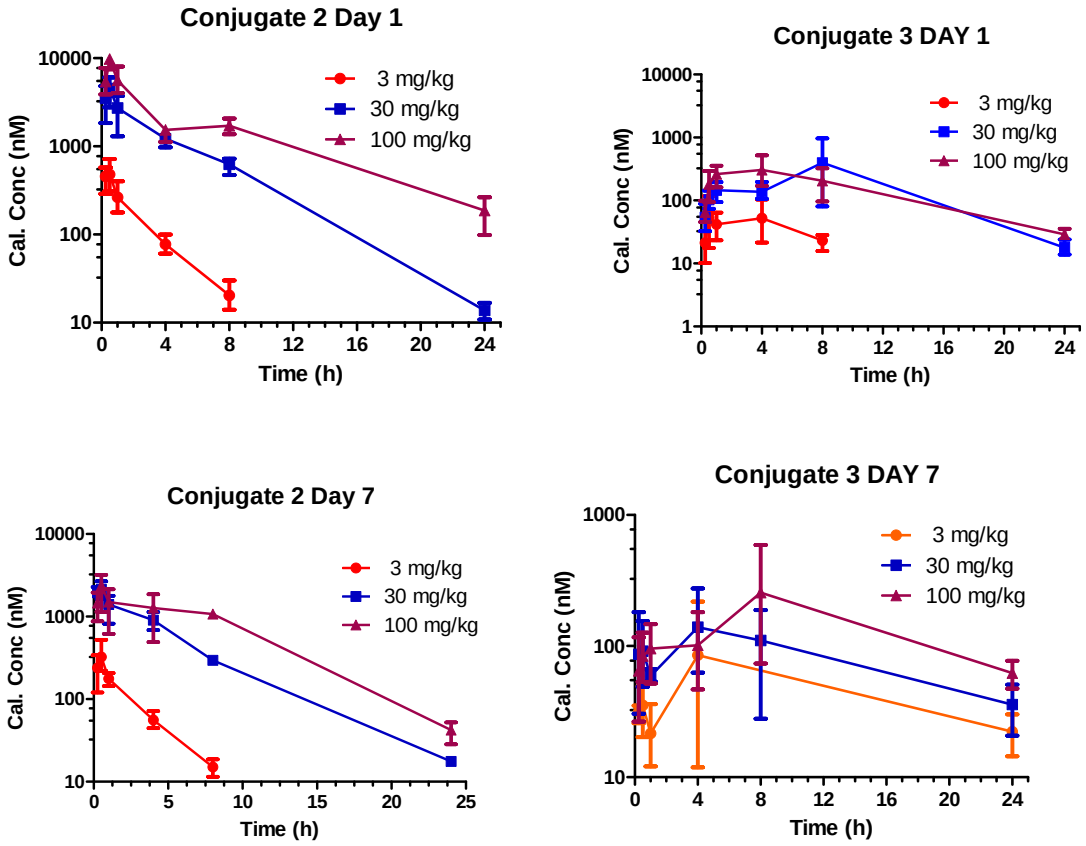
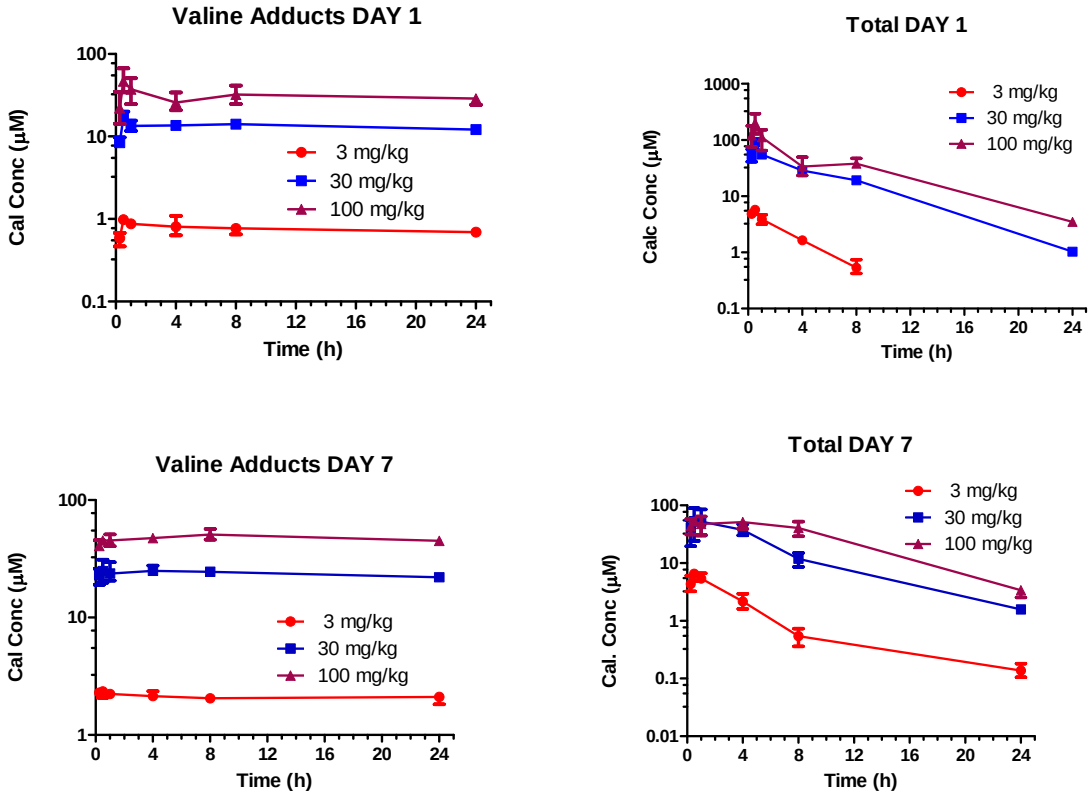


Fig. 7. Concentration profiles for valine adducts and total measurement for Day 1 and 7 after oral administration of 1



713 **Table 1. Toxicokinetic parameters for the conjugate 2**

Parameter	Period	Male (n =3)		
		Dose (mg/kg/day)		
		3	30	100
AUC0-t (h×µM) Mean [Range]	Day 1	0.969 [0.857-1.18]	13.9 [11.1-15.8]	33.0 [28.5-38.4]
	Day 7	0.623 [0.55-0.713]	7.74 [5.75-9.03]	15.4 [11.1-18.4]
Cmax (µM) Mean [Range]	Day 1	0.511 [0.309-0.717]	4.54 [2.76-6.05]	9.75 [7.48-11.4]
	Day 7	0.335 [0.229-0.522]	2.19 [1.77-2.69]	2.38 [1.14-3.21]

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Table 2. Toxicokinetic parameters for conjugate 3

Parameter	Period	Male (n =3)		
		Dose (mg/kg/day)		
		3	30	100
AUC0-t (h×µM) Mean [Range]	Day 1	0.310 [0.216-0.437]	3.34 [1.53-6.60]	3.40 [2.20-4.12]
	Day 7	0.950 [0.102-2.27]	1.40 [0.775-2.57]	2.63 [0.778-5.45]
Cmax (µM) Mean [Range]	Day 1	0.095 [0.064-0.118]	0.419 [0.144-0.966]	0.379 [0.295-0.517]
	Day 7	0.101 [0.037-0.049]	0.175 [0.063-0.275]	0.285 [0.117-0.590]

Table 3. Toxicokinetic parameters for hemoglobin valine adduct of 1

Parameter	Period	Male (n =3)		
		Dose (mg/kg/day)		
		3	30	100
AUC0-t (h×µM) Mean [Range]	Day 1	18.1 [15.7- 20.9]	317 [308- 327]	730 [573-914]
	Day 7	50.5 [49.3- 51.5]	567 [545- 578]	1140 [1090-1230]
Cmax (µM) Mean [Range]	Day 1	0.982 [0.908-1.10]	17.1 [15.4- 20.0]	46.7 [34.4-67.1]
	Day 7	2.44 [2.36-2.55]	29.0 [26.3- 31.0]	50.9 [45.9-56.8]

Table 4. Toxicokinetic parameters for the total measurement 1 bound to sulfhydryl moiety and free fraction of 1

Parameter	Period	Male (n =3)		
		Dose (mg/kg/day)		
		3	30	100
AUC_{0-t} (h×µM) Mean [Range]	Day 1	16.0 [15.7-16.3]	375 [350-388]	698 [517-917]
	Day 7	24.6 [22.0-27.6]	345 [305-398]	611 [530-677]
C_{max} (µM) Mean [Range]	Day 1	5.74 [5.32-5.97]	91.9 [78.9-98.8]	201 [129-295]
	Day 7	6.54 [4.88-7.82]	71.8 [41.0-89.3]	59.8 [57.0-64.1]