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Impairment of silymarin metabolism in liver cells subject to injury induced by reactive oxygen species

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Abstract

Introduction: *Silybinins, the main components of silymarin, are lipid-soluble and widely used for the treatment of liver diseases. Diltiazem also exhibits hepatoprotective properties via interaction with intracellular membranes. Both silymarin and diltiazem reduce hepatocyte injury caused by reactive oxygen species (ROS). Silybinins have low bioavailability due to poor water solubility and rapid metabolism. In patients treated with silymarin, the blood concentration of silybinins often varies considerably between individuals, suggesting that silybinin metabolism is altered in diseased livers. The aim is to determine whether, in injured liver cells, silybinin metabolism is inhibited, and whether this inhibition is affected by diltiazem.*

Materials and methods: *H4IIE rat liver cells were treated with H_2O_2 to induce cell injury, assessed by measuring extracellular lactate dehydrogenase spectrophotometrically. Silybinins and their metabolites were quantitated using LCMS.*

Results: *Silybinin B, but not silybinin A, was metabolized to silybinin glucuronide as the main product. This metabolism was substantially inhibited in injured cells, and this inhibition was not reversed by diltiazem.*

Conclusion: *It is concluded that injury to liver cells induced by ROS decreases the metabolism of silybinins. In patients with diseased livers, the bioavailability of silybinins may be altered by the extent and nature of injury to hepatocytes induced by the disease.*

Keywords: *silymarin, liver cells, metabolism, cell injury, LCMS*

Abbreviations: *ROS: Reactive oxygen species; DMEM: Dulbecco's Modified Eagles Medium*

Introduction

Silymarin, a purified natural product derived from milk thistle (Silybummarianum), is used world-wide for the treatment of liver diseases, including drug-induced hepatotoxicity, non-alcoholicsteatohepatitis, hepatitis C and cirrhosis [1-5]. Milk thistle has been used for about 2,000 years as a remedy for liver and other ailments [6]. The active components of silymarin are flavonolignans. These are principally silybinins A and B, but also include isosylibinin, silychristin, silydianin and taxifolin [6,7]. While numerous intracellular targets for the action of silybininshave been identified, one of the principle actions of these agents is to decrease reactive oxygen species (ROS) and scavenge free radicals [5,6,8,9]. Since silybinins have significant lipid solubility, they provide an advantage over some other antioxidants in that their activity is exerted in a lipid as well as an aqueous environment [7]. In the absence of an enhancing agent, silybinins have a low bioavailability due to low water solubility and low absorption, as well as rapid first pass metabolism and excretion [10-12]. In the liver, silybininsare principally metabolized by phase II conjugative glucuronidation and sulphation, and the resulting glucuronides and sulphates excreted from hepatocytes via the canalicular efflux bile acid transporters [7,13,14].

Treatment with silymarin, even at high doses, appears safe and there are few side effects [6,15]. However, clinical trials employing high doses of silymarin have often not produced the degree of disease improvement predicted based on studies in vitro. This is most likely because their rapid metabolism and excretion in bile leads to a relatively low concentration of the active components in blood and the liver [2,7,12,16]. To improve the effectiveness of silymarin treatment, current studies are being directed towards the development of new bioenhancers to improve bioavailability, and to gain a better understanding of the factors, which influence the rate of silybinin metabolism and excretion [7,10,12,17].

Most studies of silybinin metabolism have been conducted with microsomes isolated from normal livers, and with normal livers in vivo [8,13,14,18,19]. While the results of some recent clinical trials conducted with patients with liver disease suggest that the metabolism of silybinins is altered in diseased hepatocytes, the effect of liver injury on silybinin metabolism remains poorly understood [7,17]. We previously showed that silymarin protects

liver cells from damage induced by ROS. A similar degree of protection was provided by d(+)-cis-diltiazem [20]. This protection from ROS-induced damage is principally mediated through the lipid environment of mitochondrial and other intracellular membranes [20]. The aim of the present study is to investigate the effect of ROS-induced injury to liver cells on their ability to metabolisesilybinin, and to determine whether diltiazem alters silybinin metabolism in injured liver cells.

Materials and methods

Materials

Silymarin, *d(+)-cis*-diltiazem HCl, H_2O_2 (30%), NAD, NADH, EDTA, Trition X-100, D- (+) – glucose, and pyruvic acid were obtained from Sigma-Aldric; Dulbecco's Modified Eagles Medium (DMEM) from Invitrogen and Gibco; fetal bovine serum from Bovogen; penicillin, streptomycinovalbuim, and EZQ protein quantification reagent from Invitrogen; trypsin (2.5% solution) from Gibco. Silymarin and diltiazem were prepared as stock solutions in water.

Culture of H4IIE liver cells

The source and subculture of H4IIE rat liver cells were as described previously [21]. The cells were incubated at 37°C under 5% CO_2 and 95% O_2 in either 6-well plates or T25cm^2 flasks. For incubation in 6-well plates, approx. 10^6 cells per well were plated on sterile 6-well plates with dimensions 13 × 8.5 × 2 cm (Greiner Bio-one, cat no. 657160). Approximately 1.5ml of DMEM supplemented with 10% (v/v) fetal bovine serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml) and 10 mM Hepes was added to each well. The cells were incubated overnight to ensure sufficient cell confluence (50-70%). H_2O_2, water, silymarin and/or diltiazem were added under sterile conditions as described in the legends to figures. For incubation of H4IIE cells in T25cm^2 flasks, approx. 2.0 ml cell suspension was plated in T25 cm^2 culture flask with dimensions 8 × 4 × 2 cm (Greiner Bio-one, cat no.690160). Approximately 6.0ml of DMEM supplemented with 10% (v/v) fetal bovine serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml) and 10 mMHepes was added into each T25 cm^2 culture flask.The cells were incubated overnight, to ensure sufficient cell confluence (50-70%), for the further experiment.H_2O_2, water, silymarin and/or diltiazem were added under sterile conditions as described in the legends to figures. Except where indicated otherwise, all incubations the medium used for all cell incubations was DMEM. For the incubation of cells during the measurement of the metabolism of silymarin, a Krebs Ringer Hepes medium was employed. This consisted of NaCl, 136 mM; KCl, 4.7 mM; $CaCl_2$,1.3 mM; $MgCl_2$, 1.25 mM; glucose, 10 mM; and HEPES, 10 mM, adjusted to pH 7.4 with NaOH.

Assessment of liver cell injury by measurement of lactate dehydrogenase leakage

H4IIE liver cell injury was assessed by measuring the amount of lactate dehydrogenase (LDH) released to the extracellular medium. LDH, which is located in the cytoplasmic space of hepatocytes, is released to the extracellular medium following cell injury and/or cell death [22]. For each experimental condition, the amount of LDH in the extracellular medium was expressed as a percentage of the total (intracellular plus extracellular) after subtraction of the amount of LDH in the extracellular medium of control cells (no H_2O_2 present). Total LDH activity was determined by lysing all the cells using digitonin(100 µmol/L).LDH activity was measured spectrophotometrically using either a Cobas Fara autoanalyser and standard Cobas Fara protocols, or on 96-well plates (Nunclon TM Δ Surface) using a Victor™ X4 2030 Multilabel Plate Reader together with a PerkinElmer 2030 workstation at 355nm and 30oC. LDH activity was expressed as optical density (OD) units/min per 10µl or 80µl sample.

Protein estimation

Protein concentration was determined using a fluorescence-based protein assay with an EZQ®Protein Quantitation Kit (Invitrogen) with ovalbumin as a standard.

Measurement of concentrations of silymarin and metabolite of silymarin

Concentrations of diltiazem, silymarin. and silymarin metabolites present in samples of extracellular medium were measured by LCMS using a Micromass Quattro Micro LCMS triple quadruple mass spectrometer (Mass Range: 2-2000 m/z) complete with ESI probe, Edwards E2M28 vacuum pump, Pentium IV PC and Mass Lynx 4.0 software.

Statistical analysis

Mean values between groups were compared using independent Student's t-test.

Results

We first established a model of ROS-induced liver cell injury based on the treatment of cells with H_2O_2 to generate ROS [23-25]. Cultured H4IIE rat liver cells were incubated in the presence of H_2O_2 for 1 h, then the medium containing H_2O_2 was removed and replaced with fresh DMEM which did not contain H_2O_2. The cells were subsequently incubated for 12 h, the culture medium separated from the adhering cells, and the activity of LDH in both the culture (extracellular) medium and in extracts of the adhering cells was measured.The results show that pre-treatment with 0.5 mM H_2O_2 increased the amount of LDH in the extracellular medium and decreased the amount in the adhering cells (Figure 1a and 1b). Leakage of LDH (amount of LDH in the extracellular medium expressed as a percentage of total LDH) was substantially increased by 0.5 mM H_2O_2 (Figure 1c). Results obtained following pre-treatment with 1.0 mM H_2O_2 showed a slightly higher increased leakage of LDH (Figure 1d-1f). For pre-treatment with 1.0 mM H_2O_2, the leakage of LDH was 75% compared with 65% for 0.5 mM H_2O_2 and about 5% for control (no H_2O_2) cells (Figure 1f cf 1c).

To assess the ability of normal liver cells to metabolisesilybinins, H4IIE cells were incubated in the presence of silymarin, and metabolic products in the extracellular medium measured by LCMS. A standard (un-metabolised) silymarin solution showed two major peaks which were identified as silybinins A and B (Figure 2a). Analysis of the extracellular medium from liver cells incubated for 4h with 20µMsilymarin showed the presence of silybinins A and B and a peak identified as silybininglucuronide (Figure 2b cf control, no silymarin, Figure 2c). Compared to analysis of the standard (unmetabolized) sample of silymarin, there was a substantial decrease in the silybinin B peak (Figure 2b cf 2a). A time course conducted from 5 min to 4 h showed that the concentration of glucuronide increased in a linear manner, while that of silybinin B decreased, and there was essentially no change in silybinin A (Figure 3 and Table 1). These results indicate that,

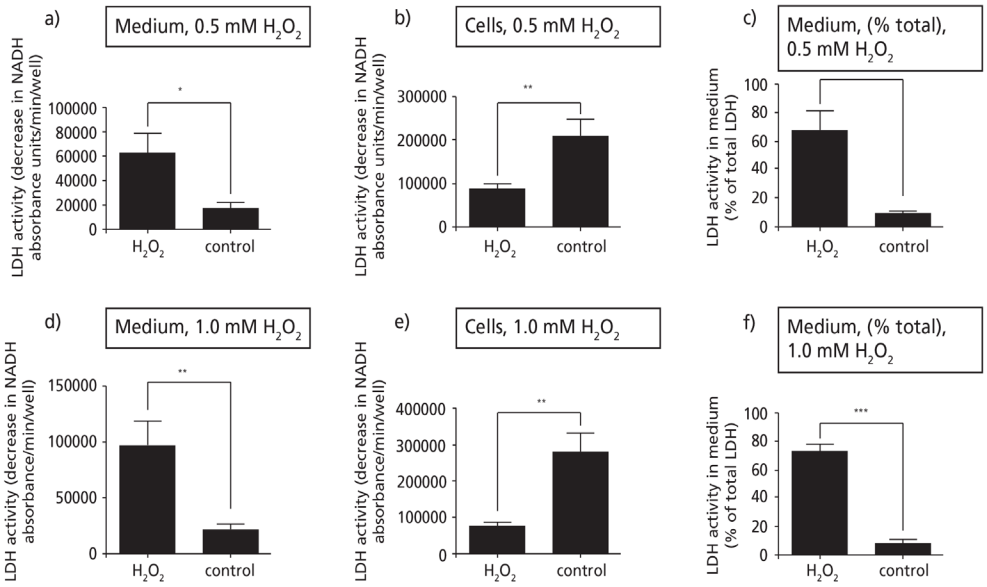

Figure 1. *Effect of pre-treatment with H_2O_2 on liver cell injury and viability assessed using lactate dehydrogenase leakage (LDH).(a,b) and (d,e). The amount of LDH in the extracellular medium (a,d) and in cells attached to the culture dish (b,e), respectively. (c,f). The amount of LDH in the extracellular medium expressed as a percentage of total LDH (medium plus cells). H4IIE rat liver cells were pre-treated for 1 h with 0.5 mM (a-c) or 1.0 mM (d-f) H_2O_2, the medium changed, the cells incubated for 12 h, then the extracellular medium and cells were collected separately for the measurement of LDH activity. The results are the means ± SEM for 6 separate experiments. The degrees of significance, determined using the independent Student's t-test for unpaired samples, are:*p<0.05, **p<0.01, ***p<0.001.*

		Area of peak (arbitary units x min)		
Peak	Retention time (min)	5 min	2 h	4 h
Silybinin B	15.5	7.870 ± 610	7.240 ± 510	6.420 ± 1230
Silybinin A	14.3	7.220 ± 600	9.060 ± 530	9.610 ± 880
Glucuronide	13.2	0	3.320 ± 190	5.510 ± 430

Table 1. *Time course for the metabolism of silybinins to silybininglucuronide by rat liver cells.The area under each of the identified peaks (silybinin A and B, and silybininglucuronide) (Figure 3) was determined as described in Methods. The values are the means ± SEM (n=4).*

		Area of peak (arbitary units x min)		
Peak	*Retention time (min)*	*Silymarin standard (20 µM)*	*Pre treatment control*	*Pre treatment with H_2O_2*
Silybinin B	*15.5*	*7.870±390*	*5.520±1.880*	*11.820±2.020*
Silybinin A	*14.3*	*4.990±780*	*10.710±2.160*	*10.880±1.740*
Silybinin Glucuronide	*13.2*	*0*	*8.380±700*	*4.560±320*

Table 2. *Cell injury, induced by H_2O_2, inhibits the metabolism of silybinins to silybininglucuronide by rat liver cells.The area under each of the identified peaks (silybinin A and B, and silybininglucuronide) (Figure 4) was determined as described in Methods. The values are the means ± SEM (n=4).*

		Area under the peak (arbitrary units x min)			
Peak	Retention time (min)	Silymarin standard (20 µM)	Pre-treatment control	Pre-treatment with H_2O_2	Pre-treatment with H_2O_2 diltiazem present with silymarin
Silybinin B	15.5	9.420	1.150 ± 950	4.620 ± 1.840**	5.230 ± 2.810*
Silybinin A	14.3	6.820	4.960 ± 540	5.890 ± 1.800	6.760 ± 1.140*
Silybininglucuronide	13.2	0	8.280 ± 220	4.800 ± 960**	6.620 ± 1.370
Diltiazem	13.7	-	-	-	1.580 ± 450

Table 3. *Effect of diltiazem on the metabolism of silybinins to silybininglucuronide by rat liver cells.The area under each of the identified peaks (silybinin A and B, and silybininglucuronide) (Figure 5) was determined as described in Methods. The values are the means ± SEM (n=4). The degree of significance, determined using the independent Student's t-test for unpaired samples are:*p<0.05, **p<0.01.*

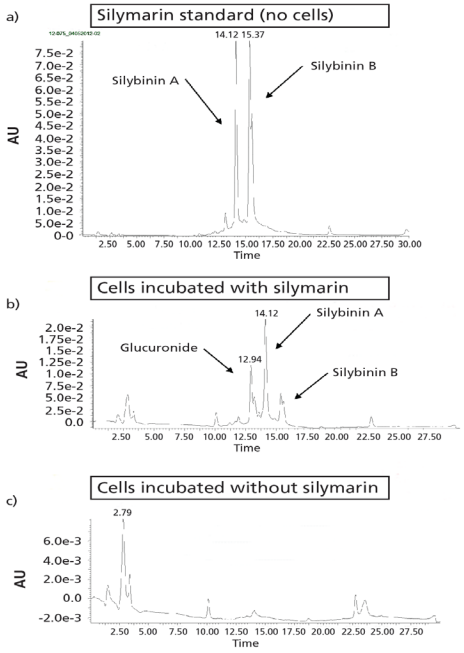

Figure 2. *Metabolism of silybinins to silybininglucuronide, measured using LCMS, by rat liver cells.(a) Standard (un-metabolised) silymarin solution (20 μM). (b and c) Liver cells incubated in the presence (b) or absence (c) of silymarin for 4 h. H4IIE cells were incubated in KRH medium and the amounts of silybinins and silybininglucuronide in the standard silymarin solution or in the culture medium were measured by LCMS.The chromatographs shown are representative of one of 3 separate experiments which each give similar results.*

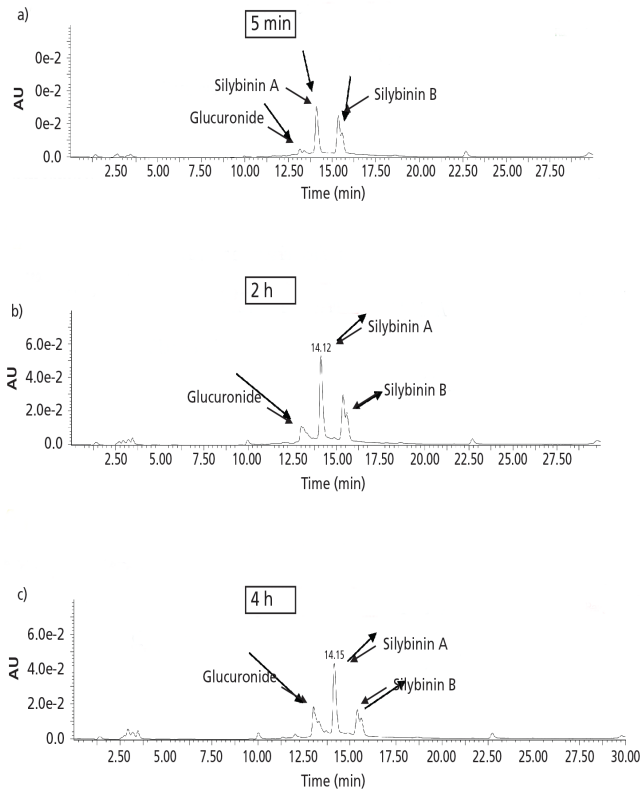

Figure 3. *Time course for the metabolism of silybinins to silybininglucuronide by rat liver cells. Liver cells were incubated in the presence of silymarin(20μM) for 5min (a), 2h (b) or 4h (c). H4IIE cells were incubated in KRH medium and the amounts of silybinins and silybininglucuronide in the culture medium measured by LCMS. The chromatographs shown are representative of one of 2 experiments which each give similar results*

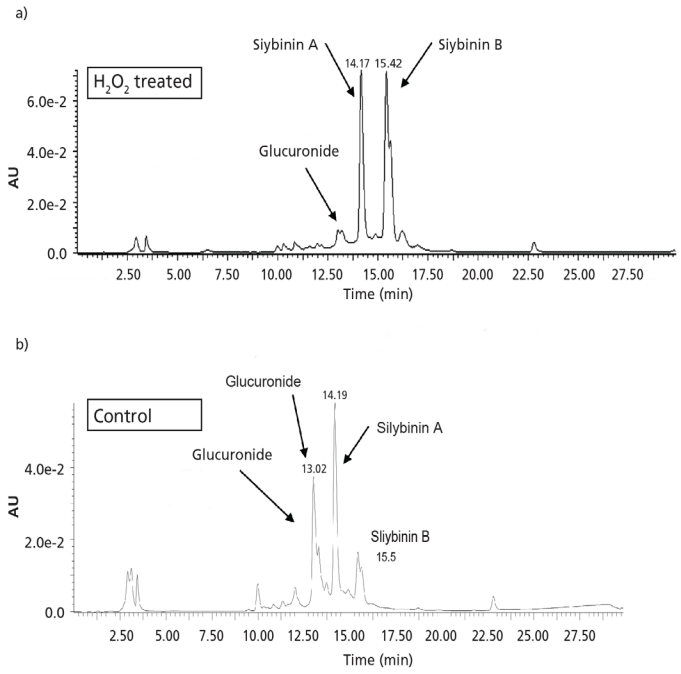

Figure 4. *Cell injury, induced by H_2O_2, inhibits the metabolism of silybinins to silybininglucuronide by rat liver cells. H4IIE liver cells were pre-treated with 1.0mM H_2O_2 (a) or water (control) (b) for 1 h, incubated in DMEM for 12 h, then incubated in KRH medium in the presence of silymarin for 6 h, and silymarin and its metabolites assayed by LCMS. The chromatographs shown are representative of one of 3 separate experiments which each give similar results.*

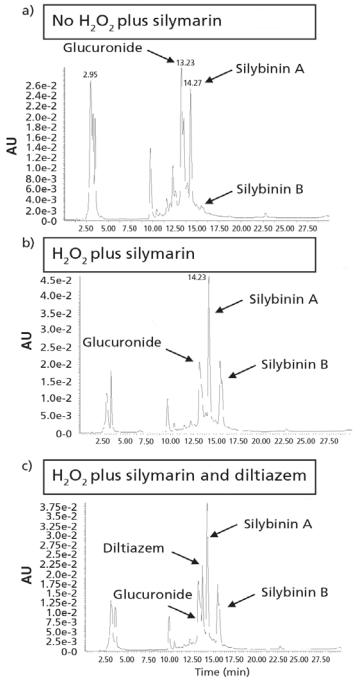

Figure 5. *Effect of diltiazem on the metabolism of silybinins to silybinin-glucuronide by injured rat liver cells. H4IIE liver cells were pre-treated with water (control) (a) or with 1.0mM H_2O_2 (b,c) for 1 h, incubated in DMEM for 12 h, then incubated in KRH medium in the presence of 20μM silymarin (a,b) or 20μM silymarin plus 20μM diltiazem (c) for 6 h, and silymarin and its metabolites assayed by LCMS. The chromatographs shown are representative of one of 2 separate experiments which each give similar results.*

under the conditions employed, silybinin B, but not silybinin A is metabolized to glucuronide as the main product.

To investigate the effect of liver cell injury on silybinin metabolism, H4IIE cells were first pre-treated with 1 mM H_2O_2 for 1 h to induce cell damage, then incubated in DMEM for 12 h, as described above. The resulting cells were then incubated in the presence of silymarin for 6 h. Silymarin and its metabolites present in the extracellular medium were assayed by LCMS. In cells pre-treated with H_2O_2 and subsequently incubated in the presence of silymarin, the formation of glucuronide and utilisation of silybinin B were substantially reduced compared to controls (Figure 4a cf 4b and Table 2).

To test whether diltiazem alters the metabolism of silymarin in injured H4IIE cells, diltiazem was included with silymarin in the 6 h incubation period. Co-incubation of diltiazem (final concentration 20μM) with silymarin had little effect on glucuronide formation in H_2O_2 pre-treated cells (Figure 5c (silymarin plus diltiazem, H_2O_2 pre-treatment) cf 5b (silymarin alone, H_2O_2 pre-treatment) and 5a (silymarin alone, no H_2O_2 pre-treatment) and Table 3). In incubations containing diltiazem, an additional peak at 13.7 min retention time, identified as diltiazem, was observed (Figure 5C).

Discussion

The results have shown that in normal untreated H4IIE rat liver cells, silybinin B is metabolized principally to glucuronide, with little metabolism of silybinin A. In liver cells injured by exposure to ROS the conversion of silybinin B to glucuronide is substantially inhibited. In injured liver cells, diltiazem did not enhance the conversion of silibinin B to glucuronide.To our knowledge, there have been few studies of the metabolism of silybinins in liver cells in culture. Most previous studies have been conducted with isolated microsomes and with livers in vivo [14,18,19]. The results of the present study, in which liver cells were incubated with silymarin for 4 or 6 h, are broadly consistent with those reported previously for microsomes which showed glucuronides as the major metabolic product, and a faster rate of metabolism for silybinin B compared to silybinin A [19]. By contrast, studies conducted with intact livers have shown the formation of a much wider range of metabolites formed from silybinins A and B [14].

The model of ROS-induced cell injury employed involved the treatment of liver cells with H_2O_2 for 1 h. This induced cell damage as indicated by release of LDH to the extracellular medium. It has previously been shown that similar models employing liver cells in culture exhibit a spectrum of injured cells, including those with damaged plasma membrane and intracellular membranes, and cells undergoing apoptosis and necrosis [26,27]. This spectrum of injury matches that observed in diseased livers in vivo such as in patients with non-alcoholichepatosteatosis and hepatitis C [28,29].

The observation that the metabolism of silybinin B is reduced in injured liver cells suggest that, in patients with hepatitis C, non alcoholicsteatohepatitis, and other liver diseases treated with high doses of silymarin, the presence of damaged hepatocytes may help to decrease the rapid metabolism and removal of silibinins from the blood, and hence promote a higher blood concentration of

pharmacologically active silybinins for a longer period.This may enhance the antioxidant and free radical scavenging actions of silybinins, but on the other hand may also increase the risk of side effects such as inhibition of organic anion transporters responsible for, in part, drug absorption and elimination [17,30,31].Thus, our results have implications for determining the dose of silymarin to be administered.

Under the conditions of the present experiments, diltiazemwas found to have no effect on the metabolism of silymarin in liver cells injured by the generation of ROS initiated by H_2O_2. However, interpretation of the present results is slightly complicated by the overlap of the peaks for diltiazem and silybininglucuronide in the LCMS profile. The lack of effect of diltiazem on silybinin metabolism is consistent with this being a process located principally within the aqueous milieu of the cytoplasmic space whereas diltiazem, as a lipophilic molecule, has been shown to have antioxidant effects within cell membranes [32]. Further experiments are warranted to investigate the actions of diltiazem on silymarin metabolism in damaged liver cells, including investigation of different concentrations and time of exposure to diltiazem.

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