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Development and Validation of HPTLC and RP-HPLC Methods for the Estimation of Berberine in *Coscinium Fenestratum* Extract and its Formulation

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ABSTRACT

Two simple, sensitive and precise HPTLC and RP-HPLC methods were developed for the estimation of berberine from *Coscinium fenestratum*, and its formulation. For the determination of berberine by HPTLC method, precoated silicagel $60F_{254}$ on aluminium sheets and a mobile phase system comprising of n-butanol: glacial acetic acid : water (8:1:1 % v/v/v) was selected. After development the plate was scanned and quantified at 350 nm. Linearity was found in the concentration range of 10 to 50 ng/spot (r=0.9992). Limit of detection was found to be 5 ng/spot and limit of quantification was found to be 10 ng/spot. In RP-HPLC method, separation was achieved on a Phenomenex, Luna, C_{18} column (150 x 4.6mm internal diameter, 5µ particle size) using a mobile phase consisting of potassium dihydrogen phosphate (pH - 2.5) (A) : acetonitrile (B), where B was run in gradient programme (20% for 0.01-20min, 50% for 20-25min, 50% for 25-26min, 20% for 26-30min), at a flow rate of 1ml/min and the elute was monitored at 220nm. The calibration curve was obtained in the range of 100 - 500 µg/mL. The slope, intercept and correlation coefficient values were found to be 57588, 6041and 0.9959, respectively. The method was validated in compliance with ICH guidelines. Low relative standard deviation and good % recovery values of both the methods showed that the developed methods were highly precise, accurate and can be employed for the routine analysis of formulations containing berberine.

Keywords: *Coscinium fenestratum*, Berberine, HPTLC, RP-HPLC, Frok capsules**,** Validation.

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INTRODUCTION

Medicinal plants play an important role in the development of potent therapeutic agents. The use of herbal medicines is becoming popular due to toxicities and side effects associated with allopathic medicines. In order to better address the inherent holistic nature of herbal medicines, analysis and quality control of herbal medicines are absolutely required. An innovative research effort to bring out the advantages of traditional system of medicine with respect to their safety and efficacy could result in the better utilization of the complementary and indigenous systems of medicine for affording cheaper and safer health care to the people of our country [1].

Standardization of plant extracts and poly herbal formulations with the help of markers would lead to increased global acceptance for them. Standardization is an essential process for ensuring the quality control of the herbal drugs. Standardization is defined as "formulation of standards for a substance or for a procedure" [2].

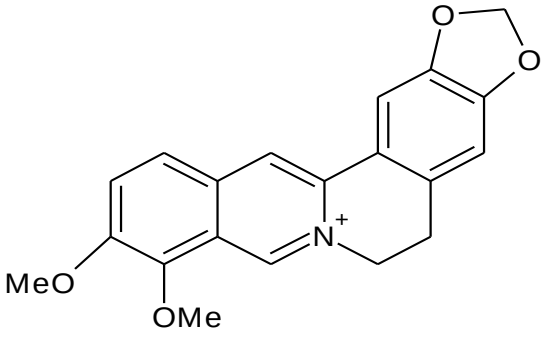

Figure 1: Structure of Berberine

*Coscinium fenestratum***,** Menispermaceae is a medicinal plant which has proven to be useful for the treatment of leprosy, urinary disorder, anaemia, fever, indigestion, dyspepsia, burning sensation, ulcer, chloasma of face, acne vulgaris, worm infestation. It is also found to be possess antihyperglycemic, anti-inflammatory and antigonococcal activities [3-7].The plant was found to contain different types of alkaloids out of which berberine, was found to be the major active principle having biological activity [8-10]. Chemically it is 5,6-dihydro-9,10-dimethoxybenzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium with the molecular formula $C_{20}H_{18}N0_4$ and molecular weight 336.36122 (Figure 1). Literature survey revealed that berberine can be estimated by TLC [11], HPLC [12-17] LC-MS [18]. The quality control of herbal medicines is therefore highly desired to ensure their authenticity, stability and consistency. HPTLC and RP- HPLC methods are becoming a routine analytical technique because of their advantages, high sample throughput, simplicity, speed, reproducibility, accuracy, reliability and robustness. In the present study a

suitable, sensitive and reliable quantitative HPTLC and RP-HPLC methods were developed for quality control and determination of berberine from *Coscinium fenestratum* plant and its formulation.

MATERIALS AND METHOD

Chemical and reagents

Berberine was purchased from Sigma Aldrich, India. The marketed formulation, Frok capsule, was purchased from local market. AR/HPLC grade methanol, n-butanol, glacial acetic acid, acetonitrile and potassium dihydrogen phosphate were supplied by S.D. Fine Chemicals Ltd., and Merck Pvt. Ltd., Mumbai.

Instruments

HPTLC was performed in Camag HPTLC System equipped with Linomat 5 sample applicator twin trough plate development chamber, TLC Scanner 3 with WinCATS software. RP-HPLC was performed in Shimadzu HPLC system with LC AT10 VP Pump, SPD M 10 AT VP Detector and CLASS M 10A software. The stationary phase used was Phenomenex, Luna, C18 column (150 x 4.6mm, 5µ). (150 x 4.6mm, 5µ)

Process of extraction

The selected plant parts were collected, dried, powdered and extraction was carried out by hot percolation method using Soxhlet apparatus at 30-40°C using organic solvents of increasing order of polarity. For extraction 1000gm of plant powder and 1000ml of the solvent were used and each extraction was carried out for 3 days with each solvent. The organic solvents used were petroleum ether, chloroform, and methanol. After extraction, the extracts were collected and dried at room temperature

Preparation of standard stock solution of berberine

10mg of berberine was transferred into a 10ml standard flask and the volume was made up with methanol to 10ml to get a concentration of 1000µg/ml. From the above stock solution, a solution with a concentration of 100µg/ml was prepared.

Preparation of stock solution of extract

Each extract (10mg) i.e. petroleum ether, chloroform, methanol of *Coscinium fenestratum* was weighed and made up to 10ml with methanol to get a concentration of 1000 µg/ml solution and these solutions were filtered through Whatmann filter paper and the filtrates were collected.

Preparation of standard stock solution of Frok capsules

The content of twenty capsules, each containing 62.5mg of *Coscinium fenestratum,* was

collected, weighed and transferred into 10ml standard flask and extracted with methanol for 1hour. The resultant extract was filtered and the filtrate was evaporated to dryness. The dried extract obtained was dissolved in 5ml of methanol and the solution was filtered through Whatmann filter paper. The filtrate was collected and used for further studies.

STANDARDIZATION

HPTLC [19]

In the present study the HPTLC fingerprinting analysis and standardization were carried out on the extracts of *Coscinium fenestratum*. Various organic solvents like petroleum ether, chloroform and methanol were successively used to prepare the extracts.

The HPTLC study was conducted on aluminium sheets precoated with 250 µm layers of silicagel $60F_{254}$ 50µl standard stock solution of berberine and formulation were applied on to the plate as bands that were 6mm. wide and 6mm apart by means of CAMAG Linomat 5 sample applicator equipped with a 100 µL syringe. Linear ascending development was performed in a twin trough glass chamber, using n-butanol : glacial acetic acid : water, (8:1:1 % v/v/v) as mobile phase, after saturation of chamber with mobile phase vapour for 20 min. The development distance was 80mm and the development time was approximately 15 min. After development, the plates were dried. The scanning was performed using TLC scanner 3 in the absorbance mode 350 nm for all measurements having slit dimension of 5×0.45mm. The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum between 200 to 400 nm. The specific peak corresponding to respective marker for formulation was located and band on R_f and UV spectrum was obtained from HPTLC scanner. The peak area of berberine in formulation chromatogram was compared with that of standard chromatogram and the amount present in formulation was calculated from the calibration graph.

HPLC[20]

In the present study, the establishment of the HPLC method for the standardization of methanolic extracts of *Coscinium fenestratum*, using Phenomenex, Luna C_{18} column (150×4.6mm, 5µ) at room temperature was carried out. The separation was achieved by using mobile phase system consisting of potassium dihydrogen phosphate (pH - 2.5) (A): acetonitrile (B), where B was run in gradient programme (20% for 0.01-20min, 50% for 20-25min, 50% for 25-26min, 20% for 26-30min). The flow rate used in these studies was 1ml/min and detection wavelength used was 220nm. The extracts showed peaks in retention times (RT) of 18.75 minutes for berberine. The peak of marker matches with that of the methanol extract of the plant. A steady baseline was recorded with the fixed chromatographic conditions. This was followed by injection of the

solution prepared from the extract and formulation and the chromatograms were recorded. The amount of berberine present in the methanolic extract and formulation were calculated from the standard graph which was plotted using peak area of the standard marker against the concentration.

Validation[21]

Validation of the HPTLC and HPLC methods for the selected marker was carried out in terms of parameters like linearity, accuracy, limit of detection (LOD), limit of quantification (LOQ), inter and intraday precision, repeatability of sample application and sample measurement, stability studies and selectivity.

Linearity and Range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of the analyte in the sample. Stock solution of berberine was suitably diluted with methanol to get concentrations in the linear range of 10 to 50 ng/spot for HPTLC and 100 to 500µg/ml for HPLC analysis .The slope, intercept and correlation co-efficient values were found from the calibration graph of the marker.

Limit of detection and Limit of quantification

The Limit of detection and limit of quantification of the standard were determined by applying decreasing amounts of the drug in triplicate on the plate. The lowest concentration at which the peak is detected is called as the 'Limit of Detection' and the lowest concentration at which the peak is quantified is called as the 'Limit of Quantification'.

Precision

Precision of the method adopted in the present work was demonstrated by Intraday precision, Interday precision, Repeatability of sample application, Repeatability of sample measurement.

precision was studied by carrying out the analysis of berberine at concentrations of 30 and 50 ng/spot for HPTLC method and in RP-HPLC, concentrations of 200 and 400µg/ml of berberine were analysed three times on the same day for intraday and three days over a period of one week for interday precision. For the repeatability study, the solution was analyzed for six times and %RSD was calculated.

Stability studies

In HPTLC, stability of the analyte on the plate was studied at different time intervals and peak areas were compared with the peak area of freshly scanned plate. Sample solutions were subjected to stability studies under room conditions. Stability was studied by looking for any change in retention time, resolution, peak shape etc, when compared with the chromatogram of

freshly prepared solution for RP-HPLC method of analysis.

System suitability studies

System suitability parameters like number of theoretical plates (N), resolution (R_s) and tailing factor were studied.

RESULTS AND DISCUSSION

The current study revealed the presence of maximum amount of berberine in the stems of *Coscinium fenestratum* and its formulation. Figure 1 shows the chemical structure of berberine. The constituents were separated by extraction methods which were optimized using various organic solvents of increasing order of polarity i.e. petroleum ether, chloroform and methanol. Dried extracts were weighed and the percentage of yield was calculated. It was found that amount of the extract obtained was higher in the case of extraction with methanol when compared to chloroform and petroleum ether extractions (Table 1).

HPTLC method was carried out for the determination of berberine in the extract. As berberine was soluble in methanol it was optimized as the suitable solvent. The mobile phase system was optimized as n-butanol : glacial acetic acid : water, (8:1:1 % v/v/v).. The optimum detection wavelength was found to be 350nm.The R_f value of berberine was found to be 0.46. Amount of berberine (mg) present in successive extracts are shown in table 2. It was found that the amount of the berberine obtained was higher in the case of extraction with methanol (0.902 mg) when compared to chloroform and petroleum ether extractions. The HPTLC method was validated as per ICH guidelines. Calibration curve was obtained in the range of 10 to 50ng/spot (figure 2) and the correlation coefficient was 0.9992. Limit of detection and quantification were found to be 5ng/spot and 10 ng/spot. The precision at intraday and interday level was evaluated in terms of % RSD and was found to be less than 2%. Details of the validation of method and its results obtained are shown in table 3. Figure 3 shows the HPTLC chromatogram (50ng/spot).

In RP-HPLC method**,** the separation was carried out on C_{18} column and mobile phase selected was potassium dihydrogen phosphate (pH - 2.5) (A) : acetonitrile (B), where B was run in gradient programme (20% for 0.01-20min, 50% for 20-25min, 50% for 25-26min, 20% for 26-30min). The mobile phases were pumped into the column at a flow rate of 1ml/min. The detection wavelength used was 220nm and the method was validated. Amount of berberine present in 10 mg of methanol extract of *Coscinium fenestratum* was found to be 0.945 mg (table 2). All the validation parameters were found to be within limit. The method showed good linearity over the concentration range of 100-500 µg/ml (Figure 4). Results of validation are summarized in table 3. Adequacy of the proposed RP-HPLC method for routine analysis of

berberine was assured by system suitability tests. The results of system suitability studies, Retention time, Theoretical plates, Resolution, Tailing factor are shown in table 4 and figure 5 shows the HPLC chromatogram of berberine (500 µg/ml) and its retention time was noted 18.75.

Analysis of formulation

The developed and validated methods were used to ascertain the content of berberine in marketed formulation containing *Cosinium fenestratum*. In HPTLC, R_f value of the formulation was compared with that of the standard berberine. Recovery study was carried at 100% level. The recovery studies showed that both batch 1 and 2 exhibited more than 96% recovery and the % RSD was found to be between 1.0-1.6. The amount of marker present in each capsule of batch 1 and batch 2 of Fork capsules was found to be 0.019 mg and 0.018 mg respectively (Table 5). By RP-HPLC method, the formulation showed peaks with retention times (RT) of 18.75. The recovery rates ranging from 95% to 97% for batch 1 and 2 proved good accuracy of the method. The low % RSD value (0.8-1.4) indicates that the method is also precise. Details of recovery study by HPTLC and RP-HPLC are given in table 6. The HPTLC analysis of Fork capsule were carried out in two batches, R_f values of formulation matches with that of standard berberine and chromatograms obtained are shown in figure 6 for batch 1 and figure 7 for batch 2 formulation. RP-HPLC chromatograms of berberine in batch 1 and batch 2 are shown in figure 8 and 9.

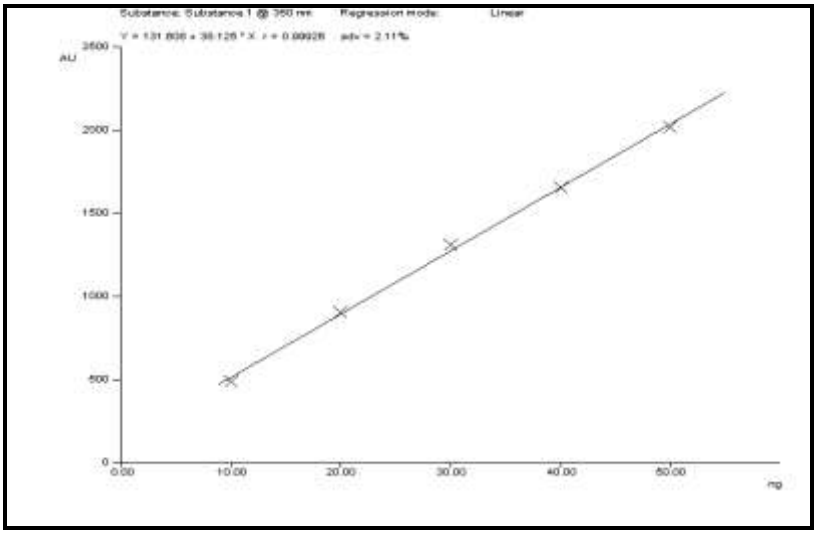

Figure 2: HPTLC calibration graph of beriberine (10-50 ng/spot)

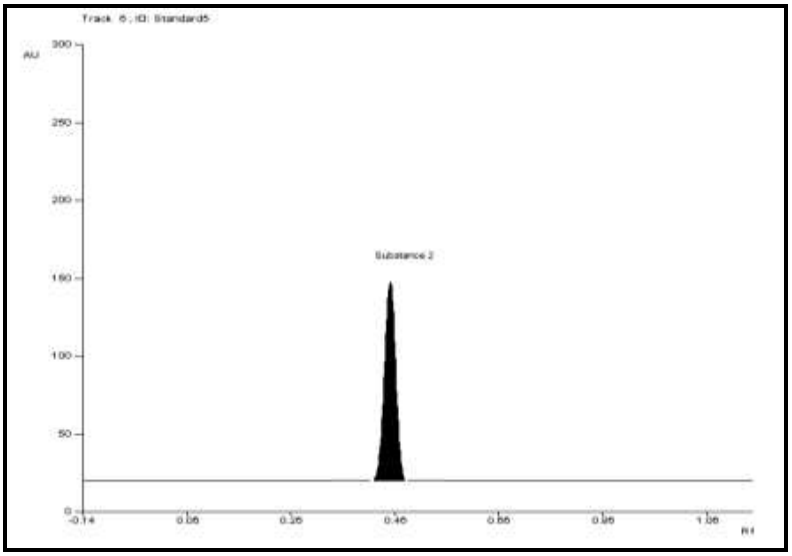

Figure 3: HPTLC chromatogram of berberine (50ng/spot)

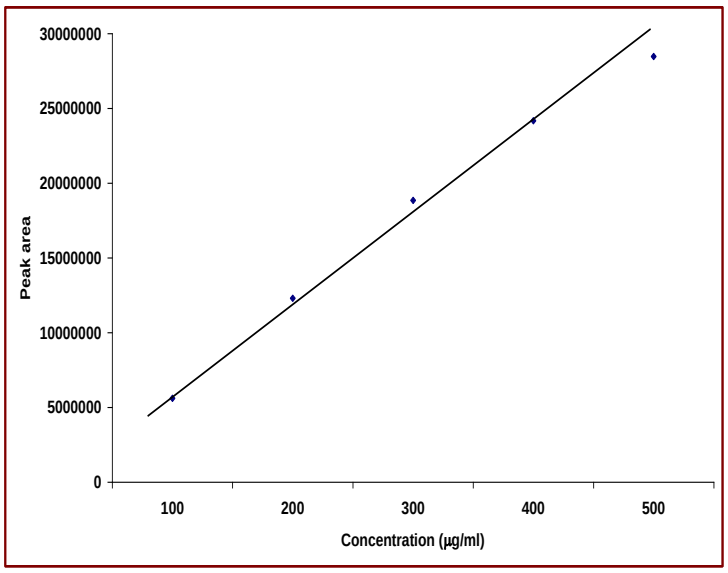

Figure 4: HPLC calibration graph of berberine (100-500 µg/ml)

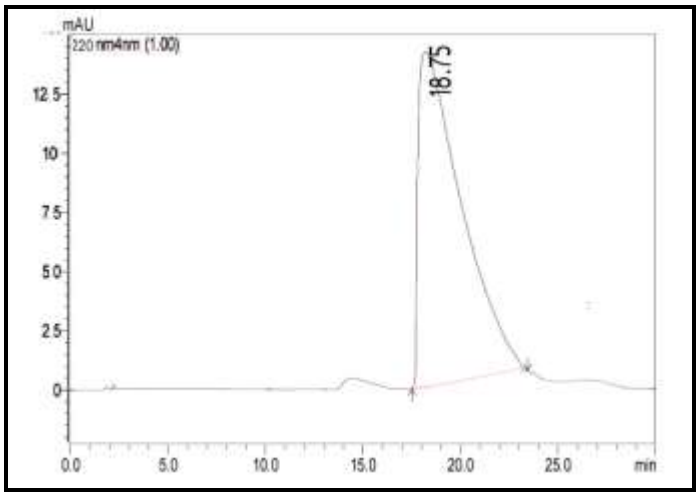

Figure 5: HPLC chromatogram of berberine (500 µg/ml)

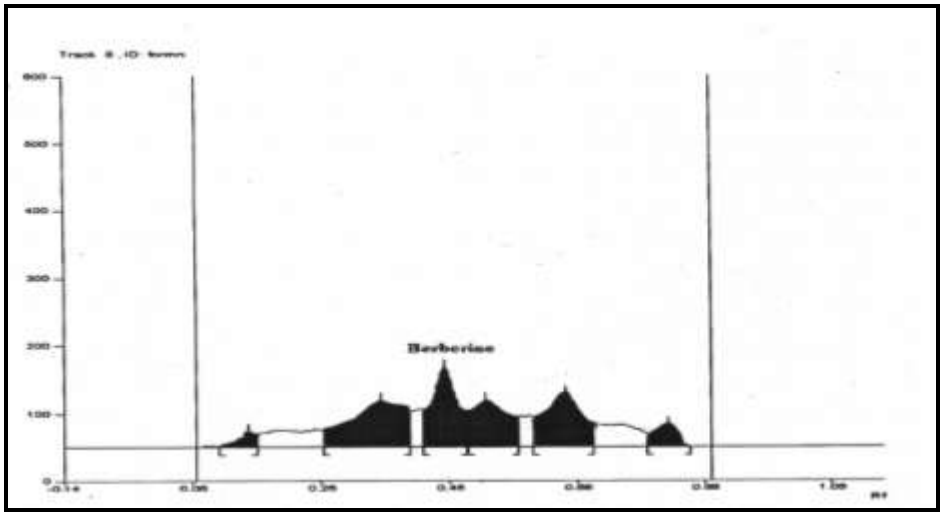

Figure 6: HPTLC chromatogram of Frok capsule (Batch 1)

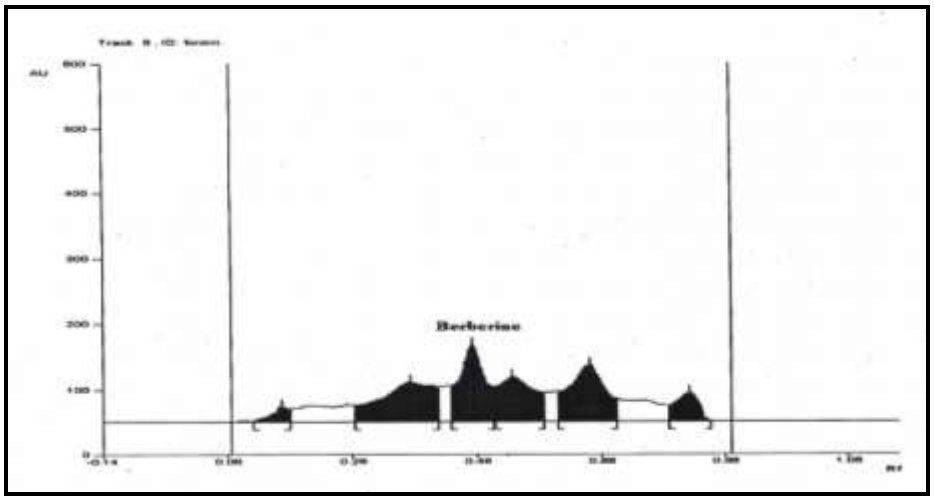

Figure 7: HPTLC chromatogram of Frok capsule (Batch 2)

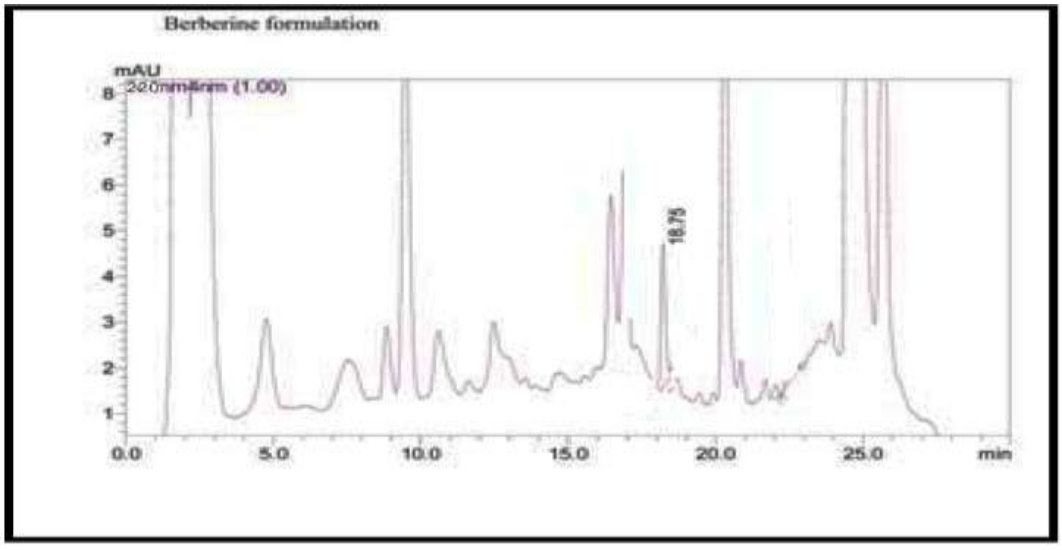

Figure 8: HPLC chromatogram of berberine in Frok capsule (Batch 1)

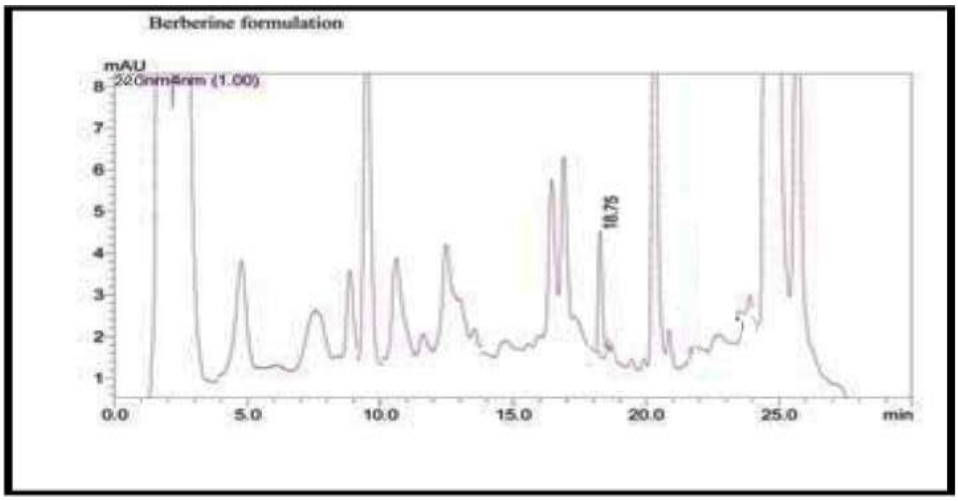

Figure 9: HPLC chromatogram of berberine in Frok capsule (Batch 2)

Table 1: Amount and percentage yield of successive extracts from1000gm of powdered drug

Name of the plant	Extracts	Amount of extract obtained (gm)	Percentage of yield (%w/w)
Coscinium fenestratum	Petroleum ether	11.23	1.12
	Chloroform	18.65	1.86
	Methanol	52.30	5.23

Table 2: Estimation of marker present in the extracts

Plant /Marker	Methods	Extracts	Amount of markers (mg) present in each extracts	%RSD*
***Coscinium fenestratum* (Berberine)**	HPTLC	Petroleum ether (10mg)	0.0075	1.32
		Chloroform (10mg)	0.244	1.41
		Methanol (10mg)	0.902	1.28
	HPLC	Methanol (10 mg)	0.945	1.4

***Mean RSD of six observations**

Table 3: Validation of methods

Parameters	Berberine	
	HPTLC	RP-HPLC
Linearity	10-50 ng/spot	100-500 µg/ml
Slope	38.125	57588
Intercept	131.608	60413
r	0.9992	0.9959
LOD	5 ng/spot	30 µg/ml
LOQ	10 ng/spot	100 µg/ml
Precision (%RSD)		
Intraday	0.39	0.15
Interday	0.32	0.12
Repeatability of application	0.40	0.43
Repeatability of measurement	0.48	-
Stability	60 min	3 hrs

Table 4: System suitability studies for proposed RP-HPLC method

Parameters	RP-HPLC
Retention time	18.754
Theoretical plates	12319.11
Resolution	10.4
Tailing factor	1.50

Table 5: Estimation of markers present in formulations

Polyherbal formulation	Marker	Labelled amount of extract (mg/capsule)	Amount of marker present (mg/ capsule)		%RSD*	
		Batch 1/Batch 2	Batch 1	Batch 2	Batch 1	Batch 2
Frok	Berberine	62.5	0.019	0.018	1.21	1.24
			0.0059	0.0062	1.36	1.61

***Mean RSD of six observations**

Table 6: Recovery study

Formulation	Level	Method	%Recovery		%RSD*	
			Batch 1	Batch 2	Batch 1	Batch 2
Frok capsule	(100%)	HPTLC	97.3	96.3	1.24	1.61
		RP-HPLC	96.1	95.3	0.81	1.43

***Mean RSD of six observations**

CONCLUSION

In this current study determination of berberine was carried out using HPTLC and RP- HPLC method. In HPTLC method the R_f values of plant extract, formulation matches with that of standard berberine and in RP-HPLC method, the retention time of berberine was found to be same in plant extract and formulation. The developed and validated HPTLC and RP-HPLC methods were simple, rapid, accurate, reproducible, selective and economical and can be used for routine quality control analysis of *Coscinium fenestratum* and quantitative determination of beriberine in Frok capsules. This method can be applied for the standardization of products containing *Coscinium fenestratum*. Thus, these analytical standardization techniques facilitate herbal manufacturers to market their medicines globally with defined content of respective bioactives to ensure their quality.

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