Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Chromatography B, 794 (2003) 303–310
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Application of liquid chromatography–electrospray ionization-ion trap mass spectrometry to investigate the metabolism of silibinin in human liver microsomes

Chandrani Gunaratna*, Tianyi Zhang

Bioanalytical Systems, Inc. 2701 Kent Avenue, West Lafayette, IN 47906, USA

Received 24 February 2003; received in revised form 23 May 2003; accepted 10 June 2003

Abstract

Silibinin is the main isomer of a group of flavanoids extracted from the seeds of the milk thistle weed, a common herb that is widely used to maintain liver health and for the treatment of liver disorders. Silibinin when incubated with human liver microsomes produced one major metabolite and at least two minor metabolites. Tandem mass spectrometry (MS) was used to identify the metabolite structures partially. MS studies confirmed that the major metabolite is demethylated silibinin and the two minor metabolites are mono-hydroxy and di-hydroxy silibinin. The K_m value for the demethylation shows that silibinin has a strong affinity for the cytochrome P450 enzymes.
© 2003 Elsevier B.V. All rights reserved.

Keywords: Silibinin

1. Introduction

Silibinin (Fig. 1) is the main isomer of a group of flavanoids extracted from the seeds of the milk thistle weed, *Silybum marianum* [1]. The purified extract, known as silymarin is widely used to maintain liver health and for the treatment of liver disorders [2]. Milk thistle is one of the most commonly used medicinal plants worldwide. Silidianin, silicristin and isosilibinin are the other minor compounds in silymarin. Silibinin is the most biologically active and is largely responsible for the anti-hepatotoxic activity [3].

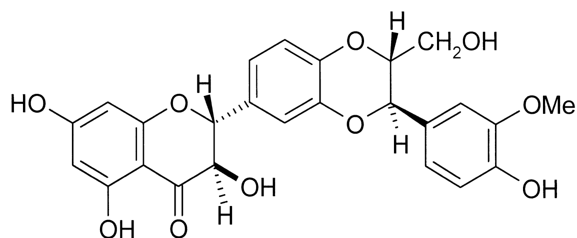

Fig. 1. Structure of silibinin.

Since Food and Drug Administration (FDA) approval is not required to regulate the herbal products, they are not tested as conventional drugs for safety and efficacy. Therefore, the adverse effects and drug interactions of the herbal preparations are mostly unknown. Cytochrome P450 mediated phase I metabolism of drugs, herbals or other foreign com-

*Corresponding author. Tel.: +1-765-497-5824; fax: +1-765-497-1102.
E-mail address: prema@bioanalytical.com (C. Gunaratna).

1570-0232/03/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S1570-0232(03)00484-7

pounds (xenobiotics) in liver is one of the primary pathways of drug elimination from the body. The cytochrome P450 enzyme system consists of several isoforms each of which can metabolize multiple substrates. This multiple enzyme–substrate interaction is the major cause of drug interactions. When the unknown compound is a substrate to a specific isozyme or when it inhibits or induces the specific isozyme, it can interact with the drugs that are metabolized by that isozyme.

Beckmann-Knopp et al. investigated the inhibition of human cytochrome P-450 enzymes by silibinin [4]. Although silibinin had a minor effect on the metabolism of a standard CYP3A4 marker, erythromycin, it clearly inhibited the dinitronifedipine oxidation by CYP3A4. Since herbal remedies can either influence the expression of drug-metabolizing enzymes or inhibit certain isoforms, they could potentially lead to deleterious drug interactions when co-administered with prescription drugs. Therefore, a thorough study on the metabolism of the active components of herbal remedies is warranted. As a part of this major goal we have investigated the metabolism of silibinin by human liver microsomes.

In milk thistle plant silibinin exists as two diastereoisomers. Several chromatographic methods have been developed to separate the individual diastereoisomers. Rickling et al. have developed a combination of two stereoselective assays using column-switching LC with electrochemical detection for the determination of free (unconjugated) silibinin and reversed-phase LC with UV detection for the measurement of total (free and conjugated) silibinin in human plasma [5]. In another study, Ding et al. have developed a LC–MS method to separate the two diastereoisomers of silibinin [6]. In our study we used LC with UV detection for metabolism studies and LC with electrospray ionization-ion trap MS detection for structure identification.

2. Materials and methods

2.1. Chemicals and reagents

Silibinin, NADPH tetra sodium salt, magnesium chloride, were all purchased from Sigma (St. Louis, MO, USA). HPLC grade solvents, acetonitrile, methanol and ethyl acetate were from Burdick and Jackson (Muskegon, MI, USA). Distilled, deionized water was obtained from a Barnstead Nanopure system (Barnstead Thermolyne, IL, USA). Pooled human and rat liver microsomes were purchased from XenoTech (Kansas City, MO, USA).

2.2. Apparatus

The chromatographic separation was achieved by a BAS 200 chromatographic system equipped with a column oven and a built-in UV detector (Bioanalytical Systems, West Lafayette, IN, USA). A Labline Environ shaker (Barnstead Thermolyne, Dubuque, IA, USA) at 37 °C was used for microsomal sample incubations. LC–MS studies were carried out using a Finnigan LCQ Deca ion-trap mass spectrometer with electrospray ionization (ThermoSeparations, San Jose, CA, USA) equipped with a BAS PM-80 gradient pump and a BAS Sample Sentinel autosampler with a 20-μl injection loop.

2.2.1. Microsomal incubations

Each incubation sample contained 1.0 mg/ml human or rat liver microsomes, 10 m*M* $MgCl_2$, 50 m*M* potassium phosphate buffer (pH 7.4) and silibinin in a total volume of 100 μl. Samples were preincubated at 37 °C for 5 min in the orbital shaker. The reactions were initiated by adding NADPH to a final concentration of 2 m*M*. Incubations were carried for 60 min and terminated by adding 400 μl of ethyl acetate. After vortexing for 2 min, the samples were centrifuged for 5 min at 10 000 *g*. The supernatant was dried under N_2, reconstituted in 50 μl of mobile phase for assay, and injected 10 μl to the LC system. Preliminary experiments were performed to determine the incubation time and the microsomal protein concentration under which the metabolite production is linear. For enzyme kinetic studies, silibinin concentrations ranging from 1 to 50 μ*M* were incubated as described previously for 20 min.

2.2.2. Chromatographic methods

In preliminary metabolism studies silibinin and the metabolites were separated isocratically on a C_{18}, Symmetry Shield™ 5 μm, 3.9×150 mm column (Waters, Milford, MA, USA) with a mobile phase

consisting of formic acid in water (pH 2.6)/ACN/methanol (75:20:5, by vol.) at a flow-rate of 1 ml/min at 40 °C and detected by UV at 289 nm. Metabolite identification was carried out by LC–MS–MS. In this case the metabolites and the parent were separated on a 3.5-μm, 50×2.1 mm C_8 Symmetry Shield column (Waters, Milford, MA, USA) at room temperature (25 °C). The mobile phase (pH 2.75) was of isocratic composition containing 25% acetonitrile and 0.1% formic acid in water. The flow-rate was 0.8 ml/min. Samples were injected by an autosampler (Sample Sentinel, BAS, West Lafayette, IN, USA) with a 20-μl injection loop.

2.2.3. LC–MS conditions

The LC–MS system for metabolite identification was equipped with a BAS PM-80 pump coupled to a Finnigan LCQ Deca ion trap mass spectrometer (ThermoQuest, San Jose, CA, USA) with an electrospray ionization (ESI) source. The MS was operated in negative ESI mode. Nitrogen was used as both the sheath and auxiliary gas at a pressure of 80 and 20 arbitrary units, respectively. The spray voltage was set at 5.0 kV and the capillary temperature was at 350 °C. Both full-scan and product ion mass spectra of silibinin and its metabolites were acquired. In LC–MS–MS experiments, helium was used as the target gas for collision-induced dissociation at 24% collision energy.

3. Results and discussion

In preliminary metabolism studies the chromatographic conditions used in combination with UV detection at 289 nm were capable of resolving the diastereoisomers of silibinin and of the metabolites produced. With the optimized organic composition of the mobile phase consisting of formic acid in water (pH 2.6)/ACN/methanol (75:20:5, by vol.) at a flow-rate of 1 ml/min at 40 °C, both diastereomers of silibinin could be detected. The standard curve for silibinin was linear in the concentration range 0.01–10 μ*M* (r^2=0.996). Since MS detection does not require complete separation of peaks, in metabolite identification studies by LC–MS chromatographic conditions were optimized to reduce the run time. Under these conditions the separation of diastereoisomers was not observed.

3.1. Silibinin metabolism

Pooled rat liver microsomes showed very little metabolism of silibinin whereas incubations with

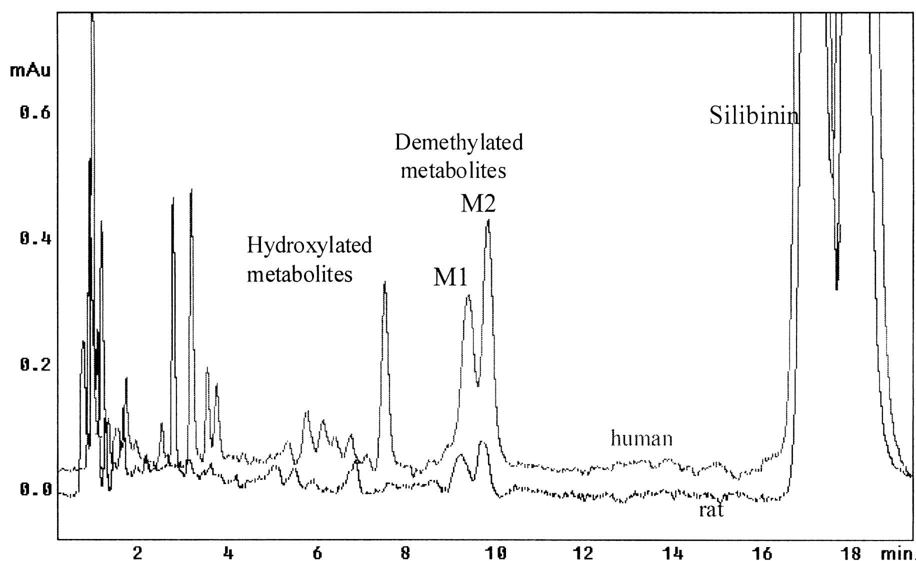

Fig. 2. Difference in metabolism of silibinin in rat and human microsomes. Silibinin (50 μ*M*) was incubated with 1 mg/ml of microsomes at 37 °C for 60 min.

human liver microsomes produced several significant metabolite peaks as shown in Fig. 2. Optimum incubation conditions with respect to the incubation time, NADPH concentration, and amount of microsomal protein were established before proceeding to the mass spectrometric identification of metabolites. Silibinin metabolism was found to be NADPH dependent and linear up to about 40 min after which the product formation decreased. However, when the ratio of product peak area to the parent peak area is plotted with time the ratio did not show an optimum, indicating the decline in the curve is due to the degradation of the parent silibinin (Fig. 3). Stability tests showed that silibinin degrades (22% in 2 h) slowly at 37 °C and this could partially account for the decline of product formation. The other reasons for the decline can include the depletion of NADPH as the reaction proceeds, and the inhibitory effect of

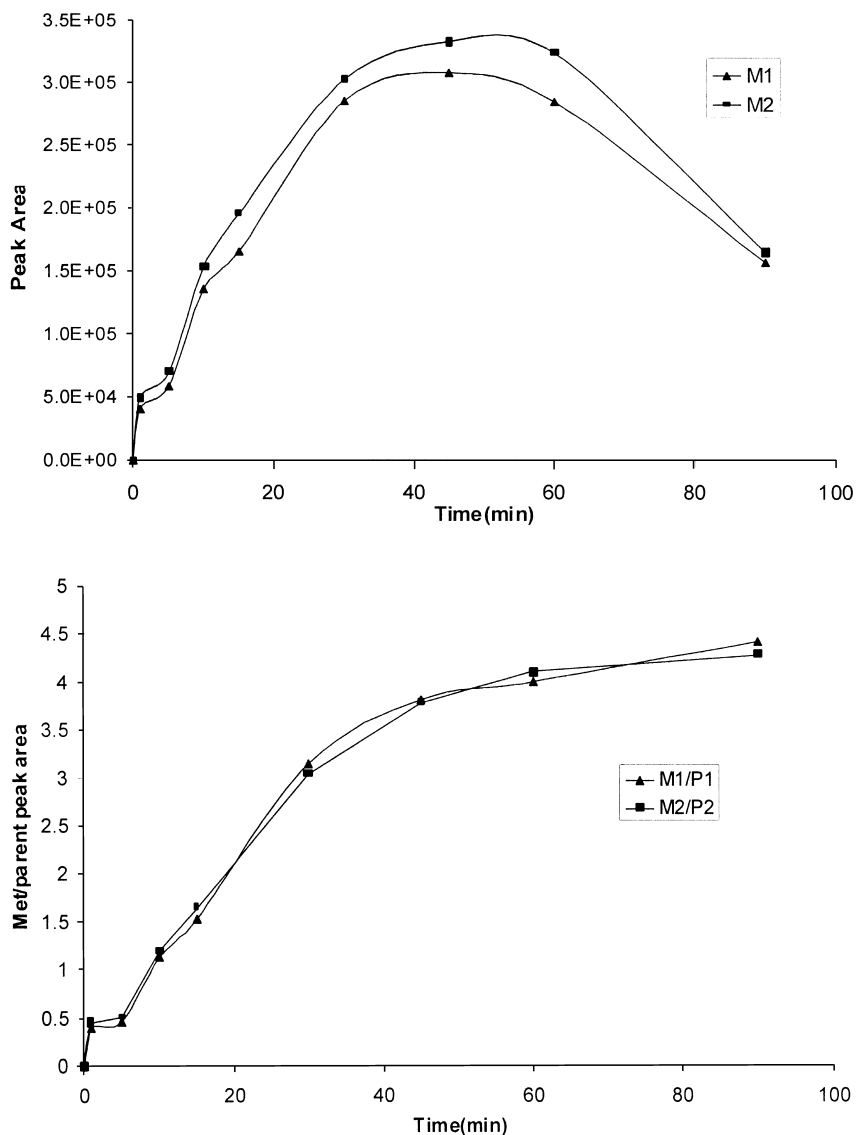

Fig. 3. Time profile for an incubation of 50 μ*M* silibinin with 1 mg/ml human liver microsomes. The top graph shows the product peak area with time and the bottom graph shows the peak area ratio of product to parent with time.

silibinin on cytochrome P450 enzymes in the microsomes.

3.2. Metabolite identification by LC–MS

The full MS scan of a human liver microsomal incubate containing silibinin and its metabolites is shown in Fig. 4. Silibinin (MW 482) is observed at m/z 481 $(M-H^{+})^{-}$ in negative ion mode. The major metabolite eluting at 3.17 min corresponds to a m/z of 467. This indicates that this metabolite is due to the demethylation of the methoxyphenyl group in silibinin. Two minor metabolites of mass at m/z 497.3 and 513.3 were also observed. These metabolites correspond to mono-hydroxylated and di-hydroxylated silibinin, respectively. The mono-hydroxylated peaks observed at 1.27, 1.53 and 2.93 min indicate that there are three possible mono-hydroxy metabolites. The di-hydroxy metabolite eluted at 1.64 min.

The LC–MS–MS chromatograms of each of these metabolites are shown in Fig. 5. From the LC–MS–MS data the scheme shown in Fig. 6 could be suggested for the metabolism of silibinin.

Since both the demethylated metabolite (m/z 467.2) and the mono-hydroxy metabolite at 2.93 min (m/z 497.3) produce a daughter ion peak at m/z 303.2 (fragment A), it can be concluded that in this monohydroxy metabolite the hydroxylation is not on the benzopyranone ring and could be on the phenyl ring or on benzodioxin ring. The mono-hydroxy metabolites at 1.27 and 1.53 min produce a fragment peak at m/z 317.1 corresponding to fragment B in Fig. 6, which indicates that in these metabolites the hydroxylation is possibly on the benzopyranone ring. The dihydroxy metabolite produces a fragment at m/z 285 (fragment C), which could result from a loss of a water molecule from fragment A, indicating that the double hydroxylation could be on the phenyl ring.

Although silibinin has as many as eight positions available for hydroxylation only a few may get hydroxylated due to steric hindrance. Sterically favorable positions for hydroxylation are the C5 and C6 positions on the phenyl ring and C6 and C8 positions on the benzopyranone ring. LC–MS–MS alone is not sufficient to identify the exact sites of hydroxylation. Unambiguous identification of the hydroxylation sites requires NMR or isotope-labeled techniques.

3.3. Enzyme kinetics

Silibinin shows a hyperbolic substrate saturation curve. The metabolite formation was found to be linearly increasing with concentration of silibinin up

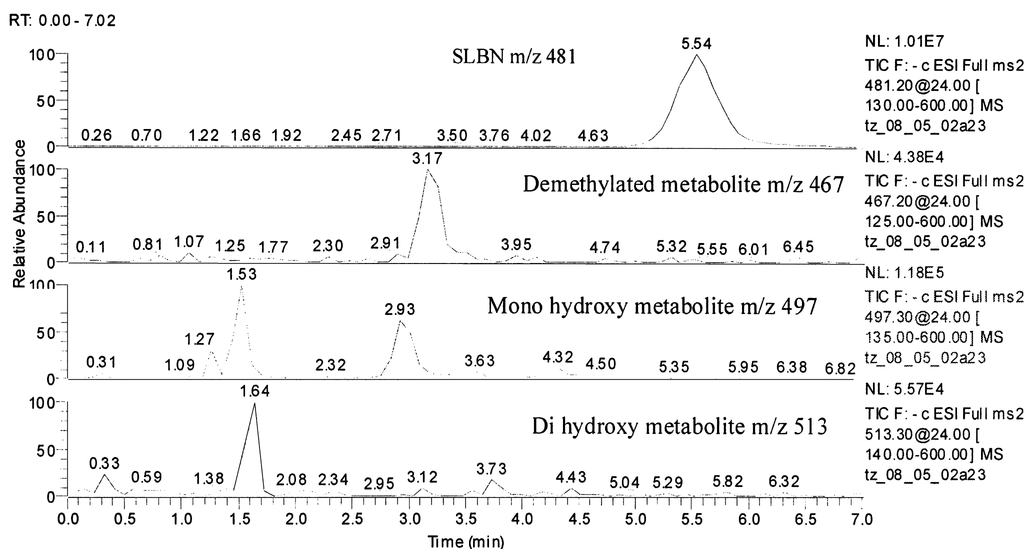

Fig. 4. The full ion scan of an incubate of 50 μ*M* silibinin with 1 mg/ml human liver microsomes.

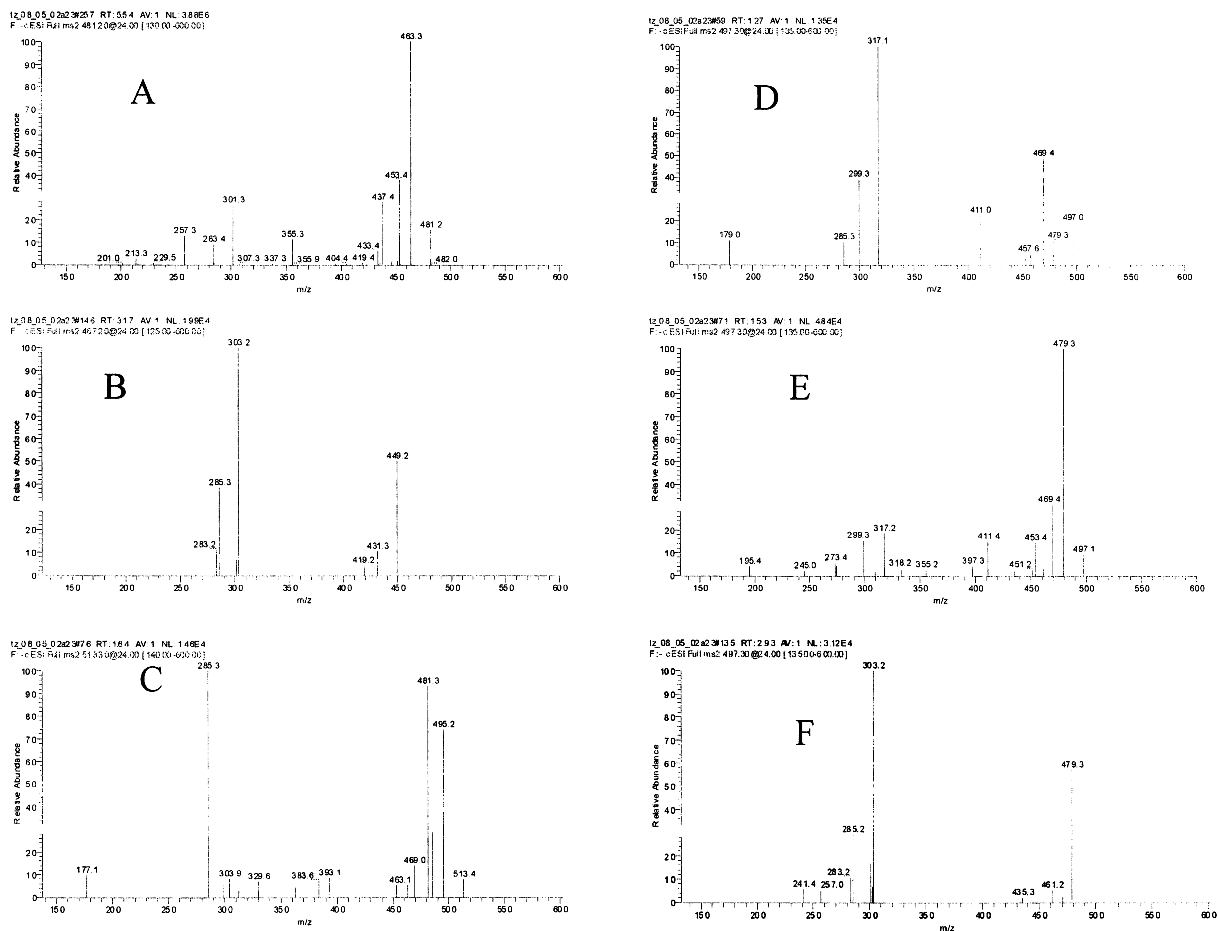

Fig. 5. The LC–MS–MS fragmentation spectra of (A) parent silibinin (m/z 481), (B) demethylated metabolite (m/z 467), (C) dihydoxy metabolite (m/z 513), (D) monohydroxy metabolite at t_R 1.27 min (m/z 497), (E) monohydroxy metabolite at t_R 1.27 min (m/z 497), (F) monohydroxy metabolite at t_R 2.93 min (m/z 497).

to 50 μ*M* of silibinin. Above 50 μ*M* the amount of metabolite decreases indicating that at higher concentrations, silibinin can inhibit the enzyme activity. Although several metabolites are formed during silibinin metabolism, the only quantifiable metabolite was the demethylated silibinin. Therefore, Michaelis–Menten enzyme kinetic parameters were evaluated only for the demethylation. Since a demethylated silibinin standard was not available to obtain product concentration, the chromatographic peak area for the product was used as the rate, V, in the Lineweaver–Burk plot [Eq. (1)] of $1/V$ vs. $1/[S]$.

$$\frac{1}{v} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \quad (1)$$

Both demethylated metabolite isomers (M1 and M2) showed linear enzyme kinetics. The K_m values were obtained from the slopes. Apparent V_{max} was obtained by a different approach where substrate depletion with time was used instead of product formation [7]. Once K_m is obtained, apparent V_{max} can be calculated from the intrinsic clearance, CL_{int}, according to Eq. (2),

$$CL_{int} = \frac{V_{max}}{K_m} \quad (2)$$

CL_{int} was obtained from Eq. (3),

$$CL_{int} = \frac{\nu^* \ln(C_{initial}/C_{final})}{time} \quad (3)$$

where v is the incubation volume.

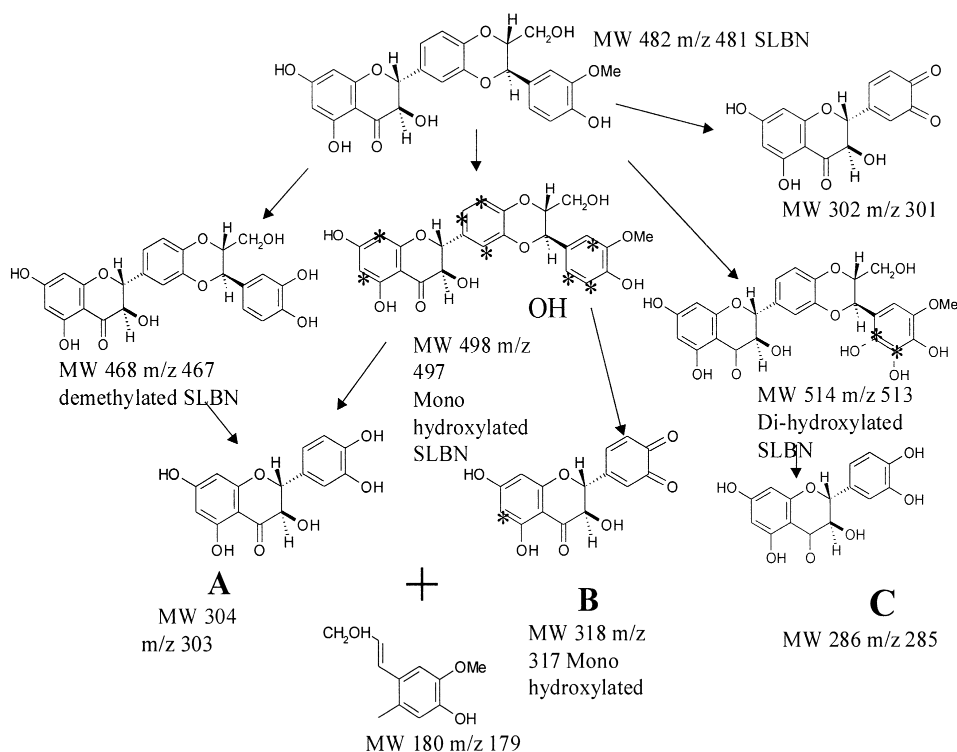

Fig. 6. A schematic illustration of possible MS fragments of silibinin. Possible hydroxylated sites are denoted by an asterisk.

The K_m value for the demethylation was found to be 14.1±4.5 μ*M* for M1 and 19.9±5.0 for M2 ($n=4$). The apparent V_{max} is 0.191±0.012 nmol/min per mg for M1 and 0.289±0.03 nmol/min per mg for M2 ($n=4$). Michaelis–Menten parameters, K_m and V_{max} define the kinetic behavior of an enzyme as a function of substrate concentration. K_m is an approximate measure of the affinity of the substrate for the enzyme and V_{max} is a measure of the rate of the enzyme reaction. The low K_m for silibinin indicates that silibinin is a high affinity substrate for the cytochrome P450 enzymes and could cause drug interactions when co-administered with other drugs.

Despite the wide use of milk thistle extract as a treatment for liver disorders [8–10] the metabolism of its main constituent, silibinin, is not well studied. Although several studies have been reported to study the inhibitory effects of silibinin [4,11] none of them have studied the metabolism of silibinin. Many flavanoids found in herbal extracts are known to inhibit CYP3A4 [12,13] leading to possible drug–drug interactions since CYP3A4 is a major isozyme involved in drug metabolism. Identification of specific cytochrome P450 isozymes responsible for the metabolism of silibinin is therefore necessary. Our studies on the isozyme identification will be presented in a separate publication.

4. Conclusion

The work described here demonstrates the application of a LC–MS method utilizing negative ESI-ion trap MS to detect and identify the in vitro metabolites of silibinin by human liver microsomes. MS studies have shown the formation of one major demethylated metabolite, three minor mono-hydroxy metabolites and one minor di-hydroxy metabolite. Although possible structures were proposed for the hydroxylated metabolites, it is evident that LC–MS–MS alone is not able to determine the exact sites of the hydroxylation. Additional structure identification techniques such as NMR or isotope-labeling are needed to provide a complete identification when multiple isomers are formed. Michaelis–Menten parameters obtained for the enzyme reaction suggests

that silibinin has the potential to interact with other cytochrome P450 substrates.

Acknowledgements

This research was supported in part by a SBIR Phase I grant (1R43AT00792-01) from the National Institutes of Health, National Center for Complimentary and Alternative Medicine (NCCAM).

References

[1] H. Wagner, L. Horhammer, R. Munster, Arzneimittelforschung 18 (1968) 688.
[2] K. Flora, M. Hahn, H. Rosen, K. Benner, Am. J. Gastroenterol. 93 (1998) 139.
[3] M.P. Miguez, I. Anundi, L.A. Sainz-Pardo, K.O. Lindros, Chem. Biol. Interact. 91 (1994) 51.
[4] S. Beckmann-Knopp, S. Rietbrock, R. Weyhenmeyer, R.H. Bocker, K.T. Beckurts, W. Lang et al., Pharmacol. Toxicol. 86 (2000) 250.
[5] B. Rickling, B. Hans, R. Kramarczyk, G. Krumbiegel, R. Weyhenmeyer, J. Chromatogr. B 670 (1995) 267.
[6] T. Ding, S. Tian, Z. Zhang, D. Gu, Y. Chen, Y. Shi et al., J. Pharm. Biomed. Anal. 26 (2001) 155.
[7] D.J. Carlile, A.J. Stevens, E.I. Ashforth, D. Waghela, J.B. Houston, Drug Metab. Dispos. 26 (1998) 216.
[8] P. Ferenci, B. Dragosics, H. Dittrich, H. Frank, L. Benda, H. Lochs et al., J. Hepatol. 9 (1989) 105.
[9] L.A. Giese, Gastroenterol. Nurs. 24 (2001) 95.
[10] R. Saller, R. Meier, R. Brignoli, Drugs 61 (2001) 2035.
[11] H.W. Leber, S. Knauff, Arzneimittelforschung 26 (1976) 1603.
[12] P.C. Ho, D.J. Saville, S. Wanwimolruk, J. Pharm. Pharm. Sci. 4 (2001) 217.
[13] C. Ioannides, Xenobiotica 32 (2002) 451.