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Short communication

Determination of active component in silymarin by RP-LC and LC/MS

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Abstract

Silybin, isosilybin, silydianin and silycristin in silymarin were separated and quantitatively determined by RP-HPLC. Two diastereoisomers of silybin and isosilybin were respectively separated by RP-HPLC and confirmed by LC/MS. Chromatographic condition consisted of column: Shim-pack VP-ODS (150 × 4.6 mm i.d. 5 μm) and Pre-column (10 × 4.6 mm i.d. 5 μm); mobile phase: methanol and solvent mixture (water: dioxane = 9:1) by gradient; flow rate: 1.5 ml/min; column temp.: 40°C; detector wavelength: 288 nm; The recovery of 99.66% for silycristin, 99.48% for silydianin, 100.0% for silybin and 98.72% for isosilybin was respectively obtained.

Keywords: Diastereoisomers; Silymarin; LC/MS; RP-LC

1. Introduction

Silymarin, an antihepatotoxic substance isolated from fruits of *Silybum marianum*, has been studied by pharmacological, biochemical and chemical tests [1–6]. Silymarin was considered as a pure compound with the structure of 7-chromanol-3-methyl-taxifolin [7], but after the introduction of more accurate methods of analysis and separation it was shown that silymarin contains silybin, silydianin and silycristin. In 1979, Merlini et al. [8,9] and Arnone et al. [10] isolated isosilybin and suggested that silybin and isosilybin are mixtures of two diastereoisomers having the same configuration at C2 and C3 and an opposite configuration at C2′ and C3′.

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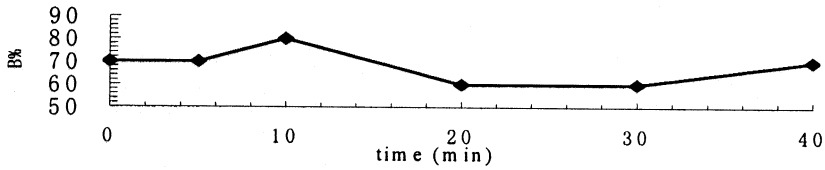

Fig. 1. Gradient of assay analysis of silymarin.

About separation and assay of silymarin, up to now the individual diastereoisomers can not be separated on a preparative scale. Direct spectrophotometric assay has been applied to determine total flavone in formulation. Spectrophotometric determination after reaction with 2,4-dinitrophenyl-hydrazine has been applied to determine total flavone in Legalon. Silybin concentrations in body fluids have been determined using either thin layer chromatography (TLC) with fluorometric detection or high performance liquid chromatography (HPLC) with UV detection [11–15], Techniques employing TLC lack both sensitivity and specificity thus, limiting their use. Previous HPLC assays are either not stereo selective or not sensitive enough to monitor silybin and isosilybin. Up to now, these have been separated and determined main effective components in silymarin and preparations of silymarin by MECC [16]. In this paper, separation and assay of four main effective components in silymarin and preparations of silymarin have been studied by RP-HPLC and the different diastereoisomers of silybin and isosilybin have been respectively separated and identified by LC/MS and three-dimensional UV scanning (TDS).

2. Experimental

2.1. Materials

Methanol and dioxane were of HPLC grade from Shanghai Chemical Supply (Shanghai, China). Silymarin 1 and Legalon capsules (silymarin and excipient) were provided by MADAUS AG (Germany). Silymarin 2 were provided by Panjing Second Pharmaceutical Factory (Liaoling, China). Yiganling tablets (silymarin and excipient) were provided by Beijing Forth Pharmaceutical Factory (Beijing, China). Standards of silybin, isosilybin, silycristin and silydianin were kindly provided by MADAUS AG (Germany) and National Institute for the Control of Pharmaceutical and Biological Products, PRC.

2.2. Instrumentation

LC-analysis was performed on Shimadzu LC-10ATVP pump equipped with SCL-10A VP sys-

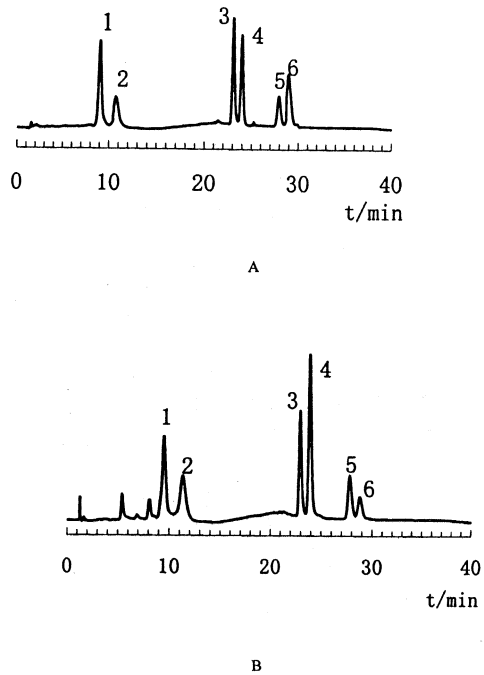

Fig. 2. Chromatograms of (A) mixture standard of silybin, isosilybin, silycristin and silydianin and (B) silymarin sample (peak 1 is silycristin, peak 2 is silydianin, peaks 3.4 are silybin and peaks 5.6 are isosilybin).

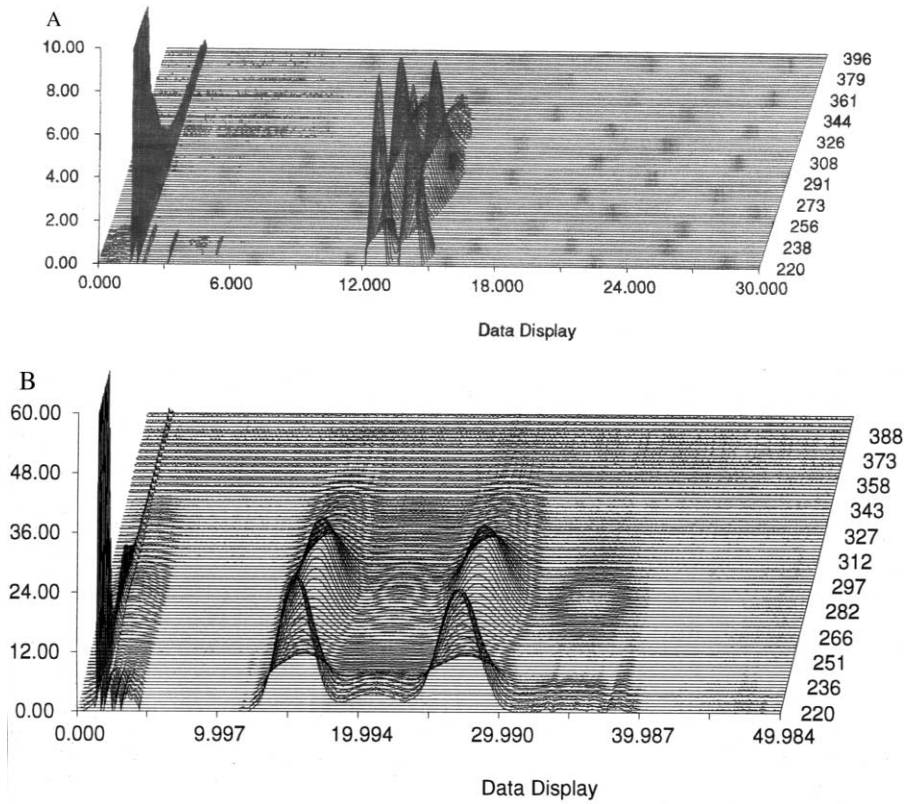

Fig. 3. (A) is 3-D-gram of silybin and (B) is 3-D-gram of isosilybin.

tem controller, SPD-10A VP UV–VIS detector, SIL-10AVP autoinjector, CTO-10ASVP column oven and IBM G42 computer with Shimadzu class-VP ver 5.02 software. DAD scanning was performed on SP8800 pump with Focus detector. LC/MS analysis was performed on Perkin–Elmer Sciex API-3000 triple quadrupole mass spectrometer equipped with Perkin Elmer series 200 pump and Perkin Elmer 785A UV–VIS detector.

2.3. Chromatographic condition

2.3.1. Chromatographic condition for assay

The analysis column was Shim-pack VP-ODS (150 × 4.6 mm, i.d. 5 μm) and pre-column (10 × 4.6 mm, i.d. 5 μm). The mobile phase consisted of methanol (A) and aqucous dioxane (B) (90% water + 10% dioxane) with gradient eluation (see Fig. 1). The flow rate was 1.5 ml/min The column was maintained at a temperature of 40 ± 1°C and the UV detector was set at a wavelength of 288 nm. The total run time was 35 min.

2.3.2. Chromatographic condition for LC/MS

The analysis column was Shim-pack VP-ODS (150 × 4.6 mm, i.d. 5 μm) and pre-column (10 × 4.6 mm, i.d. 5 μm). The mobile phase consisted of methanol and water (3:7). The flow rate was 1.5 ml/min. The column was maintained at a room temperature and the UV detector was set at a wavelength of 288 nm. The total run time was 55 min.

3. Results and discussion

3.1. Chromatograms of mixture standards and sample of silymarin

Dissolved a standard mixture containing silybin, silycristin, silydianin and isosilybin and sam-

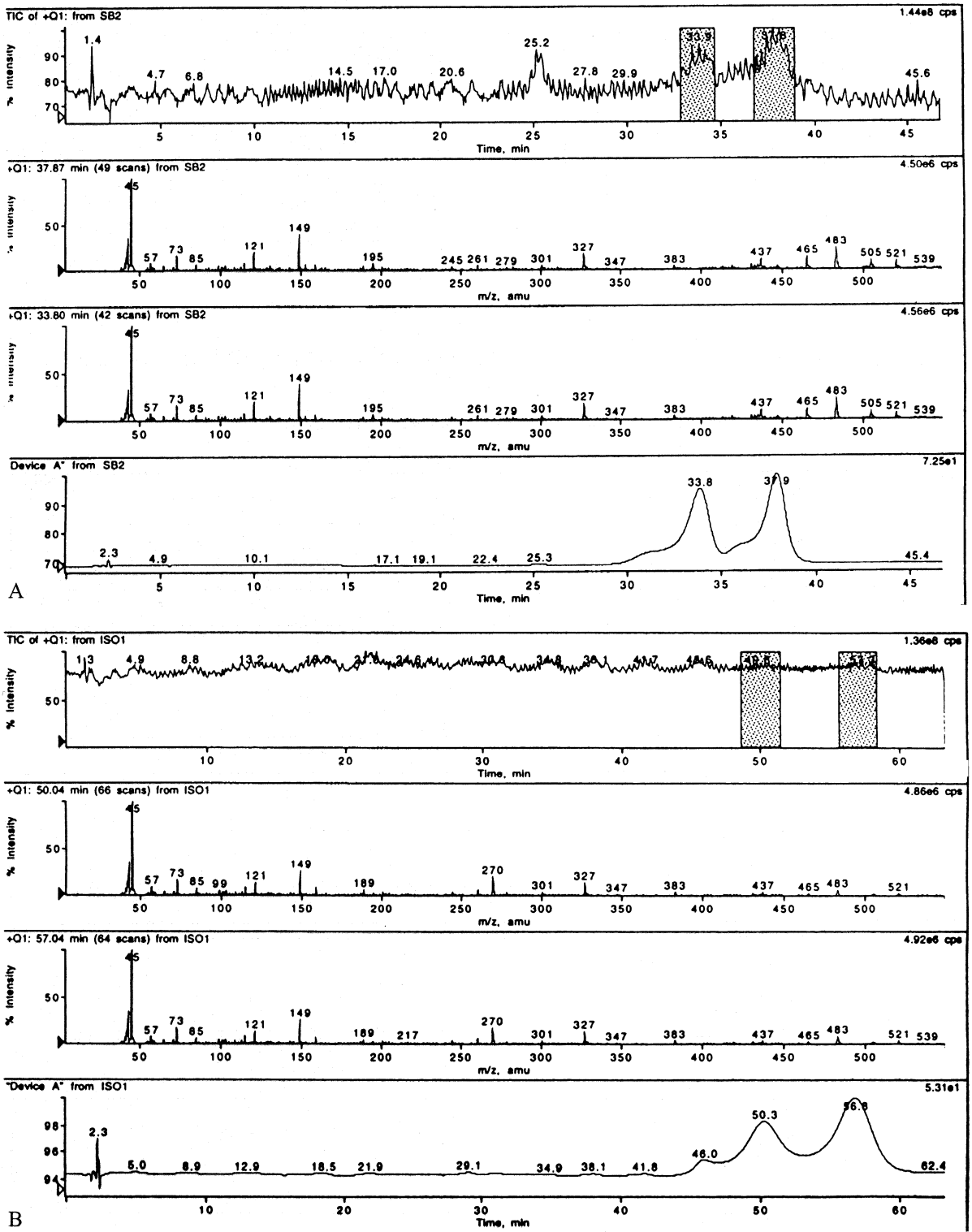

Fig. 4. LC/MS analysis of two diastereoisomers of silybin (A) and isosilybin (B).

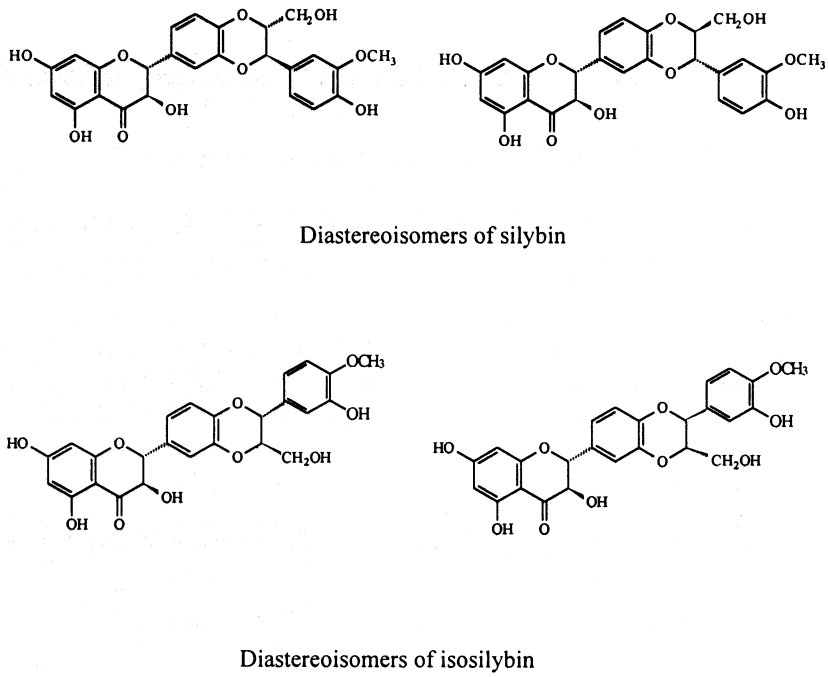

Fig. 5. Structures of diastereoisomers of silybin and isosilybin.

Table 1
Linearity of silycristin, silydianin, silybin and isosilybin

Components	Regression equation	Correlation coefficient	Concentration range (μg/μl)
Silycristin	$Y = 1397620X - 10198$	0.99958	0.1398–1.398
Silydianin	$Y = 755927X + 8791$	0.99933	0.0846–0.846
Silybin	$Y = 162551X - 88924$	0.99960	0.1437–1.437
Isosilybin	$Y = 1228090X + 21931$	0.99959	0.0885–0.885

ple of silymarin in 10 ml methanol, respectively. Injected separately about 10 μl of the two solutions into the chromatograph and recorded the chromatograms (see Fig. 2). Dissolved a standard silybin and isosilybin in 10 ml methanol, respectively. Injected separately about 10 μl of the two solutions into the chromatograph, recorded the chromatograms and scan peak with TDS method (see Fig. 3).

3.2. LC/MS analysis

The silybin was resolved into two different peaks, and the same was found with the isosilybin, DAD scanning shows that their UV absorbency spectra were the same, the mass spectra of both substances show the molecular peak at

Table 2
Recovery and repeatability of silybin, isosilybin, silycristin and silydianin

Components	Mean ± R.S.D. (%)	Recovery ± R.S.D. (%)
Silybin	27.2 ± 1.3	102.1 ± 1.4
Isosilybin	11.3 ± 1.1	100.7 ± 1.0
Silycristin	12.0 ± 0.9	102.3 ± 1.3
Silydianin	16.0 ± 1.1	99.76 ± 1.3

Table 3
Assay of silymarin, legalon and yiganling (%) ($n = 3$)

Sample	Batch	Silycristin	Silydianin	Silybin[a]	Isosilybin[b]
Silymarin 1	65536	12.07	16.18	27.22(0.572)	11.33(1.481)
	65810	12.11	15.87	28.74(0.576)	10.90(1.555)
Silymarin 2	980303	11.83	3.79	26.34(0.585)	7.12(2.068)
	980805	13.10	4.06	27.86(0.582)	7.79 (2.016)
	980802	13.28	4.24	30.95(0.586)	7.76(1.997)
Legalon[c]	821055	23.0	28.3	48.9	22.3
	825121	23.3	28.8	52.7	22.7
	830153	23.2	27.5	53.6	19.5
Yiganling[c]	980409	6.2	2.0	14.4	4.0
	980411	6.1	2.0	13.4	4.0
	980412	6.3	1.8	14.3	3.9

[a] Ratio of peak area of two diastereoisomers of silibin.
[b] Ratio of peak area of two diastereoisomers of isosilybin.
[c] Assay of one yiganling tablet or legalon capsule (mg).

433 and very similar fragmentation. Most fragments are found in each mass spectrum and differ only in intensity (see Fig. 4). Therefore, according to our results, it seems that the silybin was a mixture of two different diastereoisomers at C2′, C3′; isosilybin was a mixture of two different diastereoisomers at C2′, C3′ (see Fig. 5).

3.3. Quantitative analysis

3.3.1. Linearity of method

Table 1 shows the linearity for the determination of silybin, isosilybin, silydianin and silycristin was constructed by analyzing a series of standards of known concentration. A significant linear relationship between peak area and solute concentration was found.

3.3.2. Recovery and repeatability of the method

The reliability of the analytical method was estimated by the small coefficients of variation and good recovery. The inter-day repeatability of the method was assessed for the determination of silybin, isosilybin, silycristin and silydianin by repeated analysis of their standards at three concentration levels. The recovery of each effective component was assessed by determination of the peak area obtained by adding standards into the known sample. It is shown in Table 2.

3.4. Application

The assay method was applied to determine silymarin 1 and Legalon capsules (Preparations of silymarin made in MADAUS AG, Germany) and silymarin 2 (made in Panjin Second Pharmaceutical Factory, China) and Yiganling tablets (made in Beijin Forth Pharmaceutical Factory, China). Injected test solution and standard solution into chromatograph, according to the chromatographic condition for assay, recorded the peak area. The results were shown in Table 3.

4. Discussion

The ratio of peak area of two diastereoisomers of silybin is different between standard and silymarin. Isosilybin is also the same. It is very interesting that the ratio of peak area of two diastereoisomers of silybin and isosilybin is also different in silymarin between Germany and China. It is maybe due to the influence of geographical environments.

5. Conclusion

To sum up, diastereoisomers of silybin and isosilybin have respectively been identified by LC/

MS. Additionally, this RP-HPLC method with a reversed phase ODS column is an analytical aid not only for the separation of the diastereoisomers of silybin and isosilybin, but also for determination of silybin (two diastereoisomers), isosilybin (two diastereoisomers), silydianin and silycristin in silymarin and preparations of silymarin.

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