

## Application of LC-MS based glutathione-trapped reactive metabolites in the discovery of toxicity of traditional Chinese medicine

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### Highlights

Metabolic bioactivation of certain drugs can generate reactive metabolites (RM) that are capable of covalently modifying cellular biomolecules and are implicated in drug induced toxicity. These electrophilic metabolites, also known as intermediates, cannot easily be detected due to the chemical instability and shorter half-life. As a natural nucleophilic agent, glutathione (GSH) can capture these electrophilic RM, forming GSH covalent conjugates. GSH conjugates screening by liquid chromatography-mass spectrometry (LC-MS) can be applied for rapid characterization and detection of potential toxicity of traditional Chinese medicine (TCM). In this paper, we briefly introduced the principle of RM trapped by GSH, as well as mentioned some qualitative and quantitative methods by LC-MS for GSH conjugates screening over recent years. Moreover, the application of this technology in the discovery of toxicity of TCM, especially those containing pyrrolizidine alkaloids, furans and quinoid species, were also reviewed. In short, the approach of LC-MS based GSH-trapped RM can be employed for rapid and sensitive detection of potential toxic RM in TCM, with a good prospect in application.

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## Abstract

We summarized the discovery of liquid chromatography mass spectrometry (LC-MS) based glutathione (GSH) capture of reactive metabolites in traditional Chinese medicine (TCM), which provides scientific basis for further research and clinical application of Chinese medicine toxicity discovery. This dissertation overviews relevant literatures of GSH capture of reactive metabolites in recent years. And then we review the principles and methods of LC-MS based GSH capture of reactive metabolites, as well as the research progress in the discovery of toxicity of TCM including pyrrolizidine alkaloids, furans and quinoid species. The review shows that the representative compounds of TCM includes adonifoline, lasiocarpine, diosbulbin B and safrol are well detected by LC-MS based GSH capture technique. And the main analytic systems of LC-MS are triple quadrupole and Q-Trap mass spectrometer. Constant neutral loss scan (CNLS), precursor ion scan (PIS) and multiple reaction monitor (MRM) are main detection methods to monitor the characteristic GSH conjugate fragmentations. The approach of LC-MS based GSH-trapped metabolites has a good application prospect in the discovery of toxic components of TCM.

**Keywords:** glutathione conjugation, Liquid Chromatography Mass Spectrometry, Reactive metabolites, Toxicity, traditional Chinese medicine

## 摘要

某些药物在体内代谢过程中会生成反应性代谢物，并与体内的大分子物质结合产生毒副作用。这些亲电代谢产物或中间体，具有化学不稳定性和较短的半衰期，往往不易被检测到。谷胱甘肽作为天然的亲核物质，可以捕获亲电的反应性代谢产物，与之形成 GSH 共价结合物。利用液质联用技术对 GSH 结合物进行检测和分析，即可快速筛查和发现中药潜在毒性物质。本文简要介绍了谷胱甘肽捕获反应性代谢物的原理，列举了近年来 LC-MS 检测 GSH 结合物的定性和定量分析方法，例如用于检测谷胱甘肽特征碎片的中性丢失扫描、前体离子扫描和多反应监测等方法，并就该技术在一些含有吡咯里西啶生物碱类（阿多尼弗林碱、毛果天芥菜碱）、呋喃类（黄独乙素）、醌类（黄樟素）中药毒性物质的发现进行了综述。概而述之，基于 GSH 捕获反应性代谢物的 LC-MS 检测方法，可快速、灵敏地发现中药潜在的毒性反应性代谢物，具有较好的应用前景。

**关键词：**谷胱甘肽结合物；液相色谱-质谱法；反应性代谢物；毒性；中药

**Abbreviation:** LC-MS: Liquid Chromatography Mass Spectrometry; GSH: glutathione; CID: Collision-induced Dissociation; CNLS: Constant Neutral Loss Scan; PIS: Precondition Ion Scan; XoPI: Extraction of Product Ion; Q-Trap: Quadrupole linear ion trap mass spectrometry; MRM: Multiple Reaction Monitoring; EPI: Enhanced Product Ion; Q-TOF: Quadrupole-time-of-flight mass spectrometer; LTQ-FTMS: Linear Ion Trap Instruments-Fourier transform mass spectrometer; MDF: Mass Defect Filtering; MS<sup>n</sup>: Multiple-stage mass spectrometry; ICP-MS: Inductively Coupled Plasma Mass Spectrometry; PAs: Pyrrolizidine Alkaloids; d-GSH: dansyl Glutathione; HPAs: Hepatotoxic Pyrrolizidine Alkaloids; 7-GSH-DHP: 7-glutathione-6,7-dihydro-1-hydroxymethyl-5H-pyrrole-azine; 7,9-di-GSH-DHP: 7,9-diglutathione-6,7-dihydro-1-hydroxymethyl-5H-pyrazine; DMF: 2,5-dimethylfuran; BBA: 4-bromobenzylamine; BDA: cis-2-butene-1,4-diol; RMs: Reactive Metabolites; MBI: Mechanism-based Inhibition; TCM: Traditional Chinese Medicine; FAB-MS: fast atom bombardment mass spectrometry

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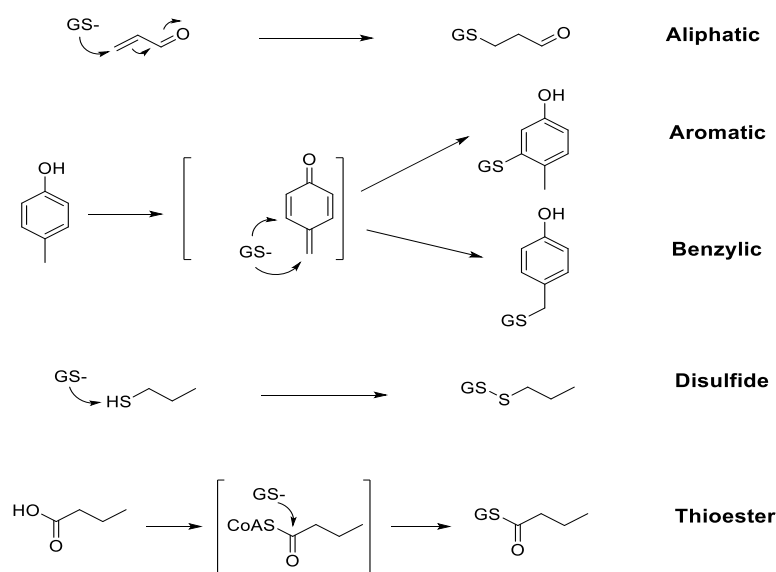
## Background

Some drugs are converted into chemically reactive metabolites by enzyme-mediated bioactivation. These reactive metabolites can covalently modify cellular macromolecules such as proteins and DNA causing cell damage and ultimately lead to drug induced toxicity and subsequently negative outcomes [1]. Some of traditional Chinese medicine (TCM), especially those containing pyrrolizidine alkaloids, quinoid species and furans, can produce toxic reactive metabolites. In order to reduce the elimination rate of candidate drugs, it is necessary to conduct drug candidate compounds for reactive metabolite screening in drug design and early development of new drugs to predict possible interactions between drugs and organisms. As a ubiquitous substance, glutathione (GSH) can capture reactive metabolites and prevent damage to important proteins and nucleic acid [2]. Adding GSH to liver microsome or hepatocyte incubation system, and detecting or identifying GSH conjugates of reactive metabolites by liquid chromatography-tandem mass spectrometry (LC-MS) is the basic method for studying reactive metabolites [3].

## GSH captures reactive metabolites

Many exogenous compounds are absorbed in the body, and then an electrophilic active intermediate is formed by metabolic reaction. These metabolites can interact with cellular components in a variety of way, such as covalent bonding with biomacromolecules and stimulate lipid peroxidation [4]. This process of converting relatively inert compounds into active intermediate metabolites is commonly referred to as metabolic activation or biological activation [5, 6]. The biological activation

process is the root cause of many TCM poisonings. However, due to the short half-life and unstable nature of reactive metabolites, they are not easily detected. Most of the reactive metabolites are electrophilic and can react with nucleophilic substances [7]. In vitro capture methods are generally used to examine the bioactivation potential of these candidate drugs. The capture agents are mainly thiols (GSH, cysteine or N-acetylcysteine), amines (semicarbazide and methoxyamines) and cyano anions, among them GSH is generally used to trap reactive metabolites. GSH, a tripeptide consisting of glutamate, cysteine and glycine, is the most ubiquitous non-protein intracellular thiol in mammalian systems and its nucleophilic cysteinyl thiol group allows GSH to react with a variety of electrophilic species to form GSH conjugates [8]. An important physiological defense mechanism against chemically reactive intermediates *in vivo* is GSH conjugation. Based on the site of conjugation, GSH conjugates are mainly divided into five structural classes, namely aliphatic, aromatic, benzylic, disulfide, and thioester, shown in Figure 1. The GSH S-transferase orients the substrate and the GSH in a position that increases nucleophilicity of -SH towards the substrate. GSH conjugates are excreted in bile and its breakdown product (the N-acetylcysteine conjugate or mercapturic acid) is excreted in urine [9]. In the liver, dipeptidases can hydrolyze glycine and glutamate from the GSH conjugate to form a cysteine conjugate, and then this conjugate is transported to the bile or blood. In the kidney, N-acetyltransferase acetylates the primary amine of cysteine to form the N-acetylcysteine conjugate [10]. Furthermore,  $\beta$ -lyase can cleave the carbon-sulfur bond to form a free thiol in the kidney. Excretion of mercapturic acid is often taken as a sign of the formation of the reactive species.

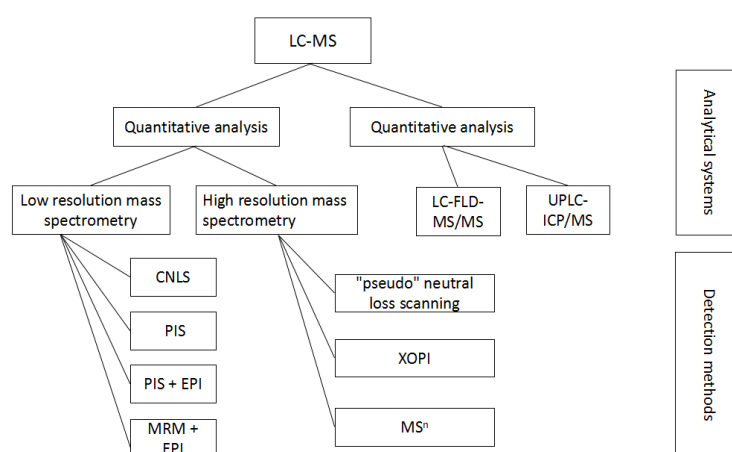


**Figure 1.** Different conjugation sites of GSH with reactive species

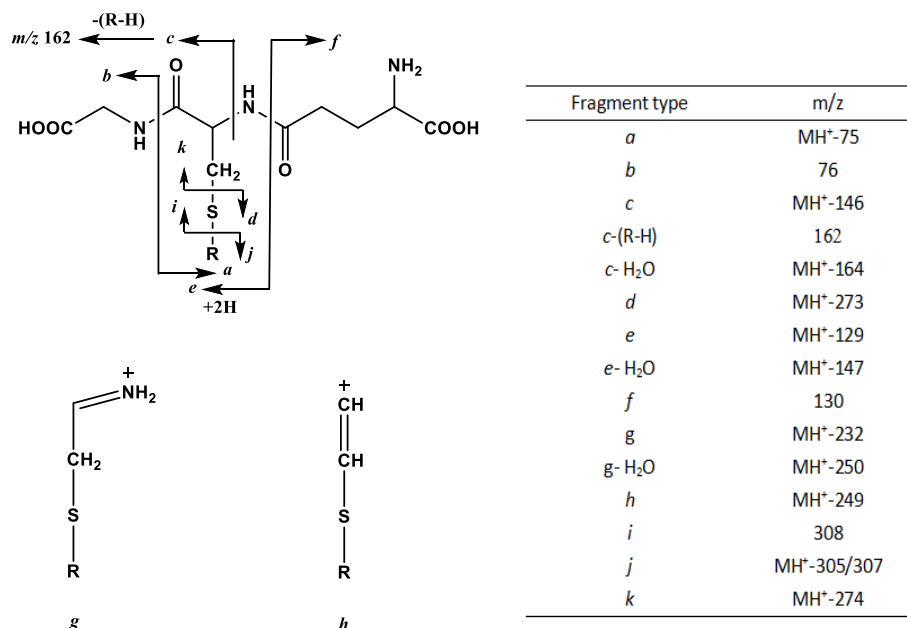
## Technology for capturing toxic substances based on GSH

Although drug metabolism is a complex process in terms of metabolic pathways and biochemical reactions, the detection of GSH conjugates can identify most of the reactive metabolites formed by drugs. GSH is a tripeptide in mammalian systems and its nucleophilic cysteinyl thiol group can trap electrophilic species to form GSH conjugates. Reactive metabolite detection using

microsomal incubations *in vitro* with GSH is one of the most crucial steps in assessing potential toxicity of pharmaceutical compounds [11]. And the identification of GSH conjugates by LC-MS (Figure 2) techniques is also an integral part of pharmaceutical research. The indirect but valuable information regarding the nature of reactive species and supporting pharmaceutical lead optimization was provided by the structural characterization of the resulting stable conjugates.



**Figure 2.** Qualitative and quantitative analysis techniques by LC-MS for capturing toxic substances based on GSH.



**Figure 3.** Characteristic fragment ions of glutathione conjugates under collision-induced dissociation [15]

### Qualitative analysis by LC-MS

The constant neutral loss method is a classic one which is based on the observation of the pyroglutamic acid moiety ( $m/z$  129 Da) cleaving from GSH conjugates upon collision-induced dissociation (CID). Therefore, neutral loss scanning of 129 Da in positive ion mode has been widely embraced as the gold standard for GSH conjugate screening. The fragments in the GSH conjugate are mainly derived from the cleavage of the peptide backbone in the structure. Although the relative abundance of different types of fragment ions sometimes depends on the nature of the bound species, GSH binders generally lose 129 Da (pyroglutamic acid) neutrally, producing e-type fragment ions [12], showed in Figure 3. Therefore, GSH conjugates can be detected by 129 Da neutral loss scan (CNLS) [13]. However, this method may lead to a false negative result due to its poor selectivity. In order to avoid this situation, Dieckhaus et al. demonstrated that negative ion MS/MS is promising to overcome this limitation because the MS/MS spectrum of the deprotonated molecule  $[M-H]^-$  of GSH and the major class GSH conjugate has a common anion fragment at  $m/z$  272 (deprotonated  $\gamma$ -glutamyl-dehydroalanyl-glycine) [14]. Therefore, the precursor ion  $m/z$  272 can be scanned in negative ion mode to detect the GSH conjugate and then switch the polarity to the positive ion mode to obtain the full scan product ion mass spectrum of the conjugate  $MH^+$ . In structural analysis, the combination of these two modes may provide more information. According to the above description of neutral loss scan and precursor ion scan, such a strategy can be adopted. Wen *et al.* developed a method for high-throughput detection of GSH conjugates by polarity switching on a quadrupole linear ion trap mass spectrometry (Q-Trap) [15]. In the same LC-MS/MS test, this method not only exerts the advantages of high selectivity and sensitivity of negative ion precursor ion scanning, but also exerts the structural analysis ability of full scan of product ions in positive ion mode, which enhances the throughput of screening GSH conjugate and greatly shortens the analysis time. Zheng *et al.* used a new method of Q-Trap mass spectrometer combined with multiple reaction detection (MRM) as a probing screening [16]. An enhanced product ion (EPI) scan was also performed to screen for reactive metabolites [17]. According to the common P450 metabolic activation reaction, a series of MRM transformations were constructed from protonated molecules of potential GSH conjugates by losing 129 Da, 305 Da and 307 Da (Figure 3) to their product ions. Due to the high scanning speed of the Q-Trap mass spectrometer, the probe scan can be set to track up to more than 100 MRM conversions without significant loss of sensitivity. However, it can only be used to detect conventional predictable GSH conjugates, while for those unconventional conjugates, neutral loss and precursor ion scanning are more effective.

In recent years, Castro-Perez *et al.* reported a method for LC/MS. Accurate neutral loss of 129.0426 Da

(corresponding to the exact mass of pyroglutamic acid) was used in a triple quadrupole-time-of-flight mass spectrometer (Q-TOF) to detect GSH conjugates to eliminate false positives [18]. Q-TOF mass spectrometry does not have a true neutral loss scan, and the detector mass spectrum is obtained by successively switching the collision energy at 5-20 eV. Precursor ions are obtained at low energy (5 eV) and product ions are obtained at high energy (20 eV) for "pseudo" neutral loss scanning. This method allows for the selective detection and identification of GSH conjugates, providing greater selectivity and greatly reducing analysis time. GSH conjugates produce anionic fragments in the negative ion mode (deprotonated  $\gamma$ -glutamyl-dehydroalanyl-glycine). Using this property, Zhu *et al.* detected product ions with a charge-to-mass ratio in the range of  $m/z$  269.5-274.5 under CID conditions and collected corresponding high-resolution mass spectrometry data. Extracting  $m/z$   $272.0888 \pm 5$  ppm of product ions from these product ions, such that the deprotonated  $\gamma$ -glutamyl-dehydroalanyl-glycine ( $m/z$  272.0888) will have a major peak and almost no interference peaks [19]. Therefore, the peaks in this range are mainly derived from the binding products of GSH. GSH conjugate precursor ions were determined by precursor ion full-scan mass spectrometry and their structures were identified by MS/MS mass spectrometry. MS data from MS and MS/MS scan are not sufficient to determine the structure of the metabolites and how they are broken. Therefore, multi-stage full-scan mass spectrometry is required to provide sufficient data to determine the fragmentation pattern and structure of metabolites or metabolite fragments, and also infer the biotransformation process of the drug in the body.

For the screen of active metabolites, the development of Q-TOF, LTQ-FTMS, and LTQ Orbitrap high resolution mass spectrometry promotes the improvement in analytical methods [20]. These instruments have high sensitivity with fast data acquisition capability, and also have the function of multi-stage mass spectrometry scanning ( $MS^n$ ). However, these mass spectra differ from traditional triple quadrupole mass spectrometers in that they do not have true neutral loss, precursor ion, and MRM scan modes, which limit their usage in detecting reactive metabolites in complex biological matrices. So as to overcome this defect, Zhu *et al.* combined high-resolution mass spectrometry with post-data processing technology mass defect filtering (MDF) for unconventional and unanticipated metabolite screening [21]. With the development of data mining technology, scholars have developed some new MDF technologies based on the conventional method. MMDF technology, multi-quality loss filtering, is to set up multiple structural filtering templates based on MDF technology to reveal different types of GSH conjugates [22]. Quality loss filtering is a kind of data processing technology after acquisition, followed by background deduction and noise reduction software [23]. The software has proven to be very



effective in detecting metabolites in complex matrices. Metabolite detection and identification can be achieved by comparing the detected sample data with control data using accurate mass data and subtracting background-related signals. Zhu *et al.* improved the background deduction procedure by adding noise reduction algorithms to further clean up residual matrix noise. The program effectively reduces matrix ions and cleans up the ion chromatogram, and the processed mass spectral data helps identify the molecular ions of the metabolite.

### Quantitative analysis by LC-MS

Gan *et al.* used LC-FLD-MS/MS method to quantify reactive metabolites in liver microsome incubation solution with dansyl glutathione (d-GSH). d-GSH is formed by a series of reactions between GSH and the fluorophore dansyl [24]. This method has been successfully applied to the quantitative detection of active metabolites in some model drug liver microsome incubation solutions. d-GSH and GSH have similar reactivity, but d-GSH is not a coenzyme of glutathione transferase. Thus, screening for compounds that require glutathione transferase catalysis is not applicable.

The concentration of drug metabolites in organisms and their pharmacokinetic parameters are useful for characterizing the toxicity of candidate drugs. GSH conjugates are key metabolites in two-phase metabolism. If GSH does not bind to one-phase metabolites, it will cause toxicity in response. Therefore, it is particularly important to monitor the level of GSH conjugates early in drug development. Stable isotope-labeled internal standard compounds have been widely used in the detection of two-phase metabolites. Li *et al.* used an isotope-labeled GSH conjugates as an internal standard, and quantitatively determined the two-phase metabolite GSH conjugate in the liver microparticle incubation solution by LC-MS [25]. The precision and accuracy were good.

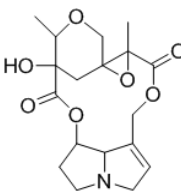
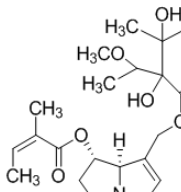
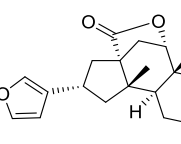
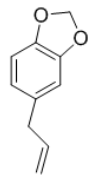
Quantification of drug-related substances in biological samples is a problem in the absence of radiolabeled drugs or standard controls. Liquid chromatography coupled with inductively coupled plasma mass spectrometry (ICP MS) can provide a novel method for determining GSH conjugates. MacDonald *et al.* quantified the GSH conjugate of clozapine in human liver microsome incubation solution by UPLC/ICP MS [26]. However, ICP MS cannot provide relevant structural information and it's not applicable to screen GSH conjugates.

In summary, most findings about the TCM reactive metabolic toxicity were qualitatively studied by LC-MS. The quantitative study of reactive metabolites is not perfect enough, and still need further improvement.

### Application in the discovery of toxic components of TCM

Metabolism of TCM can produce some metabolites with physicochemical and pharmacological properties that significantly different from the parent drug. Therefore, in order to reduce the risk of expensive clinical stage loss due to the production of toxic reactive metabolites by drug candidates, reliable and efficient methods are needed to capture and detect reactive metabolites. GSH is a nucleophile that binds well to most reactive metabolism. GSH is incubated *in vitro* with hepatic microsomes or hepatocytes to capture the TCM metabolites, and then the GSH conjugates are detected by qualitative analysis and quantitative analysis by LC-MS (Table 1). This method has been widely applied to discover and detect the toxic components of TCM, and the elimination rate of drug candidates is reduced. Some TCM toxic components captured by LC-MS-based GSH are summarized as follows.

**Table1.** Summarized characteristic of the adonifoline, lasiocarpine, diosbulbin B and safrole were detected by LC-MS.

Compound type	Representative compound	Compound structure	Trapping agents	Analytic system	Detection method	Characteristic ions	Plant origin	Reference
Pyrrolizidine alkaloids	adonifoline		GSH	LC-MS <sup>n</sup> Q-TOF	1. PIS 2. polarity switch mode	308 Da 290 Da 138 Da 120 Da	<i>Senecio scandens</i> ; <i>S. dolichodoryius</i> .	[29]
	lasiocarpine		GSH	LC-MS/MS	PIS	504 Da 666 Da 360 Da 130 Da	<i>Symphytum officinale</i>	[31]
Furan-containing compounds	diosbulbin B		GSH/BBA	Q-Trap MS Q-TOF MS ICP-MS LC-MS/MS	1.polarity switch mode 2.NLS 3.PIS 4.MRM scan 5.EPI+MRM 6. EPI+IDA	861 Da 863 Da 129 Da 272 Da 169 Da 171 Da 79 Da 81 Da	<i>Dioscorea bulbifera</i> L.	[36]
Quinoid species	safrole		GSH	LC-MS/MS FAB-MS	PIS	456 Da 130 Da 307 Da	<i>Sassafras albidum</i> (Nutt.) Nees	[42]

## Pyrrolizidine alkaloids

Pyrrolizidine alkaloids (PAs) is widely found in plants with flowers, mainly distributed in Boraginaceae, Compositae, Legum inosae and Orchidaceae. At least 40 TCM or folk medicines in China contain PAs. Some of them, such as senecio, coltsfoot flower, perrin and comfrey, are recorded in the Chinese Pharmacopoeia, and they are widely used in Chinese patent medicine preparations. PAs is called hepatotoxic pyrrolizidine alkaloids (HPAs) because of its significant liver toxicity [27]. The toxicity of PAs depends to a large extent on their metabolic activation through liver enzymes, including cytochrome P450, which forms covalent adducts with cellular nucleophiles to become chemically reactive pyrrole derivatives to exhibit toxicity. Capturing reactive metabolites with nucleophilic GSH to produce structurally stable GSH conjugates has been widely exploited to evaluate preclinical bioactivation potential drug candidates [28]. Research has shown that GSH captured their pyrrole metabolites to form GSH conjugates including 7-glutathione-6,7-dihydro-1-hydroxymethyl-5H-pyrrole-azine(7-GSH-DHP); and 7,9-diglutathione-6,7-dihydro-1-hydroxymethyl-5H-pyrazine (7,9-di-GSH-DHP); to compare the metabolic activity of PAs, and those GSH conjugates were quantified by LC-MS/MS [29]. This method was highly sensitive and selective for the estimation and quantification of GSH-DHP conjugates. Using the most powerful quantitative MRM method, the metabolites can be separated into two major pyrrole metabolites by LC-MS/MS, namely 7-GSH-DHP and 7,9-di-GSH-DHP. A large number of studies have been conducted on the metabolism of PAs, but they are mainly concentrated *in vitro*, and there are relatively few studies *in vivo*. Aster contains a variety of hepatotoxic pyrrolizidine alkaloids, of which clivorine is the main component. The combination with pyrrole to form a pyrrole conjugate during metabolism in the body is an important indicator of its toxicity. There are experimental studies using *Ligularia hodgsonii* Hook to extract a certain amount of clivorine [30]. Hepatic toxicity was studied by detecting serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, binding to pyrrole and reduced GSH in liver tissue. Senecionine is a 12-membered macrolide 1,2-unsaturated PA, which has attracted extensive attention all over the world because of its strong hepatotoxicity and genotoxicity. It is also one of the earliest and most studied PAs. Among these metabolites in bile, urine and feces of rats after oral senecio, GSH-DHP conjugate, 7-GSH-DHP, 7,9-diGSH-DHP and dehydroretronecin were inferred by retention time and neutral loss of 129 Da using LC-MS<sup>n</sup>. The DHP formed by dehydrogenation of sub-alkali is a poisonous intermediate of PAs, and its binding to DNA and protein will directly cause irreversible damage. Therefore, the identification of DHP indirectly proves the mechanism of the toxicity of Senecionine. Comfrey contains as many as 14 PAs, including 7-acetylintermediate, 7-acetyllycopsamine, echimidine,

intermediate, lasiocarpine, lycopsamine, myoscorpine, symlandine, symphytine, and symviri-dine. However, the mechanism of geranium-induced genotoxicity and carcinogenicity is still not fully understood. Available evidence suggests that active metabolites of comfrey PA interact with DNA in liver endothelial cells and hepatocytes, leading to DNA damage mutations that induce cancer progression [31]. Since the limited conditions, the GSH can be used to capture the PAs metabolite of comfrey. This method requires further verification. HPAs exists in many medicinal plants, but there are few studies on its toxicity and safety. The research on its mechanism of toxicity is not deep enough, and there is no detoxification drug at present. Therefore, LC-MS based GSH captured reactive metabolites are useful for detecting and identifying the metabolic toxicity of PAs. However, methods based on LC-MS detection need further optimization.

## Furan-containing compounds

Furan compounds are abundant in foods and herbs. Many furan-containing compounds have been reported to be cytotoxic and carcinogenic agents [32]. The toxicity of the furan compound is mainly from the electrophilic intermediate produced by furan epoxidation reaction [33]. The intermediate is an epoxide or *cis*-enedione which can react with nuclear proteins such as DNA or DNA to initiate toxicity. Previous studies have shown that furan is oxidized to reactive R,  $\beta$ -unsaturated dialdehyde, *cis*-2-butene-1,4-diol (BDA), which is catalyzed by cytochrome P450 in the reaction. Lu *et al.* proposed seven additional characteristics of urinary furan metabolites, and the presence of abundant furan metabolites in urine was determined by LC-MS/MS, indicating that oxidized furans were followed by BDA and BDA with cellular cysteine and lysine residues. The base reaction may represent an important *in vivo* pathway for furan biotransformation. The metabolites are markers of furan exposure and biological activation because they are derived from cellular BDA reaction products. And these metabolites can be explored as potential biomarkers in human studies. Metabolism of hepatotoxic furans results in the formation of protein adducts in target organs. An important step in furan toxicity may be the protein adduct formation. *In vitro*, semicarbazide or GSH can capture furan active metabolites and they are identified as BDA. Studies in rat hepatocytes have shown that BDA cross-link GSH with various amines, including proteins, in a metabolically dependent process. Phillips *et al.* analyzed the tryptic digests of alkylated proteins by LC-MS/MS, indicating that most adducts occur on lysine residues, and the selectivity of BDA reactions is lower than that of GSH-BDA [34]. This type of adducts may contribute to the toxic effects of furan experimented by the use of LC-MS/MS and NMR as an adduct of O-methyl hydrazine of the open-loop furan moiety of L-739,010 with liver microsomes [35]. The furan moiety undergoes oxidative cleavage to highly reactive 2-butene-1,4- dialdehyde, representing the major site biotransformation of L-739,010. Many furan-containing



compounds are biologically activated to produce toxicity, so the furan portion of L-739,010 can be considered undesirable. Wang et al. reported that the crude extract obtained from the TCM *Dioscorea bulbifera* L. contains furan dioxime, and the GSH/BBA-derived pyrrole can be produced by incubation of 2,5-dimethylfuran (DMF) in rat liver microsomes with GSH and 4-bromobenzylamine (BBA) as capture agents [36]. A neutral loss scan of 129 Da and a precursor ion scan of  $m/z$  272, 169 were obtained by LC-MS, and then the incubation mixture was monitored for 171 polarity switching mode. And the formation of bromine-labeled pyrrole derivatives by ICP MS. Previous LC-MS may be sufficient to detect those furans of known structure, but it has a limiting effect on screening complex mixtures of samples, especially those containing no structural information. However, new applications of LC-ICP MS, as well as the use of bromine-labeled compounds as capturing and labeling agents, are particularly unique, allowing us to quickly detect the formation of active metabolites.

### Quinoid species

An important toxicity of herbal medicines stems from their metabolic biotransformation into reactive metabolites (RMs). CYP catalyzes the synthesis of xenobiotics into chemically reactive metabolites or intermediates and stable metabolites. An *in vitro* assay using pulsed ultrafiltration and LC-MS/MS has been developed to screen plant extracts for the formation of electrophilic and potentially toxic quinoid species following hepatic cytochrome P450 bioactivation [37]. Some of the constituent compounds in herbal medicines can be activated by P450 as electrophilic intermediates capable of alkylating cellular biomolecules or participating in a redox cycle reaction and causing cell damage [38]. Covalent binding of hepatic macromolecules can lead to liver toxicity. Plant electrophiles contain phenols substituted with alkoxy- or o-hydroxy groups that can be metabolized to quinone methides or o-quinones, respectively [39]. The human body's important defense against this reactive intermediate is the nucleophile GSH. GSH is covalently bound to an electrophile to form a stable hydrophilic binder. Safrole is the main component of the volatile oil of Xixin and has antifungal effects. However, it has been shown that safrole has a certain relationship in the carcinogenesis of hepatocytes. Methylenedioxypheyl and allyl-benzene substructures of safrole can cause inhibition of enzyme activity and toxic effects, as a result of the mechanism-based inhibition (MBI) of CYP450 enzymes (CYPs) and producing RMs. Safrole combines with GSH to form two reactive metabolites of 1,2-dihydroxy-4-allylbenzene (M1) and 1'-hydroxyxanthine (M2). Yang et al. used the UPLC-MS/MS method to identify RMs by analysis of the safrole lysis process and GSH-M1 adduct. It can be concluded that the hepatotoxicity mechanism of jaundice is related to CYP1A2-mediated RMs and the cleavage process of GSH-M1/M2 adduct is analyzed in detail. Significant information has been obtained for predicting

drug-induced liver damage *in vivo* [40]. Bolton et al. selectively oxidized hydroxychavicol to the corresponding o-quinone (HC-quinone, 4-allyl-3,5-cyclohexadiene-1,2-dione) or p-quinone methide (HC-QM, 2-hydroxy-4-allylidene-2,5-cyclohexadien-1-one) and used GSH trapped these reactive electrophiles. And then used UV, NMR, and mass spectrometry fully characterized the GSH conjugates. By altering the GSH incubation conditions and other factors, experiments have shown that Safrole has another biological activation pathway that leads to toxicity. The toxic effects is initial O-dealkylation of the methylenedioxy ring forming hydroxychavicol, 2-electron oxidation to the o-quinone, and isomerization forming the more electrophilic p-quinone methide [41]. *Sassafras albidum* (Nutt.) Nees extract (sassafras), *Symphytum officinale* L. (Purple Grass) and *Rosemary* (*Rosmarinus officinalis* L.) contain compounds that are carcinogenic or toxic to mammals. All of them can produce GSH adducts during this screening assay. Johnson et al. used database search and other LC-MS/MS studies to identify several compounds that form GSH conjugates, including new metabolites of rosmarinic acid. These results demonstrate the feasibility of using pulsed ultrafiltration LC-MS/MS for the screening of botanical extracts and dietary supplements for compounds. Not knowing the chemical composition of the sample does not affect LC-MS detection, as only GSH adducts can be detected during selective LC-MS/MS analysis using precursor ion scanning and the chemical diversity in the extract does not interference with the identification of active metabolites [42]. After detection of the GSH adduct, LC-MS/MS with product ion scanning can be used for further characterization to facilitate structural resolution, and a search database (such as the NAPRALERT database) can help identify active metabolites and their plant precursors.

### Conclusion

TCM reactive metabolites, especially Chinese medicines containing pyrrolizidine alkaloids, furans and quinoid species may cause liver toxicity, leading to liver failure or other adverse reactions [43]. Therefore, this is an important topic worthy of discussion. Screening of the reactive metabolites of candidate drug is a key part of the discovery and development of TCM. The early study of reactive metabolites is beneficial to determine the structural information of candidate Chinese medicines, thus effectively reducing the cost of TCM development. Mass spectrometry based GSH capture of reactive metabolites has become an important research method for screening and identifying active metabolites. At present, mass spectrometry techniques for capturing GSH binding reactive metabolites include triple quadrupole, ion trap, quadrupole-linear ion trap, high resolution mass spectrometry, and data processing methods MDF and background subtraction, which greatly promote these methods. Studies of reactive metabolites and high throughput screening were achieved. Recently, the

application of predictive software in the structure of reactive metabolites has helped to identify the structure of candidate drugs, thereby reducing the chances of drug development failure. Due to the complexity of the TCM ingredients and the multi-target effect, the mass spectrometric response of the conjugate is usually different from the original shape after the drug metabolism intermediate is combined with GSH. Therefore, the conventional mass spectrometry method cannot quantitatively determine the reactive metabolite. The scale of this metabolic pathway is also unknown. Consequently, quantification of reactive metabolites remains a huge challenge. Accurately and rapidly quantitative analysis of reactive metabolites still requires sustained efforts.

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