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Research Article

ISOLATION AND HPLC METHOD DEVELOPMENT FOR FILIXIC ACID PBP FROM *DRYOPTERIS FILIX-MAS*

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ABSTRACT

A simple isolation method and rapid, specific high performance liquid chromatographic method was developed and validated for filixic acid PBP in *Dryopteris filix-mas* extract. HPLC analysis was performed on C_{18} column using ~~a~~ 90:10 (*v*/*v*) mixtures of acetonitrile and methanol as isocratic mobile phase at a flow rate 1.0 ml/min. UV detection was at 254 nm for filixic acid PBP. Filixic acid shown retention time at 4.02 min. Method was validated for accuracy, precision, linearity, specificity and sensitivity in accordance with International Conference on Harmonisation guidelines. Validation studies revealed that the method is specific, accurate, precise, reliable and reproducible. Good linear correlation coefficient ($r^2 > 0.993$) was obtained for calibration plots in the range tested. The limit of detection was 2.25 µg/ml and limit of quantification was 7.53 µg/ml for filixic acid PBP. Intra and inter-day relative standard deviation of precision was less than 1.50 %. Recovery was between 94.57 and 101.05 % filixic acid. The method can be successfully used for quantitative analysis of filixic acid PBP in *D. filix-mas* for day-to-day studies.
Keyword: *Dryopteris filix-mas*; male fern; filixic acid PBP; HPLC; isolation.

1. INTRODUCTION

Male fern consist of dried rhizomes and fond bases of *Dryopteris filix-mas* and other species of *Dryopteris*, belonging to family Polypodiaceae[1]. In the ancient literature *Dryopteris filix-mas* is reported to be used against helminthiasis by tape warm[2]. Drypoteris acylphloroglucinols also shows tumor inhibitory effect[3]. *D. filix-mas* contain number of ether soluble phloroglucinol derivative contributing to anthelmintic property. *D. filix-mas* were use as a vermicide, vermifuge and to remove tapeworm. Indian male fern yield 8-10% ether extractive value of which 30 % is filicin. It is the mixture of six homologous compound filixic acid, i.e. filixic acid BBB, ABB and PBP (*fig. 1*), filicinic acid, aspadinol, albaspidine and flavaspidic acid[1,4]. Filixic acid PBP is pale yellow plates with melting point in the range 184-186°C[5-7]. Pamttila and Sundman have done isolation work for filixic acids[8]. Thin layer chromatography (TLC) and High Performance Thin Layer Chromatography (HPTLC) method for qualitative analysis of male fern is available in literature[9].

In present study attempt was made to isolate the filixic acid PBP from the resin. The specific, reliable and reproducible HPLC method was developed and validated according to International Conference on Harmonization (ICH) guidelines[10].

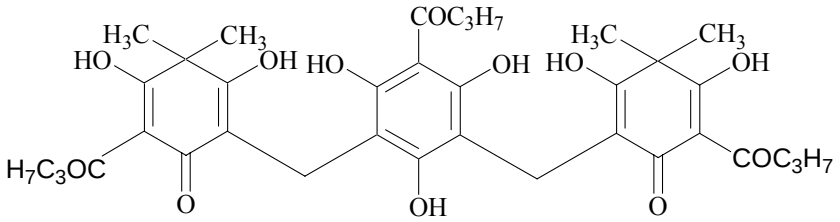

Fig. 1 Structure of filixic acid PBP

2. EXPERIMENTAL

2.1 Plant Material and Chemical

Air dried rhizomes and fond bases of *D. filx-mas* were collected in January (Three samples) from Mumbai, authenticated and voucher specimen was deposited in Institute of Chemical Technology, Mumbai. Analytical grade acetonitrile, methanol and chloroform were purchased from Merck, India for HPLC study. All chemicals were of analytical grade purchased from Sigma-Aldrich. All solvents for HPLC study were filtered through 0.45 µm pore size filter (Milipore Bedford).

2.2 Method

2.2.1 Isolation of filixic acid PBP

Dried rhizomes were powdered (1 kg) and refluxed with methanol (thrice, 1 L x 1 h each time). The extracts were combined and evaporated by using vacuum Rota evaporator to obtain dried residue (300 g). Residue was suspended in water and kept overnight which leads to separation of precipitate. Precipitate was washed twice with the water (250 ml each time) and dried. Precipitate was loaded on column filled with silica gel (80-120 mesh size). Column was eluted with chloroform. Each fraction of 200 ml was collected using 100 % chloroform as eluent. Fractions from 5 to 15 showing mixture of three different compounds were pooled and concentrated to dryness (34 g). The residue was reloaded on silica gel column and eluted with 100 % chloroform, and fractions number 1 to 5 each of 100 ml collected and pooled togather on the basis of TLC profile. For TLC, Toluene: ethyl acetate (9:1) was used as a solvent system and vanillin-sulphuric acid as a derivatising agent. Isolated compound was confirmed as filixic acid PBP (1.3 g) by melting point, UV, IR, NMR and Mass spectroscopic studies. The NMR spectral analysis was done in CDCl3, using TMS as internal reference.

2.2.2 Chromatography

HPLC analysis was performed with a JASCO (Hachioji, Tokyo, Japan) system consisting of an intelligent pump (PU-1580, PU-2080), a high-pressure mixer (MX-2080-31), a manual sample injection valve (Rheodyne 7725i) equipped with a 20 µL loop, and a UV–visible detector (UV-1575). RP-18 endcapped column (250 mm × 4.6 mm i.d., 5 µm particle, Hibar Lichrocart Purospher Star, Merck, Darmstadt, Germany).

2.3 Validation of chromatographic method

HPLC method development and validation studies carried out using Acetonitrile: Methanol (90:10) as isocratic mobile phase at flow rate of 1 ml/min, at UV at 254 nm. The analysis was performed at ambient temperature and data were analyzed on a computer equipped with Borwin software.

5 g drug sample was extracted with 100 ml methanol by Soxhlet apparatus for 24 h. Extract was filtered and transferred to 100 ml volumetric flask and volume was made with methanol (Sample stock solution). Standard stock solution (1 mg/ml) of filixic acid PBP was freshly prepared in methanol. Calibration curve was prepared by using standard solutions of different concentrations (10, 20, 30, 40 and 50 µg/ml). In validation study calibration plots were constructed for standard filixic acid, after triplicate analysis of each solution.

LOD and LOQ were experimentally verified by diluting known concentration of filixic acid until the average response were approximately three to ten times the standard deviation of response for six replicate determinations.

Precision was determined as the intra-day and inter-day variation of results from analysis of three different standard solutions. Intra-day precision was determined by triplicate analysis of each solution on a single day. Inter-day precision was determined by triplicate analysis of the solutions on two successive days.

The accuracy of the method was determined by application of the standard addition method. Known amounts of the standard (1ml of 10, 50, and 100 µg/ml standard solutions) were added to the 1 ml of extract (sample stock solution) and analyzed in triplicate as described above. The total amount of filixic acid was calculated from the corresponding calibration plot and the recovery of filixic acid was calculated by using following equation:

Recovery (%) = (amount found - amount contained) / amount added x 100

3. RESULT AND DISCUSSION

3.1 Characterization of Isolated Compound

Isolated compound was pale yellow coloured flaks. Melting point of isolated compound was 184-186°C. In UV spectroscopic study compound under consideration shows UV maxima at 278 and 218 nm. IR spectra was measured in KBr and shows value at 3150 (-OH), 2950 (=CH), 1640-1610 (C=O), 1430 and 1193 cm^{-1}. Mass spectroscopy shows parent peak at 640.93 m/z and other peak at 432,404, 236, 193, 181, I65 m/z[11,12]. The NMR spectral analysis showed signal at 0.97 (3H, t), 1.11 (6H, t), 1.38 (6H, s), 1.40 (6H, s), l.74 (2H, m), 3.15 (6H, t), 3.47 (4H, s), 9.90 (2H, s), 11.29 (1H, s), 12.72 (1H, s), 15.61 (1H, s), 17.77 (2H, s). NMR spectral data is in resemblance with reported work by Hisada S, *et. al.*[12]. TLC study was done for isolated compound by using Toluene: Ethyl acetate (9:1) as solvent system and vanillin-sulphuric acid as a derivatizing agent. Filixic acid PBP showed R_f value at 0.56. Above results confirmed that isolated compound was filixic acid PBP.

3.2 Validation of chromatographic method

In HPLC study filixic acid PBP was resolved with retention time (R_t) at 4.03. Reproducible results were obtained for each of three samples. Validation of HPLC method was done for filixic acid PBP. Chromatogram obtained from standard solution shown good resolution.

3.2.1 Linearity

The linearity of the calibration curve for filixic acid PBP (10, 20, 30, 40 and 50 µg/ml concentration) was good (r = 0.993) over the concentration range investigated. Calibration curve

were prepared by plotting peak area *vs* concentration of filixic acid PBP. 20 µl standard solutions were injected and respective peak areas were recorded by following regression equation.

$$Y = 6871X + 28428.$$

Where, Y is peak area and X is concentration.

3.2.2 Sensitivity

LOD and LOQ were 2.25µg /ml and 7.52 µg/ml respectively, which were calculated by using the following formulae.

$$LOD = 3.3\ \sigma / S \text{ and } LOQ = 10\ \sigma / S.$$

Where σ is the standard deviation of the response and S is the slope of the calibration plot.

3.2.3 Precision

Analysis of filixic acid PBP was performed at different solution of 10, 20 and 30 µg/ml concentrations. No significant intra-day and inter-day variation was observed. Relative standard deviation (RSD) was always less than 1.5 % (Table I).

3.2.4 Recovery

For all drug samples after spiking with standard solution of 10, 50 and 100 µg/ml concentration, the method shown total recovery of filixic acid PBP, between 94.57 to 101.05 % (Table II)

3.3 Sample analysis

Isolated filixic acid PBP shown purity above 98 %. The retention time of filixic acid PBP was 4.07 min (Figure 1). All samples of *D. filix-mas* were analyzed under the same chromatographic condition. The analysis, including sample preparation, were performed in triplicate (Table III).

CONCLUSIONS

In conclusion, appreciable amount of filixic acid PBP from *D. filix-mas* has been isolated by above method and its structure was confirmed by UV, IR, NMR and Mass spectroscopic studies.

A simple, rapid, reproducible, and sensitive HPLC method has been developed for analysis of filixic acid PBP first time. The method was validated for linearity, LOD, LOQ, precision, inter-day and intra-day variation, and recovery study. Method is suitable for processing of many samples in limited time for day to day analysis, pharmacokinetic and bioequivalence studies.

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Table I. Precision, repeatability and stability of filixic acid PBP

Analyte		Precision (RSD, %)				Repeatability (n=3)		Stability
		Intra-day (n=3)		Inter-day (n=3)		Mean content (mg $g^{-}1$)	RSD of P_a (%)	RSD of P_a (%)
		R_t	P_a	R_t	P_a			
Filixic acid PBP	10 µg mL^{-1}	0.10	1.12	0.11	1.09	1.04	1.10	0.89
	20 µg mL^{-1}	0.13	0.89	0.11	0.25	0.91	0.92	0.71
	30 µg mL^{-1}	0.19	0.95	0.20	0.15	0.98	0.93	0.85

R_t is retention time and P_a is peak area

Table II. Recovery of filixic acid PBP from *D. filix-mas* Rhizome.

Analyte	Contained (µg)	Added (µg)	Found (µg)	Recovery (%)	Mean (%)	RSD (%)
Filixic acid PBP	42.99	100	134.90	95.05	97.95	2.25
	42.99	100	137.11	95.89		
	42.99	100	135.23	94.57		
	42.99	50	91.19	98.07		
	42.99	50	91.24	98.12		
	42.99	50	91.23	98.10		
	42.99	10	52.93	99.90		
	42.99	10	53.65	101.05		
	42.99	10	53.50	100.80		

Table III. Content (%) of filixic acid PBP in *D. filix-mas*

Sample	Filixic acid PBP content (%)
1	0.172
2	0.186
3	0.190

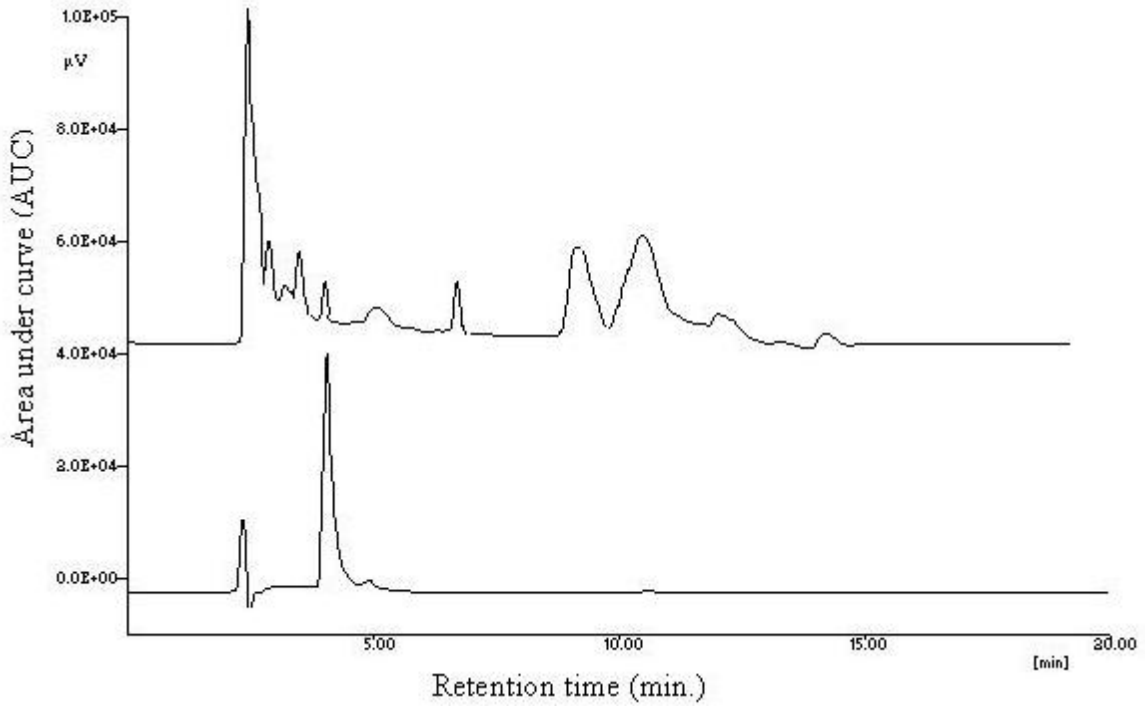

Figure 1. HPLC chromatograms for filixic acid PBP (lower chromatogram) and methanol extract of *D. filix-mas* (upper chromatogram).