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A fragmentation-based method for the differentiation of glutathione conjugates by high-resolution mass spectrometry with electrospray ionization

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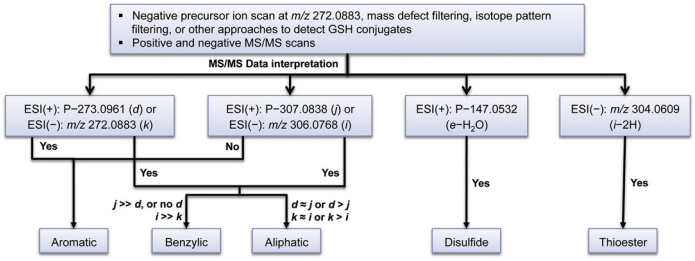
HIGHLIGHTS

- The fragmentation of five different classes of GSH conjugates were described.
- A fragmentation-based method was established to differentiate GSH conjugate types.
- Different intensities of *i*/*j* ions can discriminate aliphatic and benzylic classes.

GRAPHICAL ABSTRACT



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ABSTRACT

Idiosyncratic reactions are one of the major causes of drug treatment limitations or market withdrawals, and likely involve the formation of reactive metabolites. Because of their unstable nature, reactive species are usually discovered as stable conjugates by glutathione (GSH) trapping rather than by direct detection. The GSH conjugates are then detected by neutral loss scanning of 129 Da or precursor ion scanning at *m*/*z* 272, but the conjugation sites can only be identified by comparison with reference standards. In the present study, the fragmentation behaviors of 52 GSH conjugates belonging to five structural classes (aliphatic, aromatic, benzylic, disulfide, and thioester) were investigated in both positive and negative electrospray ionization modes by high-resolution mass spectrometry with suitable collision energies such that the relative abundance of the parent ion was approximately 50% of the most abundant product ion. Several structural-diagnostic fragmentations were identified: aliphatic conjugates gave *i*/*j*-type ions upon cleavage of the C—S bond between the drug and GSH, and *d*/*k*-type ions formed by the cleavage of the cysteinyl C—S bond, with approximately equal intensity, in both positive and negative modes, whereas aromatic conjugates only possessed *d*/*k*-type ions, and benzylic conjugates primarily yielded *i*/*j*-type ions. Disulfide conjugates typically produced dehydrogenated GS^- fragment ions ([*i*-2H]-type) in negative mode, and thioester conjugates displayed sequential losses of pyroglutamic acid and water ([*e*-H_2O]-type) in positive mode. A fragmentation-based method was thus established to facilitate the discrimination of these five classes of GSH conjugates, thereby providing insight into the bioactivation mechanisms and supporting lead optimization.

Abbreviations: CE, collision energy; CID, collision-induced dissociation; ESI, electrospray ionization; FAB, fast-atom bombardment; GSH, glutathione; HLM s, human liver microsomes; LC-MS/MS, liquid chromatography-tandem mass spectrometry; NAC, *N*-acetylcysteine; NADPH, *β*-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt; UHPL, C/Q-TOF MS, ultra-high performance liquid chromatography-quadrupole-time of flight-mass spectrometry.

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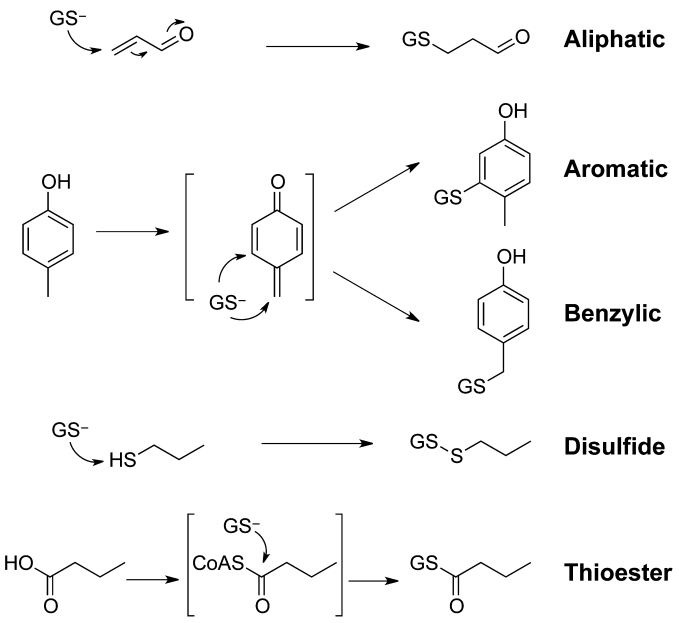

Fig. 1. Different conjugation sites of GSH with reactive species.

1. Introduction

Some drugs are converted into chemically reactive metabolites or intermediates via enzyme-mediated bioactivation. These reactive species are capable of covalently modifying cellular macromolecules such as proteins and DNA [1,2], which leads to cell damage and ultimately results in drug-induced idiosyncratic toxicity. Although there is no direct evidence that reactive metabolite formation is a causative factor in idiosyncratic toxicity, accumulating data support that reactive species are the mediators of the toxic response to certain xenobiotics [3–5]. It was reported recently that 18 of 31 drugs withdrawn from the market and 18 of 37 drugs flagged for alerts form reactive metabolites [6]. Thus, the detection of reactive metabolites may intensify the concern on the safety issues of specific drugs.

Most reactive metabolites are unstable and short-lived, and thus cannot be directly detected. However, the detection of glutathione (γ-glutamylcysteinylglycine, GSH) conjugates in biological samples, particularly in bile, can provide indirect evidence of the presence of reactive species. In vivo metabolism studies are not always conducted in the early discovery stage, and thus, in vitro GSH trapping assays are utilized as an alternative means of assessing bioactivation potential [7]. Structural characterization of the resulting stable conjugates may provide indirect but valuable information regarding the nature of reactive species, thereby providing insight into the bioactivation mechanisms and supporting pharmaceutical lead optimization.

GSH is a ubiquitous tripeptide in mammalian systems, and its nucleophilic cysteinyl thiol group allows GSH to react with a variety of soft electrophilic species to form GSH conjugates. In vivo GSH conjugation is an important physiological defense mechanism against chemically reactive intermediates. Based on the site of conjugation, GSH conjugates are mainly divided into five structural classes: aliphatic, aromatic, benzylic, disulfide, and thioester (Fig. 1).

Neutral loss scanning of 129 Da (pyroglutamic acid) in positive ion mode has been widely embraced as the gold standard for GSH conjugate screening [8]. Dieckhaus et al. demonstrated that negative precursor ion scanning at m/z 272 (deprotonated γ-glutamyl-dehydroalanyl-glycine) could allow more unbiased detection of GSH conjugates [9]. The two methods described above can be used to detect GSH conjugates. Once GSH conjugates are found, the product ion spectra can be acquired to elucidate their structures. The key problem of these methods is that fragmentation patterns have not be fully utilized for identification of the linkage of GSH adducts, and the conjugation sites can only be identified by comparison with synthesized or isolated standards [10–12]. Hence, there is a need for a simple and rapid analytical method for identifying the sites or moieties of GSH conjugation and the structures of the reactive metabolites from which they are derived.

In our previous bioactivation studies of chlorogenic acid and 4-nonylphenol [11,12], different fragmentation patterns were observed for the aromatic and benzylic classes of GSH conjugates in negative electrospray ionization (ESI) mode: the aromatic conjugates displayed a fragment ion resulting from cleavage of the cysteinyl C—S bond at m/z 272, and the benzylic conjugates yielded a highly abundant ion *at* m/z 306 (GS^-) formed by the cleavage of the C—S bond between the drug and GSH. Moreover, the neutral loss of 147 Da (pyroglutamic acid and water) from the $[M+H]^+$ species has been reported for the thioester conjugate of diclofenac [13]. These results suggest that characteristic fragmentations could be used to differentiate the classes of GSH conjugates and thus tentatively identify conjugation sites.

No report has comprehensively studied the fragmentation patterns of different structural classes of GSH conjugates and identified diagnostic ions for each class. The aim of this study, therefore, was to systematically investigate the fragmentation behaviors of 52 GSH conjugates derived from 26 drugs (Fig. 2) in both positive and negative ESI modes by ultra-high performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UHPLC/Q-TOF MS). Besides those of the GSH conjugates of 3-methylindole, the structures of the test GSH conjugates were confirmed by comparison with the reference standards in the literature or in our lab [10–30]. Based on the observed diagnostic fragmentations in the current study, a general strategy for the detection and differentiation of GSH conjugates was established, the effectiveness of which was evaluated on a liquid chromatography–tandem mass spectrometry (LC–MS/MS) platform. The results confirmed that this approach can be routinely used for the rapid identification and characterization of GSH conjugates, which will facilitate the elucidation of bioactivation mechanisms and guide structural optimization in the early discovery stage.

2. Experimental

2.1. Materials

The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA): acetaminophen, captopril, chlorogenic acid, clopidogrel, clofibric acid, clozapine, diclofenac, GSH, ibuprofen, mefenamic acid, 3-methylindole, monocrotaline, naproxen, β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH), 4-nonylphenol, oridonin, prasugrel, ticlopidine, tiopronin, triptolide, troglitazone, valproic acid, and zofenopril. Allitinib was kindly provided by Allist Pharmaceuticals, Inc. (Shanghai, China). Dauricine was purchased from Shenzhen Medherb Bio-Tech Co., Ltd. (Shenzhen, Guangdong, China). Lapatinib was purchased from TLC Pharmachem., Inc. (Vaughan, Ontario, Canada). Erdosteine and famitinib were kindly provided by Jiangsu Hengrui Medicine Co., Ltd. (Lianyungang, Jiangsu, China). Human liver microsomes (HLMs) were purchased from BD Biosciences (Woburn, MA, USA). Acetonitrile and formic acid of HPLC grade were supplied by Sigma–Aldrich. All other reagents and solvents were of analytical were purchased from China National Medicines (Shanghai, China).