Pharmacogn. Res.
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ORIGINAL ARTICLE

Method Development and Validation for the Determination of Caffeine: An Alkaloid from *Coffea arabica* by High-performance Liquid Chromatography Method

P Naveen, HB Lingaraju[1], M Deepak[1], B Medhini, K Shyam Prasad[1]

Analytical Research and Development, Vidya Herbs Pvt. Ltd., [1]Phytochemistry Lab, Vidya Herbs Pvt. Ltd., Bengaluru, Karnataka, India

ABSTRACT

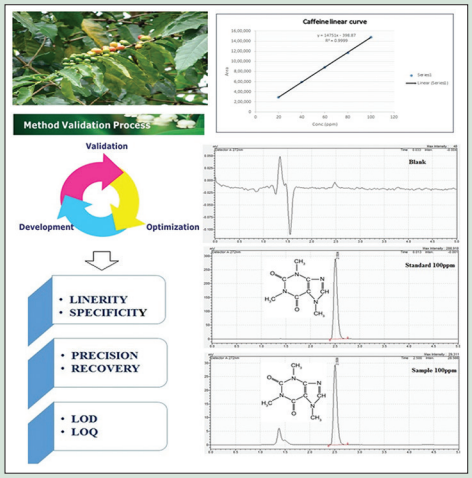
Objective: The present study was investigated to develop and validate a reversed phase high performance liquid chromatography method for the determination of caffeine from bean material of *Coffee arabica*. **Materials and Methods:** The separation was achieved on a reversed-phase C18 column using a mobile phase composed of water: methanol (50:50) at a flow rate of 1.0 mlmin-1. The detection was carried out on a UV detector at 272 nm. The developed method was validated according to the requirements for International Conference on Harmonisation (ICH) guidelines, which includes specificity, linearity, precision, accuracy, limit of detection and limit of quantitation. **Results:** The developed method validates good linearity with excellent correlation coefficient (R2 > 0.999). In repeatability and intermediate precision, the percentage relative standard deviation (% RSD) of peak area was less than 1% shows high precision of the method. The recovery rate for caffeine was within 98.78% - 101.28% indicates high accuracy of the method. The low limit of detection and limit of quantitation of caffeine enable the detection and quantitation of caffeine from *C. arabica* at low concentrations. **Conclusion:** The developed HPLC method is a simple, rapid, precisely, accurately and widely accepted and it is recommended for efficient assays in routine work.

Key words: Caffeine, *Coffea arabica,* linearity, precision, recovery, validation

SUMMARY

A simple, accurate, and sensitive high-performance liquid chromatography (HPLC) method for caffeine from *Coffea arabica* has been developed and validated. The developed HPLC method was validated for linearity, specificity, precision, recovery, limits of detection, and limits of quantification by the International Conference on Harmonization guidelines. The results revealed that the proposed method is highly reliable. This method could be successfully applied for routine quality work analysis.

Abbreviation Used: *C. arabica*: *Coffee arabica*, ICH: International Conference on Harmonisation, % RSD: Percentage Relative Standard Deviation, R2: Correlation Coefficient, ppm: Parts per million, LOD: Limits of detection, LOQ: Limits of quantification, SD: Standard deviation, S: Slope, RP-HPLC: Reverse phase high performance liquid chromatography, v/v: Volume per volume.



Correspondence:
Dr. P. Naveen,
Analytical Research and Development,
Vidya Herbs Pvt. Ltd.,
Jigani, Anekal Taluk,
Bengaluru, Karnataka, India.
E-mail: naveen2384@gmail.com

DOI: 10.4103/pr.pr_79_17

Access this article online
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INTRODUCTION

In the past years, the individuals were faced with the different ailments and the herbs are continuously used for the treatment of all the ailments. Nowadays, herbal medicine is accepted medicine as complementary and alternative therapy in combination with the main line therapies due to the unwanted toxicity and side effects.[1] Herbal medicines consist of various phytocompounds, which bared pharmacological activities.[2]

Coffee species are well identified and widespread throughout the world. It belongs to the Rubiaceae family. More than 120 coffee species have been identified worldwide.[3] The main leading coffee species are *Coffea arabica* (Arabica coffee) which is accountable for 70% of the total coffee market and *Coffea canephora* (Robusta coffee) accounts for the rest.[4]

The main phytoconstituents of *C. arabica* contain caffeine, an alkaloid,[5] chlorogenic acid, a polyphenolic compound,[6] carotenoids,[7] and enzymes.[8] *C. arabica* has been reported for antioxidant,[9] anticancer,[10] hepatoprotective,[11] anti-inflammatory,[12] immunostimulatory,[13] antidiabetic,[14] antibacterial,[15] and antiviral.[16]

Reversed-phase high-performance liquid chromatography (RP-HPLC) involves the separation of molecules on the basis of hydrophobicity. Extensive literature survey revealed that few methods were reported for the estimation of caffeine by RP-HPLC. Hence, an attempt has been

Cite this article as: Naveen P, Lingaraju HB, Deepak M, Medhini B, Prasad KS. Method Development and Validation for the Determination of Caffeine: An Alkaloid from *Coffea arabica* by High-performance Liquid Chromatography Method. Phcog Res 2018;10:88-91.

made to rapid development and validates a new, simple, accurate, precise, and economically feasible RP-HPLC method for the quantification of caffeine in *C. arabica*.

MATERIALS AND METHODS

Caffeine standard (99.8%) was obtained from Sigma Aldrich (India). All reagents and solvents were analytical and HPLC grade. The bean material of *C. arabica* was procured from Sargod Trading Co., Chikmagalur, Karnataka, India.

Preparation of the extract

Hydroalcoholic extract of coffee bean was prepared by Soxhlet extraction of 500 g of powdered coffee bean material of *C. arabica* using 70% ethanol at 75°C in four successive batches. The first batch extraction was done by adding 800 ml solvent for 3 h and further three successive extractions were done by adding 500 ml per batch for 3 h. After completion of extraction, the extracted liquid was concentrated by evaporating in rotary evaporator, with an approximate yield of 15% w/w.

Instrumentation and chromatographic conditions

HPLC was performed on a Shimadzu LC2030 C Prominence I (Japan) system equipped with a quaternary low-pressure gradient solvent delivery LC2030 pump with high-pressure switching valves, online LC2030 degasser unit, a high-sensitivity LC2030 ultraviolet (UV) detector, high-speed drive LC2030 autosampler with a 100 µl loop, and it accommodates 216 samples at a time with direct access rack system and large capacity column oven. The system controlled and data analyzed by LabSolutions software. Before choosing the chromatographic condition, a number of trials were carried out with different ratios of solvents, flow rate, and temperatures to check the retention time (RT), peak shape, tailing factor (peak symmetry), and theoretical plates of the analyte. A separation was carried out in Kinetex C18 column (100A0, 150 × 4.6 mm, 5 µm pore size). The mobile phase consists of isocratic elution with a low-pressure gradient using double-distilled water:methanol (A:B): (50:50) with a flow rate of 1.0 ml/min and the injection volume of 5 µl. All solutions were degassed and filtered through 0.2 µm pore size filter. The column was maintained at 26°C throughout analysis, and the UV detector was set at 272 nm. About 70% methanol used as a diluent for assay by HPLC analysis, and the total LC run time was 5 min. The instrument was calibrated and qualified before the analysis. Using these chromatographic conditions, it was possible to confirm the RT of caffeine by injection of corresponding standard separately. In addition, the system suitability parameters such as tailing factor (T) and theoretical plates (N) are calculated to the caffeine standard solution (100 ppm).

Preparation of standard solution

Accurately weighed known amounts of caffeine reference standard were mixed and dissolved in 70% methanol in a 100 ml volumetric flask to obtain a stock solution of 100ppm. Before analysis, the solution was filtered through 0.20 µm nylon membrane filters.

Preparation of sample solution

The dried coffee bean extract was prepared in 70% methanol to achieve the final concentration of 100 ppm. Before analysis, the solution was filtered through 0.20 µm nylon membrane filters.

Preparation of spiked sample solution

Three different volumes (2, 4, 6 ml) of standard stock solution and 1 ml of sample (100 ppm) solution are added into 10 ml of volumetric flask separately. The standard stock solution was spiked into the sample to determine recovery. Before analysis, the solutions were filtered through 0.20 µm nylon membrane filters.

Validation of the method

The validation of the developed analytical method was done according to the International Conference on Harmonisation (ICH) guidelines.[17] The method is validated for specificity, linearity, recovery, precision, and the limits of detection (LOD) and limits of quantification (LOQ).

Specificity

Specificity of a method was determined by testing standard substances against potential interferences. It was demonstrated by injecting six replicate injections of 100% test (sample) solution.

Linearity

Linearity was determined by different known concentrations of caffeine standard solution in triplicate by diluting the standard stock solution. For the determination of linearity, five aliquots were pipetted out from standard stock solution (i.e., 100 ppm). 2-10 ml of standard stock solution was pipetted out in to a series of 10 ml volumetric flasks and volume was made up with the solvent to obtain concentration ranging from 20 ppm to 100 ppm of caffeine. The calibration curve was constructed by plotting the peak areas against concentration, and the linear regression equations were calculated. The correlation coefficient was also computed.

Precision

Precision was determined by studying the repeatability (intraday) and intermediate (interday) precision. The repeatability and intermediate precision of the method was determined by calculating the percentage relative standard deviation (% RSD) of three different concentration levels of caffeine standard 20, 40, and 60 ppm. The repeatability was examined on the same day, whereas intermediate precision examined on different days by the different analyst.

Accuracy

The accuracy of the method was tested by performing the recovery studies at three different levels of standard stock solution added to the samples. The standard stock solution was spiked into the samples to determine recovery. Three different volumes (2, 4, 6 ml) of standard stock solution and 1 ml of sample (100 ppm) solution are added into 10 ml of volumetric flask separately. Triplicate injections were made with all the spiked samples.

% of Recovery = (b − a)/c × 100.

Where,

- "a" is the amount of drug found in the sample before addition of standard drug
- "b" is the amount of drug found after addition of standard drug
- "c" is the amount of standard drug added.

Limit of detection and limit of quantification

The LOD was calculated by based on standard deviation of y-intercepts of the regression lines (SD) and the slope (S), using the equation LOD = 3.3 (SD/S). LOQ was also calculated by the equation LOQ = 10 (SD/S).

RESULTS AND DISCUSSION

Validation of analytical procedure is the process for proving that an analytical procedure is suitable for its intended purpose. Results obtained from method validation study can be used to judge the quality, reliability, and consistency of analytical results. An RP-HPLC method was developed and validated for the determination of caffeine in *C. arabica*. Several mobile phase compositions were tried, and a satisfactory separation was obtained using the mobile phase composition water:methanol (A:B): (50:50 v/v). An optimized chromatogram of blank, standard, and sample was shown in Figure 1.

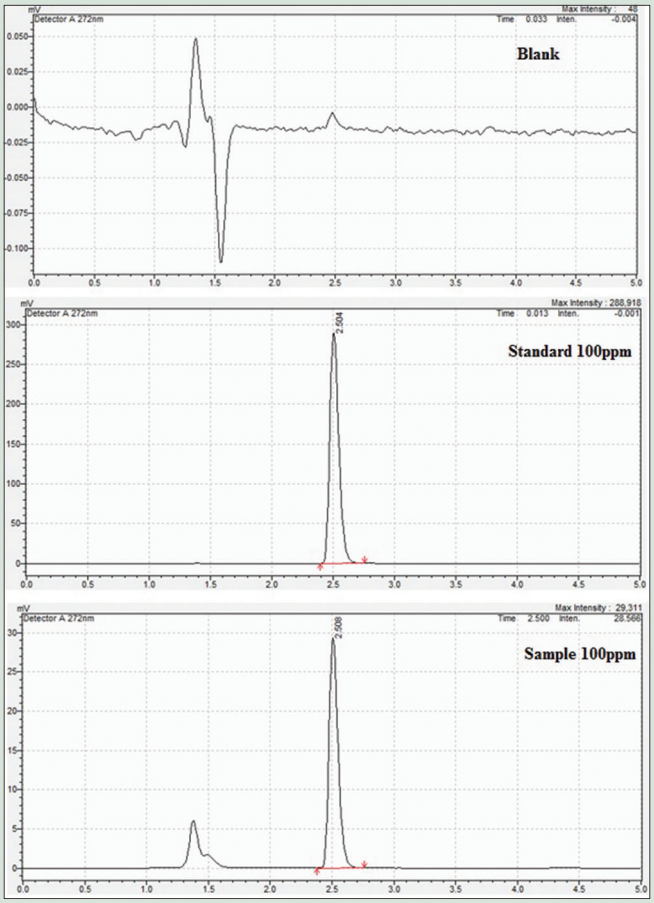

Figure 1: Optimized high-performance liquid chromatography of caffeine

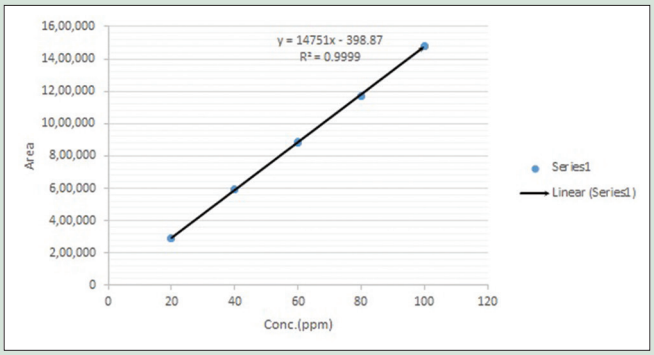

Figure 2: Calibration curve of caffeine

Table 1: Results of precision (*n*=6)

Concentration(ppm)	Repeatability			Intermediate precision		
	Mean area	SD	% RSD	Mean area	SD	% RSD
Standard 20	291671	62	0.021	291188	198	0.068
Standard 40	595294	307	0.052	589490	321	0.054
Standard 60	885256	462	0.052	883354	509	0.058

Values are expressed as mean±SD (*n*=6). SD: Standard deviation; RSD: Relative standard deviation

Table 2: Results for accuracy (*n*=3)

Recovery study of Caffeine					
	Volume of reference standard stock solution added(mL)	b	a	c	% Recovery
20% spiked sample	2	445372	149940	291671	101.28
40% spiked sample	4	737982	149940	595294	98.78
60% spiked sample	6	1029340	149940	885256	99.33

Values are expressed as mean±SD (n=3). SD: Standard deviation; RSD: Relative standard deviation

The RT of standard and sample peak was found to be at 2.504 and 2.508 min, respectively. The system suitability parameter of theoretical plates and tailing factor of standard solution (100 ppm) were found to be 4564 and 1.297, respectively, which indicated column efficiency is satisfactory.

The ICH defines specificity as "the ability to assess the analyte for the presence of various components which may be expected to be present". The 100% test (sample) chromatograms confirm the presence of caffeine RT at 2.508 without any interference. The RT of sample solution is overlay with the standard solution, so the method was specific.

To check the linearity of the analytical method, calibration curves were plotted by peak area versus concentration of caffeine standard in the range 20–100 ppm, which is summarized in Figure 2. The calibration plots of caffeine standard solution were linear, and the regression equation is represented by y = 14751* × −398.87. This indicated that good fitting of the curve. The correlation coefficient (R^2) of caffeine standard solution was 0.999. The acceptance criteria for linearity are that the correlation coefficient (R^2) should not be less than 0.990.[18] This indicates that the method showing good linearity.

Precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Table 1 shows that in repeatability, the % RSD of peak area of caffeine standard concentration of 20, 40, and 60 ppm was found to be 0.021%, 0.052%, and 0.052%, respectively. The data [Table 1] pertaining to intermediate precision revealed that the % RSD of peak area of caffeine standard concentration of 20, 40, and 60 ppm was found to be 0.068%, 0.054%, and 0.058%, respectively. These precision presented % RSD values are less than 1.0%, so the method was found to be highly precise and reproducible.

Accuracy is the closeness of agreement between the values found. The value accepted as a conventional true value or the accepted reference value. The recovery values obtained were given in Table 2. The % recovery of standard added to the preanalyzed sample was calculated. The average % recoveries at three different levels (20, 40, and 60 ppm spiked sample) of caffeine were found to be 101.28%, 98.78%, and 99.33%. This indicates high accuracy of the method.

The LOD and the LOQ for caffeine were found to be 2.62 ppm and 7.94 ppm, respectively. Low LOD and LOQ of caffeine enable the detection and quantitation of this alkaloid in *C. arabica* at low concentrations.

CONCLUSION

The developed HPLC method is a simple, rapid, precisely, accurately, and widely accepted, and it is recommended for efficient assays in routine work. Therefore, the method was proved to be suitable for caffeine, alkaloid determination in *C. arabica*. Further explorations are needed to investigate the standardization of individual phytoconstituents of *C. arabica*.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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