

## Validated HPLC Method for Cynarin Determination in Biological Samples

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**SUMMARY.** A simple and sensitive method for the determination of cynarin is described and partially validated. The assay was used to determine the pharmacokinetic profile of cynarin after Wistar rat intravenous administration. Cynarin was isolated from rat plasma by solid-phase extraction. Separations were performed by reversed-phase high-performance liquid chromatography with ultraviolet detection.

**RESUMEN.** “Validación de un Método para la Valoración por HPLC de Cinarina en Muestras Biológicas”. En el presente trabajo se describe y valida parcialmente un método simple y con una adecuada sensibilidad para la determinación de cinarina en plasma. Este método fue utilizado para determinar el perfil farmacocinético de la cinarina luego de la administración endovenosa a ratas wistar. La cinarina fue aislada del plasma de rata por extracción en fase sólida. Las muestras obtenidas se analizaron por HPLC en fase reversa con detección ultravioleta.

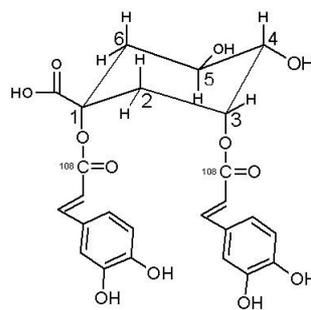
### INTRODUCTION

The artichoke (*Cynara scolymus* L.) originally comes from the mediterranean region of Europe, and is also cultivated around the world. The flower is used worldwide with nutritious purposes and the leaves, with medicinal purposes, broadly used in phytoterapic preparations with special indication in hepatic affections.

The artichoke leaves are characterized by the composition and high content in bitter phenolic acids, whose choleric, hypocholesteremic and hepatoprotector activities are attributed.

In France, Germany, Italy and Hungary the legislation allows the use of several plant derived medications such as leaves in form of hidroalcoholic extracts, alone or in association with other herbs, for therapeutic indications: choleric, cholagogue and to promote the elimination of water for the kidneys.

At least some of these effects are due to the antioxidant potential of artichoke extracts and/or their phenolic constituents (around 2%), such as caffeic acid, chlorogenic acid and cynarin, flavonoids (0.1 to 1%) <sup>1</sup> and essential oils.



cynarina

**Figure 1.**  
Structure of  
cynarin.

The percentage of phenolic acids in the vegetable extract varies considerably with the recolection time, form of drying leaves and the extractive process used. Pharmacological studies demonstrated that extracts of artichoke (*Cynara scolymus*, Asteraceae) and its principal active principle, cynarin (1,3-dicaffeoylquinic acid, C<sub>25</sub>H<sub>24</sub>O<sub>11</sub>, Fig. 1), possess choleric <sup>2,3</sup> and hypocholesteremic activities. Extracts of these plant also protect hepatocytes treated with carbon tetrachloride from hepatic cellular necrosis

**KEY WORDS:** Artichoke leaf extracts, Biological samples, HPLC.

**PALABRAS CLAVE:** CLAR, Extractos de hoja de alcaucil, Muestras biológicas.

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(activity related to the power antioxidant effect of the phenolic acids) <sup>4,5</sup>. Pharmacological investigations and clinical reports recently published <sup>6</sup> showed the efficacy and safety of artichoke extracts in the treatment of hepato-biliary disfunctions and abdominal pain.

In order to define the pharmacokinetic profile of cynarin, a sensitive and selective analytical method was needed, which allowed for the determination of cynarin in biological samples in the nanogram range. Moreover, the need for an efficient samples clean-up and chromatographic separation should not be underestimated.

In the present study, a method for determination of cynarin in plasma of Wistar rats was developed. The procedure, based on the use of solid-phase extraction and reversed-phase liquid chromatography with ultraviolet detection, is simple and rapid and provides accurate and precise results.

## MATERIAL AND METHODS

### **Chemicals and standards**

Methanol, acetic acid and phosphoric acid (Merck, Argentina) were used. Alumina and Tris/HCL were from Sigma (USA). Water employed to prepare stock and working solution was of ultrapure quality (Milliq). Stock solution 0.1 mg/ml of cynarin was prepared in Methanol: water (1:1,v/v). Working solutions of cynarin were made by dilution with the same solvent and used to prepare aqueous standards and spiked plasma samples on standards curves.

### **Chromatography**

The chromatographic system comprised a Varian 9012 ternary unit (USA) with a 20 µl loop injection valve (Rheodyne USA) and a variable-wavelength ultraviolet Varian 9050 detector (USA) set at 316 nm. A Ib-Sil ODS (250 mm x 4.6 mm x 5 µm dp) reversed-phase column (Phenomenex USA) was used. The chromatographic procedure was performed at room temperature (18-25 °C). The column was eluted with water / methanol / acetic acid (78.5:20:2.5, v/v/v). The mobile phase was delivered with a flow rate of 1.3 ml/min. Data were processed by means of Varian star 5.5 software from Varian (USA).

### **Extraction**

In the present method, biological samples were purified by solid-phase extraction. A 70 µl aliquot of rat plasma was mixed with 30 µl

Tris/HCL buffer (pH 8.75; 2M) and 20mg alumina, shaken 5 min and centrifuged (10000 g, 5 min). The precipitate was washed once with water (1 ml). Cynarin was eluted with 70 µl phosphoric acid (0.4 M), shaken and centrifuged (10000 g, 5 min). Aliquots of 20 µl were injected into the chromatographic apparatus.

### **Calibration curve and method validation**

Cynarin was injected into the column and identified by retention time. Calibration curves were obtained by plotting the peak area versus the theoretical concentration of analyte added to drug-free rat plasma. Curves correspond to 2 replicate measurements of six concentrations of cynarin, over a range of 0.038 -1.25 µg /ml. Data was subjected to least-squares regression analysis.

Precision of the bioanalytical method was evaluated on the basis of the coefficients of variation occurring intra-day assay. The accuracy was expressed in terms of Bias % = (Measured concentration - Nominal concentration) /Nominal concentration X100.

Specificity of the analytical method was assessed by comparison of chromatograms for the presence of interfering peaks and changes in retention time. Chromatograms of extracted plasma samples, derived from treated and non treated animals were examined.

### **Extraction efficiency**

The mean recovery of cynarin from spiked plasma rat samples was evaluated to test the efficiency and reproducibility of the extraction procedure. The responses of these standards taken by means of the extraction procedures were compared with those from standard solutions at the same concentration injected directly into the liquid chromatographic apparatus. The peak-area were compared to standard aqueous samples without extraction responses.

### **Animal treatments**

Male Wistar (280-300 g) rats were housed three per cage in stable conditions of humidity (60 ± 5 %) and temperature (22 ± 2 °C) according to local regulations (SENASA), and allowed free access to food and water. The animals were, maintained on 12-h light, 12-h dark cycle.

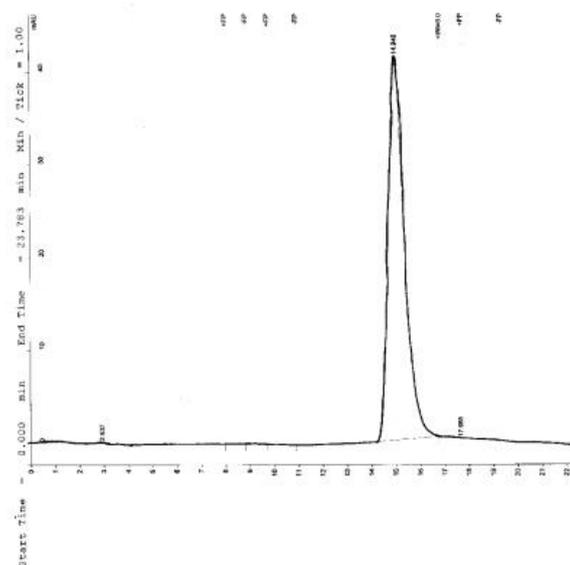
Cynarin was dissolved in saline solution and administered intravenously (0.17 mg/kg ) to 4 male rats. Blood samples were taken from animals by indwelling catheter T-4 at 5, 10, 20 and 30 min, after iv administration of the drug. Can-

nula was filled with sterile heparinized (20 UI/ml) saline solution. Rats were previously anesthetised with chloral hydrate (150 mg/Kg ip). Blood cells were removed by centrifugation and the separated plasma was stored at -20 °C until used. In pharmacokinetic study data from plasma levels were analysed with Topfit 2.0 software with a non compartmental method.

**RESULTS AND DISCUSSION**

**Detection and sensitivity**

Figure 2 shows the retention time of the tested compound. The drug were detected at 316 nm and the retention time was 15.0 ± 1.0 min. The limit of detection (LOD), with a signal-to noise ratio of 3, was taken as 0.02 µg/ml in plasma. The lower limit of quantification (LOQ) was taken as 0.038 µg/ml for plasma in calibration standard curves, with cv% and Bias % lower than 5%.



**Figure 2.** Representative chromatogram of cynarin in the analytical procedures; the relative retention time is 15 min.

**Linearity**

A linear response was observed in plasma samples over the examined concentration range (0.04 µg/ml - 1.25 µg/ml). Mean regression coefficient was 0.9961 and its mathematical expression was:  $y = 8117.3x - 81.476$  concentration = f(areas).

**Precision, accuracy and extraction efficiency**

Table 1 reports the Cynarin precision and accuracy of intra assay plasma measurements (n=3-5).

Plasma			
Nominal concentration (µg/ml)	Measured concentration (µg/ml)	CV%	Bias%
0.039	0.04	4.80	2.56
0.078	0.09	5.42	15.38
0.625	0.65	2.21	4.00
1.25	1.33	1.08	6.40
2.5	2.41	3.21	-3.60

**Table 1.** Precision and accuracy measurements of Cynarin in plasma. CV%: coefficient of variation, RE%: relative error: Bias% (Measured concentration - Nominal concentration)/ Nominal concentration X 100.

Table 2 reports the extraction efficiency, with alumina, expressed as mean recovery of Cynarin from plasma samples. The recovery in plasma ranged from 63% to 76%. The calculated extraction efficiency in plasma samples was independent from the concentration.

Plasma added concentration (µg/ml)	Measured concentration (µg/ml)	Recovery %
0.039	0.024	63.5
0.078	0.059	75.6
0.250	0.183	73.2
0.500	0.355	71.0
1.000	0.710	71.0
1.250	0.930	74.4

**Table 2.** Recovery measurement of Cynarin from plasma.

**Application to a pharmacokinetic study**

The present method has been applied to the pharmacokinetic study of Cynarin after iv administration to Wistar rats. Fig. 3A shows the chromatogram from drug-free rat plasma, where it is evident that no interfering peaks are present at the retention time of Cynarin. Fig 3B shows the chromatogram from rat plasma sample obtained after 5 minutes of iv administration of Cynarin.

Figure 4 shows the time profile of plasma concentrations after iv administration of Cynarin. Experimental results were analyzed with the TOPFIT non compartmental analysis, as shown in Table 3.

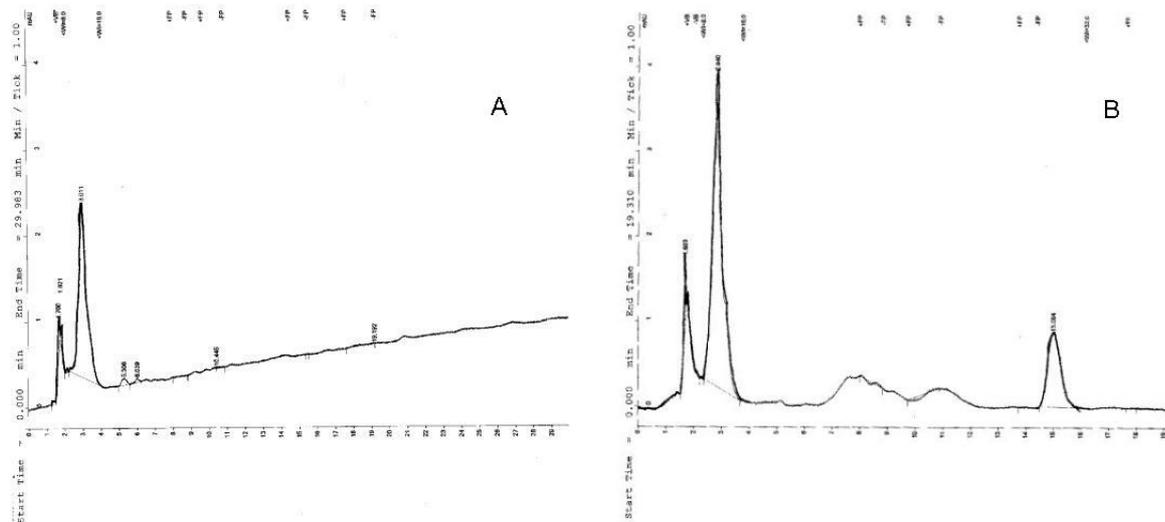
**CONCLUSIONS**

The method here described resulted sensitive and specific for the quali-quantitative determination of active Artichoke extract component (cynarin) in rat plasma.

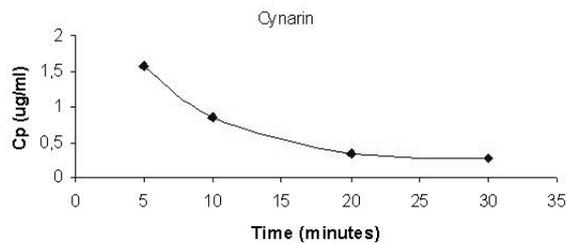
The extraction procedures demonstrated an acceptable recovery. This work represents a partially validated method and seems to be ade-

	$\lambda z$ (h <sup>-1</sup> )	T1/2 z (h)	Vd z (ml)	Cl (ml/min)	MRT(h)
1	5.78	0.12	34.10	3.29	0.17
2	2.35	0.29	24.20	0.95	0.32
3	2.66	0.26	30.30	1.34	0.31
4	4.98	0.14	30.70	2.55	0.16
X ± $\sigma$ -1	3.94 ± 1.69	0.20 ± 0.09	29.82 ± 4.12	2.03 ± 1.08	0.24 ± 0.09

**Table 3.** TOPFIT non compartmental analysis: Plasma. Kinetics values obtained in rat sample.  $\lambda z$ : terminal rate constant, T1/2 z: half life, Vd z: apparent volume of distribution, Cl: total clearance, MRT: mean residence time.



**Figure 3.** A) Chromatogram of drug-free rat plasma; B) Chromatogram from a rat plasma sample with Cynarin.



**Figure 4.** The ordinate indicates the plasma level and abscissa represents time after the administration of the drug. (n= 4).

quate for the analysis of biological samples allowing the pharmacokinetic study of Cynarin in Wistar rat plasma.

The preliminary pharmacokinetics values indicated a low elimination half-life and a distribution in the extracellular fluid. The clearance of 2 ml/min in animals ranging from 280 to 300 g represents a clearance of 7 ml/ min.Kg. This high depuration indicated a possible important liver metabolism and perhaps a high first-pass metabolism with low oral bioavailability. The Vd of 30 ml is equivalent to 0.1 l/Kg indicating that the drug reach the circulatory system and extracellular fluid (7,8).

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