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Journal of Pharmaceutical and Biomedical Analysis
34 (2004) 1071–1078
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Stereoselective metabolism of silybin diastereoisomers in the glucuronidation process

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Received 2 September 2003; received in revised form 22 November 2003; accepted 1 December 2003

Abstract

A separation method for the hepatoprotective drug silybin and its metabolites by RP-HPLC was described. Based on this separation, the stereoselectivity of the metabolism of silybin was investigated by incubation of the drug and its two diastereoisomers with bovine liver microsomes. Information about the structures of these metabolites was obtained, using UV, HPLC/MS and NMR spectra. Four major metabolites (M_1, M_4 of silybin A and M_2, M_5 of silybin B), were prepared by preparative HPLC, and their configurations were accomplished by NMR spectra. A HPLC method was used to quantify the metabolites. The results showed that silybin was extensively metabolized and the major sites for glucuronidation were the C-20, C-7, at phenolic OH groups. Furthermore, the results obtained reveal that there was significant stereoselectivity in the glucuronidation process of silybin. Silybin B was glucuronidated at a more efficient rate than its diastereoisomer, and glucuronidation of silybin B was much preferred at the 20 position, while that of silybin A was similar at both 7 and 20 position.

Keywords: Stereoselective metabolism; Silybin; UV, HPLC/MS, NMR spectrometry; Glucuronidation

1. Introduction

Stereoselective pharmacological activity and pharmacokinetic behavior of drugs have attracted an increasing interest in recent years since the development of methods for stereospecific. A large percentages of drugs used in clinic are chiral compounds and most of them are used as racemate (stereoisomeric mixtures) [1]. It is commonly agreed that the information of pharmacokinetic and pharmacodynamic behavior of enantiomers is very important for a safty and reasonable use clinically of racemic drugs.

Flavonoligan silybin is an important hepatoprotective drug, isolated from the seeds of milk thistle *Silybum mariana*, widely used in therapy of various liver damages [2]. Natural silybin is a mixture of two diastereoisomers A and B having configurations 2R, 3R, 10R, 11R and 2R, 3R, 10S, 11S in a 1:1 ratio (Fig. 1). These forms are distinguished by HPLC and are temporarily designed as SA and SB, respectively according to their retention time in HPLC as shown in Fig. 2B [3,4], but their absolute configuration is

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doi:10.1016/j.jpba.2003.12.002

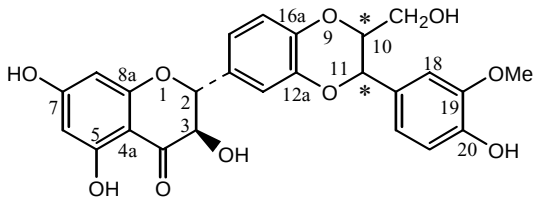

Fig. 1. Chemical structure of silybin.

not known, and they have been separated on preparatory scale only in the form of acetylated glycosides [5].

Although previous studies have suggested that the main metabolites of silybin are its glucurated conjugates, it is still uncertain whether the glucuronidation is preferred by one of its diastereoisomers. Pure silybin A and B are only available in minimal quantities, which makes the possible pharmacodynamic differences unable to be studied [6]. Therefore there was no material reported on the stereoselectivity in the glucuronidation process of silybin up to now.

In the present study, metabolism of silybin and its two diastereoisomers in glucuronidation process was investigated by bovine liver microsomes. The goal of this study was to definitively identify the formed metabolites and to determine the kinetics of formation, including with respect to stereoselectivity.

2. Experimental

2.1. Materials

The crude extract from the seeds of milk thistle *Silybum mariana* was purchased from Panjin pharmaceutical Co. Ltd. (Liaoning Province). Silybin A (SA, with shorter retention time) and silybin B (SB, with longer retention time) were prepared by preparative HPLC from the crude extract. The purity of the diastereomers was determined on a Phenomenex (Torrance, CA) C_{18} Luna analytical column (ODS 4.6 mm × 250 mm), and accounts to 98.4% for silybin B, 95.2% for silybin A, respectively. Their structures were demonstrated by HPLC and NMR spectra. Uridine 5-diphosphoglucuronic acid trisodium salt (UDPGA) was purchased from Sigma (St Louis, MO). The HPLC-grade methanol and acetonitrile were purchased from Milipore (Milford, MA, USA).

2.2. Instrumentation

HPLC analysis was carried out on a chromatographic system consisting of a Waters 600 multi-solvent delivery system and a Waters 996 photodiode Array Detector (Waters Association, Milford, MA, USA), on line-degasser. The separation and quantification of the glucuronidation metabolites and parent drug were achieved by isocratic reversed phase

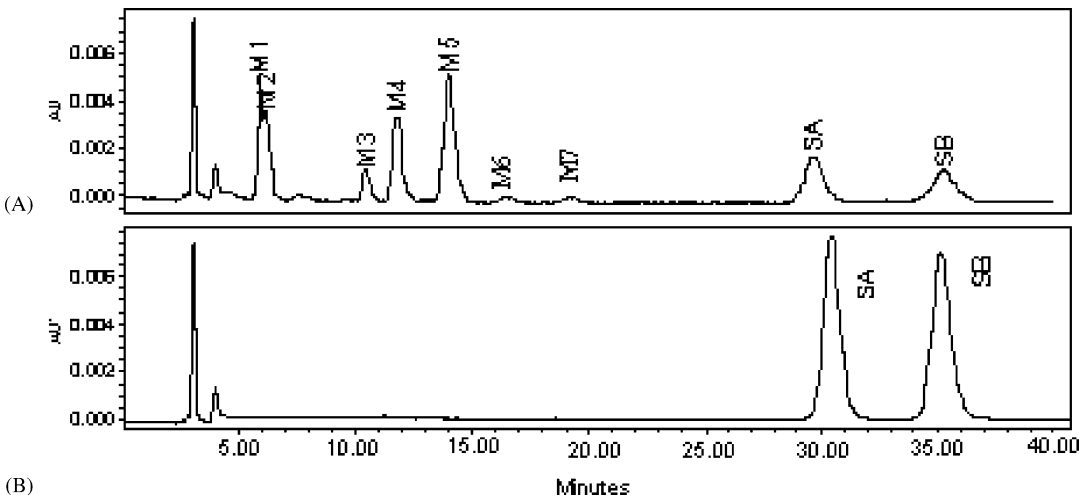

Fig. 2. Chromatograms of silybins (SA and SB) and their metabolites in incubation mixture (A) a sample incubated for 16 h and (B) a control sample. Chromatographic conditions are described in text.

chromatography on a Phenomenex (Torrance, CA) C_{18} Luna analytical column (ODS 4.6 mm × 250 mm), and mobile phase were MeCN/MeOH/H_2O (2.4:45:52.6) at a flow rate 1 ml/min, 35 °C. Data acquisition and analysis were performed using Waters Millennium (version 4.0).

Preparative HPLC was carried out on a semi-preparative system comprising a Waters 600 E gradient pump (Millipore, Watford, UK), manual loop injector 3725i-119, 5 ml loop volume (Rheodyne Inc., Cotati, CA), spectra 996 photodiode Array Detector (set at 286 nm). The column (25 mm × 100 mm) was packed with Delta^{TM}-pak C_{18} reversed phase 5 μm material and eluted with methanol/water/acetic acid (42:58:0.1) at a flow rate 5 ml/min, 25 °C.

HPLC/ESI-MS: Separation was performed using a Phenomenex C_{18} Luna analytical column (250 mm × 4.6 mm). A mobile phase was programmed as follows: MeCN/MeOH/H_2O (2.4:45:52.6). The characterization of the metabolites was performed on a Quattro tandems quadrupole mass spectrometer equipped with a megaflow electrospray interface (Micromass, Manchecter, UK) operating in positive and negative mode ion-trap mass. After UV detection, the HPLC effluent was split postcolumn to 250 μl/min and introduced into the electrospray source. Samples were analyzed using a spray voltage of 4.5 kV, capillary heater temperature of 450 °C, sheath gas of 35 psi (N_2) and an auxiliary gas flow (N_2) of 447 l/h. Mass spectra were acquired with a scan range from 50 to 1100 *m/z* (full scan analysis) in 2 s in both ion modes. All data were processed by Masslynx (Micromass, Manchester, UK) software.

NMR spectra 1D- and 2D-NMR data were measured on a Bruker DRX-500 NMR spectrometer in CD_3OD (or in DMSO) at 30 °C with TMS as internal standard.

2.3. Incubation and assay

The bovine liver microsomes were prepared by differential centrifugation according to literature procedure [7], protein concentrations were determined by the Lowery assay [8].

Metabolism of silybin in glucuronidation process was investigated by bovine liver microsomes. Additionally, to identify whether a metabolite was from silibin A or silybin B, the silybin diasrereomers were incubated separately. The incubates were analyzed by the RP-HPLC-DAD system. The quantification was done by analyzing the peak area of each diastereoisomers.

According to the literature [9], the incubation conditions of pooled microsomes initially were optimized with respect to protein concentration, co-solvent and incubation time (data not shown).

A mixture of 2.1 mg UDPGANa_3 salt (3.2 μmol), with a protein concentration at 0.22 mg/ml, and a co-solvent of DMSO/MeCN = 1:4, an incubation time of 16 h were used as optimized conditions for studying the metabolism of silybin and its two diastereoisomers in the bovine liver micrsomes. The volume was adjusted to 200 μl with Tris buffer (pH 8.0, containing 6 mM $CaCl_2$, 3% w/v BSA, and 1 mM dithiothreitol). The mixture was preincubated for 5 min at 30 °C in a shaking water bath. The reaction was initiated by adding 8 μl 0.2 M stocking solvent of variable substrates to a final concentration of 8 μM, respectively, including silybin, SA and SB. Control incubations were performed in the absence of UDPGANa_3 salt or microsomes. Reactions were allowed to proceed in the shaking water bath for 16 h at approximately 30 °C, and were terminated by the addition of 200 μl acetonitrile. The incubation mixtures were then centrifuged to remove participated protein. Supernatants obtained were analyzed directly by HPLC and HPLC/ESI-MS for metabolites identification. The control samples were treated in the same way.

The optimized analytical-scale reaction was scaled up to a larger reaction volume (25 ml), with substrate (SB, 97 mg, 0.2 mmol, mw 482.44; 8 mM final concentration), and UDPGANa_3 salt (260 mg, 0.4 mmol) and a final concentration of 0.22 mg/ml microsomal protein were added to the Tris buffer (25 ml, 50 mM, pH 8.0, containing 6 mM $CaCl_2$, 3% w/v BSA and 1 mM dithiothreitol) at 30 °C. After 16 h, EDTA was added to the reaction mixture and the reaction was terminated by adding acetonitrile (75 ml) and by cooling on ice.

The reaction mixture was diluted with three volumes of acetonitrile, and was centrifuged (5000 rpm, 15 min) to remove precipitated protein. The precipitates resuspended in 5 ml 50% methanol, reprecipitated with three volumes of acetonitritle, and recentrifugated. The filtrates were combined and the solvent removed in vacuo. The residue was dissolved in 50% methanol, filtered, and two major metabolites

were prepared by preparative HPLC as described under general Section 2, giving yields of 9.2 mg of M_2 and 27 mg of M_5.

Further purification of the metabolites from SA led to the isolation of M_1 8.0 mg and M_4 6.5 mg, respectively.

3. Results and conclusions

3.1. Optimization of incubation

A most interesting thing found was that different concentration of bovine liver microsomes and incubation time lead to a difference not only in amount but also in the conjugate sites. The optimum concentration of bovine liver microsomes appeared to be 0.22 mg/ml, too little enzyme or too much enzyme resulted in much reduced yields after incubation, and the best reaction time was 16 h, prolongation of the incubation time, the yields of the main metabolites were lowered.

3.2. Metabolites identification and quantification

A reverse phase HPLC method was developed to separate metabolites of silybin and its two diastereoisomers. A typical HPLC chromatogram of silybin and its metabolites was shown in Fig. 2.

In the incubation of silybin, as shown in Fig. 2, seven metabolite peaks (M_1–M_7) besides parent silybin (peak 8 and 9) were detected in the incubation mixture. They had almost the same UV spectra as that of the parent. Thus, it could be speculated that they were the metabolites of silybin. Metabolites 1–5, which eluted at approximately 5.91, 6.18, 10.44, 11.81 and 14.03 min, respectively, were the five major metabolites. They corresponded to 14.5, 12.5, 4.01, 19.15 and 35.35% of the total peak area, respectively. Other two peaks (M_6 and M_7) accounted for about 0.42, 0.59, respectively. About 13% unchanged silybin (SA and SB) remained, indicating that silybin was extensively metabolized. Furthermore, metabolite 1 had identical UV spectra as that of metabolite 2, with a maximum absorbance at 286.3 nm. This suggested that they were two diastereoisomers. It was also supported by the ES/MS spectra. Analogously, M_4 and M_5, and possibly M_6 and M_7, were diastereoisomeric pairs. The HPLC of the two diastereoisomers also confirmed that metabolites 1, 4 and 7 were from SA (Fig. 4B) while metabolites 2, 5 and 6 were from SB (Fig. 4C).

The structural identification of silybin metabolites was performed by negative (positive)-ion HPLC/MS. Metabolites 1–7 had identical ion mass spectra, which yielded a molecular ion of m/z 681($M+Na^+$) (Fig. 3). The product ion mass spectrum did show fragment

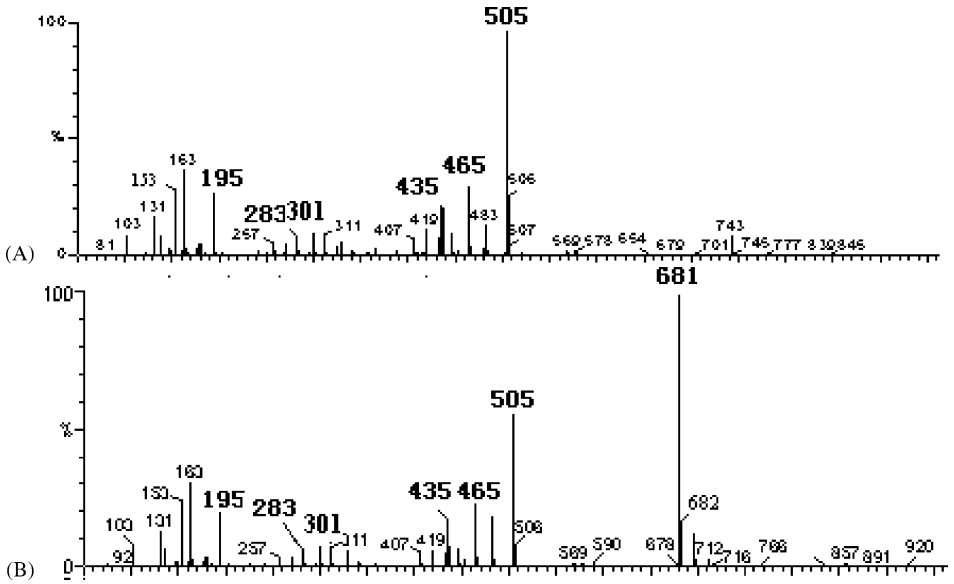

Fig. 3. ESI-MS spectra of silybin and its metabolites (A) SB and (B) M_2, 7-*O*-β-D-glucuronide of SB.

Table 1
^{1}H NMR data of SA, SB and their main glucuronides (500 MHz, 30 °C)

Protons	Sa[a]	M_1[a]	M_4[a]	SB[b]	M_2[b]	M_5[b]
2	5.06 1H, d, $J = 11$ Hz	5.10 (1H, d, $J = 11$ Hz)	5.08 (1H, d, $J = 11$ Hz)	5.0 (1H, d, $J = 11.5$ Hz)	5.02 (1H, d, $J = 11.5$ Hz)	4.97 (1H, d, $J = 11.5$ Hz)
3	4.58 1H, d, $J = 11$ Hz	4.70 (1H, d, $J = 11$ Hz)	4.62 (1H, d, $J = 11$ Hz)	4.54 (1H, d, $J = 11$ Hz)	4.56 (1H, d, $J = 11.5$ Hz)	4.51 (1H, d, $J = 11.5$ Hz)
6	5.89 (1H, d, $J = 2$ Hz)	6.17 (1H, S)	5.91 (1H, S)	5.94 (1H, d, $J = 2$ Hz)	6.22 (1H, S)	5.92 (1H, d, $J = 2$ Hz)
8	5.84 (1H, d, $J = 2$ Hz)	5.91 (1H, S)	5.88 (1H, S)	5.91 (1H, d, $J = 2$ Hz)	6.22 (1H, S)	5.89 (1H, d, $J = 2$ Hz)
10	4.08 (1H, s)	4.18 (1H, dd, $J = 8, 3$ Hz)	4.21 (1H, dd, $J = 8, 3$ Hz)	4.10 (1H, d, $J = 4$ Hz)	4.08 (1H, dd, $J = 8$, 3.5 Hz)	4.08 (1H, dd, $J = 7$, 3.5 Hz)
11	4.91 (1H, d, $J = 8$ Hz)	4.98 (1H, d, $J = 8$ Hz)	4.99 (1H, d, $J = 8$ Hz)	4.94 (1H, d, $J = 8$ Hz)	4.92 (1H, d, $J = 8$ Hz)	4.96 (1H, d, $J = 7$ Hz)
13	7.08 (1H, d, $J = 2$ Hz)	7.11 (1H, s)	7.12 (1H, s)	7.11 (1H, d, $J = 2$ Hz)	7.10 (1H, s)	6.99 (1H, s)
15	7.05 (1H, d, $J = 8$ Hz)	7.02 (1H, s)	7.10 (1H, s)	7.07 (1H, d, $J = 8$ Hz)	7.05 1H, dd, $J = 10$, 1.5 Hz	7.03 (1H, dd, $J = 8$, 1.5 Hz)
16	6.97 (1H, d, $J = 8$ Hz)	6.99 (1H, s)	7.01 (1H, s)	7.02 (1H, d, $J = 10.5$ Hz)	7.0 (1H, d, $J = 10$ Hz)	7.10 (1H, d, $J = 8$ Hz)
18	7.0 (1H, d, $J = 2$ Hz)	6.99 (1H, s)	7.03 (1H, s)	7.03 (1H, d, $J = 1.5$ Hz)	7.03 (1H, d, $J = 1.5$ Hz)	7.05 (1H, d, $J = 1.5$ Hz)
21	6.81 (1H, d, $J = 8$ Hz)	6.82 (1H, d, $J = 8$ Hz)	6.82 (1H, d, $J = 8$ Hz)	6.85 (1H, d, $J = 8$ Hz)	6.83 (1H, d, $J = 8$ Hz)	7.20 (1H, d, $J = 7.5$ Hz)
2	6.86 (1H, d, $J = 8$ Hz)	6.86 (1H, d, $J = 8$ Hz)	6.98 (1H, d, $J = 8$ Hz)	6.93 (1H, d, $J = 8$ Hz)	6.90 (1H, d, $J = 8$ Hz)	7.01 (1H, d, $J = 7.5$ Hz)
23d		3.78 (1H, d, $J = 11.5$ Hz)	3.56 (1H, d, $J = 11.5$ Hz)	3.73 (1H, dd, $J = 12, 2$ Hz)	3.72 (1H, dd, $J = 12.5$, 2 Hz)	3.71 (1H, dd, $J = 12.5$, 2 Hz)
23u	Overlapped with H_2O	3.53 (1H, dd, $J = 4$ Hz)	3.38 (1H, d, $J = 4$ Hz)	3.51 (1H, dd, $J = 12, 6$ Hz)	3.48 (1H, d, $J = 5$ Hz)	3.48 (1H, d, $J = 12.5$ Hz)
19-OMe	3.79 (3H, s)	3.84 (3H, s)	3.80 (3H, s)	3.89 (3H, s)	3.86 (3H, s)	3.87 (3H, s)
1′		4.99 (1H, s)	4.97 (1H, s)		4.97 (1H, s)	5.00 (1H, s)
2′		3.36 (1H, d, $J = 6$ Hz)	3.29 (1H, dd, $J = 11, 6$ Hz)		3.56 (1H, m)	3.58 (1H, m)
3′		3.30 (1H, dd, $J = 11$, 6 Hz)	3.28 (1H, dd, $J = 11$, 6 Hz)		3.52 (1H, m)	3.49 (1H, m)
4′		3.43 (1H, d, $J = 6$ Hz)	3.43 (1H, d, $J = 6$ Hz)		3.65 (1H, m)	3.65 (1H, m)
5′		3.48 (1H, m)	3.52 (1H, m)		3.52 (1H, m)	3.52 (1H, m)

[a] Dissolved in DMSO.
[b] Dissolved in CD_3OD.

ions at *m*/*z* 465,435, 301, 283 and 195, which were present in the spectrum of the parent silybin (*m*/*z* 505, $M+Na^+$). And the loss of 176 amu from the molecular ion of *m*/*z* 681 to 505 indicated that these metabolites were consistent with glucuronide conjugates.

M_2 and M_5 were unambiguously identified by detail analysis of their NMR spectral data (Tables 1 and 2). M_2 and M_5 have a same molecular weight. Their ^1H NMR data and ^{13}C NMR data also showed that they are glucuronide conjugates. The linkage between the silybin and glucuronic acid was detemined by HMBC. The correlation of the proton at δ 4.97 ppm with the carbon at δ 167.3 (C-7) in HMBC determined that M_2 was silybin B-7-*O*-β-D-glucuronide. The chemical shift of C-6, C-7 and C-4a, by comparison with that of silybin B caused by the glucuronation at C-7 position (Table 2) also supported this conclusion. The correlation of the proton at δ 5.00 ppm with the carbon at δ 148.3 (C-20) in HMBC determined that M_5 was silybin B-20-*O*-β-D-glucuronide.

Analogouely, M_1 and M_4 were determined as silybin A-7-*O*-β-D-glucuronide and silybin A-20-*O*-β-D-glucuronide. Their NMR spectral data was shown in Tables 1 and 2. The structures of M_6 and M_7 were not determined further for little samples obtained.

As shown in Fig. 4, Metabolites 1, 4 and 7 were formed only from silybin A, on the contrast,

Table 2
^{13}C NMR data of SA, SB and their main glucuronides (125 MHz, 30 °C)

Carbons	SA[a]	M_1[a]	M_4[a]	SB[b]	M_2[b]	M_5[b]
2	85.15	85.39	85.24	84.69	84.81	84.66
3	74.06	74.17	74.06	73.71	73.89	73.70
4	199.74	201.32	200.15	198.27	199.18	198.33
4a	102.74	104.81	102.97	101.84	103.57	101.84
5	165.97	165.38	166.01	164.42	164.72	165.32
6	98.95	99.47	98.90	97.42	98.50	97.39
7	170.55	167.81	170.11	168.80	167.29	168.73
8	97.91	98.03	97.90	96.35	97.12	96.34
8a	165.04	165.03	165.14	168.03	164.14	164.41
10	80.78	80.81	80.79	80.07	80.05	79.93
11	78.47	78.54	78.32	77.74	77.72	77.39
12a	145.86	145.98	145.84	145.16	145.15	144.98
13	118.91	119.07	119.03	117.64	117.66	117.90
14	132.74	132.59	132.91	131.58	131.37	133.03
15	123.81	124.11	124.12	122.11	122.19	122.24
16	118.05	118.02	117.83	117.85	117.86	117.68
16a	146.24	146.40	146.32	145.49	145.52	145.40
17	130.16	130.19	132.91	129.48	129.48	131.63
18	114.57	114.32	114.43	112.14	112.11	112.85
19	150.32	150.33	151.74	149.27	149.25	151.17
20	149.75	149.72	149.54	148.39	148.35	148.28
21	119.08	119.39	119.34	116.31	116.29	118.24
22	123.16	123.23	122.76	121.71	121.71	121.54
23	62.86	62.87	62.85	62.11	62.11	61.97
19-OMe	58.44	58.39	58.49	56.51	56.52	56.76
1′		101.76	102.46		101.25	102.72
2′		74.78	74.68		74.43	74.69
3′		77.46	77.49		77.72	77.51
4′		73.94	73.79		73.88	73.70
5′		75.43	75.77		76.43	77.39
6′		173.68	170.11		Not prepared	Not prepared

[a] Dissolved in DMSO.
[b] Dissolved in CD_3OD.

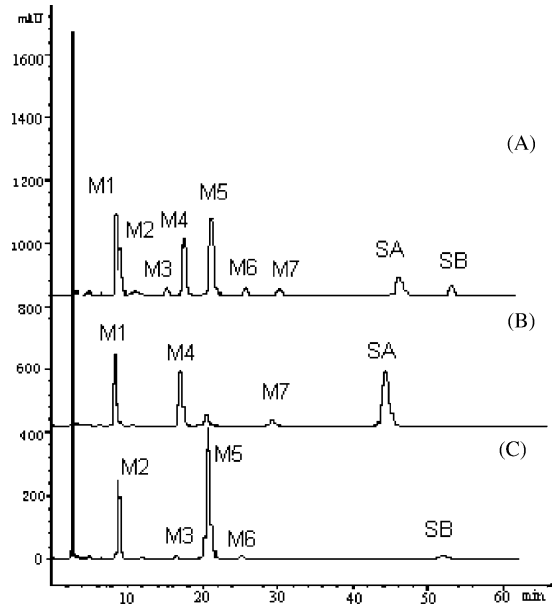

Fig. 4. Chromatograms of silybin (SA + SB), SA, SB and their metabolites. (A) Chromatograms of silybin (SA + SB) and its metabolites; (B) chromatograms of SA and its metabolites and (C) chromatograms of SB and its metabolites.

metabolites 2, 3, 5 and 6 were obtained only from silybin B.

The enzymatic stereoselectivity was also represented in different yields of the metabolites of silybin A and B, respectively. From the disappearance rate of the substrates (Fig. 5), we could deduce that silybin B was glucuronidated at a more efficient rate than that of its diastereoisomer. Similar observation was also refered by Křen et al. [6] and Weyhenmeyer et al. [3].

It was also found in the present study that when the two diastereoisomers were incubated individually, with the time going on, the decreased rates of both isomers were in great difference, while when they incubated together, the difference reduced (Fig. 5). It was commonly agreed that the rate of metabolism of each enantiomer was always greater when incubated separately, than that when incubated together as a racemic mixture. However, in this study, the rate of metabolism of silybin A was smaller when incubated separately than when incubated together, which indicated that the two diastereoisomers interacted with each other when incubated together, the mechanism should be further studied.

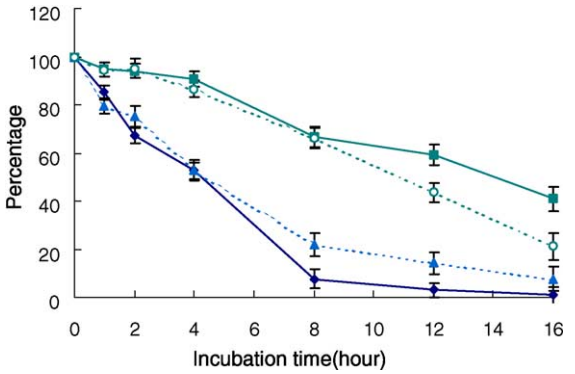

Fig. 5. Decrease: when incubated individutely (■); in SA in SA and SB in bovine liver microsomal incubation (○); in SA when incubated together (▲); in SB when incubated together (◆); in SB when incubated individually. Data represent the mean value of triplicate determinations obtained from one representative experiment.

In the previous report, Křen et al. had obtained three β-glucuronides of optically pure silybin A using bovine liver glucuronyl transferase, in which 7-*O*-β-D-glucuronide was the main product, while the most abundant conjugate in humans was its 20-*O*-β-D-glucuronide [6]. But in the present study, as shown in Fig. 6, glucuration of silybin B was much preferred at the 20 position, while for silybin A, the transformation rate was similar both at 7 and 20 positions. It was unusual because the position of

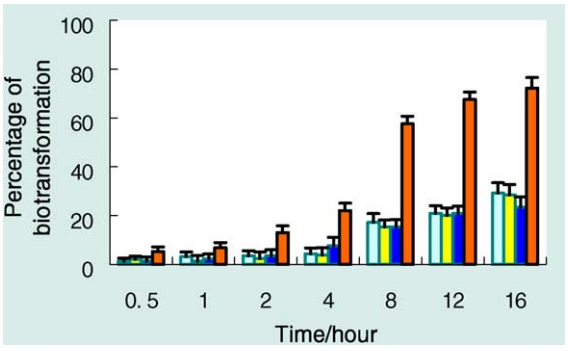

Fig. 6. Formation of glucuration of silybin A and silybin B. (□) 7-*O*-β-D-glucuronide of silybin A, (□) 20-*O*-β-D-glucuronide of silybin A, (■) 7-*O*-β-D-glucuronide of silybin B, (■) 20-*O*-β-D-glucuronide of silybin A. Data represent the mean±S.D. of triplicate experiments.

C-20 was considered rather unreactive than the C-7 position due to H-bond formation with the adjacent methoxy group. The reasonable explain was that different species had different isoenzyme systems and had different stereoselectivity for substrates.

An interesting thing found in this study was that M_3 had been formed within the first 3 h in the incubation mixture of silybin B, but the yield became reduced as the time prolonged, and at last, it could only be detected by HPLC/MS. The structure could be deduced to be a mono-glucuronide of silybin B by the characteristic of its mass speoctra, but the structure was not determined due to its low yield. The reason for this phenomenon probably was that the phenolic OH or the alcoholic group at initial period was easily activated, but under the incubation condition, the glucuronide was degraded as time prolonged.

Acknowledgements

This work was supported by National Fund of Natural Science of China (No. 30070885). The authors would like to thank Dr. G.Z. Tu for his help in NMR measurement and excellent technical assistance.

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