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Original article

Characterization of a highly sensitive and selective novel trapping reagent, stable isotope labeled glutathione ethyl ester, for the detection of reactive metabolites

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ABSTRACT

Introduction: Glutathione (GSH) trapping assays are widely used to predict the post-marketing risk for idiosyncratic drug reactions (IDRs) in the pharmaceutical industry. Although several GSH derivatives have been introduced as trapping reagents for reactive intermediates, more sensitive and selective reagents are desired to prevent the generation of erroneous results. In this study, stable isotope labeled GSH ethyl ester (GSHEE-d_5) was designed and its detection capability was evaluated.

Methods: GSHEE-d_5 was synthesized and its detection potential was compared with stable isotope labeled GSH ([$^{13}C_2$,^{15}N]GSH) as a reference trapping reagent. The trapping reagents were added to human liver microsomes as a 1:1 mixture with GSHEE or GSH, respectively, and incubated with seven IDR positive drugs and three IDR negative drugs. The adducts formed between the reagents and reactive metabolites were analyzed by unit resolution mass spectrometer (MS) using isotope pattern-dependent scan with neutral loss filtering.

Results: A single-step reaction of GSH and ethanol-d_6 produced GSHEE-d_5 with a yield of 85%. The GSHEE-d_5 assay detected adducts with all seven IDR positive drugs, and no adducts were detected with the three IDR negative drugs. In contrast, the [$^{13}C_2$,^{15}N]GSH assay failed to detect adducts with three of the IDR positive drugs. In the case of diclofenac, the GSHEE-d_5 assay showed a 4-times greater signal intensity than the [$^{13}C_2$,^{15}N]GSH assay.

Discussion: GSHEE-d_5 enabled the detection of reactive metabolites with greater sensitivity and selectivity than [$^{13}C_2$,^{15}N]GSH. These results demonstrate that GSHEE-d_5 would be a useful trapping reagent for evaluating the risk of IDRs with unit resolution MS.

1. Introduction

Despite extensive safety testing of drug candidates, extremely rare adverse events still occur after approval. These types of toxicity are called idiosyncratic drug reactions (IDRs). Drugs have been withdrawn from the market or given black box warnings because of IDRs. Although the mechanisms of IDRs are poorly understood, reactive metabolites are thought to play a role in the onset of some IDRs such as severe hepatotoxicity, epidermal necrolysis, and agranulocytosis (Evans, Watt, Nicoll-Griffith, & Baillie, 2004; Ikeda, 2015; Kalgutkar & Dalvie, 2015). Owing to the lack of predictivity of IDRs and their associated safety risks, the development of better assays for the detection of reactive metabolites is needed.

Reactive metabolites are unstable biotransformants of some drugs and readily generate a toxic complex with macromolecules in the body. Minimizing the potential for the formation of reactive metabolites by drug candidates during the drug discovery process would be beneficial for the development of safer drugs with reduced IDR potential (Evans et al., 2004). Several reports have been published to distinguish safe compounds from toxic ones using radioisotope labeled compounds. Three groups independently reported that it is key to know the clinical daily dose and the in vitro extent of covalent binding of reactive metabolites since the correlation between these two factors is required to assess the risk of IDRs (Nakayama et al., 2009; Obach, Kalgutkar, Soglia, & Zhao, 2008; Usui, Mise, Hashizume, Yabuki, & Komuro, 2009). Although these assays were informative, radiolabeling of test compounds is required, and this is not a practical approach in early stage drug development. Thus, these assays cannot be applied until the compound is far enough along in the drug development process to be a promising development candidate.

Glutathione (GSH) trapping assays are widely used in the pharmaceutical industry. The formation of GSH adduct determined by [^{35}S]GSH with non-labeled compounds correlated well with the amount of covalent binding to microsomal proteins determined by radiolabeled compounds (Masubuchi, Makino, & Murayama, 2007). LC-MS/MS analysis enabled the detection of GSH adducts without using [^{35}S]GSH or radiolabeled compounds. The neutral loss (NL) scan is commonly used to analyze adducts of reactive metabolites. However, this assay

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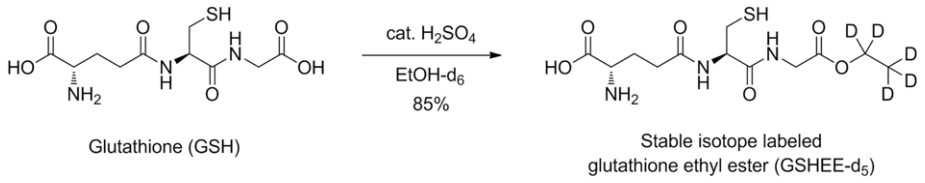

Fig. 1. Synthesis of stable isotope labeled glutathione ethyl ester (GSHEE-d_5).

sometimes produces inaccurate results because of its low sensitivity and selectivity. Because the NL scan of 129 Da is not specific for GSH adducts, a compound that does not produce reactive metabolites is sometimes categorized to toxic (false positive). For example, testosterone exhibited three positive responses in the NL scan although it does not generate any reactive metabolites when incubated with human liver microsomes (Yan & Caldwell, 2004). On the other hand, the assay sometimes fails to detect the reactive metabolites, and compounds that are potentially toxic are categorized as safe (false negative). In one study, no adducts of mefenamic acid were detected in the NL scan whereas three GSH adducts were detected in the multiple reaction monitoring-enhanced product ion scan (Zheng et al., 2007). These results show that there is a clear need for a more sensitive and selective trapping reagent.

Several GSH derivatives have been reported to reduce the erroneous results. GSH ethyl ester (GSHEE) has greater sensitivity than GSH by improving the efficiency of solid phase extraction (SPE) and ionization in MS due to increasing hydrophobicity (Soglia et al., 2004). The GSHEE adduct of a reactive metabolite of acetaminophen is 80-fold more sensitive to detection by LC-MS than the corresponding GSH adduct (Soglia et al., 2004). Stable isotope labeled GSH ([$^{13}C_2$,^{15}N]GSH) is another useful analogue (Huang, Huang, & van Breemen, 2015; Ma, Wen, Ruan, & Zhu, 2008; Mutlib et al., 2005; Yan & Caldwell, 2004; Yan, Maher, Torres, Caldwell, & Huebert, 2005). A 1:1 mixture of GSH and [$^{13}C_2$,^{15}N]GSH results in an isotopic doublet which allows greater selectivity for the detection of adducts than GSH alone by using isotope pattern-dependent MS/MS scan with NL filtering (NLF). Stable isotope labeled bis-methyl GSH (bisMeGSH-d_6) has also been used for the detection of reactive metabolites (Defoy, Dansette, Neugebauer, Wagner, & Klarskov, 2011). Because both of the carboxylic acid moieties in GSH are esterified, bisMeGSH is more hydrophobic than GSH, conferring it greater sensitivity in adduct detection by MS. However, the improvement in signal intensity was found to be dependent on the type of adducts present. In the most extreme case, bisMeGSH-d_6 failed to detect one of the adducts of carbamazepine that was detected by GSH. It was considered that the α-carboxylic acid group at the γ-glutamyl residue of GSH was important in GSH S-transferase (GST) mediated reactions (Armstrong, 1997), and that the formation of adducts would be limited to chemical reactions.

In this article, we report a new trapping reagent, stable isotope labeled GSHEE (GSHEE-d_5). GSHEE-d_5 was synthesized and its ability to trap reactive metabolites was evaluated. Similar to bisMeGSH-d_6, GSHEE-d_5 is expected to have greater sensitivity than [$^{13}C_2$,^{15}N]GSH in adduct detection by LC-MS. Moreover, since GSHEE-d_5 has a carboxylic acid group at the γ-glutamyl residue, it is expected to be recognized by GST.

2. Methods

2.1. Materials

Acetaminophen, diclofenac, clozapine, trovafloxacin, testosterone, midazolam, and methanol-d_4 were purchased from Sigma-Aldrich (St. Louis, MO). Tienilic acid was purchased from Cypex (Dundee, UK). Imipramine, magnesium dichloride, and dithiothreitol were purchased from Wako Pure Chemical Industries (Osaka, Japan). Mefenamic acid was purchased from LKT Laboratories (St. Paul, MN). Terfenadine was purchased from Acros (Pittsburgh, PA). Reduced GSH was purchased from Sigma-Aldrich, Acros, and Wako Pure Chemical Industries. Concentrated sulfuric acid, triethylamine, ethanol, and diisopropyl ether were purchased from Kanto Chemical (Tokyo, Japan). 2 M hydrogen chloride in methanol was purchased from Kokusan Chemical (Tokyo, Japan). Ethanol-d_6 was purchased from Isotec (Miamisburg, OH). β-NADPH was purchased from Oriental Yeast (Tokyo, Japan). Stable isotope labeled GSH (γ-glutamyl-cysteinyl-glycine-$^{13}C_2$,^{15}N, [$^{13}C_2$,^{15}N]GSH) was purchased from Cambridge Isotope Laboratories (Andover, MA). Pooled human liver microsomes and pooled rat liver microsomes were purchased from Corning Life Sciences (Tewksbury, MA). Oasis® HLB solid phase extraction (SPE) 96-well plate (30 μm, 10 mg) was purchased from Waters (Milford, MA). SensoLyte® GST Activity Assay Kit *Fluorimetric* was purchased from AnaSpec (Fremont, CA).

2.2. Synthesis of GSH derivatives

Fisher's esterification procedure was used for the chemical synthesis of GSHEE-d_5. Reduced GSH (503.8 mg, 1.64 mmol) was suspended in 5 mL of ethanol-d_6 (99.5 atom% D), and concentrated sulfuric acid (0.137 mL, 2.56 mmol) was added to the reaction mixture. The reaction mixture was stirred for 30 min at room temperature. After it stood for 21 h at room temperature without stirring, triethylamine (0.714 mL, 5.12 mmol) was added to the solution for neutralization. Crystallization was then performed by the addition of ethanol (10 mL) and diisopropyl ether (15 mL). The reaction mixture was stored for 16 h at 4 °C and the precipitated crystal was filtered. The crystal cake was dried for 3 h at 35 °C under reduced pressure to obtain GSHEE-d_5 (472 mg, 85% yield) as a white powder (Fig. 1); product was characterized by electrospray ionization MS (Positive) and ^{1}H NMR analysis. m/z 341.2 $[M + H]^+$, ^{1}H NMR (D_2O, 400 MHz) δ 1.98–2.04 (2H, m), 2.35–2.42 (2H, m), 2.79–2.81 (2H, m), 3.63 (1H, t, J = 6.08 Hz), 3.87–3.88 (2H, m), 4.41 (1H, t, J = 6.12 Hz). Non-labeled GSHEE was synthesized by the similar method for GSHEE-d_5.

Non-labeled bisMeGSH was synthesized using the following procedure. Reduced GSH (350 mg, 1.14 mmol) was dissolved in 5 mL of 2 M hydrogen chloride in methanol. The reaction mixture was stirred for 14 h at room temperature. The reaction mixture was evaporated under reduced pressure. Appropriate volumes of a 25% ammonium aqueous solution and methanol were added to the residue on an ice bath to adjust the pH to 9. The mixture was then evaporated and purified by column chromatography to obtain bisMeGSH (110 mg, 29% yield) as a white powder. Since the yield was unsatisfactory, bisMeGSH-d_6 was synthesized by an improved procedure. Reduced GSH (300 mg, 0.976 mmol) was suspended in 1.5 mL of methanol-d_4 (99.8 atom% D), and concentrated sulfuric acid (0.104 mL, 1.95 mmol)

Fig. 2. Analysis of adducts of diclofenac in the GSHEE-d_5 assay. Diclofenac (10 μM) was incubated with GSHEE/GSHEE-d_5 (1:1 mixture, 1 mM total) in human liver microsomes. After solid phase extraction, samples were injected into UPLC/MS and total ion chromatograms (TICs) were obtained (A–C; no drug control, D–F; diclofenac). Each assay has three chromatograms; the TIC of full MS scans (A, D), the TIC of isotope pattern-dependent MS/MS scans (B, E), and the TIC of isotope pattern-dependent MS/MS scans with 129 Da of NLF (C, F). The TIC of isotope pattern-dependent MS/MS scans with 129 Da of NLF identified two adducts, DIC-1 (4.44 min) and DIC-2 (4.58 min). The MS spectra from full MS scans of DIC-1 (G) and DIC-2 (H) had a unique isotopic doublet with 5 Da differences. The MS/MS fragments were detected in isotope pattern-dependent MS/MS scans for DIC-1 (I) and DIC-2 (J). The structures of the adducts were estimated based on molecular weight, MS/MS fragments, and literature information (Zhou, Chan, Duan, Huang, & Chen, 2005).