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Short communication

A novel rapid method for simultaneous determination of eight active compounds in silymarin using a reversed-phase UPLC-UV detector

Hong Liu [a], Zhenxia Du [b], Qipeng Yuan [a,*]

[a] State Key Laboratory of Chemical Resource Engineering, Beijing University of Chemical Technology, Beijing 100029, China
[b] School of Science, Beijing University of Chemical Technology, Beijing 100029, China

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ABSTRACT

A novel rapid chromatographic method based on utilization of UPLC column was developed for the analysis of eight active compounds in silymarin. The analysis was performed on a Waters Acquity UPLC system with an Acquity UPLC^{BEH} C18 column (5 mm × 2.1 mm I.D., 1.7 μm) and a gradient elution of methanol and water containing 0.01% formic acid with a run time of 9 min, in which the retention time of the last analyte was 5.8 min. And all eight active compounds achieved complete separation. Comparison of system performance with conventional HPLC was made with respect to analysis time, efficiency and sensitivity. The results indicated that the type of column, the type of mobile phase and the modified addition were significant to the separation of isomeric compounds in herb extracts.

1. Introduction

Silymarin has been widely used as a therapeutic agent for a variety of acute and chronic liver diseases [1–3]. It is mainly composed of a series of flavonolignans: silydianin and three groups of diastereoisomeric flavonolignans (silychristin and isosilychristin, silybin A and silybin B, and isosilybin A and isosilybin B) and a flavonoid: taxifolin. Silybin consisting of silybin A and silybin B is the main biologically active component. Recently, each flavonolignan isomer of silymarin has been reported for different biological activities [4–7]. Therefore, the development of quality control methods for qualitative and quantitative determinations of the major active compounds in silymarin is an essential issue for the effective and safe use of this traditional herb.

Up to now, there are many chromatographic methods, such as thin layer chromatography (TLC) [8], capillary electrophoresis [9], HPLC equipped with UV [9–13], electrochemical [9], DAD [14] or MS detection [15–17], etc., for the determination of flavonolignans in silymarin. However, these methods suffered from long analysis time, low resolution and sensitivity and/or few analytes. Shibano et al. [18] introduced a gradient mobile phase of methanol–water containing 0.1% formic acid on a Nucleosil 100-3 C18 HD (2.0 mm × 125 mm I.D.). Complete separation of all active compounds in silymarin needed 35 min. At the same time, it consumed large amounts of organic solvents, which were often expensive and potentially harmful. Therefore, the researchers would be interested in the improved application of liquid chromatography for the determination of silymarin.

Recently, ultra-performance liquid chromatography (UPLC) has been considered to be a new direction of liquid chromatography [19–23]. The principle advantages reported for UPLC are the increase in the signal-to-noise ratio (S/N) (narrower peaks), a reduction in the analysis time and an enhancement in peak resolution [24,25]. UPLC has been widely used in the fields of quality control, pharmaceutical, toxicological and clinical analysis for increasing analysis throughout and reducing analysis costs.

The aim of this work was to develop a simple and rapid chromatography method for the quality control of silymarin in plant extract and diary supplement. Moreover, comparison of system performance with conventional HPLC was made with respect to analysis time, efficiency and sensitivity. This UPLC method could also be compatible with mass spectrometry detection by splitting flow.

2. Experimental

2.1. Instrumentation and chromatographic conditions

UPLC analyses were performed using a Waters Acquity Ultra-Performance LC (UPLC) system (Waters, Milford, MA, USA). UPLC separation was achieved using an Acquity UPLC^{BEH} C18 column (50 mm × 2.1 mm, 1.7 μm, Waters, Milford, MA, USA). The mobile phase consisted of (A) methanol and (B) water containing 0.01% formic acid. A gradient elution program was applied as follows: 0–0.5 min linear increased from 25% to 30% A; 0.5–2.8 min linear increased from 30% to 35% A; 4.5–6.0 min hold on 45% A; 6.1 min

* Corresponding author. Tel.: +86 10 64437610; fax: +86 10 64437610.
E-mail address: yuanqp@mail.buct.edu.cn (Q. Yuan).

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linear decreased from 45% to 25% A; the composition was held at 25% A for a further 3 min for reequilibration, giving a total run time of 9 min. The retention time of the last eluted analyte was 5.8 min. Flow rate was o.4 mL/min. The injection volume was 1 μL. Column temperature was 30 °C. The detective wavelength was 288 nm. The pressure limit was set as follows: low, 0 kPa; high, 103 MPa during the elution process, the highest pressure was about 91.76 MPa.

The HPLC was used on a Shimadzu LC-20AVP system with two LC-20AT solvent delivery units, an SPD-20A UV/vis detector, a CTO-10ASVP column oven (Shimadzu, Kyoto, Japan) and a T2000P workstation (Beijing, China). HPLC separation was achieved using a reversed-phase C18 column (250 mm × 4.6 mm, 5 μm, Diamodsil™). The mobile phase consisted of (A) methanol and (B) water containing 0.1% formic acid. A gradient elution program was modified according to the paper in press [26]: 0–3 min hold on 47% A; 3–13 min linear increased from 47% to 50% A; 13–25 min linear increased from 50% to 60% A; 25–35 min hold on 60% A; 35.01 min linear decreased from 60% to 47% A; the composition was held at 47% A for a further 10 min for reequilibration, giving a total run time of 45 min. The elution time of the last analyte was 31 min. Flow rate was 1 mL/min. The injection volume was 10 μL. Column temperature was 30 °C. The detective wavelength was 288 nm.

The pressure limit was set as follows: low, 4.0 MPa; high, 9.5 MPa during the elution process, the highest pressure was about 8.9 MPa.

2.2. Chemicals

The standard compounds of taxifolin, silydianin, silychristin, isosilychristin, silybin A, silybin B, isosilybin A and isosilybin B were isolated by pre-LC in our laboratory as unpublished data. The purities were confirmed by chromatographic methods. The structures were identified by the analysis of the spectroscopic data (IR, NMR, ESI/MS–MS, CD). The data were compared with published spectroscopic data [16,27]. Methanol and formic acid are of HPLC grade and purchased from Dikma Technology Inc. (USA). The co-product of silybin recrystallization was prepared in our laboratory. The serum was obtained from Anzhen Hospital (Beijing, China). The samples containing serum were purified by solid phase extraction for deproteinization.

2.3. Sample preparation

Dry crude extracts (0.1 g) was weighed and dissolved in 10 mL methanol. 100 μL above solution was adjusted to 10 mL with the initial mobile phase (A:B, v/v) and mixed thoroughly. Prior to injection, an adequate volume (2 mL) was passed through a 0.2 μm membrane filter. The first 1.0 mL was discarded and the remaining volume was collected as the sample for LC analysis.

2.4. Preparation of standard solution

The individual stock solutions of the standard compounds were prepared at the concentration of 2.0 mg/mL in methanol. Each standard solution was mixed together at the concentration of 50 μg/mL taxifolin, 100 μg/mL silychristin, 200 μg/mL silydianin, 400 μg/mL isosilychristin, 50 μg/mL silybin A, 50 μg/mL silybin B, 50 μg/mL isosilybin A and 400 μg/mL isosilybin B. And the mixture was further diluted to 5 times, 10 times, 20 times, 40 times, 50 times and 100 times for UPLC analysis. And the mixture of standards for HPLC analysis did not include 400 μg/mL isosilychristin.

2.5. Validation procedure

The newly developed UPLC method was validated in terms of precision, accuracy and linearity according to ICH guidelines [9]. Assay method precision was carried out using six independent tests. The accuracy of the assay method was evaluated in triplicate using three concentration levels of low, middle and high.

The limit of detection (LOD) and limit of quantification (LOQ) were determined by injecting a series of dilute solutions with known concentrations. LOD and LOQ were defined as the signal-to-noise ratio equal to 3 and 10, respectively.

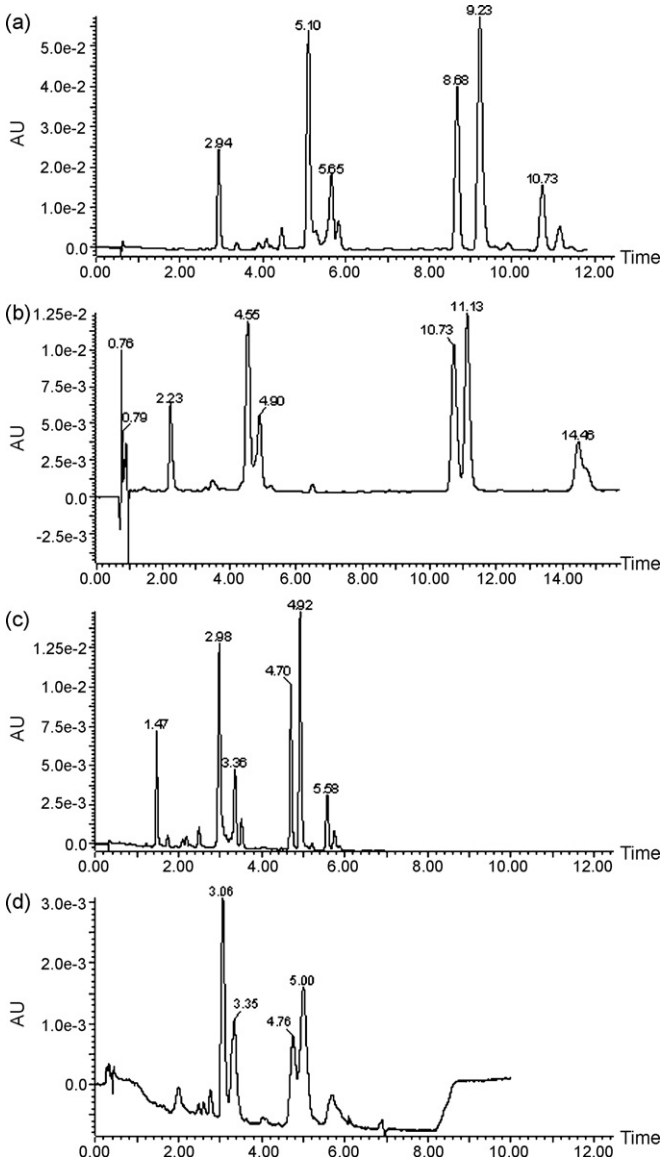

Fig. 1. Chromatograms of silymarin extract obtained by different UPLC conditions: (a) the analysis was achieved using an Acquity UPLC^{BEH} C18 column (100 mm × 2.1 mm, 1.7 μm). Flow rate was 0.4 mL/min. Column temperature was 30 °C. The mobile phase consisted of (A) methanol and (B) water containing amount of formic acid. A gradient elution program was applied as follows: 0–1 min was kept at 30% A, 2–3 min linear increased from 30% to 35% A, 3–4 min linear increased from 35% to 40% A, 6–9 min linear increased from 40% to 45% A, 9–12 min hold on 45% A, 12–13 min linear decreased from 45% to 30% A. (b) The analysis was achieved using an Acquity UPLC^{BEH} C18 column (100 mm × 2.1 mm, 1.7 μm). Flow rate was 0.3 mL/min. Column temperature was 40 °C. The mobile phase consisted of (A) acetonitrile and (B) water containing amount of formic acid. A gradient elution program was applied as follows: 0–5 min was kept at 20% A, 5.01 min linear increased from 20% to 22.5% A, 5.01–16 min hold on 22.5%, 16.01 min linear decreased from 22.5% to 20% A. (c) The analysis was achieved using an Acquity UPLC^{BEH} C18 column (50 mm × 2.1 mm, 1.7 μm). A gradient elution program was applied as follows: 0–0.5 min linear increased from 25% to 30% A, 0.5–2.8 min linear increased from 30% to 35% A, 4.5–6.0 min hold on 45% A, 6.1 min linear decreased from 45% to 25% A. (d) The mobile phase did not contain formic acid, the other UPLC conditions were the same as in (c).

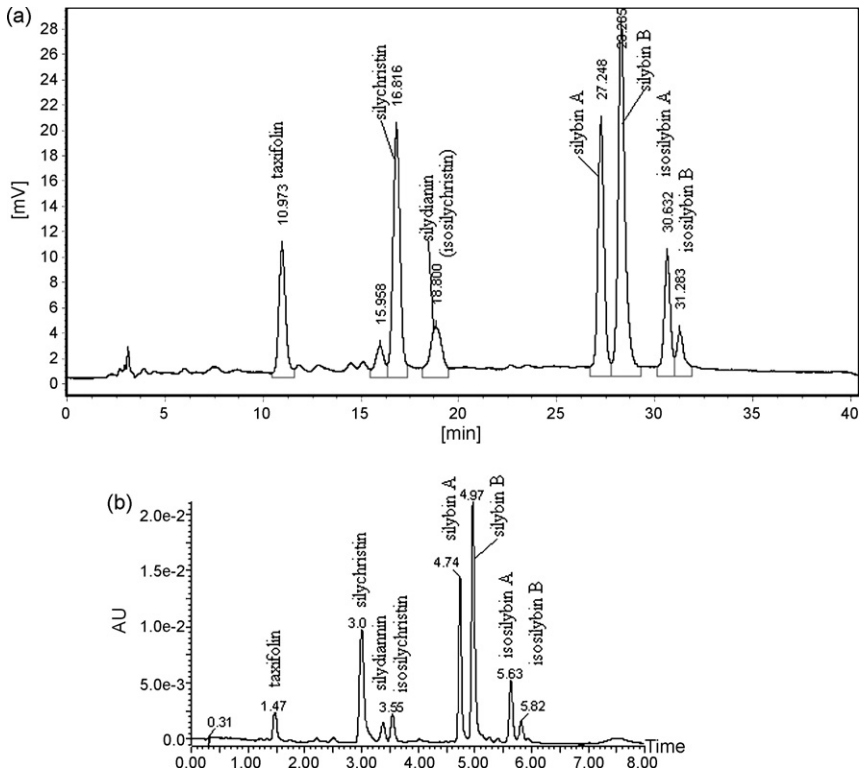

Fig. 2. Comparison of chromatograms of silymarin extract by (a) HPLC and (b) UPLC at wavelength 288 nm.

3. Results and discussion

3.1. Chromatographic conditions optimization

Optimal chromatographic condition was obtained after running different mobile phases with reversed-phase C18 column. The different columns were tried on Acquity UPLC^{BEH} C18 (100 mm × 2.1 mm I.D., 1.7 μm) (Fig. 1a) and Acquity UPLC^{BEH} C18 (50 mm × 2.1 mm I.D., 1.7 μm). All the chromatograms of silymarin were partially separated using acetonitrile as mobile phase component (Fig. 1b). Methanol was preferred to acetonitrile as the mobile phase. The best result (Fig. 1c) was observed with UPLC^{BEH} C18 column (50 mm × 2.1 mm I.D., 1.7 μm) using methanol and water containing 0.01% formic acid as the mobile phase. The addition of formic acid was advantageous to the separation of peaks (Fig. 1d). Many different gradient systems were tried for the best separation of peaks.

Comparison of chromatographic performance of UPLC (gradient) with HPLC (gradient) was preformed. The run time for all the analytes in silymarin was about 9 min in the UPLC system, while the run time for all the analytes from silymarin was about 45 min in our HPLC system. The UPLC method allowed shortening the analysis time up to 5-fold compared to that on our HPLC method and in the literature [18]. In our HPLC method, silychristin and isosilychristin were overlapped, while in UPLC system all the analytes were completely separated. LOD of the UPLC method was lower twice than that of our HPLC method.

The typical chromatograms obtained from final HPLC and UPLC conditions of chemical fingerprint analysis of silymarin are depicted in Fig. 2.

3.2. UPLC method validation

The validation study allowed the evaluation of the method for its suitability for routine analysis.

3.2.1. Specificity

The specificity of the UPLC method was determined by injecting individual samples, wherein no interference with taxifolin, silychristin, silydianin, silybin A, silybin B, isosilybin A and isosilybin B from five different sources of silymarin extracts, the co-product of silybin recrystallization (Fig. 3a) and silymarin containing serum (Fig. 3b and c (blank serum)) was observed. The chromatograms were checked for the appearance of any extra peaks. The purities of the principle and other chromatographic peaks were found to be satisfactory. The UPLC–MS–MS method for the determination of the active components of silymarin in blood sample was being further investigated in our laboratory.

Table 1
Simultaneous detection of eight main active compounds of silymarin of retention time, regression equation, correlation coefficient, LOD and LOQ by UPLC.

Main active compounds	t_R/min	Regression equation	R	LOD/ng	LOQ/ng
Taxifolin	1.48	$y = 79.40x + 2.23$	0.9998	0.68	2.5
Silychristin	3.02	$y = 70.06x - 2.71$	0.9995	1.25	5
Silydianin	3.40	$y = 28.52x + 4.23$	0.9994	2.5	10
Isosilychristin	3.56	$y = 15.52x + 5.66$	0.9990	5	20
Silybin A	4.75	$y = 66.34x + 1.22$	0.9992	0.68	2.5
Silybin B	4.97	$y = 189.41x + 2.62$	0.9990	0.68	2.5
Isosilybin A	5.64	$y = 14.34x + 11.72$	0.9997	1.25	5
Isosilybin B	5.83	$y = 8.16x + 2.11$	0.9991	2.5	10

Table 2
Results of UPLC analysis of silymarin samples.

Samples	Content (g/100 g)								
	Taxifolin	Silychristin	Silydianin	Isosilychristin	Silybin A	Silybin B	Isosilybin A	Isosilybin B	Total
Silymarin (No. 1)	2.04	11.50	2.43	6.54	12.53	22.84	10.27	7.23	75.39
Silymarin (No. 2)	1.79	11.31	2.43	9.95	13.33	24.42	11.92	7.53	82.67
Silymarin (No. 3)	1.09	6.62	1.74	6.53	6.97	13.14	6.21	3.96	46.27
Silymarin (No. 4)	1.76	9.93	1.69	5.17	11.98	18.68	7.35	6.47	63.02
Silymarin (No. 5)	0.75	7.20	3.41	2.83	7.47	13.95	7.39	4.68	47.70

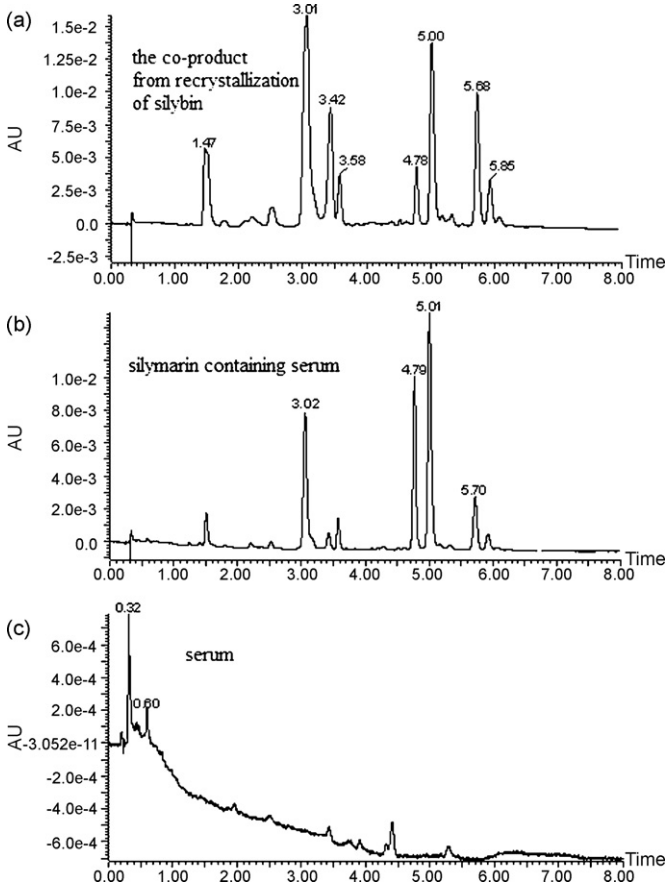

Fig. 3. Chromatograms of all active compounds in co-product of silybin recrystallization and silymarin containing serum by UPLC: (a) co-product of silybin recrystallization, (b) silymarin containing serum and (c) blank serum at wavelength 288 nm.

3.2.2. Limit of quantification and limit of detection

All standards and samples were injected in triplicate. They were determined by serial dilution of sample solution using the described UPLC conditions. The results are shown in Table 1.

3.2.3. Linearity

The quantitative capability of the system employing UPLC method was tested in the assay. Each calibration curve was performed with six different concentrations in triplicate. Table 1 shows the results of the standard calibration curves of integrated peak area ($n = 3$) and linearity (R^2). Calibration curves were linear with correlation coefficients >0.999 for all the analytes. The results showed excellent correlation between the peak area and concentration.

3.2.4. Recovery and precision

Recoveries were performed employing the method of standard addition. Nine portions of silymarin extracts were spiked with the mixed standards of eight active compounds of silymarin. An RSD% was within ±0.3% for taxifolin, ±1.8% for silychristin, ±0.9% for silydianin, ±1.0% for isosilychristin, ±1.7% for silybin A, ±2.6% for silybin B, ±2.3% for isosilybin A and ±1.2% for silybin B. Recoveries of the three concentration levels (low, medium and high: 1.26, 1.58, 2.36 μg/mL for taxifolin; 2.49, 3.06, 4.59 μg/mL for silychristin; 4.93, 6.16, 9.24 μg/mL for silydianin; 9.07, 12.0, 18.0 μg/mL for isosilychristin; 3.38, 4.22, 6.32 μg/mL for silybin A; 9.96, 12.4, 18.7 μg/mL for silybin B; 9.88, 12.35, 18.52 for isosilybin A and 2.95, 3.68, 5.52 μg/mL for isosilybin B) were 93.8%, 109.9%, 116.5% for taxifolin; 75.8%, 87.8%, 102.9% for silychristin; 80.1%, 97.9%, 100.9% for silydianin; 90.6%, 100.9%, 103.7% for isosilychristin; 104.9%, 86.4%, 82.3% for silybin A; 85.9%, 96.0%, 108.2% for silybin B; 89.0%, 97.4%, 104.2% for isosilybin A and 86.8%, 92.1%, 107.0% for isosilybin B. All recoveries were very well indicating the good recovery of the method.

The precision of the assay method was evaluated by carrying out six independent assays. All the RSD of assay of the standards of main active components in silymarin was within the acceptable limit of 2.0%. The intra-day RSD was 1.1% for taxifolin, 1.5% for silychristin, 1.1% for silydianin, 1.4% for isosilychirstin, 2.0% for silybin A, 1.4% for silybin B, 1.7% for isosilybin A and 1.9% for isosilybin B. The inter-day RSD was 1.7% for taxifolin, 1.7% for silychristin, 1.5% for silydianin, 1.7% for isosilychirstin, 2.0% for silybin A, 1.6% for silybin B, 1.9% for isosilybin A and 2.0% for isosilybin B. Multiple injections showed that the results were highly reproducible and showed a low standard error.

3.3. Application

This method has been applied successfully for the quantitative analysis of the eight constituents in the five different silymarin extracts in China, all of which were purchased in Beijing and Chengdu, China. The results of the quantitative analyses are summarized in Table 2. The heterogeneity of the various commercial samples was quite evident.

4. Conclusion

The newly developed UPLC method for all active compounds in silymarin was found to be capable of giving shorter retention time and maintaining better resolution than that with conventional HPLC method. The developed UPLC method was suitable for rapid analysis of silymarin extract and was successfully applied to quality control of all active compounds of silymarin in diary supplement.

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