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Usnic acid reactive metabolites formation in human, rat, and mice microsomes. Implication for hepatotoxicity

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ABSTRACT

Usnic acid is a lichen compound which is extensively studied due to its cytotoxic, antiproliferative, antimicrobial, antiviral, antiprotozoal, and anti-inflammatory activities. Despite a broad spectrum of biological properties, usnic acid is a hepatotoxic agent, thus its potential use as a drug is limited. Certain hepatotoxic drugs may act by generating reactive metabolites that damage the liver. The aim of the study was to predict the biotransformation of usnic acid enantiomers to reactive products using a trapping assay with glutathione in human, rat, and mice liver microsomes. Our results indicate that each enantiomer forms two reactive metabolites; in turn, these metabolites form adducts with glutathione, which may partially explain the toxicity of usnic acid. In silico analysis indicated structural alerts for the generation of reactive metabolites in usnic acid formula. This study proposes a novel mode of the hepatic toxicity of usnic acid enantiomers; it also provides some useful suggestions for designing safer usnic acid derivatives.

1. Introduction

Usnic acid (Fig. 1) is a lipophilic and optically active dibenzofuran derivative that is naturally found in lichens, especially within the *Cladonia*, *Usnea*, *Lecanora*, *Ramalina*, *Evernia* and *Parmelia* genera (Araújo et al., 2015). Both enantiomers of usnic acid have been reported to have cytotoxic, antiproliferative (Galanty et al., 2017), antimicrobial (Maciag-Dorszyńska et al., 2014; Studzińska-Sroka et al., 2015), antiviral (Sokolov et al., 2012), antiprotozoal (Si et al., 2016) and anti-inflammatory (Su et al., 2014) properties. Right-handed usnic acid has been studied more often and seems to be more active compared to its left-handed form, but exceptions exist (Bazin et al., 2008; Yilmaz et al., 2004).

Despite such a broad spectrum of interesting biological activities, usnic acid is a known hepatotoxic agent (Araújo et al., 2015). This property has been reported both *in vitro* (Chen et al., 2017; Han et al., 2004; Pramyothin et al., 2004; Sonko et al., 2011) and *in vivo* in rodents (Pramyothin et al., 2004). Hepatotoxicity due to usnic acid has also been observed in humans ingesting weight-reducing supplements containing this compound (Brown, 2017), therefore its potential application in medicine is limited.

One of the mechanisms of action of hepatotoxins is their metabolic modification, which results in the formation of reactive, usually electrophilic metabolites which covalently bind intracellular nucleophiles (e.g. glutathione) and macromolecules such as proteins and DNA, leading to their dysfunction. The toxicity of reactive metabolites may be also associated with the production of immunogenic complexes (Cho and Uetrecht, 2017). This mode of hepatotoxicity is distinctive for drugs like acetaminophen (paracetamol) (Hinson et al., 2010), as well as a number of phytoconstituents such as safrole (Yang et al., 2017), myristicin (Yang et al., 2015) and bakuchiol (Chi et al., 2016).

In order to identify possible new metabolites from usnic acid, we utilized a trapping assay; this is a standard procedure for detecting reactive metabolites in which a tested compound is subjected to incubation with a mixture of metabolizing enzymes and a trapping nucleophilic agent such as glutathione. The potential reactive compounds that are generated during metabolism are captured by the trapping agent and form adducts, which can then be detected by mass spectrometry (Yang et al., 2015).

In this study, usnic acid enantiomers were subjected to a trapping assay in human, rat, and mice microsomes. The *in silico* method allowed structural alerts of usnic acid for the generation of reactive metabolites

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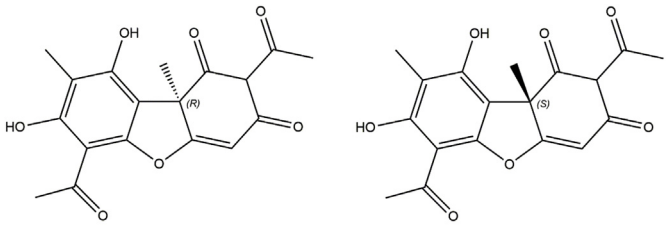


(+)-usnic acid (-)-usnic acid

Fig. 1. Usnic acid enantiomers in keto forms.

to be indicated. The results allow us to propose a novel mechanism of usnic acid hepatotoxicity and provide some new implications for the process of developing safer usnic acid derivatives.

2. Materials and methods

2.1. Chemicals

(+)-Usnic acid, reduced glutathione, human, rat, and mice liver microsomes, $NADP^+$, glucose-6-phosphate dehydrogenase, glucose-6-phosphate, buffers, LC/MS-grade acetonitrile, LC/MS-grade methanol, formic acid puriss. p.a., HPLC-grade chloroform were from Sigma-Aldrich. HPLC-grade water was obtained from an HLP 5 (HYDROLAB Poland) apparatus and was filtered through a 0.2 μm filter before use.

2.2. Isolation and identification of (-)-usnic acid

(-)-Usnic acid was isolated from *Cladonia uncialis* subsp. *uncialis* (L.) Weber ex F.H. Wigg., collected in northern Poland in dry, non-coastal European Scots pine forests in July 2008; its identity was verified by one of the authors (AG, a specialist in pharmacognosy and lichenology). The voucher specimen was deposited in the herbarium of Jagiellonian University Department of Pharmacognosy (Ref. No. KFg/2008/L2). Isolation of the compound was performed as described previously (Galanty et al., 2017) using a chloroform extract of dried lichen material (80 g). The identity of the compound was assessed by measuring the melting point and by TLC and HPLC analyses, according to previously published methods (Galanty et al., 2017). The enantiomeric form of isolated usnic acid was confirmed using a Jasco P-2000 polarimeter.

2.3. Microsomal incubations

A usnic acid primary stock solutions were prepared using acetonitrile (7.27 mM), which was further diluted in a phosphate buffer (second stock) to a concentration of 1.25 mM.

The assay was performed as previously described (Yang et al., 2015), with small modifications. The reaction mixtures consisted of (+) or (-)-usnic acid (50 μM), glutathione (5 mM), and human, rat, or mice microsomes (1 mg protein/mL) in a phosphate buffer (pH = 7.40; 0.1 M); they were preincubated for 10 min on an Eppendorf Thermoblock (37 °C, 350 rpm). Next, a NADPH-regenerating system ($NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase in phosphate buffer) was added to initiate the reaction. The total volume of the reaction mixture was 250 μL. Control reactions were performed without glutathione, microsomes, or the NADPH-regenerating system. The final concentration of acetonitrile did not exceed 0.68%. After 1 h, the reaction was terminated by adding an equal volume of ice-cold methanol and the tubes were transferred for 1 h to −20 °C. Samples were then centrifuged (13.000; 10 min) and the resulting supernatants were transferred to new microcentrifuge tubes and subjected to UPLC-MS/MS analyses. Three separate repeats of the experiments were conducted.

2.4. UPLC-MS/MS analyses

The UPLC-MS/MS system consisted of a Waters ACQUITY® UPLC® (Waters Corporation, Milford, MA, USA) coupled to a Waters TQD mass spectrometer (electrospray ionization mode ESI and tandem quadrupole). Chromatographic separations were carried out using the Acquity UPLC BEH (bridged ethyl hybrid) C_{18} column (2.1 × 100 mm, and 1.7 μm particle size) equipped with Acquity UPLC BEH C18 VanGuard pre-column (2.1 × 5 mm, and 1.7 μm particle size). The column was maintained at 40 °C and eluted under linear gradient conditions using 95%–0% of eluent A over 10 min, followed by isocratic elution using 100% of B over 2 min and linear gradient elution from 0% to 95% of A over 0.5 min, at a flow rate of 0.3 mL min^{-1}. Eluent A was water/formic acid (0.1%, v/v); eluent B was acetonitrile/formic acid (0.1%, v/v).

Chromatograms were recorded using a Waters eλ PDA detector. Spectra were analysed in the range 200–700 nm with 1.2 nm resolution and a sampling rate of 20 points/s.

MS detection settings of the Waters TQD mass spectrometer were as follows: source temperature 150 °C, desolvation temperature 350 °C, desolvation gas flow rate 600 L h^{-1}, cone gas flow 100 L h^{-1}, capillary potential 3.00 kV, cone potential 20 V. Nitrogen was used for both nebulizing and drying gas. The data were obtained in a scan mode ranging from 50 to 1000 *m/z* at 0.5 s intervals; 8 scans were summed to get the final spectrum. Collision activated dissociation (CAD) analyses were carried out with an energy of 20 eV and 50 eV using argon as the collision gas. Consequently, the ion spectra were obtained by scanning from 50 to 660 *m/z* range.

Data acquisition software was MassLynx V 4.1 (Waters).

2.5. Determination of structural alerts in ToxAlert

ToxAlert web database (http://ochem.eu/alerts) (Sushko et al., 2012) was used to examine usnic acid for structural alerts in relation to the formation of reactive metabolites. For this purpose, SMILES formulas of both usnic acid enantiomers and tautomers were introduced to the software and the structures were correctly generated. Analysis was performed with “Idiosyncratic toxicity (RM formation)” as an endpoint, while Molecule Preprocessing parameters were default.

3. Results

3.1. (-)-Usnic acid isolation

The applied isolation procedure yielded 108 mg of 95% pure (-)-usnic acid with a melting point of 202 °C; $[\alpha]_D^{24}$ - 375° ($CHCl_3$). The expected values for a reference substance were 203 °C for melting point and $[\alpha]_D^{24}$ - 495° for optical rotation [Huneck and Yoshimura, 1996].

3.2. Assessment of usnic acid structural alerts in silico

ToxAlerts generated two structural alerts of usnic acid: the presence of “ortho- or para-alkylphenols” and “Michael acceptors” (Kalgutkar and Soglia, 2005). There were no differences between the enantiomeric and tautomeric forms.

3.3. Trapping assay of usnic acid enantiomers

Incubation of usnic acid enantiomers with the tested microsome systems resulted in the formation of several metabolites. In the experiments performed in the absence of glutathione, two metabolites were detected: products of usnic acid hydroxylation (*m/z*: 361; retention time: 4.47 min) and dehydrogenation (*m/z* = 343; retention time: 6.33 min). A different metabolic profile was observed in the samples containing reduced glutathione. The peaks which appeared in the chromatograms of samples with (+)-usnic acid and glutathione were eluted in 4.58 min (M1) and 4.74 min (M2) (Fig. 2). The same eluting

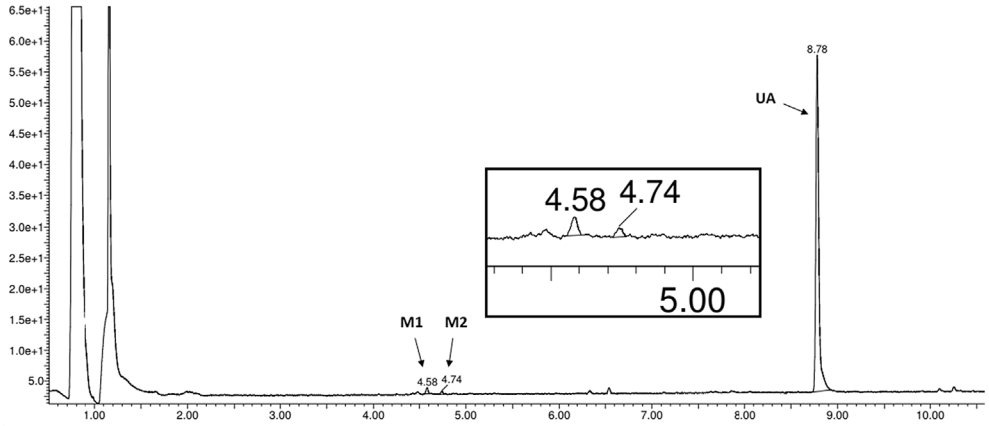


Fig. 2. UPLC chromatogram of the sample from the incubation mixture, consisting of (+)-usnic acid and glutathione in human liver microsomes. Metabolites (M1 and M2) and (+)-usnic acid (UA) are indicated by arrows. Magnified in the inset.

times were found for (-)-usnic acid metabolites - M3 and M4, respectively.

The subsequent mass spectrometry analyses of the samples indicated *m/z* values of M1 and M3 as 652, and M2 and M4 as 650. The fragmentation patterns of all the metabolites obtained using a collision energy of 20 eV showed a loss of 129 Da; this is characteristic of glutathione adducts and is associated with the loss of pyroglutamic acid. Loss of 75 Da was found in metabolites M1 and M3, which is associated with the loss of glycine (652- > 577). Also, in the fragmentation of both metabolites, peaks at *m/z* = 308 were observed; this is the mass of glutathione (Fig. 3). Due to equal retention times, *m/z* and fragmentation patterns of M1/M3 and M2/M4 pairs, these metabolites were identified as enantiomers.

3.4. Structure elucidation

Further analysis of mass spectra using a collision energy of 50 eV showed only minor changes of fragmentation patterns obtained for the metabolites compared to usnic acid; this suggests that the usnic acid underwent only minor modification during the metabolism. Metabolites M1 and M3 seem to be hydrogenated counterparts of M2 and M4; the acetyl substituent in the position 2 was reduced to 1-hydroxyethyl. Alternative reduction of the acetyl substituent in position 6 would lead to a product with a labile hydroxy substituent, but this was not observed on the MS/MS spectra. Even when using low collision energy, usnic acid's carbon–glutathione sulfur bond was the first to undergo fragmentation, therefore the “place of connection” between usnic acid and glutathione cannot be determined with MS/MS. However, based on structure alerts generated by ToxAlerts in usnic acid formula, it may be implied that the activation is associated with the presence of ortho-alkylphenols (Kalgutkar and Soglia, 2005), therefore the most probable site of reactivity is the methyl group. The potential structures of the metabolites and their fragmentation patterns are shown in Table 1 and Schemes 1–3.

3.5. Semi-quantitative analysis of metabolite formation

Although a precise quantitative analysis of the obtained metabolites was not possible, the differences in adduct formation between samples were compared by juxtaposing the metabolites' areas under the peaks (Table 2).

4. Discussion

Usnic acid is a lichen compound with potent biological activity, but its hepatotoxicity limits its clinical significance. Several liver damage cases have been reported in humans taking usnic acid or supplements containing usnic acid. Liver-related injuries included liver necrosis, jaundice, fulminant hepatic failure, hepatic encephalopathy and hepatitis. Some patients required liver transplants, while in a few cases liver failures were fatal (Brown, 2017).

Usnic acid hepatotoxicity is believed to have an idiosyncratic

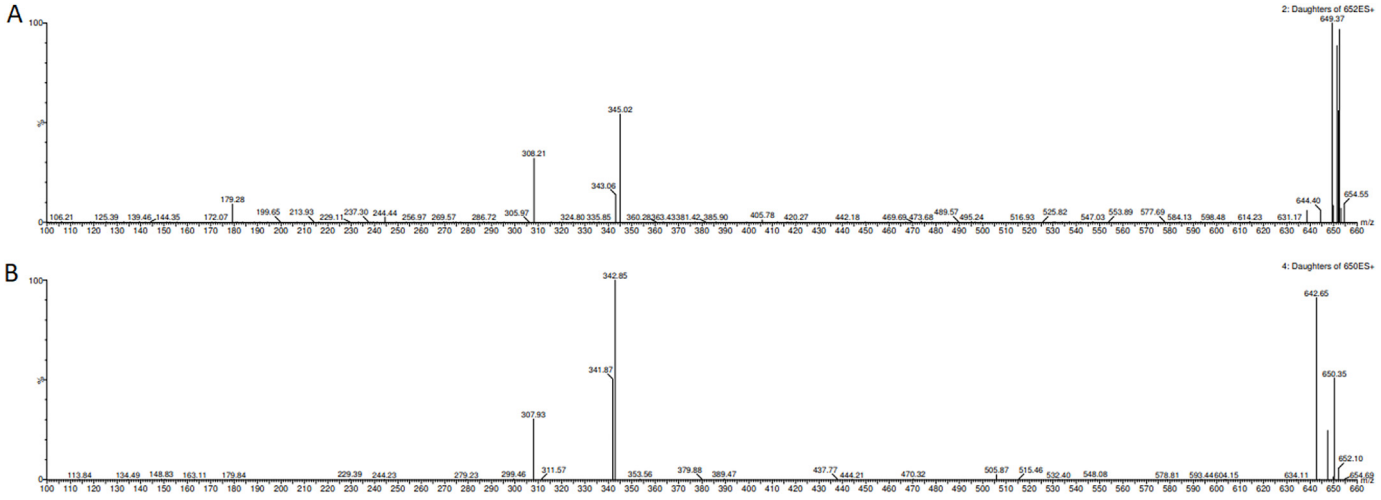
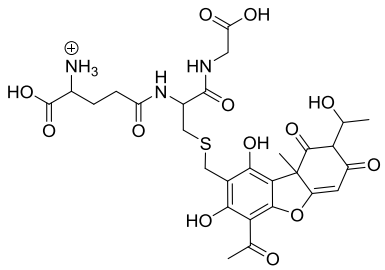
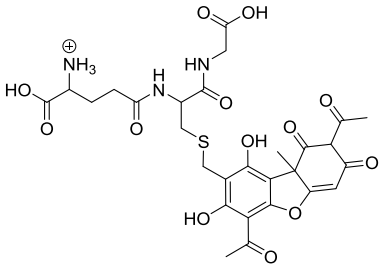
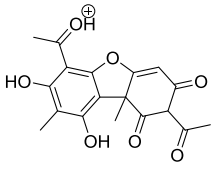


Fig. 3. Fragmentation of metabolites M1 (A) and M2 (B).

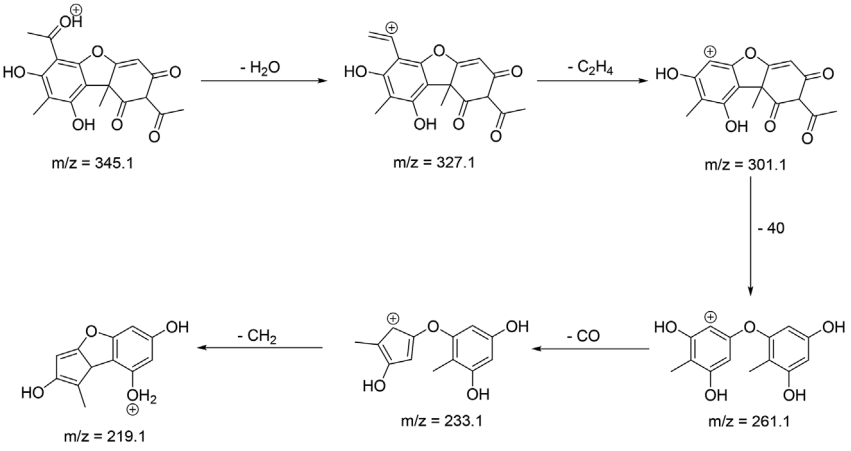
Table 1
Elution times, *m/z* values, fragmentation ions, structure of usnic acid, and potential structures of metabolites identified as glutathione adducts formed by (+) and (-)-usnic acid incubation in human liver microsomes.

Compound	RT [min]	*m/z*	Fragmentation ions	Structure
M1/M3	4.58	652.2	179.0, 233.1, 261.1, 308.1, 345.1	
M2/M4	4.74	650.2	179.0, 233.1, 261.1, 308.1, 343.1	
usnic acid	8.78	345.1	219.1, 233.1, 261.1, 301.1, 327.1	

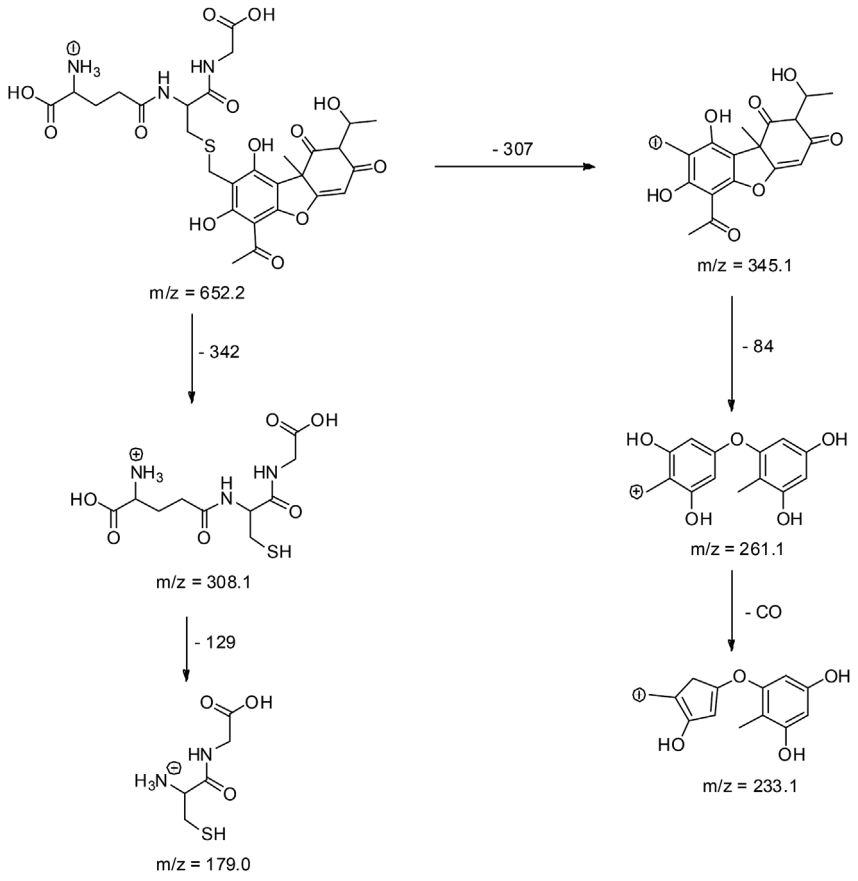
character and the exact mechanism of toxicity has not yet been defined. It has been suggested that usnic acid exerts cytotoxicity against rat hepatocytes by inducing the loss of membrane integrity and disruption of mitochondrial functions (Pramyothin et al., 2004). Oxidative stress induction has been indicated as a possible mode of toxicity of usnic acid in mouse hepatocytes (Han et al., 2004), while in human hepatocellular carcinoma cells (HepG2) the compound induced DNA damage apoptosis and cell cycle arrest (Chen et al., 2017). Most cellular studies imply that usnic acid causes necrosis and affects mitochondrial functions (Araújo et al., 2015).

In this study the metabolism of usnic acid was investigated as a potential cause of hepatotoxicity. Incubation of usnic acid enantiomers with human, rat and mice liver microsomes resulted in the formation of dehydrogenated and hydroxylated metabolites. Hydroxylated metabolites have previously been found in studies concerning the metabolism of usnic acid in S9 fraction (Foti et al., 2008), while the products of the dehydrogenation of usnic acid were detected for the first time. However, the most important result of our study concerns the detection by UPLC-MS/MS of the presence of usnic acid metabolite adducts with glutathione; this reveals a novel mechanism of usnic acid hepatotoxicity through the generation of reactive metabolites. A potential role of reactive metabolites of usnic acid in hepatotoxicity has previously been suggested (Pramyothin et al., 2004), but our study documented the formation of such compounds for the first time. The concentration of usnic acid used in the experiment is comparable to that observed in rabbits' blood after oral administration of the drug (Krishna and Venkataramana, 1992), therefore this effect could be significant from the perspective of further *in vivo* experiments.

Hepatic necrosis may also be due to reactive metabolites associated with glutathione depletion and oxidative stress (Hinson et al., 2010).



Scheme 1. Proposed fragmentation pattern of usnic acid.



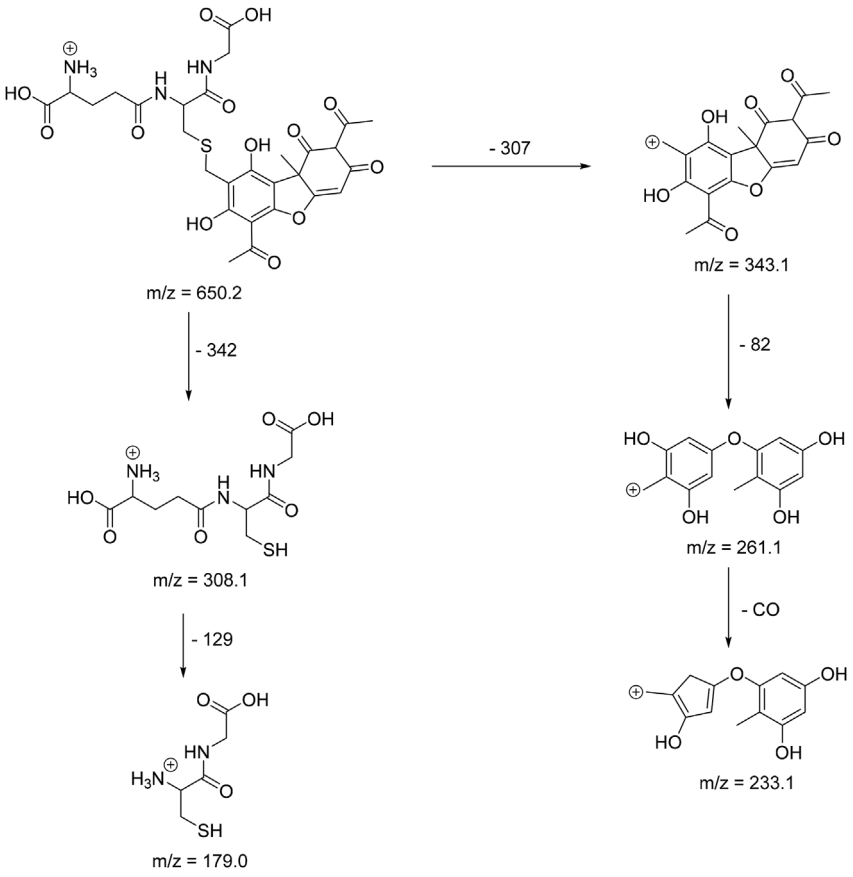
Scheme 2. Proposed fragmentation pattern of M1/M3.

Reactive metabolites may also act on cell membranes (Attia, 2010), DNA and proteins (Peterson, 2013), leading to loss of functions. As mentioned above, all these reactive metabolite-mediated effects have been observed in previous research concerning usnic acid hepatotoxicity (Araújo et al., 2015; Chen et al., 2017; Pramyothin et al., 2004). However, mechanistic examination of usnic acid's influence on hepatocytes has previously been mainly performed *in vitro*, while reactive metabolites may also covalently modify proteins and produce immunogenic haptens, which, after recognition by the immune system, are targeted by toxic immune reaction (Cho and Uetrecht, 2017). Such an effect cannot be observed in a simple *in vitro* experiment, therefore there is a need to establish if this phenomenon really occurs and whether it is responsible for the usnic acid-associated idiosyncratic reaction.

There is one report in the available literature that claims the metabolism of usnic acid has a detoxifying character. Shi et al. (2014) indicated that inhibitors of some CYPs may increase usnic acid cytotoxicity against rat hepatocytes, suggesting that the compound is more toxic than its metabolites. However, the results of this single study, performed in an *in vitro* model, cannot disprove the toxicity of the observed reactive metabolites, especially in humans, or that other mechanisms of the observed interaction may also be involved. Also, the response of the immune system to immunogenic adducts cannot be included in an *in vitro* study (Cho and Uetrecht, 2017). Moreover, the concentrations of usnic acid used in the experiment of Shi et al. were lower in comparison to pharmacokinetics data (Krishna and Venkataramana, 1992). Frequently a drug has two routes of biotransformation, detoxifying and toxifying; such a phenomenon may occur in the case of usnic acid, especially since metabolites other than reactive ones are known.

Although no quantitative determination of the formation of usnic acid metabolites was performed due to a lack of analytical standards of metabolites, some general conclusions on the differences in metabolic profile between species and enantiomers have been drawn based on the estimation of the area under the peak of adducts (Ryan et al., 2015). In human liver microsomes, peak areas of metabolites M1 and M3 were higher than M2 and M4, respectively. The opposite result was observed in rat and mice liver microsomes. There were also some differences between the adducts produced from both usnic acid enantiomers. (-)-Usnic acid transformed more preferentially to metabolite M3 than M4, in comparison to (+)-usnic acid, which metabolized to M1 and M2 in human liver microsomes. The area under the M3 metabolite peak was found to be over 10 times higher compared to the M4 compound in human liver microsomes, while the peak of metabolite M1 was only twice the size of that of compound M2. Enantiomers' metabolic profiles in rat liver microsomes were more similar to each other, while in mice they were the same. M2/M4 were more considerable, than M1/M3 in both species, but in mice the differences were more significant. Differences in the toxicity between both enantiomers can therefore be expected, especially in human livers, but this requires further experimental confirmation. Since no toxicological data on the possible differences of the hepatotoxic profile of usnic acid enantiomers have been published, such preliminary results would benefit the literature. The production of metabolites in different ratios between human, rat, or mice liver microsomes may imply that rodent models are less useful in studies on usnic acid metabolite-dependent hepatotoxicity, and extrapolation of experimental results from rodents to humans should be done with great caution.

Because of the labile carbon–sulfur bond, the unambiguous structures of the obtained adducts were not determined with UPLC-MS/MS. However, based on structure alerts generated by ToxAlerts in usnic acid formula, it may be implied that the activation is associated with the presence of ortho-alkylphenols. Two ortho-methyl phenol moieties may, by two-electron oxidation, form reactive ortho-quinonemethides



Scheme 3. Proposed fragmentation pattern of M2/M4.

Table 2
Ratios of the area under the peaks of adducts formed from usnic acid enantiomers in different microsome systems. HLM – human liver microsomes; RLM – rat liver microsomes; MLM – mice liver microsomes.

Parent compound	Metabolites	Ratio in HLM	Ratio in RLM	Ratio in MLM
(+)-usnic acid	M1: M2	2.18: 1	0.73: 1	0.15: 1
(-)-usnic acid	M3: M4	10.1: 1	0.45: 1	0.15: 1

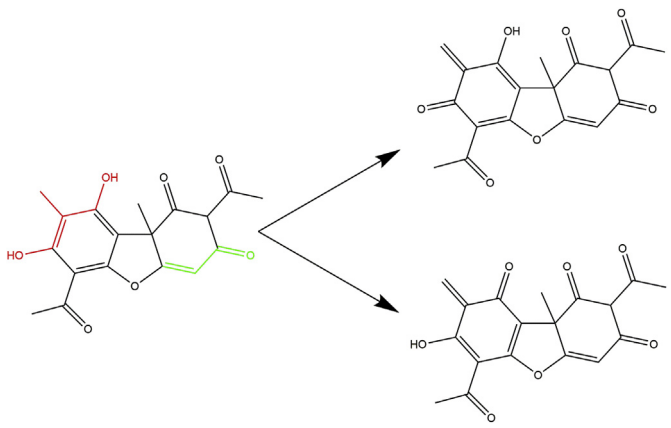


Fig. 4. Structural alerts (ortho-methyl phenols – red; Michael acceptor – green) in usnic acid and a proposed biotransformation to quinonemethides (Kalgutkar and Soglia, 2005). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Fig. 4). The theoretical mass of quinonemethide-glutathione adduct is 649 Da, which is consistent with the masses of M2/M4 metabolites (m/z = 650) from mass spectrum analyses.

Based on the m/z and fragmentation patterns obtained for the metabolites, it is suggested that M1/M3 may possess a reduced acetyl moiety in position 2 to hydroxyethyl one (see 3.4).

ToxAlert showed the presence of Michael acceptors (α,β-unsaturated carbonyl), which are reactive *per se* (Kalgutkar and Soglia, 2005), but in this study such activity was not indicated. However, the complete determination of the adducts' structure requires further, more complex studies involving chemical synthesis of the metabolites.

To avoid the alerts mentioned above, further studies are thus recommended in designing non-toxic usnic acid derivatives, lacking ortho-methyl phenols, especially since this moiety is unchanged in most of the studies on usnic acid modifications (Bazin et al., 2008; Bekker et al., 2015; Yu et al., 2016).

5. Conclusion

Detection of reactive metabolites is an important step in the drug development process. Idiosyncratic hepatotoxicity reactions, caused by the generation of reactive metabolites, not only caused several drugs to be withdrawn from the market, but also caused the deaths of many people. We suggest that formation of usnic acid reactive metabolites may be one of the mechanisms in which usnic acid induces liver injuries. The results of this study provide directions for future research concerning potential applications of usnic acid and its derivatives in medicine. It would be recommended to avoid ortho-methyl phenol moiety in further chemical modification of usnic acid pharmacophore. A novel concept for further toxicological studies on usnic acid hepatotoxicity would be to consider the differences between species and enantiomers.

Acknowledgments

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