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Determination of Silybin of Nutraceutical Herbal Preparations Using HPLC-PDA

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Abstract – Silymarin is an antihepatotoxic substance isolated from the fruits of *silybum marianum*. Possibly due to their antioxidant and membrane stabilizing properties, the compounds was shown to protect various organs and cells against a number of insults (Kvasnička *et al.,* 2003). Among the main silymarin components, [silybin (SB_A, SB_B), isosilybin (ISB_A, ISB_B) silydianin (SD) and silychristin (SC)], silybin is the major pharmacologically active compound. Korean Pharmaceutical Codex (2nd ed.) describes silybin as the main substance of Cardus Marianus extract as supportive treatment of chronic inflammatory liver disorders. The aim of this work was to analyze silybin from various preparations containing cardus marianus extract, nicotinamide, and riboflavin (CNR). Nine commercial products were tested using reversed-phase HPLC-PDA assay. The limits of detection and quantification were 0.2 μg/ml and 1 μg/ml, respectively. Calibration curve showed a good linearity ($r^2 = 1.00000$) in the range of 1~500 μg/ml of silybin standard solutions.
Keywords – silybin, HPLC-PDA, nicotinamide, riboflavin, pyridoxine, thiamine

Introduction

Silymarin, derived from the milk thistle plant, *Silybum marianum*, has been used for centuries as a natural remedy for diseases of liver and biliary tract. Currently the most important medicinal applications of milk thistle is its use as a hepatoprotectant and as supportive treatment of chronic inflammatory liver disorders such as cirrhosis, hepatitis, and fatty infiltration due to alcohol and toxic chemicals (Flora *et al.,* 1998). Silymarin was considered as a pure compound with the structure of 7-chromanol-3-methyl-taxifolin (Hahn Von *et al.,* 1968). However, after the introduction of more accurate methods of analysis and separation, it was shown that silymarin consisted of several flavonolignans, including silybin diastereomers, isosilybin diastereomers, silydianin and silychristin (Kvasnička *et al.,* 2003; Quaglia *et al.,* 1999). Kvasnička *et al.* (2003) analyzed the components of silymarin. Silymarin components were determined by HPLC according to the Czech Pharmacopeia 97 and by proposed capillary zone electrophoretic method. Analysis by HPLC was carried out on Purospher RP18 (150 × 4 mm, 5 μm) using mixture of 85% phosphoric acid : methanol : water (0.5 : 46 : 64) as mobile phase at a flow rate 1 ml/min. Flavonolignans of silymarin were detected at 288 nm. Silychristin (SC), silydianin (SD), silybinA (SB_A), silybinB (SB_B), isosilybin (ISB_A), and isosilybin (ISB_B) were isolated and showed respective retention time in chronological order. By the way, the reversed-phase HPLC-PDA condition was modified from the multivitamin-mineral preparation analysis method by Lam *et al.* (1984).

Among the flavonolignans, silybin (silibinin) is the principal bioactive component of silymarin. It has been reported to work as antioxidants scavenging free radicals and inhibiting lipid peroxidation (Mira *et al.*, 1994; Carini *et al.*, 1992). Studies also showed that it protects against genomic injury, increases protein synthesis of hepatocyte, decreases the activity of tumor promoters, stabilizes mast cells, chelates iron, and slows calcium metabolism (Takahara *et al.*, 1986; Pietrangelo *et al.*, 1995; Lecomte and j., 1975). Nutritional supplements derived from milk thistle extracts account for over 180 million dollars in sales annually in Germany and 1.6 billion dollars annually in the Unites States (Brevoort, 1996). As the popularity of treatment with this and other herbs increases, intensive quality control studies are necessary. In this paper, we tried to identify and determine quality and quantity of each of the various CNR preparations sold in Korea, by analyzing silybin with HPLC-PDA.

Experimental

The commercial products were purchased randomly from various pharmacies in Korea. All products were made from different pharmaceutical companies (Table 1).

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Table 1. Composition of products

company	composition	
A	Cardus Marianus extract 200 mg (silymarin 140 mg, silybin 60 mg) thiamine HCl 8 mg	pyridoxine HCl 8 mg nicotinamide 24 mg calcium pantothenate 16 mg cyanocobalamine 2.4 μg riboflavin 8 mg
B	Cardus Marianus extract 200 mg (silymarin 140 mg, silybin 60 mg) thiamine nitrate 4 mg	pyridoxine HCl 4 mg nicotinamide 12 mg calcium pantothenate 8 mg cyanocobalamine 1.2 μg
C	Cardus Marianus extract 200 mg (silymarin 140 mg, silybin 60 mg) thiamine HCl 8 mg	pyridoxine HCl 8 mg nicotinamide 24 mg calcium pantothenate 16 mg cyanocobalamine 2.4 μg riboflavin 8 mg
D	Cardus Marianus extract 200 mg (silymarin 140 mg, silybin 60 mg) thiamine HCl 8 mg	pyridoxine HCl 8 mg nicotinamide 24 mg calcium pantothenate 16 mg cyanocobalamine 2.4 μg riboflavin 8 mg
E	Cardus Marianus extract 200 mg (silymarin 140 mg, silybin 60 mg) thiamine nitrate 8 mg	pyridoxine HCl 8 mg nicotinamide 24 mg calcium pantothenate 16 mg cyanocobalamine 2.4 μg riboflavin 8 mg
F	Cardus Marianus extract 200 mg (silymarin 140 mg, silybin 60 mg) thiamine HCl 8 mg	pyridoxine HCl 8 mg nicotinamide 24 mg calcium pantothenate 16 mg cyanocobalamine 2.4 μg riboflavin 8 mg
G	Cardus Marianus extract 200 mg (silymarin 140 mg, silybin 60 mg) thiamine nitrate 4 mg	pyridoxine HCl 4 mg nicotinamide 12 mg calcium pantothenate 8 mg cyanocobalamine 1.2 μg
H	Cardus Marianus extract 200 mg (silymarin 140 mg, silybin 60 mg) thiamine nitrate 4 mg	pyridoxine HCl 4 mg nicotinamide 12 mg calcium pantothenate 8 mg cyanocobalamine 1.2 μg
I	Cardus Marianus extract 200 mg (silymarin 140 mg, silybin 60 mg) thiamine nitrate 4 mg	pyridoxine HCl 4 mg nicotinamide 12 mg calcium pantothenate 8 mg cyanocobalamine 1.2 μg

Chemicals and Reagents – Standards of silymarin, silybin, and vitamins, and heptanesulfonic acid sodium salt were obtained from Sigma-Aldrich. Methanol and acetic acid used in this work were of HPLC grade and other reagents were of analytical grade. Ultra pure water system (Barnstead, USA) treated water (with resistivity more than 18 MΩ) was used throughout the experiment.

Instrumentation – HPLC were performed on Waters Alliance System instrument consisting of Separation Modules (Waters 2695, USA) and Photodiode Array Detector (Waters 2996, USA) controlled by Empower.

Working Standard Solution and Sample Solutions – For quantitative analysis, silybin was used as external standard at four concentration levels 1, 10, 100, and 500 μg/ml. The standards and each sample were dissolved in methanol.

The weight variation was tested according to Korean Pharmacopoeia to measure the average weight per capsule. Another 20 capsules were weighed accurately from each of the nine preparations respectively, opened, transferred content, and mixed thoroughly. The portion of the content, equivalent to about 6 mg of silybin, was added to a 100 ml volumetric flask, was weighed. Methanol (70 ml) was added

and stirred for 30 minutes. Additional methanol was added to make 100 ml, and each extracted solution was filtered through a membrane filter with pore size of 0.45 μm. The first 10 ml of the filtrate was discarded and the test was performed with 10 μl each of the sample solutions and the standard solutions as directed under the HPLC-PDA assay.

HPLC-PDA assay – The separation of silybin diastereomers of the nine CNR's commercially available products were carried out using stationary phase Waters Symmetry C_{18} (3.9 × 150 mm, 5 μm). Heptanesulfonic acid sodium salt (5 mM) with 1% acetic acid (solvent A) and methanol (solvent B) were served as mobile phase at a flow rate 0.5 ml/min. Samples were eluted by a gradient method which was : 0 to 3 min, 85.0% A; 3 to 15 min, from 85.0 to 50.0 %; 30 to 35 min, from 50.0 to 85.0 and detected using photo diode array (PDA). Total elution time was forty minutes and column conditioning time was twenty minutes.

Results

Linearity and recovery rates – Four solutions containing silybin at concentrations ranging from 1.0 to 500.0 μg/ml were analyzed. The silybin was resolved into two peaks. Silybin concentration is expressed as a sum of both diastereomers, because it is expected that antioxidative potencies should not be much different from each other (György *et al.,* 1992). The calibration functions of silybin standard calculated with peak area (y, mAU) and concentration (x, μg/ml) was $y = 53928.34875x + 2499.72871$ ($r^2 = 1.00000$), over the concentration range 1 to 500 μg/ml (Fig. 1). For recovery test, known amounts of silybin standards were added to the known concentration of sample solutions and analyzed three times by HPLC-PDA. The efficiency of recovery by the HPLC-PDA was shown in Table 2. The recovery rates of silybin diastereomers were between 95.2% and 100.5%.

Impurity and spectrum – The result of the impurity test was plotted. Generally, the value of the purity angle of the target peak should be lower than that of the purity threshold. It means if the purity angle is less than the purity threshold, the peak is spectrally homogeneous. As the purity angles were lower than the purity threshold of sample solution and spiked solution, the results these impurity tests were suitable. The first isolated silybin diastereomer (SB I) and second isolated silybin diastereomer (SB II) were named in sequence of retention time according to the reversed-phase HPLC-PDA.

A library match of spectrum of spiked solution to standard solutions was performed according to Waters empower PDA guideline. The spectrum of spiked solution corresponded with the spectrum of silybin standard. While the silybin was isolated into two peaks and their

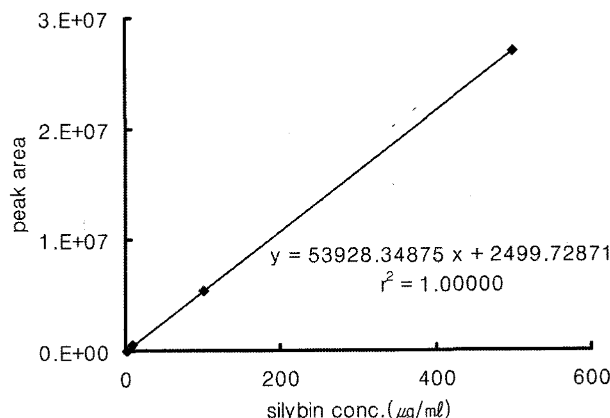


Fig. 1. Linearity study of silybin standard solutions at 287 nm. (Silybin concentration is expressed as a sum of both diastereomers).

Table 2. Recovery rates of silybin diastereomers from commercial CNR products (each value is mean of n = 3)

company	silybin added (μg/ml)	silybin found (μg/ml)	recovery rate (%)	SD	RSD (%)
A	158.0	150.5	95.2	3.2	3.4
B	160.0	153.5	95.9	3.6	3.8
C	158.0	158.2	100.1	4.1	4.1
D	159.0	157.5	99.1	3.4	3.4
E	157.0	154.1	98.2	5.5	5.5
F	159.0	155.9	98.1	1.8	1.8
G	161.0	161.8	100.5	4.3	4.3
H	160.0	152.9	95.6	2.2	2.3
I	158.0	153.4	97.1	1.9	2.0

RSD = SD/mean value ×100

Table 3. PDA result of purity angle and purity threshold

name	spiked solution	standard solution	sample solution
silybin diastereomer I	$0.154 < 0.264^{\alpha}$	$0.118 < 0.295$	$0.219 < 0.367$
silybin diastereomer II	$0.148 < 0.260$	$0.125 < 0.302$	$0.311 < 0.334$

$^{\alpha}$Left value is purity angle and right is purity threshold.
In terms of purity angle and purity threshold angle, silybin diastereomer I showed 0.154, 0.264 and silybin diastereomer II showed 0.148, 0.260, respectively. The results of purity of the standard solution of silybin 100 μg/ml were 0.118, 0.295 of silybin diastereomer I and 0.125, 0.302 of diastereomer II, respectively and in sequence. The results of sample solution indicated 0.219, 0.367 of silybin diastereomer I and 0.311, 0.334 of diastereomer II, respectively.

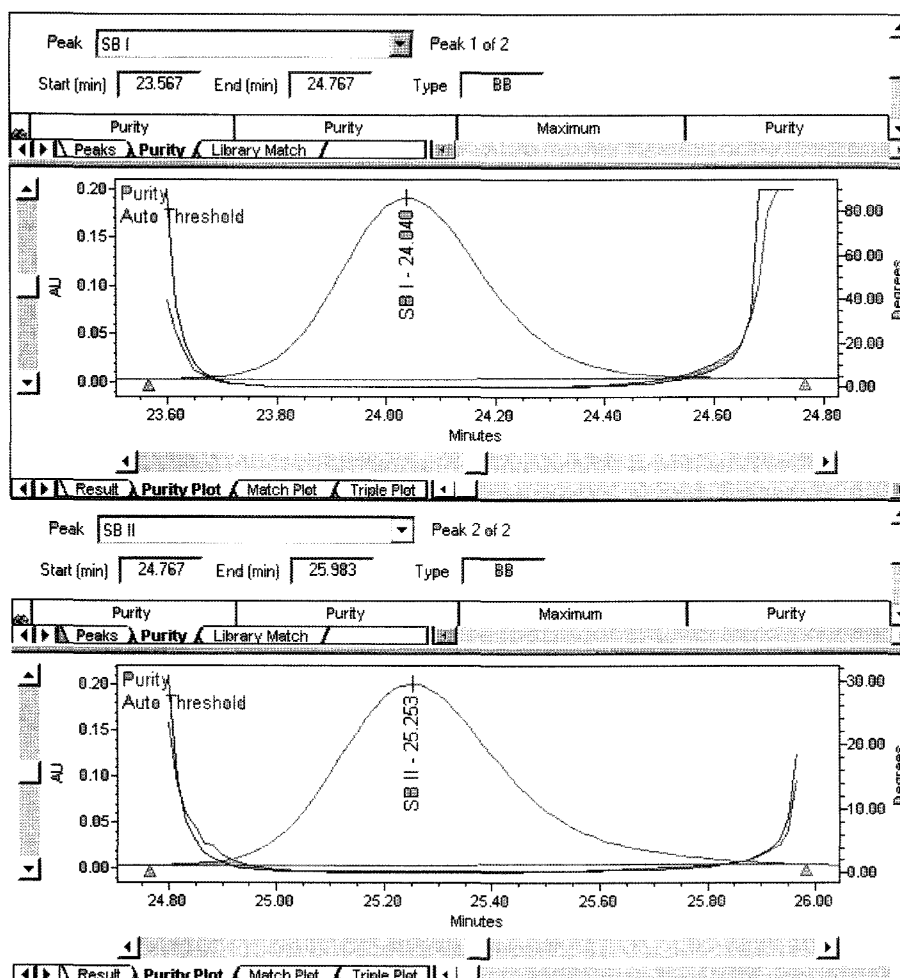


Fig. 2. Impurity test of spiked solutions. SB I : First isolated silybin diastereomer. SB II : Second isolated silybin diastereomer.

retention times were different, 3D plot showed that their UV absorbance spectra were similar (Fig. 3).

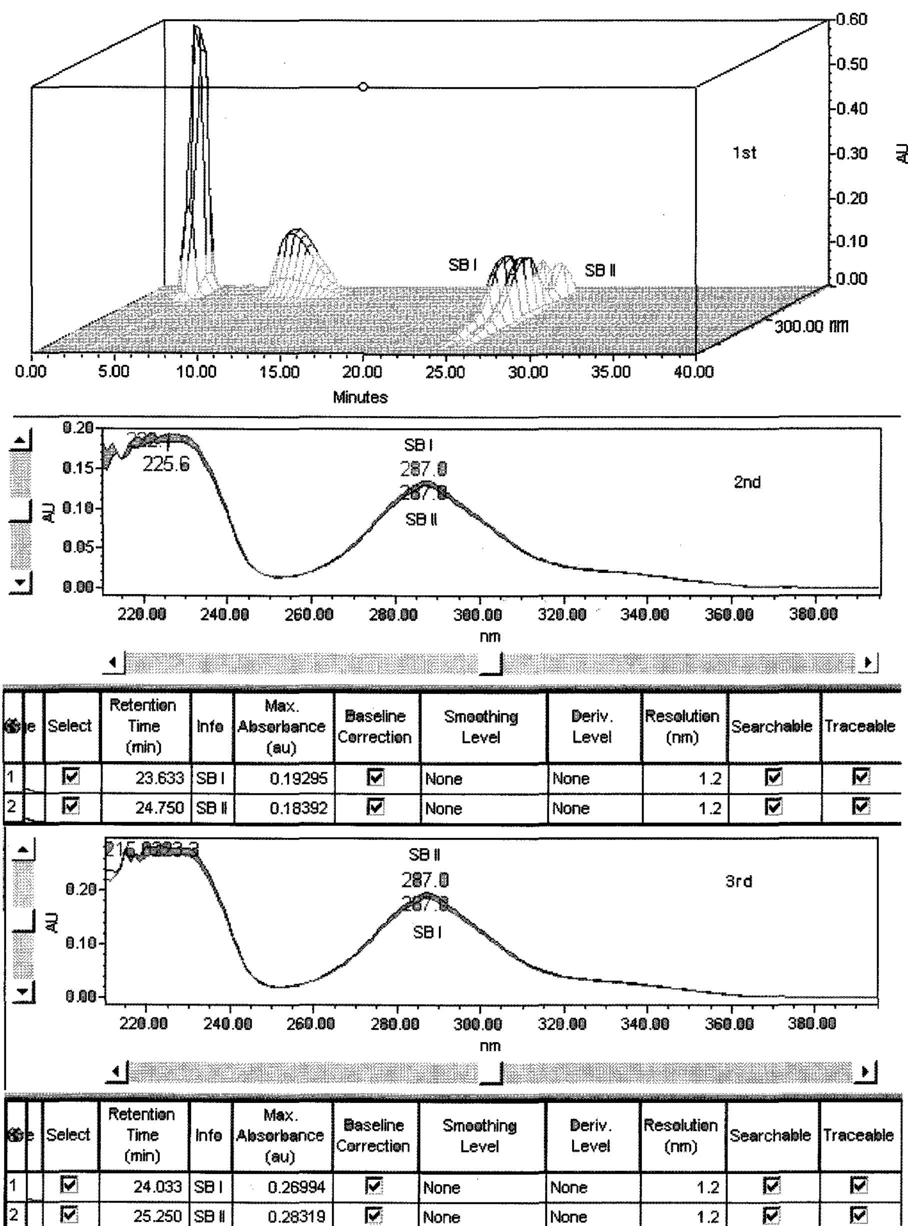
The results having same spectrum but different retention time from the spiked solution concurred with Arnone *et al* (1979) that natural silybin is a diastereoisomeric mixtures of two compounds with the same configuration at C-2 and C-3, and opposite at C-2', C-3' through ^{1}H NMR and ^{13}C NMR spcetra (Arnone *et al*,. 1979). The absolute assignment was achieved by X-ray analysis of the natural diastereomeric mixture showing that isomerism occurs only in the benzodioxane (Lotter and Wagner, 1983). Ding *et al.* (2001) confirmed the diasteroisomeric nature of silybin and isosilybin and, reported that the mass spectra of both substances showed the molecular peak at 433 and very similar fragmentation, however most fragments were found in each mass spectrum and differ only in intensity.

These reports confirmed that both silybin and isosilybin are mixtures of diastereoisomers and are difficult to separate. We successfully determined two diastereoisomers of silybin without peak interruption of vitamins by HPLC-PDA in this study. The observed linearity, recovery, and impurity indicated this method is suitable and applicable for qualitative and quantitative evalution of the silybin diastereomers in the compound cardus marianus extract, nicotinamide and riboflavin commercial products (CNR).

The chromatogram of the sample solution obtained as directed in the HPLC-PDA method (Fig. 5) exhibits a major peak for silybin diastereomers, retention time of which, relative to that of external standard, corresponds to that exhibited in the chromatogram of the standard solution obtained as direted in the HPLC-PDA method. The third chromatogram was obtained by adding standards into the known sample and its retention time corresponded to that of standard solution, as well.

Discussion

Recently, main components of silymarin, namely silybin



	Select	Retention Time (min)	Info	Max. Absorbance (au)	Baseline Correction	Smoothing Level	Deriv. Level	Resolution (nm)	Searchable	Traceable
1	☑	23.633	SB I	0.19295	☑	None	None	1.2	☑	☑
2	☑	24.750	SB II	0.18392	☑	None	None	1.2	☑	☑

	Select	Retention Time (min)	Info	Max. Absorbance (au)	Baseline Correction	Smoothing Level	Deriv. Level	Resolution (nm)	Searchable	Traceable
1	☑	24.033	SB I	0.26994	☑	None	None	1.2	☑	☑
2	☑	25.250	SB II	0.28319	☑	None	None	1.2	☑	☑

Fig. 3. 3D-Plot of silybin standard solution (1st) and spectra of silybin standard solution (2nd) and spiked solution (3rd). SB I : The first isolated silybin diastereomer. SB II : The second isolated silybin diastereomer.

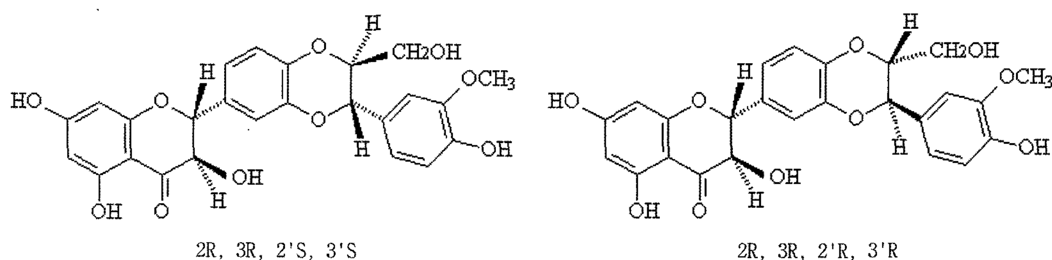


Fig. 4. Structure of silybin diastereomers

diastereomers, isosilybin diastereomers, silydianin and silychristin from *Silybum marianum* have been determined and separated using column switching with electrochemical detection reversed-phase HPLC, and recrystallization (Rickling

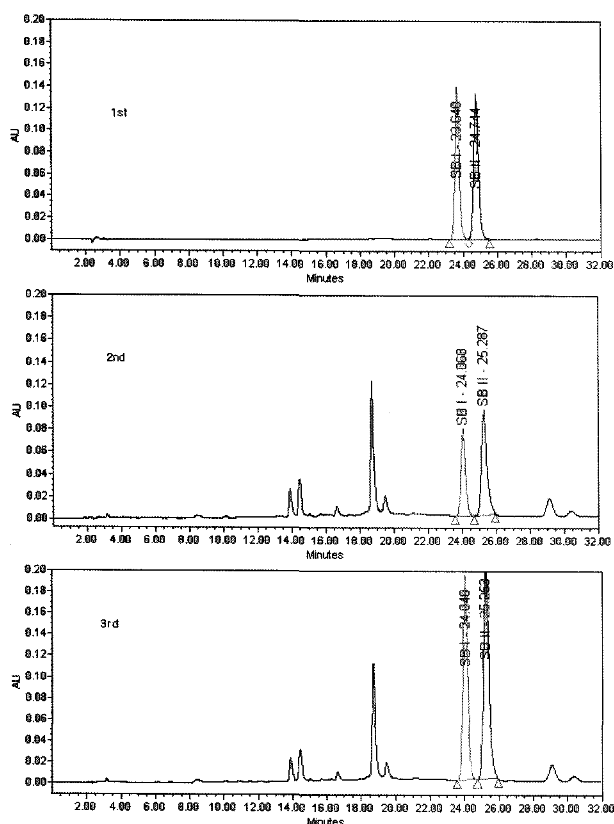


Fig. 5. Chromatograms of silybin standard solution (1st), sample solution (2nd),and spiked solution (3rd) at 287 nm. SB I : The first isolated silybin diastereomer. SB II : The second isolated silybin diastereomer.

et al,. 1995; David *et al,*. 2003). *Campodónico et al.* (2001) employed dissolution tests of silymarin tablets and capsules. Their HPLC assay showed silymarin contains taxifoline, silycristine, silydianine, silybin, and isosilybin. The extraction process used moderate sonication for 5 minutes to avoid exposing samples to heat generated from prolonged treatment. We extracted silybin from the sample preparations by using magnetic stirring apparatus. Although silybin is soluble in methanol, ethanol, acetone, ethyl acetate, we adopted methanol as many other references (Anonymous, 2001).

According to Korean Pharmaceutical Codex second edition, the identification and assay of silybin from commercially available CNRs are based on HPLC method using aminopropylsilyl silica column and eluted with acetonitrile : acetic acid : water (95 : 2 : 3). The codex shows vitamin mixture containing thiamine, nicotinamide, riboflavin, and pyridoxine could be analyzed using one general HPLC method. However, determination of silybin from CNR products containing vitamins proves difficult due to its compositions as well as its excipients containing soybean oils, lecithin, beeswax, preservatives, and other ingredients which contribute to a sustain suspension state during sample preparation.

Although there are many references with regards to the analysis of silymarin, successful separation of various flavonolignans (Kvasnička *et al.,* 2003; Quaglia *et al,*. 1999) and separating silybin diastereomers and isosilybin diastereomers (Ding*et al.,* 2001; Rickling *et al,*. 1995), literature search did not yield conditions for separation of silybin from nutraceuticals derived from Cardus marianus extracts preparations. We surveyed analysis of samples containing both silybin and vitamins concurrently by use of ion-pairing effect of heptanesulfonic acid in a gradient mobile phase system. Although the UV max of silybin is 287 nm, UV setting of 270 nm was used due to widely favorable absorbance of nicotinamide, pyridoxine, riboflavin, and thiamine. It was possible to identify components of silybin diastereomers and vitamins in commercial CNR products. However it was difficult in quantifying vitamins themselves. For example, the retention time of thiamine was similar to that of peak3 for silymarin. The chromatogram of silymarin showed that silymarin consisted of various

Table 4. Analytical results of silybin diastereomers in each preparation

company	labeled amount	content of silybin diastereomers (%) No. of experiment			mean	RSD
A	60 mg/cap	90.1	92.5	90.9	91.2	1.3
B	60 mg/cap	90.4	93.8	92.5	92.2	1.9
C	60 mg/cap	100.7	102.9	103.4	102.3	1.4
D	60 mg/cap	88.9	90.4	92.9	90.7	2.2
E	60 mg/cap	95.1	90.9	91.1	92.4	2.6
F	60 mg/cap	115.4	110.0	114.3	113.2	2.5
G	60 mg/cap	101.8	100.9	101.5	101.4	0.5
H	60 mg/cap	104.9	110.2	109.4	108.2	2.6
I	60 mg/cap	90.7	100.1	98.5	96.4	5.2

* Areas are calculated as a sum of both diastereomers peak area.

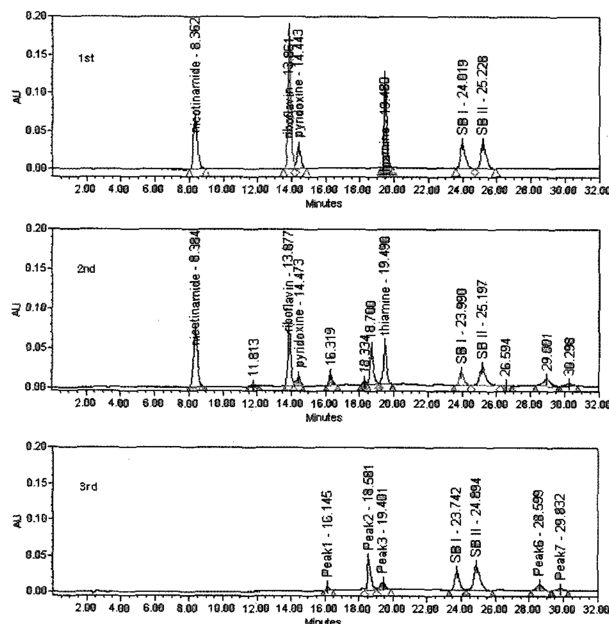


Fig. 6. Chromatograms of standard mixture of four vitamins and silybin(1st), sample solution (2nd), and silymarin standard solution at 270 nm. SB I : The first isolated silybin diastereomer. SB II : The second isolated silybin diastereomer.

components including silybin. (Fig. 6) The first and second chromatograms showed that silybin diastereomers were successfully separated, and the third one showed that silybin was isolated from silymarin through the reversed-phase HPLC-PDA condition.

Throughout this study, the HPLC-PDA mode using reverse phase Symmetry column is an analytical aid for not only the separation of nicotinamide, pyridoxine, riboflavin, thiamine, and silybin but also for the determination of two diastereomers of silybin. Qualitatively, we were able to simultaneously analyze silybin diastereomers and vitamins found in commercially available CNR products. Each CNR product contained more than 90 percent of the labeled amount of silybin diastereomers ($C_{25}H_{22}O_{10}$: 482.44). They all conformed to the Korean Pharmaceutical Codex second edition regulation.

Conculsions

The determination of silybin diastereomers in compound cardus marianus extract, nicotinamide and riboflavin commercial products were developed by HPLC-PDA. 3D-PDA method provides a linear response, is precise, and may be used for quality assesment and study isolation and identification. The linearity, recovery, and impurity of silybin diastereomers indicates that this method may be used as pharmacotechnical quality indicator.

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