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Original Article

The Study of Silymarin Release Kinetic in Free and Hydrogel Bound Micellar Forms: Qualitative and Quantitative Analysis Using RP-HPLC.

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Abstract

Silymarin is a safe herbal medicine; however, it has some undesirable properties such as short half-life and poor aqueous solubility. To the best of our knowledge, this study is the first to report utilizing a dual-drug delivery system (DDDS) to enhance the release profile of silymarin from both micelles and hydrogels. In this experimental study, the release profile of micellar silymarin and micelle-hydrogel bounded silymarin during 21 days was examined using Knauer K2600A liquid chromatography. The calibration curve was plotted using the peak-areas of the silymarin at different concentrations.The RP-C18 column allowed a good separation of the components of standard silymarin.LOD and LOQ were 16.5 and 55.02 µg/ml, respectively. The *in vitro* release profiles of the two compounds showed a rapid release of silymarin, especially in the absence of hydrogel. The cumulative release graph revealed that the hydrogel-bound form has more constant release kinetics than the free micelle form; this means that the hydrogel-bound form may sustain for longer durations.In this study, a dual-drug delivery system based on hydrogel/micelle composites was introduced. The results showed that the Puramatrix hydrogel plays an important role in the constant release of silymarin. Furthermore, the RP-HPLC method presented in this study can be used by other researchers to overcome the difficulties associated with the *in vitro* separation and quantification of silymarin.

Keywords: Micelles, Puramatrix hydrogel, Release, RP-HPLC, Silymarin.

1. Introduction

Silymarin, an extract isolated from the seeds and fruits of the milk thistle plant *Silybum marianum*, consists of some very important compounds, e.g., taxifolin, silychristin, silydianin, silybin A, silybin B, isosilybin A and isosilybin B [1].

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Historically, silymarin is widely used as a safe, nontoxic herbal medicine for the treatment of various disorders such as liver diseases [2], cancers [3] and diabetes [4, 5].

Different studies have demonstrated that the efficacy of silymarin is restricted by its poor aqueous solubility, its oral bioavailability, and poor intestinal permeability resulting from its distraction by enterohepatic circulation, rapid excretion and its short half-life [6–8]. To overcome these impediments, higher doses have been applied in treatment patterns. Though elevated doses of silymarin can trigger insulin resistance and stimulate inflammatory reactions, low doses do not have any side effects [3, 9, 10].

Various analytical methods have been introduced for the separation or quantitative measurement of silymarin extracts. These methods are TLC, high performance liquid chromatography (HPLC) and UV-V spectrophotometry [11, 12]. HPLC provides the possibility of the separation of substances and the capability to find out their quality and quantity [13-15].

Many pharmaceutical systems have been investigated in order to enhance both bioavailability and solubility of silymarin including its complexation with phosphatidylcholine, cyclodextrins, and phospholipids as well as incorporating it in more soluble carriers [16].

Among the different types of drug delivery systems (DDSs), hydrogels that are extremely permeable to various kinds of drugs have been used as carriers for the drugs [17, 18].

Polymeric micelles are an important DDS, and they are formed by self-assembly from amphiphilic block or graft Copolymers [19, 20].

The core-shell construction of the micelles can enhance the solubility of hydrophobic agents and save the entrapped drug. One of the important problems of combined therapy is determining how to control the release profiles of different drugs, and, to date, simple DDSs have been unable to solve this problem. Therefore, advancement is necessary in the development of dual-drug delivery systems (DDDSs) that can manage the release profiles of various drugs. To date, very few studies of DDDSs have been published [18].

To the best of our knowledge, this study is the first to report utilizing a DDDS to enhance the release profile of silymarin from both micelles and hydrogels. The data presented in this paper serve as a benchmark for other researchers in their development of additional studies of drug delivery systems.

2. Materials and Methods

2.1. Materials

In this experimental study, HPLC-grade methanol and water were purchased from Merck. HPLC-grade trifluoroacetic acid and high-purity silymarin were purchased from

Sigma Aldrich (St. Louis, MO, USA). Puramatrix peptide hydrogel was purchased from BD Biosciences (San Jose, CA, USA). The silymarin-containing micelle was kindly provided by Dr. Mohammad Jafari, at the Mashhad University of Medical Sciences.

2.2. Apparatus

A Knauer K2600A liquid chromatography (Berlin,Germany) equipped with a Eurospher-100 C18 column (150 × 4.6 mm I.D, 5 μm) was used for silymarin analysis.

2.3. Chromatographic Conditions

HPLC conditions were as follows: mobile phase, methanol:water:0.1% TFA (50:50, v/v) with isocratic elution at room temperature (20–24°C); detection wavelength, 288 nm; flow rate, 1 ml/min; injection volume, 20 μl; and run time, 15 min.

2.4. Standard Solutions Preparation

Quantitative measurement of silymarin was performed using the external standard method. Ten milligrams of pure silymarin powder was dissolved and serially diluted in methanol to achieve nine different concentrations including 1000, 500, 250, 125, 62.5, 31.25, 15.6, 7.8 and 3.9 μg/mL.

2.5. In Vitro Release Study

Micellar silymarin was loaded into the hydrogel to form a complex, and its release profile over the next 21 days was evaluated. Specifically, 50-μlof silymarin micelles were dissolved in 1000 μl of PBS (pH 7.4). Then, 200 μl of this solution was added to a microtube containing 50 μl hydrogel, while another 200 μl was added to a microtube containing 50 μl PBS. After brief vortexing, the microtubes were incubated at 37°C for1 hour. Every day, an aliquot of supernatant was withdrawn from each tube and an equal volume of PBS was added back. Finally, 20 μl of collected supernatant was injected into the RP-HPLC, and silymarin content was calculated using the following equation: y=7929x-42845, where y is the peak area, 7929 is the slope of the curve, and 42845 is the y-intercept.Statistical analysis was performed by SPSS (version 16). Data presented as median and interquartile range (IQR) by using Wilcoxon test.

3. Results and Discussion

The data presented in this article were obtained from an experimental study. They show the *in vitro* release profile of two forms of silymarin, micellar silymarin and micelle–hydrogel–bounded silymarin.

Fig.1 shows the chromatogram obtained from the extract with RP-C18. As can be seen, the RP-C18 column allowed good separation of the components of standard silymarin**.**

The calibration curve was plotted based on the areas of the silybin B peaks against the concentration of silymarin solutions, and a regression line was obtained for the calibration (correlation coefficient=0.999)

The calibration curve using the peak-areas of the silymarin at different concentrations is given in Fig. 2.

Concentrations of silymarin in the samples were calculated using the standard calibration

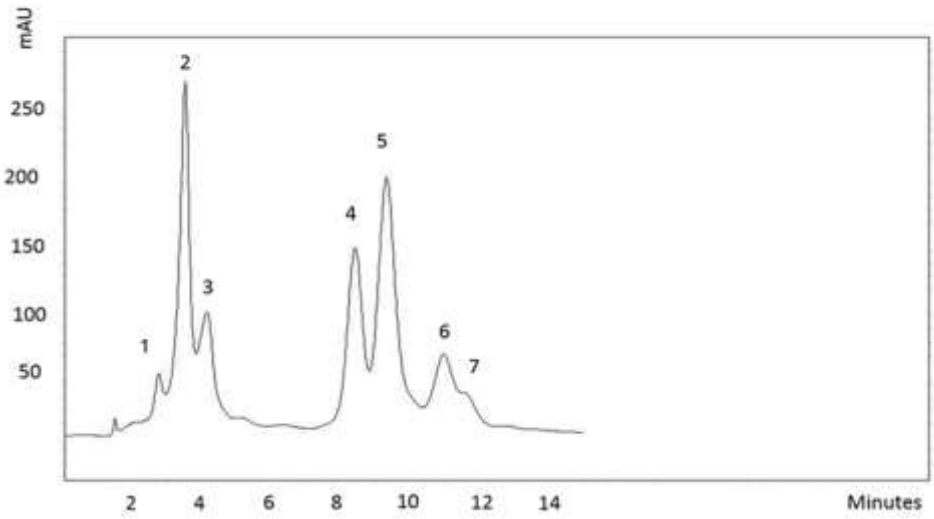

Figure 1. Chromatogram obtained from the extract with a RP-C18 stationary phase. Mobile phase: as described in material and method section.

1: toxifolin, 2: silychristin, 3: silydianin, 4: silybin A, 5: silybin B, 6: isosilybin A, 7: iso-silybin B

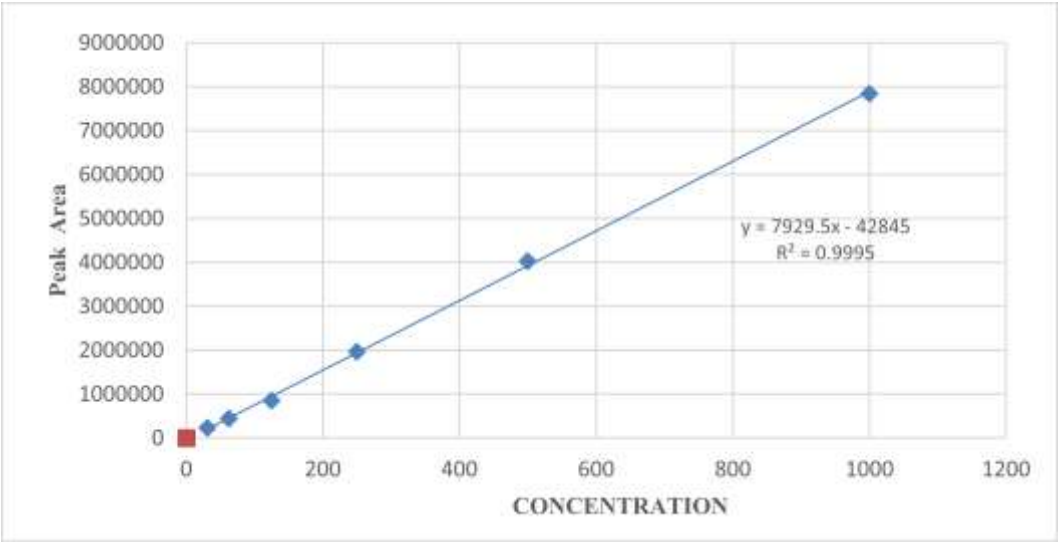

Figure 2. The calibration curve was plotted using the peak-areas of solutions at different concentrations of silymarin.

curve. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated by using the standard deviation of the calibration curve intercept (SD) and its slope (M). The LOD is equal to (3* SD⁄ M), where SD is the value of the calculated intercept, and the LOQ is equal to (10* SD⁄ M). The LOD and LOQ were 16.5 and 55.02 µg/mL, respectively

The in vitro release profiles of the two compounds are shown in Fig.3. A fast release

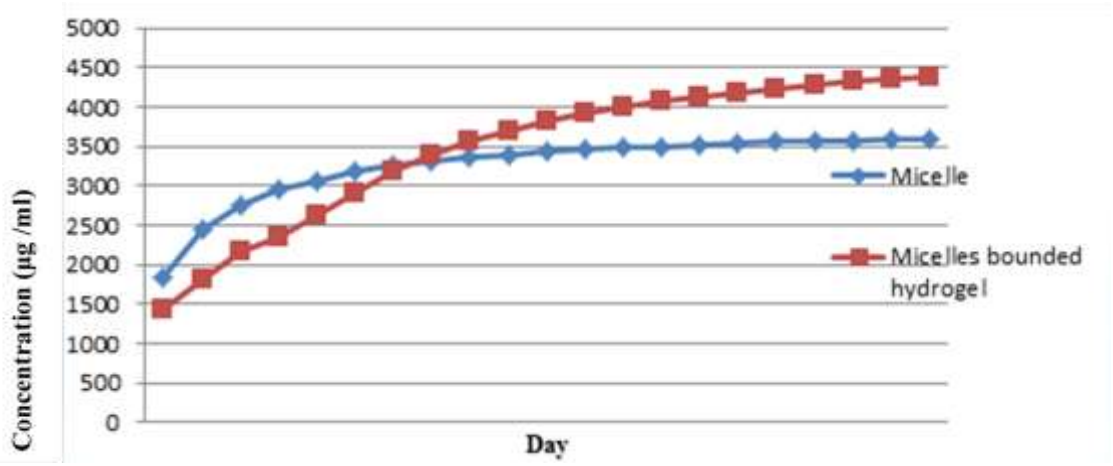

Figure 3. The in vitro cumulative release profiles of silymarin from free micelles and hydrogel bounded micellar form.

of silymarin was seen primarily, especially in the absence of hydrogel. In the following days, the release amount of silymarin in both methods decreased steadily, with some fluctuations, especially for free micelle forms. The cumulative release graph shows that the silymarin release kinetic from the hydrogel-bounded form is more constant than from the free micelle form and probably will be sustained for a longer duration (P value= 0.01).

In this study, we have presented an example of a dual-drug delivery system based on hydrogel/micelle composites. As a result, the hydrogel/micelle structure is shown to be a proper candidate as a dual-drug delivery vehicle with controlled release profiles for any water-insoluble drug [18].

Silymarin is a water insoluble herbal remedy that has been widely used in pharmaceutical applications. With respect to challenges in measuring silymarin, a gradient RP-HPLC method was developed in this study, which can be used by other researchers for *in vitro* separation and quantification of silymarin. This method gave precise and accurate separation between Silybin A and B, the two major components in silymarin extracts. An advantage of this method is the use of a UV detector, which is accessible in almost all research laboratories. Another advantage is a short testing cycle for rapid, accurate results [21].

Ding et al. determined the silymarin constituents by RP-HPLC. They used a Shim-pack VP-ODS column (150×4.6 mm i.d. 5m) and chromatographic condition was; 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 [22].

Graf and et al reported the first UHPLC MS-MS method for quantitative analysis of these compounds. The advantage of the method was the excellent resolution attainable to give a complete analysis in less than seven minutes. The method validation could be performed by using both UV and MS detectors so, it is appropriate for different types of analytical instruments [23].

In another report, separation, isolation, and structural characterization of the constituents of the *Silybum marianum* were performed using a RP-HPLC method. Moreover, 2D NMR and CD spectroscopy were applied for the confirmation of structures, including absolute stereochemistries [24].

Silymarin efficacy is restricted by its poor aqueous solubility and oral bioavailability, as well as poor intestinal permeability associated with extraction by enterohepatic circulation and fast excretion. To overcome these limitations, applying the micellar silymarin complex is expected to be a promising and effective strategy for systemic delivery [25].

The cumulative release graph shows that silymarin release kinetics of bounded hydrogels is more constant than the free micellar form and will likely sustain for longer durations. It means that the hydrogel plays a crucial role in the constant release of silymarin, while its safety and biodegradability have been proven in several studies [26-29].

In another study, the release profiles of insulin from a Puramatrix hydrogel were investigated. The results indicated a linear correlation between higher concentrations of Puramatrix hydrogel and increased release profiles of insulin [25].

4. Conclusion

In conclusion, the *in vitro* release patterns of silymarin micelles from the Puramatrix hydrogel proved that the Puramatrix hydrogel enhances the bioavailability of silymarin. Furthermore, The RP-HPLC method presented in this study can be used by other researchers to overcome the difficulties associated with the *in vitro* separation and quantification of silymarin.

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