



Development of a validated HPLC method for the determination of sennoside A and B, two major constituents of *Cassia obovata* Coll.

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ABSTRACT

Introduction: *Cassia obovata* Coll is the only *Senna* species which grows wild in Iran. In the present study, an optimised reverse High Performance Liquid Chromatography (HPLC) validated method was established for quantification of sennosides A and B, the major constituents of *C. obovata* with a simple and accurate method.

Methods: HPLC analysis was done using Waters 515 pump on a Nova-Pak C18 (3.9 × 150 mm). Millennium software was used for the determination of the sennoside A and B in *Cassia* species and processing the information. The method was validated according to USP 32 requirements.

Results: The solvent impact on the selectivity factor and partition coefficient parameters evaluated. Using a conventional RP-18 L1 column, 3.9 × 150 mm, the mobile phase was selected after several trials with different mixtures of water and acetonitrile. Sennosides A and B were determined using the external standard calibration method. Using USP 35-NF 30, the LOD and LOQ were calculated. The reliability of the HPLC-method for analysis of sennoside A + B was validated through its linearity, reproducibility, repeatability, and recovery. Finally ethanol:water (1:1) extracts of *Cassia obovata* and *Cassia angustifolia* were standardized by assay of sennoside A and B through above HPLC validated method.

Conclusion: Through the above method, determination of sennosides in *Cassia* species are completely possible. Moreover, through comparing the results, even though sennosides are rich in *Cassia angustifolia* but, the results shows that *C. obovata* could be considered as an alternative source for sennosides A and B.

Implication for health policy/practice/research/medical education:

In the present study, and for the first time, we developed a validated High Performance Liquid Chromatography (HPLC) method for the determination of sennoside A and B, two major constituents of *Cassia obovata* Colladon. Through the above HPLC method, assay of sennosides in different *Cassia* species and *Cassia* adulterations as well as single or combined formulations containing *Cassia* extracts are completely possible.

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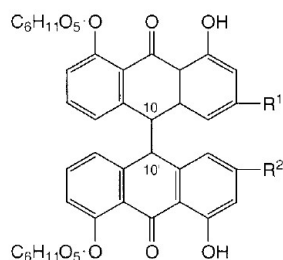
Introduction

Medicinal herbs are normally supplied in the shops without standardization. Herbalists sometimes unintentionally, supply closed morphological medicinal herbs instead of each other. So, control of herbal bulk drugs is necessary

to avoid adulterations or unwanted mistakes. *Cassia angustifolia* known as senna and *Cassia acutifolia* known as senna Mackie are widely applied in the Iranian traditional medicine to treat intestinal complications (1). One of the frequently reported adulterations of the *C. angustifolia* or

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C. acutifolia are with *C. obovata* due to their similarities. Dog senna or kaspind in Persian in the Iranian herbal Pharmacopeae is ascribed to the dried leaflets of *Cassia obovata* Coll., or its synonym name, *Cassia italica* (Mill.) Lam., containing at least 1.0-1.15% of anthraquinone derivatives (2,3). This plant is the only *Senna* species grows wild in the Hormozgan and Sistan Baluchestan provinces in the south east of Iran. It is also endemic to tropical Africa, the Middle East countries, and Pakistan (3). *C. obovata* is also cultivated in Italy from the sixteenth century (3). *Cassia Italica* is commercially available as a laxative drug in Mali and African countries (3). It is one of the plants of the Sudan used to treat intestinal complications, circulatory problems, urinary stones and sexually transmitted diseases (4). Previous phytochemical studies of *C. italica* have resulted in the isolation of β -sitosterol, stigmasterol, α -amyrin and 1,5-dihydroxy-3-methyl anthraquinone, physcion, chrysophanol and 10,10'-chrysophanol bianthrone (5-7) which could occur also as their 1,1'-diglucosides. Bianthrone are compounds with two identical or different anthrone molecules like sennidin A and B which are present in the *Cassia* species. They also could form by artifact from the converting of the monoanthrones enzymatically by drying between 20 and 50 °C (3). They have two chiral centres at C-10 and C-10', and for a compound having two identical anthrone moieties like sennidin A, two forms (the 10S,10'S and 10R,10'R) are possible together with the meso form sennidin B (Figure 1). Anthraquinone compounds suggested being responsible for the purgative effects of the plant. So, their contents should be quantified and used as an index for the valuation or even its detection in the bulk powder form. According to pharmacopeias guideline, before marketing the plant drugs, if the active constituents are known and quantifiable, the amount in which they are present in the herbal drug should be detected. In the case of lack of knowledge of active constituent(s), other tests like spectroscopic and/or chromatographic fingerprints and process validation especially for the drug substance are required for authentication and control (8).



	R ¹	R ²
Sennoside A	COOH	COOH
Sennoside B	COOH	COOH
Sennoside C	CH ₂ OH	COOH
Sennoside D	CH ₂ OH	COOH

Figure 1. Chemical structures of sennosides A-D in the *Cassia obovata* Coll

To the best of our knowledge, there is no report on the HPLC method development and determination of dianthrone like sennosides for the *Cassia obovata*. Thus, the purpose of this work was to develop a High Performance Liquid Chromatography (HPLC) validated method for determination of sennosides A and B of *Cassia obovata* with a simple, rapid and precise method.

Materials and Methods

Plant Material

The aerial parts of *Cassia obovata* Colladon (Caesalpiniaceae) were supplied from the Bandar Abbas, Iran and was identified by Dr. L. Ghaem-maghani, Department of biology, Faculty of Science, University of Isfahan. A herbarium specimen (No 1541) was deposited in the herbarium of the Faculty of Pharmacy, Isfahan University of Medical Sciences (Iran).

Instrumentation

HPLC analysis was done on the 515 HPLC pump, waters 2487 dual wavelength absorbance detector (Waters, Milford, MA, USA). The column was a Nova-Pak C18, 3.9 × 150 mm (Waters, Milford, MA, USA) and Millennium software was used for the determination of compounds and processing the data.

Chemicals

Methanol and acetonitrile HPLC-grade solvents were purchased from Caledon Company (Canada). Sennoside A and B, as analytical standards, were purchased from Sigma-Aldrich Corporation, USA.

Determination of Sennosides

Using high pressure liquid chromatography, sennosides were determined through external standard calibration method.

Solvent Effect

For evaluating the solvent impact on the selectivity factor and partition coefficient parameters which affecting on the extraction of sennosides from plant matrix, hot water and ethanol: water (50%) with different strength and selectivity's were selected.

Sample Preparation

In a volumetric flask, 1 g of the leaflets powder was weighed and 10 mL of the solvent were added to the sample powder. It was extracted, filtered, and washed three times with 10 milliliter of the solvent. Using the rotary evaporator under vacuum and at a 40 °C temperature, the filtrates were combined and evaporated till dryness. The rotary evaporated extract was washed 3 times with 2 ml HPLC-grade acetonitrile: water (1:1).

Preparation of the Standard Solutions

800 μ g analytical standards were diluted in 1 ml of HPLC-grade acetonitrile: water (1:1) to prepare the stock solution

of 800 µg/ml. Different standard solutions were made by serially diluting the stock solution to concentrations of 400, 200, 100, and 50 µg/ml.

Limit of Detection and Limit of Quantification

Limit of detection (LOD) and Limit of Quantification (LOQ) were counted on using equations, $LOD = 3 \times S/N$ and $LOQ = 10 \times S/N$. USP 35-NF 30 defines $S/N = 2h/h_n$, where h is the height of the peak corresponding to the interest component from the peak apex to the midpoint of the noise, and h_n is the difference between the largest and smallest background noise value observed over a distance equal to five times the width at the half-height of the peak of interest. The USP definition from S/N is useful in the gradient systems, in which a drift in the baseline is present. Differently, in the event of using signal to noise ratio, noise will be overestimated when drift is present (9).

Validation

The reliability of the HPLC-method for analysis of sennoside A + B was validated through its linearity, reproducibility, repeatability, and recovery (10).

The linearity means how well a calibration plot of response versus concentration approximates a straight line. It was performed by linear squares regression. Correlation coefficient (r^2) in addition to plot slope and intercept provided desired information on linearity. A linearity correlation coefficient greater than 0.999 for p -value < 0.1 or greater than 0.99 for p -value < 0.05 is thought to be acceptable (10).

Precision is the level of agreement among individual test results, when a series of determinations for the same analyte are done repeatedly. Repeatability and reproducibility could be measured as the parts of precision through intra-day, and inter-day assays followed by determination of coefficient of variance. CV is calculated utilizing the equation $CV, \% = (Stdev/X) \times 100$, where $Stdev$ is the standard deviation, and X is the mean of HPLC responses. In the in-vitro systems, with easy isolation, CV less than 3% is needed (10).

Intra-day Precision

Instrumental precision is evaluated through several times injection of the same sample within the selected range. Intra-day precision was found out by injecting two replications of different concentrations (800, 400, 200, 100, and 50 µg/ml), three times in the same day ($n = 6$). Peak area was measured and CV % was calculated (10).

Inter-day Precision

Inter-day precision was found by injecting of two replicates of different concentrations (800, 400, 200, 100, and 50 µg/

ml) on three sequential days in a week ($n = 6$). Peak area was measured and % CV was calculated (10).

Recovery

Recovery assures you that the real quantity of substances in the real sample has been evaluated. One method of determining the recovery is adding a certain amount of analytical standards to a blank matrix lacking standard. The samples are extracted as mentioned in the sample preparation method, and volumized to 6 ml. The added standard is determined through a validated HPLC method by three times and the recovery percentage is calculated in every case (10,11).

Statistical Analysis

Data analysis were performed using Waters Millennium Chromatography Software (Build 1154, Waters Corporation, Milford, USA). Data were reported as Mean \pm SD and the results were analyzed statistically by Excel 2007 software.

Results

The HPLC method carried out in this study was aimed to optimize the resolution, reduce runtime and the limit of quantification of sennosides in the crude plant material.

Optimizing the extraction method

Two extraction methods consisted of ultrasonic radiation (60 min), and maceration overnight was compared to check the effect of the procedure on the extraction (Table 1).

Method development

Using a conventional RP-18 column, 3.9×150 mm (Waters, Milford, MA, USA), the mobile phase was selected after several trials with different mixtures of water and acetonitrile. At the first using gradient method from 10% to 100% acetonitrile in water, the solvent composition which the sennosides are eluted was detected. Then shorter gradient times from 0% to 50% were selected to reduce run times and improve peaks. The water is nonabsorbing and 0-50 % ACN-water was a solvent mix with low UV absorbance and low background noise (<0.0005 at 280 nm). Finally, a gradient system of water with 0.1% phosphoric acid (A), and ACN:H₂O (50:50) with 0.1% phosphoric acid (B) was selected. It was started with A: B (100:0) for 2 min, then 0–30% B in 12 min, A: B (70:30) for 10 min, then 30→50% B in 20 min, and A: B (50:50) for 5 min following with equilibrating with A: B (100:0) for 10 min. The flow rate was 1 ml/min and

Table 1. The effect of solvents and extraction procedures for determination of sennosides in *Cassia obovata*

Sample	Extraction method		Solvent	
	Maceration overnight	Ultrasonication (60 min)	Ethanol: Water (1:1)	Hot Water (60 °C)
Sennoside A	0.28 \pm 0.011	0.19 \pm 0.008	0.28 \pm 0.011	0.064 \pm 0.0021
Sennoside B	0.26 \pm 0.010	0.18 \pm 0.007	0.26 \pm 0.010	0.069 \pm 0.0018

temperature was kept constant at 30 °C to eliminate the temperature variations. UV detector time constant was set to 0.1 and the detector sample rate to 10 pts/Sec in the single wavelength mode at 280 nm to increase the signal to noise ratio. Smaller volume of the sample was injected by reducing the loop to 5 µL to have sharp peaks with good resolution.

Determination of sennosides

Using high pressure liquid chromatography, sennosides A and B was determined using the external standard calibration method. The retention time for sennoside B and A was observed to be 19.07, and 28.35 min (Figures 2 and 3).

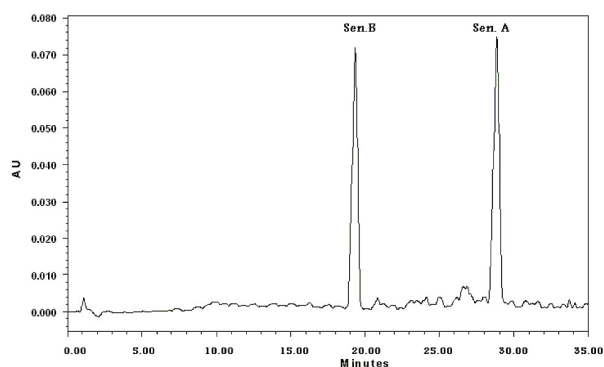


Figure 2. HPLC chromatogram of sennoside B and A (50 µg/mL) at 280 nm. The retention time for sennoside B and A was observed to be 19.07, and 28.35 min.

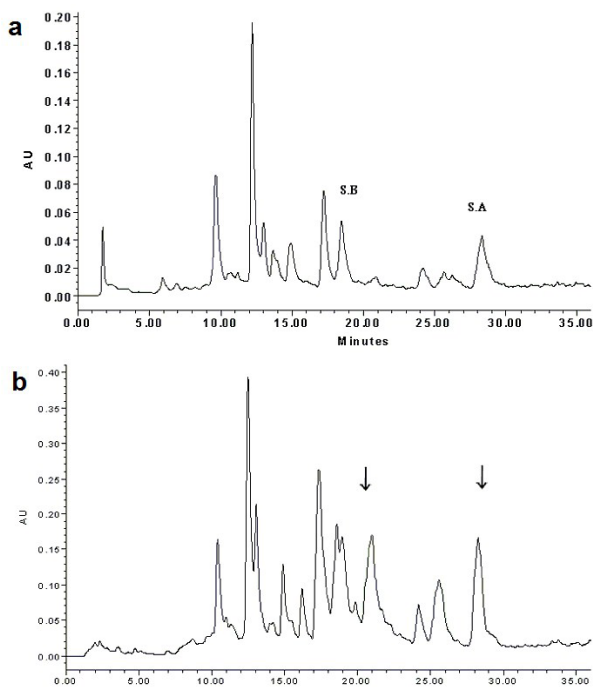


Figure 3. High performance liquid chromatography chromatograms of a) *Cassia obovata* and b) *Cassia angustifolia*. Chromatograms were carried out at 280 nm on a reversed-phase HPLC column (RP-18, 150 mm × 3.9 mm, 5 µm particle size, waters, US) using water with H₃PO₄ (0.2%) as solvent A and acetonitrile : water (50:50) as solvent B with gradient elution at 30 °C and flow rate of 1 ml.min⁻¹.

Signal, Noise, and Assay precision

Using USP 35-NF 30 definition of signal to noise ratio: $S/N = 2h/h_n$, the LOD and LOQ were calculated as 2.2 µg/mL and 7.5 µg/mL for sennoside B, and 3.75 µg/mL and 12.5 µg/mL for sennoside A, respectively (9,12). The LOD and LOQ values could be reduced more in the case of using isocratic mobile phase system, but the current gradient mobile phase system possessed better selectivity (Table 2). The instrumental precision by intra-day results and intermediate precision through inter-day results of repetitive standards quantitation in the different concentrations in the selected linear range, 50 to 800 µg/mL, as is presented in Table 3.

Recovery

The accuracy of the validated method describes the extent to which test results quantified by the method and the real amount. In this method, accuracy was evaluated as recovery test after spiking of 800 µg of sennoside B standard to 2 g of the *Myrtus communis* L. powder plant as blank sample matrix. After extraction as is mentioned in the sample preparation method it was volumized to 2 mL in each additional level. Three determinations were carried out and the recovery percentage was calculated in every case was found to be $83.69 \pm 3.28\%$.

Discussion

HPLC validated method was established for quantification of sennosides A and B, the major constituents of *C. obovata* with a simple and accurate method. The retention time for sennoside B and A was observed to be 19.07, and 28.35 min. By the aid of the Millennium software, the calibration curve was determined by linear regression in the range of 50 to 800 µg/mL. The regression equation was $y = 4351.x - 28210$ with the correlation co-factor (R^2) of 0.999 for sennoside A and $y = 3572.x + 10685$ with $R^2 = 0.998$ for sennoside B, where x is the concentration of standard in µg/mL (Figure 4).

Ethanol:water (1:1) extract obtained from 1 gram *Cassia obovata* dried powder diluted to 4 mL was standardized to contain 722.05 ± 24.30 , and 790.70 ± 29.35 µg/mL sennoside A and B. It is equivalent to % 0.29 of sennoside A and 0.32% of sennoside B in the dried plant. The extract obtained from *Cassia angustifolia* dried powder was standardized also to yield % 0.83 of sennoside A and 1.49% of sennoside B. Comparing the results shows that the amounts of both sennosides in *Cassia angustifolia* are more than *C. obovata*, even though *C. obovata* is also considered as an alternative source for sennosides A and B.

Cassia species are freely administrated without controlling the main constituents. Sennosides A and B are the main active constituents of senna or its related formulations. The validated HPLC method described in this article allowed the assay of sennosides in *cassia* extracts or pharmaceutical formulations containing these

Table 2. Linearity, LOD, and LOQ parameters of Sennoside A, and B standards

Compound	LOD $\mu\text{g/mL}$	LOQ $\mu\text{g/mL}$	R ² square	Equation	Linear range ($\mu\text{g/mL}$)
Sennoside A	3.75	12.5	0.999	$y = 4351.x - 28210$	50-800
Sennoside B	2.2	7.5	0.998	$y = 3572.x + 10685$	50-800

Table 3. Repeatability and intermediate precision assays of standards and real sample

Analytes	Concentrations ($\mu\text{g/mL}$)	inter-day n=6	RSD% (inter-day)	RSD% (intra-day)
Sennoside A	50	208215 \pm 8051	3.87	3.80
	100	425023 \pm 12201	2.87	2.71
	200	829363 \pm 20145	2.43	2.21
	400	1664790 \pm 45012	2.70	2.01
	800	3476863 \pm 125014	3.60	3.12
Sennoside B	50	263262 \pm 11820	4.00	2.80
	100	475446.5 \pm 20940	3.98	2.67
	200	843383 \pm 35343	3.76	2.06
	400	1524978 \pm 54948	3.44	1.41
	800	3111968 \pm 110862	3.20	2.34

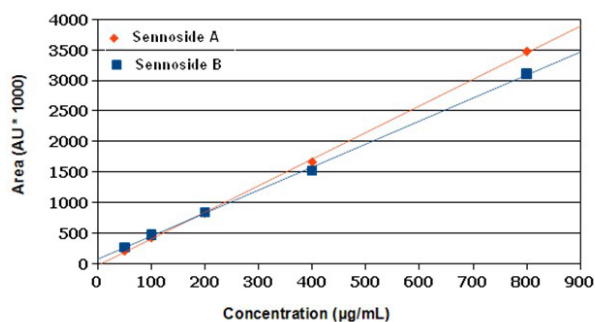


Figure 4. Calibration curve of sennoside A and B using HPLC method and acetonitrile:water as mobile phase with pH adjusted to 2.3 at 280 nm. Using the millennium processing software, the calibration curve was determined by linear regression in the range of 50-800 $\mu\text{g/mL}$. The regression equation was $y = 4351.x - 28210$ with the correlation co-factor (R²) of 0.999 for sennoside A and $y = 3572.x + 10685$ with R²=0.998 for sennoside B, where x is the concentration of standard ($\mu\text{g/mL}$).

two compounds in accordance with USP requirements. In comparison with previous validated HPLC methods on sennosides, the LOD and LOQ in this method were decreased to lower amounts and the linear range was optimized to a larger range from 50 to 800 $\mu\text{g/mL}$ (13,14). Previous reports on determination of sennosides by the HPLC method was done on *Cassia angustifolia* while this method was optimized on both *C. angustifolia* and *C. obovata*.

Authors' contributions

NGD co-ordinated the study and participated in most of the experiments. MG carried out the design and contributed in data analysis and writing and finalizing the manuscript. SA participated in most of the experiments and in manuscript preparation.

Conflict of interests

The authors declared no competing interests.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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