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Analysis of the active components of silymarin

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Abstract

Silymarin is an antihepatotoxic substance isolated from fruits of *Silybum marianum*. Possibly due to their antioxidant and membrane stabilizing properties, the compounds have been shown to protect different organs and cells against a number of insults. The content and composition of main silymarin components (silybin, isosilybin, silydianin and silychristin) in various pharmaceuticals were analysed using HPLC and newly developed capillary zone electrophoresis method. Antioxidant properties expressed as total antioxidant status (TAS) of silymarin components were studied. Results of TAS were correlated with electropherograms and chromatograms.

Keywords: Silymarin; Pharmaceutical analysis; Antioxidants; Silychristin; Silybin; Isosilybin; Silydianin

1. Introduction

In recent years, antioxidants have been subjected to many epidemiological studies that have related their consumption with a reduction in the incidence of cardiovascular diseases and cancers. Among the recognised antioxidants (carotenoids, tocopherols, vitamins C and E, etc.) there is an extensive family of diverse components known generally as polyphenolic compounds. Silymarin is an antihepatotoxic polyphenolic substance isolated from the milk thistle plant, *Silybum marianum*. Derivatives of milk thistle have been used as herbal remedies for almost 200 years. They are currently enjoying re-emergence for therapy of liver diseases, as other natural remedies have become increasingly popular in the USA. Their use has been widespread throughout Europe since preparations became officially available for clinical use there in 1969 [1]. Silymarin was considered as a pure compound with the structure of 7-chromanol-3-methyl-taxifolin, but after the introduction of more accurate methods of analysis and separation it was shown that silymarin consists of a large number of flavonolignans (see Fig. 1), including silybin[1] (SB_A, SB_B), isosilybin (ISB_A, ISB_B), silydianin (SD) and silychristin (SC). A number of other flavonolignans have also been found in the seeds including dehydrosilybin, desoxysilycristin, desoxysilydianin, silandrin, silybinome, silyhermin, and neosilyhermin. Possibly due to their antioxidant and membrane stabilizing properties, the compounds have been shown to protect different organs and cells against a number of insults. Currently the most important medicinal application of milk thistle is its use as a

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[1]One can find in literature also alternative names for silybin such as sylybinine, silybinine and silybinin.

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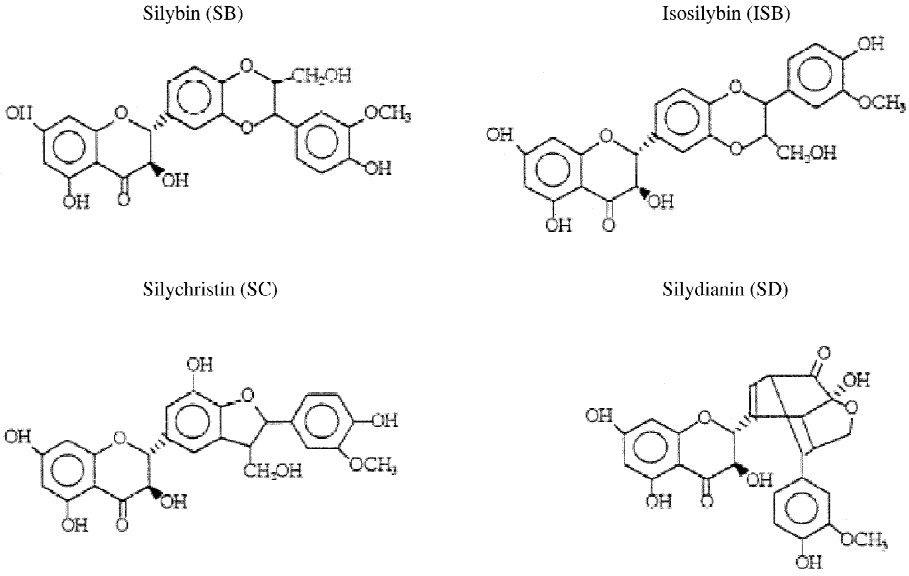

Fig. 1. Structures of main silymarin components.

hepatoprotectant and as supportive treatment of chronic inflammatory liver disorders such as cirrhosis, hepatitis, and fatty infiltration due to alcohol and toxic chemicals. It has also been used in the treatment of liver damage by poisonous mushrooms. Most liver toxins produce their damaging effects by free radical mechanisms. Silymarin protects red blood cell membranes against lipid peroxidation and haemolysis (breaking down of the red blood cells) caused by certain red blood cell poisons. Extracts of milk thistle fruits are supplied as capsules, tablets, liquids, powders and creams.

Up to now, main components of silymarin have been separated and determined by HPLC with UV, electrochemical or MS detection [2–4] and capillary electrophoresis [4]. Many different methods have been proposed for evaluating antioxidant power including DPPH (2,2-diphenyl-1-picrylhydrazyl) method [5], oxygen radical absorbance assay [6], flow injection analysis [7], Total antioxidant status (TAS) test of Randox [8] and electrochemical antioxidant test [9].

In this paper, separation and determination of four main silymarin components have been studied by HPLC and proposed capillary zone electrophoresis (CZE) methods. The basic characteristics, i.e., repeatability, accuracy, linearity and limit of detection of CZE methods were evaluated. On a series of silymarin sample the HPLC results were compared with CZE ones. The content of silymarin components were correlated with their antioxidant activity expressed as TAS.

2. Experimental

2.1. Chemicals

Standards of silychristin, silydianin, silybin, isosilybin and samples of fruit of milk thistle, silymarin and pharmaceuticals were obtained from IVAX (Opava, Czech Republic). Phosphoric acid, methanol, ammonia hydroxide was purchased from Lachema (Brno, Czech Republic), ε-aminocaproic acid (EACA), polyvinylpyrrolidone (PVP10) and hydroxyethylcellulose (HEC) were obtained from Sigma–Aldrich (Prague, Czech Republic). All chemicals were of analytical grade. The TAS test was purchased from Randox Labs. (Crumlin, UK).

2.2. Instrumentation

HPLC analyses were performed on Gynkotek HPLC instrument consisting of pump P580, UV–Vis detector UVD 170S and autosampler GINA 50T (Gynkotek, Germany) controlled by Chromeleon 6.12 software package. CZE analyses were done with an electrophoretic analyser EA 100 (Villa-Labeco, Slovak Republic) connected with a UV–Vis detector LCD 2084 (ECOM, Czech Republic).

2.3. Conditions of analyses

Silymarin components were determined by HPLC according to the Czech Pharmacopoeia 97 (Chapter 2.2.29) and by proposed capillary zone electrophoretic method. Antioxidant properties of these components were evaluated by the TAS test.

2.3.1. CZE assay

The CZE separation of main components of silymarin was made in hydrodynamically closed system. Analysis was performed in a fluorinated ethylene–propylene copolymer (FEP) capillary of a 25 cm (20 cm effective length)×320 μm I.D. The carrier electrolyte consisted of 10 m*M* EACA+100 m*M* ammonium hydroxide+0.5% PVP10+0.1% HEC and constant driving current applied to capillary was 100 μA. Samples were injected by sample valve with fixed internal loop (200 nl) and separated analytes were detected at 254 nm. One analysis requires 10 min. The silybin was used as standard for quantitative analysis and the external standard method (five concentration levels 10, 25, 50, 100 and 200 μg/ml) was used. Standard and sample were dissolved in 0.01 M ammonium hydroxide.

2.3.2. HPLC assay

The separation of silymarin flavonolignans was carried out using stationary phase Purospher RP18 (150×4 mm, 5 μm). A mixture of 85% phosphoric acid–methanol–water (0.5:46:64, v/v) served as mobile phase. The elution has been made in an isocratic mode at a flow-rate 1 ml/min and the detection at 288 nm. One analysis requires 25 min. The quantitative analysis is based on silybin standard and external standard method was used. Standard and samples were dissolved in the mobile phase.

2.3.3. Antioxidant assay

The TAS test was performed in accordance with the supplier's instruction. The principle of the test is as follows. ABTS [2,2′-azino-di-(3-ethylbenzthiazoline sulphonate)] is incubated with a peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation $ABTS^{\cdot+}$. This has a relatively stable blue–green colour, which is measured at 600 nm. Antioxidants in the added sample cause suppression of this colour production to a degree, which is proportional to their concentration. As a standard for TAS test 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid was used.

3. Results and discussion

During the CZE method development we found that polyvinylpyrrolidone has great influence to flavonolignans separation. There were found that diastereomers of silybin are not separated if no PVP10 is added into carrier electrolyte. The higher PVP10 concentration the better resolution of SB_A from SB_B and SD from SC was observed. The PVP10 addition increases analysis time. The addition of methanol has minimal effect on separation of flavonolignans and similarly as PVP10 increases analysis time. Typical electropherogram of silymarin extract is shown on Fig. 2 and Fig. 3 shows the chromatogram of the same sample. From these figures is clear that:

(1) Diastereomers of isosilybin are not separated by CZE method, but HPLC method enables their separation,

(2) CZE provides better separation of silychristin and silydianin from sample constituents than that of HPLC and

(3) CZE analysis is two times shorter than that of HPLC.

Quantitative analysis is based on silybin standard. The basic characteristics of the CZE method, i.e., linearity, precision, accuracy (recovery) and quantification limit are summarised in Table 1. Results clearly showed that the CZE method fulfils the pharmacopoeia criteria (accuracy should be within the range 98–102% and repeatability expressed as RSD<1%) and therefore it is suitable for intended purpose.

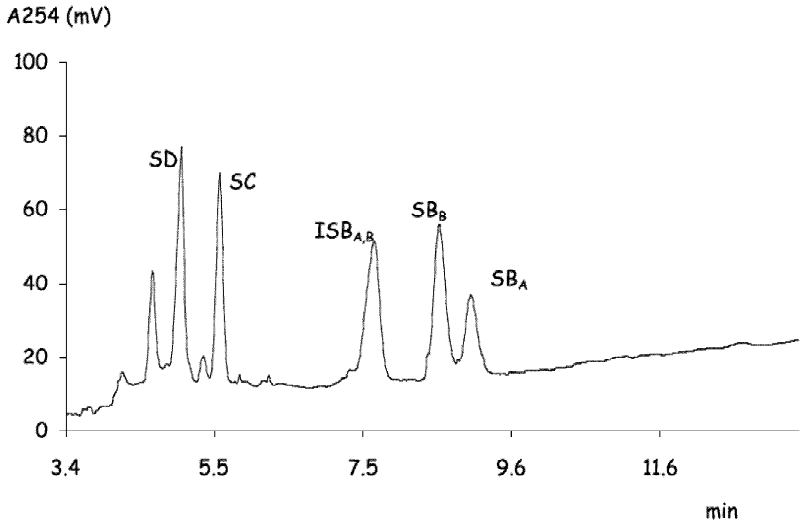

Fig. 2. Electropherogram of silymarin batch 017 (200 μg/ml). The carrier electrolyte used for CZE analysis consisted of 10 m*M* EACA+100 m*M* ammonium hydroxide+0.5% PVP10+0.1% HEC; constant driving current was set at 100 μA. Silymarin components were detected at 254 nm.

Tables 2 and 3 summarize CZE and HPLC results of real sample analysis. The CZE results are correlated with HPLC as shown on Fig. 4. From this graph is clear that HPLC method gives slightly higher total flavonolignans content than that of the CZE method. It is due to higher silydianin and silychristin levels found by HPLC comparing to CZE. It could be due to worse HPLC separation of these flavonolignans from sample constituents (see Figs. 2 and 3). The similar levels of the main

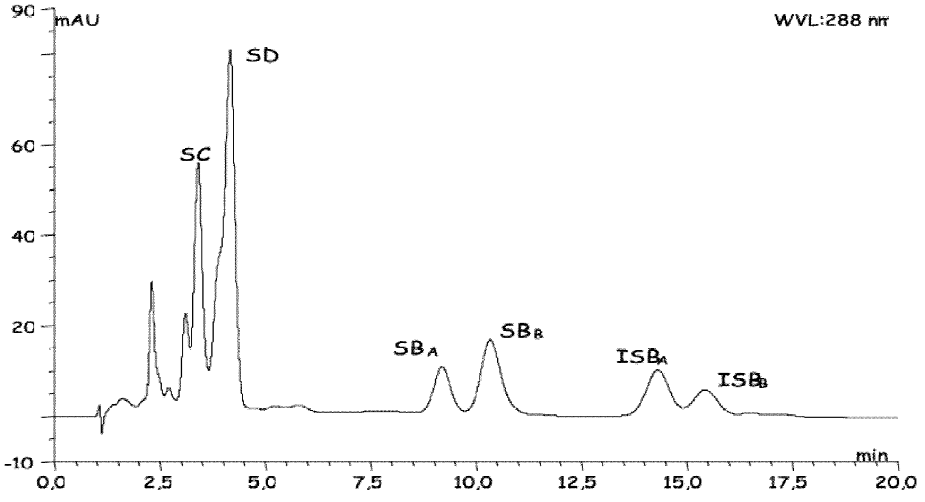

Fig. 3. Chromatogram of silymarin batch 017 (200 μg/ml). HPLC analysis was carried out on Purospher RP18 (150×4 mm, 5 μm) using mixture of 85% phosphoric acid–methanol–water (0.5:46:64, v/v) as mobile phase (isocratic elution) at a flow-rate 1 ml/min. Flavonolignans of silymarin were detected at 288 nm.

Table 1
Method characteristics for CZE analysis of silybin[a]

Characteristic	Value
Precision[b] (RSD, $n=6$)	0.88%
Accuracy (recovery)[c]	98.0±3%
Linearity[d]	10–200 μg/ml
Quantification limit ($S/N=10$)	0.5 μg/ml[e]

[a] Silybin concentration is expressed as a sum of both diastereomers SB_A and SB_B.
[b] Results based on analysis of solubilized silymarin batch 040 (200 μg/ml).
[c] Results based on analysis of silymarin batch 022 and 50 and 100% standard addition of silybin.
[d] Correlation coefficient is 0.9974.
[e] It corresponds to 0.2 g/100 g (sample weight 200 μg/ml).

silymarine component silybin were found by the CZE and HPLC.

The results of antioxidant power of samples determined by the commercial TAS test are summarized in Table 4. From the results is clear that the antioxidant power varied considerably for different samples while for standards such as silybin, isosilybin and silydianin are similar. Exception is silychristin standard, which has antioxidant power of 20% higher than the others. Model mixture of standard showed 50% higher antioxidant power than that of individual standards. The TAS results were correlated with flavonolignans content found by HPLC and CZE. From the Figs. 5 and 6 summarizing this correlation is clear that CZE results of

Table 2
Results of CZE analyses of silymarin sample and pharmaceutical

Sample	Content (g/100 g)				
	Silydianin	Silychristin	Isosilybin[a]	Silybin[b]	Total
Silymarin batch No. 022	0.61	12.67	7.13	34.68	55.10
Silymarin batch No. 004	0.75	14.07	8.10	19.52	42.44
Silymarin batch No. 017	6.88	5.89	7.50	9.68	29.96
Solubilized silymarin batch No. 040	0.60	12.24	7.33	29.87	50.03
Solubilized silymarin batch No. 060	0.40	7.32	3.87	46.90	58.49
SILYGAL batch No. 3A105024	0.19	4.21	2.46	9.11	15.97
Silybum Marianum, batch No. 502	0.05	0.38	0.25	1.16	1.83
Silybum Marianum, batch No. 139SX	0.15	0.46	0.33	1.24	2.17

[a] Sum of isosilybin diastereomers.
[b] Sum of silybin diastereomers.

Table 3
Results of HPLC analyses of silymarin sample and pharmaceutical

Sample	Content (g/100 g)				
	Silydianin	Silychristin	Isosilybin[a]	Silybin[b]	Total
Silymarin batch No. 022	3.11	17.11	6.62	37.12	63.96
Silymarin batch No. 004	4.49	17.93	7.04	19.64	49.09
Silymarin batch No. 017	14.66	8.42	7.95	9.48	40.50
Solubilized silymarin batch No. 040	2.92	14.76	5.99	28.34	52.01
Solubilized silymarin batch No 060	1.86	9.17	4.07	46.35	61.45
SILYGAL batch No. 3A105024	0.95	5.26	2.29	8.70	17.21
Silybum Marianum, batch No. 502	0.29	0.97	0.43	1.85	3.54
Silybum Marianum, batch No. 139SX	0.58	1.14	0.54	1.95	4.21

[a] Sum of isosilybin diastereomers.
[b] Sum of silybin diastereomers.

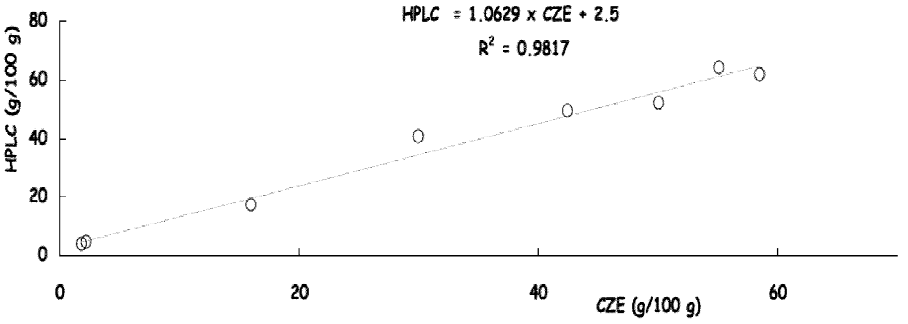

Fig. 4. Comparison of HPLC and CZE results.

Table 4
Results of TAS analyses of silymarin standard, samples and pharmaceuticals

Sample	Total antioxidant status		
	mmol/100 g of sample	mmol/100 g of flavonolignans	mmol/mmol of flavonolignans[a]
Standard of silybin batch No. SC200698/4	447.2	465.9	2.25
Standard of isosilybin batch No. 150586	426.6	426.6	2.06
Standard of silydianin batch No. 120692	448.0	448.0	2.16
Standard silychristin batch No. SC010498/10	513.0	581.6	2.81
Standard mixture[b]	576.8	605.2	2.92
Silymarin batch No. 022	402.7	730.9	3.53
Silymarin batch No. 004	392.4	924.6	4.46
Silymarin batch No. 017	391.6	1307.2	6.31
Solubilized silymarin batch No. 040	389.9	779.3	3.76
Solubilized silymarin batch No. 060	398.4	681.2	3.29
SILYGAL batch No. 3A105024	88.5	554.3	2.67
Silybum Marianum, batch No. 502	9.1	494.0	2.38
Silybum Marianum, batch No. 139SX	11.6	533.0	2.57

[a] Average relative molecular mass of flavonolignans is 482.44.
[b] 1% SD, 10% SC, 8% ISB, 40% SB, total concentration was 200 μg/l.

flavonolignans content are closely related to antioxidant power expressed as TAS than that of HPLC ones. An interesting finding is that the higher purity of milk thistle extracts the lower antioxidant power. It shows that extract probably contain ''impurities'' with higher antioxidant power than identified

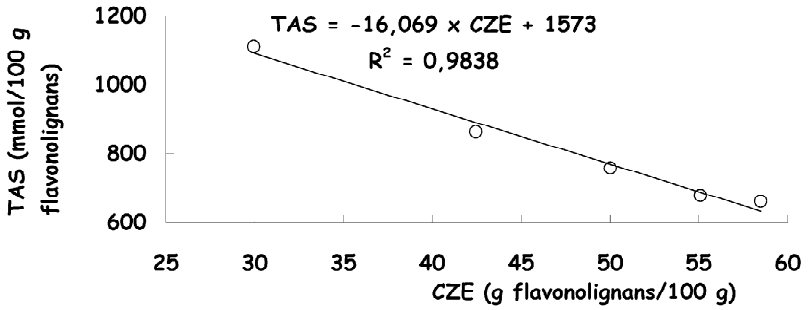

Fig. 5. Comparison of TAS and CZE results.

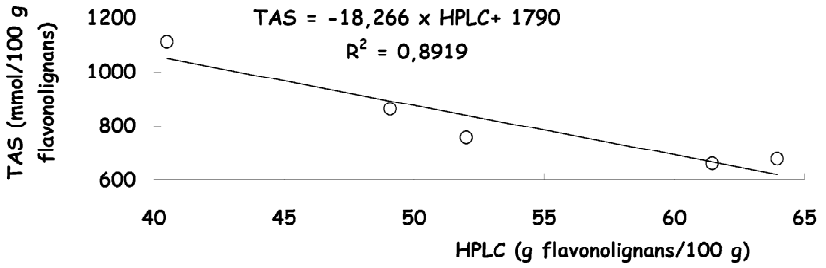

Fig. 6. Comparison of TAS and HPLC results.

flavonolignans. These ''impurities'' will be studied within ongoing project.

4. Conclusion

The determination of silymarin components in various sample made by HPLC and a newly developed CE method gave comparable results. The shorter analysis and better resolution of silydianin and silychristin from sample constituents are the main advantages of CZE method. Comparison of TAS and content of identified flavonolignans shows that with increasing purity of silymarin extract the TAS of sample decreases. The extract probably contains ''impurities'' with higher antioxidant properties than that of identified flavonolignans.

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