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Original article

An improved HPLC method for determination of colocynthin in colocynth

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Abstract

Background and objectives: Colocynthin is the major active secondary metabolite of colocynth, *Citrullus colocynthis* (L.) Schrad, which has been used in traditional and ethno medicine of many countries. It could be considered as an active marker for quality control of colocynth and its herbal products. Analysis and standardization of colocynth and its herbal preparations are a critical issue for their safe applications in phytotherapy and traditional medicine. In the present work, a simple and efficient sample preparation was developed and optimized through combination of matrix solid phase dispersion and ultrasonic assisted extraction. In addition, analytical reversed-phase HPLC method was optimized for analyzing the concentration of colocynthin in colocynth pulp. **Methods:** Powdered colocynth pulp was grinded with diatomaceous earth to obtain a homogenous mixture. The blend was mixed with methanol and extracted by sonication, followed by centrifugation and filtration. The analytical chromatographic separation was carried out using Luna C_{18} in isocratic elution with methanol: isopropanol: water: triflouroacetic acid (30:10:60:0.1 v/v). The method was validated as well. **Results:** The validation parameters were determines as follows, linear range (r^2 = 0.999, 75-500 µg/mL), precision (intra-day < 2.7%, inter-day = 4.4%) and accuracy measured via determination of recovery (90-107%). The limit of detection and quantization were calculated 8.5 and 25.7 µg/mL, respectively. **Conclusion:** Regarding the relatively high content of colocynthin in colocynth pulp, the validated HPLC method could be applied for quality control of colocynth pulp used in Traditional Persian Medicine.

Keywords*: Citrullus colocynthis* (L.) Schrad, Colocynthin, HPLC, matrix solid phase dispersion, ultrasonic assisted extraction

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Introduction

Colocynth, *Citrullus colocynthis* (L.) Schrad. (Cucurbitaceae) is a perennial plant which resembles the common watermelon. The fruits, leaves and roots of this plant have been used in traditional and ethno medicines of many countries. The use of colocynth as a drug has been documented in ancient times and religious books [1].

In Traditional Persian Medicine, colocynth has been used for variety of applications including migraine, tremor, paralysis, epilepsy, loss of memory, cramps, worms, edema, vitiligo, pain, sciatica, amenorrhea, abortion, etc. There are several traditional herbal formulations and dosage forms of colocynth such as oral, nasal, otic, ophthalmic, topical, rectal and vaginal forms [2].

Similarly, colocynth is a well-known plant in modern medicine. The fruit pulp, seeds, aerial parts and roots have been subjected to extensive pharmacological studies. The authors have reported several effects such as anti-cancer, anti-diabetic, anti-hyperlipidemic, reversible anti-fertility, central analgestic activity, anti-inflammatory, anti-bacterial, anti-fungal, anti-venom and anti- allergic activities from the plant [3-18]. Unfortunately, *Citrullus colocynthis* has been described as a highly toxic plant. Prescriptions of the plant is banned by the United States food and drug administration in 1991 [19]. Recent clinical studies have shown effectiveness of colocynth extracts as anti-diabetic and anti-hyperlipidemic [20-22]. Clinical and *in vivo* studies have also shown that colocynth is safe in anti-diabetic therapeutic dose range [19,22]. For instance, Lorenz *et al*. have demonstrated the anti-diabetic therapeutic dose of colocynth extracts, standardized based on the amount of cucurbitacins, was 660-1220 fold less than median lethal dose (LD_{50}) [22]. These findings show standardization of colocynth plant and herbal products is essential for safe and effective use in traditional and contemporary medicine.

Among the phytochemical constituents of colocynth such as flavonoids [23-25], phenolics, alkaloids [25-27] etc. cucurbitacins have been studied more than others [7,23-25]. Many of the pharmacological and toxicological properties of colocynth such as anticancer and the powerful cathartic action were attributed to these triterpene saponins [12,28].

Colocynthin, cucurbitacin E-2-*O*-glucoside (figure 1), is the main active principle present in the pulp of *Citrullus colocynthis* that exhibits cathartic, antihistaminic, anticholinergic, negative chronotropic and negative inotropic activities [7,27,29]. The considerable amount (more than 3% plant tissue) and pharmacological activity of colocynthin make it suitable as a marker for quality control and standardization of colocynth herbal preparations [30].

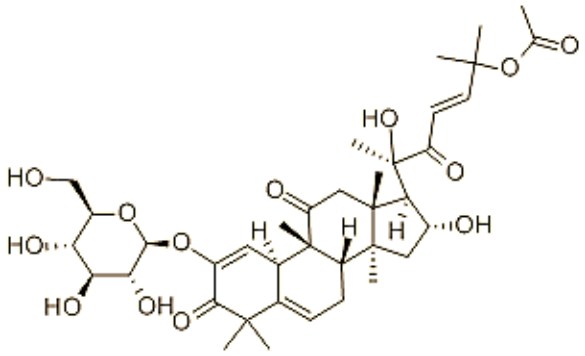

Figure1. Chemical structure of colocynthin

Reverse phase high-performance liquid chromatography (RP-HPLC) offers considerable promise for colocynth analysis due to the polar nature of the highly functionalized marker. Despite of the promising situation, few studies have been carried out about analysis of cucurbitcine glycosides such as colocynthin. Bauer and Wagner have reported C_{18}-based HPLC methods for analyzing cucurbitacin B, E, I, and L glucosides in plant extracts including *Citrullus colocynthis* [31]. Halaweish and Tallamy have reported a HPLC method for cucurbitacin D and I glucosides from *Cucurbita texana* [32]. The complexity of sample

preparation, gradient elution mode, low recovery (63.85%) and use of acetonitrile which is an expensive co-solvent compared to methanol are drawbacks of these methods.

To improve the previous works, Matsuo *et al*. reported a quantitative method to quantify colocynthin in *C. lanatus* juice and solid residues of the fruits [33]. Although this method addressed some drawbacks by using isocratic elution and simpler sample preparation, the extraction efficiency of samples were not appropriate. In addition, selection of internal standard with no structure similarity and peak overlap leaded to inconsistent results.

In the present study, a new, simple and reliable sample preparation; coupled with improved HPLC method were developed and validated with respect to validation parameters such as selectivity, linearity, precision, recovery, limit of detection (LOD) and limit of quantization (LOQ).

Experimental

Plant material

Dried fruits of *Citrullus colocynthis* (L.) Schrad, were obtained from local market in Kerman, Iran and their identity was confirmed by a botanist. The fruit pulps were separated from seeds and shells, milled and sieved by 1 mm sieve.

Chemicals

Diatomaceous earth, Celite 545, was purchased from Merck KGaA Co. (Darmstadt, Germany). Isopropanol HPLC grade was obtained from LGC Standard (Wesel, Germany) and methanol from J.T Baker (Deventer, Netherland). The colocynthin working standard used for determination of the corresponding peak in test samples was isolated from *Citrullus colocynthis* fruit pulps by preparative HPLC using method proposed by Sachdev-Gupta *et al.* with some modifications [34,35]. The structure of purified colocynthin was confirmed by comparing the ^{1}H-NMR, ^{13}C-NMR, mass and UV spectral data with those reported [35-37]. The purified colocynthin was standardized by using reference standard of colocynthin purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany).

Instrumentation

Preparative HPLC purification was performed using Knauer LC system equipped with a vacuum degasser, quaternary solvent mixer and K-2600 UV detector. The preparative column Merck Lichrocart 100 RP-18 end-capped (10 × 250 mm, 10µm) was used for colocynthin separation. The analytical HPLC system consisted of an Agilent 1100 series HPLC system consisted of G1312A binary pump, G1387A WPALS auto-sampler and G1314A UV detector (Agilent Technologies, Waldbronn, Germany). The Chemstation for LC 3D (Rev. A.10.01 [1635]) was used for data acquisition. The column was a reversed phase (Luna C_{18} 250 × 4.6 mm, 5 µm) column (Phenomenex Inc., Aschaffenburg, Germany) equipped with C_{18} security guard cartridge (Luna 4 × 3.0 mm). HPLC parameters were as follows: pump mode, isocratic; solvent system, 30:10:60:0.1 (v/v) methanol: isopropanol: water: triflouroacetic acid; solvent flow rate, 1.0 mL/min; detection wavelength, 237 nm; sample injection volume, 5 µL; run time, 20.0 min. The resulting peak areas were used to calculate the amount of analyte in sample. Each data point was based on the average of three replicate measurements.

Optimization of the extraction procedure

Solvent selection

Based on literature and sample preparation requirements in RP-HPLC, methanol and methanol/water (40:60 and 30:70 v/v) were used for extraction of the analyte.

Effect of extraction method

To determine the effect of the procedure on the extraction, soxhlet extraction, ultrasound assisted extraction and combination of ultrasound assisted extraction (USAE) with matrix solid phase dispersion (MSPD) were compared.

Sample preparation

Powdered colocynth pulp (100 mg) was placed in

a glass mortar and grinded with 100 mg diatomaceous earth to obtain a homogenous mixture. Then, 100 mg of the mixture was mixed with 2 mL of methanol, shacked slightly by vortex for 30 seconds and placed in the sonicator for 10 min. The solid material was separated by centrifugation in 8000 rpm for 10 min. A determined volume (150 µL) of clear solution was diluted with 850 µL ml of Millipore water, passed through 0.2 µm PTFE filter and analyzed by HPLC.

Precision

Six samples were analyzed on the same day and on three consecutive days; then relative standard deviations were calculated. Three injections were carried out for each sample.

Sample recovery

A determined amount of colocynth pulp (750 mg) was mixed and grinded with equal amount of diatomaceous earth (750 mg). Three replicate amounts of plant powder with diatomaceous earth (3×400 mg) were weighted and divided into four equal portions (4×100 mg pulp). One part was used as the real sample and others were spiked with colocynthin standard material (56.3, 112.6 and 168.9 µg). All samples were extracted and analyzed as described previously.

Results and Discussion

Methanol and methanol: water (40:60 and 30:70 v/v); which their compositions are compatible to mobile phase, were used as extraction solvents in preliminary method development. Despite of reduction of interferences and compatibility, these extraction solvents were not satisfactory precise in repeatability experiments (10.3-12.8% intraday and 11.3% interday), even with applying ultrasound waves.

After comparison between the different columns such as, C_8, C_{18} and NH_2, the best separation efficiency was obtained by using the C_{18} column. In order to achieve the optimum selectivity, different mobile phases were applied, starting from 2:1 (v/v) methanol: water applied by Matsuo *et al*. [34]. It was found that addition of triflouroacetic acid (0.1%) and isopropanol increased the peak symmetry and resolution of the colocynthin peak (RT=13.791 min). Each cucurbitacin peak was resolved from the neighboring peaks with good peak symmetry and separation efficiency (figure 2).

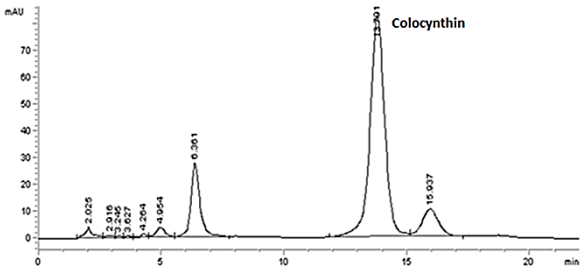

Figure 2. HPLC Chromatogram of fruit pulp extract of *Citrullus colocynthis* (L.) Schrad

Linearity was obtained in concentration 25-1000 µg/mL of colocynthin with equation y=5.21x+43.57, R^2=0.999. The overall limit of detection (LOD) and limit of quantization (LOQ) were 8.5 and 25.7 µg/mL, respectively.

Figure 3 represents the residual plot for different concentration response of colocynthin standard solutions. Based on the plot, there was a slight negative curvature in residual plot. In order to find the dosing range, the ratio of response/effect was drawn against the concentration (figure 4). The data presented in the figure indicates that applicable linear range for colocynthin calibration curve was between 75-500 µg/mL (± 0.05 mean response/factor).

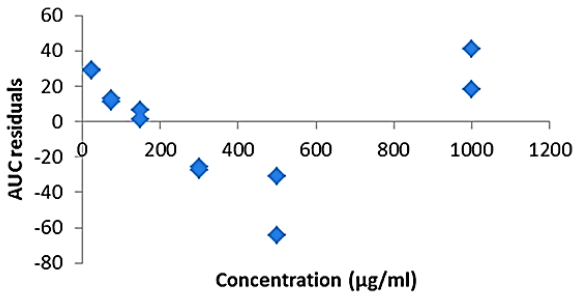

Figure 3. Residual plot of calibration curve of colocynthin

Table 1 represents the results of repeatability experiments for combination of matrix solid dispersion and ultrasound assisted extraction. The relative standard deviations (RSDs) for intraday experiments were between 2.4% and 2.7%; which were significantly lower than the preliminary study (10.3% - 12.8%). Interday precision had also decreased to 4.4%, compared to 11.3% of ultrasound assisted extraction.

The results of sample preparation recovery are shown in table 2. The extraction recoveries were calculated between 90.03% and 107.70% that was considerably higher than the reported sample preparation method (63.85%) [32].

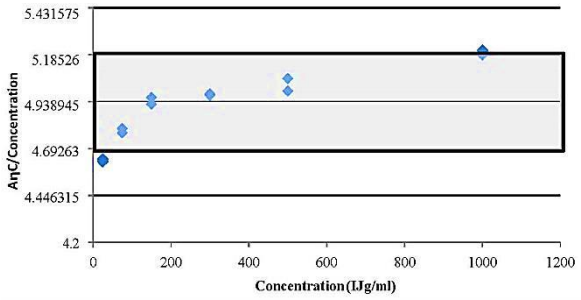

Figure 4. Response/Factor plot and dosing range

The extraction steps could be the most time consuming activity of the development process but is invaluable to the robustness of the method. Colocynthin is soluble in solvents such as methanol, ethanol, chloroform, ethyl acetate and acetone. Chloroform and methanol have been used to extract cucurbitacins and their glycosides form plant tissues [31-33]. Although chloroform extracts provided better samples in terms of presence of interferences, the extraction recovery was considerably lower than methanol [31,32].

Ultrasound assisted extraction (USAE) refers to application of ultrasonic waves (more than 16 KHz) for extraction. In this type of extraction, ultrasonic vibration exerts a mechanical effect, allowing greater penetration of solvent into the sample matrix and increasing the contact surface area between the solid and the liquid phase. Ultrasonic extraction is not dependent to the matrix and is relatively fast, and inexpensive. However, USAE have some drawbacks such as inability to change the extraction solvent, filtration requirement, inefficient extraction and decomposition of compounds in some cases [38]. Although reasonable extraction efficiencies were obtained using methanol and methanol-water solvents, the precision results were not optimal (more than 10%) even with ultrasound application. This phenomenon could be attributed to the particle size and swelling of the plant tissue by water, forming clumps which reduces solvent accessibility. Matrix solid phase dispersion is particularly suitable for obtaining a homogenous matrix and cellular disruption of plants. Indeed, the sample blending with an abrasive solid support allowed the mechanical disruption of the matrix structure, as confirmed by scanning electron microscopy [39,40]. In addition, the importance of mechanical forces (homogenization and vortex) for constant and steady extraction recovery was emphasized [32]. Therefore, a combination of MSPD and USAE was applied to ensure clean samples with consistent precision and higher recoveries.

The non-adsorptive support, diatomaceous earth, was selected for disruption and homogenization of matrix while minimizing adsorption of the analyte that could lead to a decrease in extraction efficiency. The colocynth cucurbitacins (E, I, B, L) have similar structures with differences in acetyl group and unsaturation positions. Therefore, separation of cucurbitacins, particularly cucurbitacin E and B glucosides was a challenge for method development. The isocratic elution mode of the instrument was optimized in terms of resolution and running time. Acidic pH played an important role in peak sharpness. Addition of isopropanol (10% v/v) also improved peak shape and separation of colocynthin and cucurbitacin B glucoside. The mechanism of this effect is not clear. Increasing solubility of the compounds and change in hydrogen-bond interactions of sugar moiety are proposed mechanisms that suggest further studies. The method validation procedure has

Table 1. Repeatability of colocynthin analysis in pulp of *Citrullus colocynthis*

Day	Colocynthin concentration (µg/ml)						Mean ± SD	RSD% intraday	RSD% interday
	A1	A2	A3	A4	A5	A6			
1	183.2	170.7	177.6	181.0	181.8	176.2	178.4 ± 4.6	2.6	4.4
2	169.8	176.7	174.9	180.9	180.6	180.0	177.2 ± 4.3	2.4	
3	167.1	169.8	167.2	163.7	163.9	156.8	164.8 ± 4.5	2.7	

Table 2. Extraction recoveries of colocynthin from colocynth

Added (µg)	Found (µg/50 mg)	Recovery %	Mean recovery ± SD
0	1174.7	-	-
	1184.7	-	-
	1174.0	-	-
56.3	1234.7	100.6	107.7 ± 4.3
	1238.2	108.1	
	1238.7	109.1	
112.6	1281.1	92.1	90.03 ± 2.04
	1278.0	89.4	
	1277.1	88.6	
168.9	1329.0	89.93	90.08 ± 0.29
	1329.0	89.93	
	1330.1	90.39	

been done based on our previous works on validation [41,42] and ICH protocol [43].The results obtained from the cucurbitacin method validation according to linearity, selectivity, accuracy, and precision showed that the proposed method was suitable for the analysis of colocynthin.

The selectivity of the method was determined by comparing chromatograms from analysis of the plant samples with standard solution. Colocynthin peak was evaluated for peak purity and resolution from the nearest eluting peak [RT (retention time) = 15.937 min]. The difference in RT (> 2 min) between colocynthin and the adjacent peak (RT= 15.937 min) was satisfactory for quantification of colocynthin.

Due to considerable change in concentration of secondary metabolites in plant materials, the wide concentration range (25-1000 µg/mL) is advantageous for their quality control by minimizing dilution and concentrating steps that increase sources of systematic and random errors in an analytical procedure. Although there was a slight negative curvature in residuals, it did not significantly affect the linearity. This curvature could be attributed to the wide range of concentration which extended from 25 µg/mL, and was very close to LOQ (25.7 µg/mL), to maximal concentration that was used with UV detectors (1000 µg/mL) [44]. Although plotting the ratio of response/effect versus concentration restricted the dosing range to 75-500 µg/mL, it ensured the linearity of method and made it applicable for real samples.

The precision expresses the proximity of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under stipulated conditions. The precision could be divided into three levels: repeatability, intermediate precision and reproducibility; and is usually computed as standard deviation or relative standard deviation

(RSD). In environmental and food analysis, the precision is very much dependent on the sample matrix and on concentration. It can vary from 2% to more than 20% [45].

It can be seen from the data in table 1 that RSDs in one day were from 2.4 to 2.7%, that was acceptable for these samples. The same conclusion could be drawn for interday RSD (4.4%). Therefore, the proposed method is quietly applicable in terms of repeatability.

Selection of sample size and standard additions are significant features of recovery assessment. The range for the accuracy (recovery) limit should be within the linear range. Based on the usual amount of colocynthin in plant tissues (3%) the amount of sample (50 mg of pulp/sample) and dilution factors (176.7 µg/mL = 1177.8 µg/50 mg) were selected in a way to fulfill the linear range requirement of recovery assessments. The extraction recoveries were calculated based on the average of three replicate samples (table 3), considerably more than 63.85 % reported in a similar study [32] and located in the range of classical acceptable limit 15% for bioanalytical procedures [46].

A new sample preparation method based on matrix solid phase dispersion and ultrasound assisted extraction was developed and validated. The developed sample preparation showed consistent extraction recovery for sample preparation while avoiding complex extraction procedures and change of solvents. The improved extraction method contributed to more efficient sample preparation with improved precision compared to previous studies. The optimized HPLC mobile phase and isocratic elution mode improved the selectivity. However, more studies are suggested in terms of intermediate precision, reproducibility, robustness, ruggedness and stability of the marker during extraction process. Based on marker selection criteria for herbal products such as relatively high percentage and pharmacological activity [30], the proposed method could be applied for quality control of colocynth pulp used in traditional medicine.

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Declaration of interest:

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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List of abbreviations:

ESI: Electrospray Ionization
HPLC: High Performance Liquid Chromatography
ICH: International Conference on Harmonization
LC-MS: Liquid Chromatography-Mass Spectrometry
LD_{50}: Median Lethal Dose
LOD: Limit of Detection
LOQ: Limit of Quantization
MSPD: Matrix Solid Phase Dispersion
NMR: Nuclear Magnetic Resonance
PDA: Photo Diode Array
PTFE: Polytetrafluoroethylene
RP-HPLC: Reverse Phase High-Performance Liquid Chromatography
RSD: Relative Standard Deviation
RT: Retention Time
USAE: Ultrasound Assisted Extraction
UV: Ultraviolet-Visible