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Quantification of β-Sitosterol and Puerarin from *Pueraria tuberosa* DC. by Using High Performance Thin Layer Chromatography

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Sensitive, simple and accurate high-performance thin-layer chromatographic methods have been established for determination of β-sitosterol and puerarin in root powder of *Pueraria tuberosa* DC. Methanolic extracts of root powder were used for the experimental work. β-Sitosterol and puerarin were found to be 0.03 and 2.45 %, respectively, in root powder of *Pueraria tuberosa* DC., by the proposed methods. Separation was performed on aluminium-backed silica gel 60 F_{254} HPTLC plate for both the standards. Detection and quantification were performed by densitometry at λ = 257 nm and λ = 366 nm for puerarin and β-sitosterol, respectively. Puerarin response was linear over the range 20 to 80 μg mL^{-1} where as β-sitosterol response was linear over the range 10 to 60 μg mL^{-1}. The validated HPTLC methods can be used for a routine quality control analysis of *Pueraria tuberosa* DC. and quantitative determination of puerarin and β-sitosterol.

Key Words: Quantification, β-Sitosterol, Puerarin, *Pueraria tuberosa* DC., HPLC.

INTRODUCTION

In Ayurveda, 'Vidari' is botanically equated to *Pueraria tuberosa* DC. of Fabaceae family. Reported chemical constituents in *Pueraria tuberosa* DC. are puerarin[1], β-sitosterol[1] and tuberosin[2]. *Pueraria tuberosa* is a large, perennial climber with very huge tuberous roots, used to treat many pharmacological activities of which antihepatotoxic activity[3], anti-implantation activity in rats[4], *etc*. Simple, rapid, economical, precise and accurate HPTLC methods have been established for determination of puerarin and β-sitosterol in *Pueraria tuberosa* DC.

EXPERIMENTAL

Analytical grade ethyl acetate, toluene and methanol were obtained from Qualigens Fine Chemicals, Mumbai, India. Standards, puerarin and β-sitosterol were procured from Sigma- Aldrich Chemie (Steinheim, Germany).

Roots of *Pueraria tuberosa* DC. were collected from Thane, India and were authenticated by the National Botanical Research Institute (NBRI), Council of Scientific

and Industrial Research, Lucknow, India. The authenticated herbarium is preserved in duplicate, one at NBRI and another at the place of research, *i.e.* Ramnarain Ruia College, Mumbai, India, for future reference.

Standard and sample preparation: Stock solution of puerarin (1000 μg mL^{-1}) was prepared by dissolving 10.0 mg accurately weighed puerarin in methanol and diluting to 10.0 mL with methanol. Aliquots (0.2 mL to 0.8 mL) of this stock solution were transferred to 10 mL volumetric flasks and the volume of each was made up to 10 mL with methanol to obtain working standard solutions containing 20 to 80 μg mL^{-1}.

Stock solution of β-sitosterol (1000 μg mL^{-1}) was prepared by dissolving 10.0 mg accurately weighed puerarin in methanol and diluting to 10.0 mL with methanol. Aliquots (0.1 mL to 0.6 mL) of this stock solution were transferred to 10 mL volumetric flasks and the volume of each was made up to 10 mL with methanol to obtain working standard solutions containing 10 to 60 μg mL^{-1}.

Roots of *Pueraria tuberosa* DC. were collected, washed, dried in the shade, powdered and the powder was passed through an 80-mesh sieve and stored in an airtight container at 25 °C. Solutions of different concentration of root powder were prepared for quantification of puerarin and β-sitosterol. 20 mg and 1 g of dried powder was accurately weighed and placed in stoppered tubes and 10 mL of methanol was added in both, the samples were vortexed for 1-2 min and left to stand overnight at room temperature. The contents of the tubes were filtered through Whatmann No. 41 paper (E. Merck, Mumbai, India). The clear supernatants were collected in dry volumetric flask. 2 mg/mL solution of root powder was used for the assay of puerarin while 100 mg/mL solution of root powder was used for the assay of β-sitosterol.

A Camag Linomat IV sample applicator was used for sample application. Camag Twin trough glass chamber (20 cm × 10 cm) was used for development of plates. Camag TLC scanner II equipped with cats 3 Version software was used for interpretation of data.

Chromatography

Procedure: Chromatography was performed on aluminium-backed HPTLC plates precoated with 0.2 mm layers of silica gel 60 F_{254} (Merck# 5554); prewashing of plates was carried out using methanol and plates were dried[5] in oven for 15 min. Samples (10 μL) were applied on the plates as bands of 8 mm width with the help of a Camag Linomat IV automatic sample applicator at the distance of 10 mm from the bottom edge of the plates.

For puerarin, plate was developed, at room temperature, with ethyl acetate: methanol:distilled water in the ratio 80:10:10 (v/v/v) as mobile phase in a Camag (Muttenz, Switzerland) glass twin-trough chamber, previously equilibrated with mobile phase for 5 min. The development distance was 8.0 cm. The plates were scanned at λ = 257 nm by means of Camag TLC Scanner and CATS3 software.

For β-sitosterol, plate was developed, at room temperature, with ethyl acetate: toluene in the ratio 90:10 (v/v/v) as mobile phase in a the Camag (Muttenz, Switzerland) glass twin-trough chamber, previously equilibrated with mobile phase for 5 min. The development distance was 8.0 cm. After development, the plate was dried at room temperature and derivatized with freshly prepared Libermann-Burchard reagent in a derivatization chamber for 20 s and again dried at room temperature. After drying, the plate was heated in oven at 105 ºC for 10 min before densitometric scanning. The plates were scanned at λ = 366 nm by means of Camag TLC Scanner and CATS3 software.

The chromatographic conditions were previously optimized to achieve the best resolution and peak shape for both puerarin and β-sitosterol. A typical HPTLC chromatogram of puerarin standard and plant is shown in Fig. 1 where as of β-sitosterol and plant is shown in Fig. 2. The chromatographic plate of puerarin standard and *Pueraria tuberosa* DC. root powder is shown in Fig. 3. The chromatographic plate of β-sitosterol standard and *Pueraria tuberosa* DC. root powder is shown in Fig. 4.

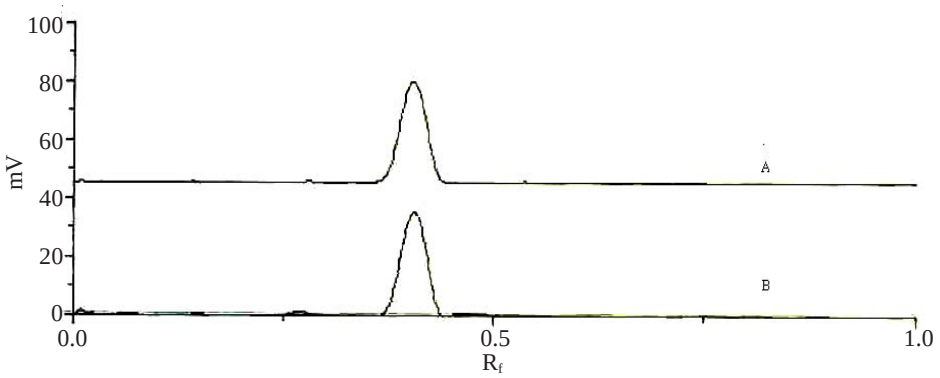

Fig. 1. Overlain HPTLC chromatogram of puerarin and *Pueraria tuberosa* DC. (A) Standard (Puerarin); (B) Sample (*Pueraria tuberosa* DC.)

Linearity of detector response: Solutions containing puerarin at 7 different concentrations (20, 30, 40, 50, 60, 70, 80 μg mL^{-1}) were prepared in methanol. β-Sitosterol solutions at 6 different concentrations (10, 20, 30, 40, 50, 60 μg mL^{-1}) were prepared in methanol. The above puerarin and β-sitosterol solutions (10 μL) were applied to different plates. The plates were developed as per the chromatographic condition mention in procedure. The detector response for the different concentrations were measured. Graphs were plotted of standard peak area against concentration of puerarin and similarly for β-sitosterol. The plot was linear in the range 20 to 80 μg mL^{-1} for puerarin and 10 to 60 μg mL^{-1} for β-sitosterol. The experiments were performed three times and the mean was used for the calculations. The linearity data is given in Table-1.

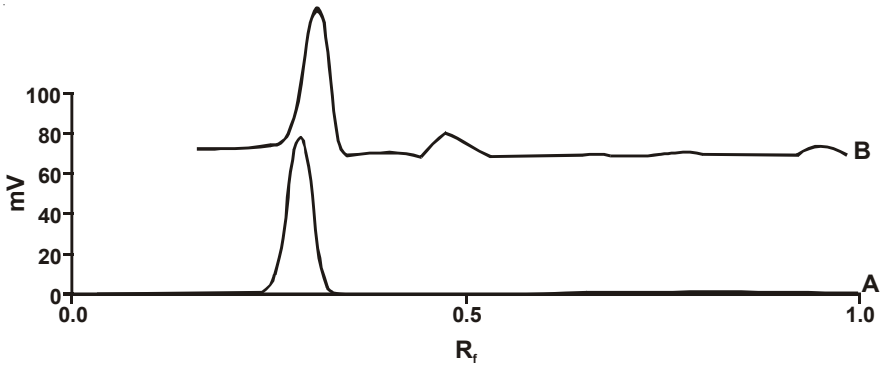

Fig. 2. Overlain HPTLC chromatogram of β-sitosterol and *Pueraria tuberosa* DC. (A) Standard (β-sitosterol); (B) Sample (*Pueraria tuberosa* DC.)

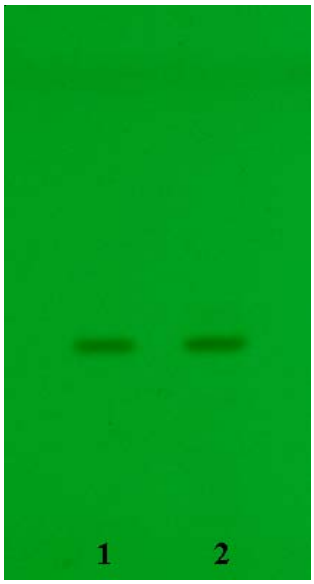

Fig. 3. Photograph of developed plate at 257 nm; Track 1) Sample (*Pueraria tuberosa* DC.); 2) Standard (Puerarin)

Fig. 4. Photograph of developed plate at 366 nm; Track 1) Standard (β-sitosterol); 2) Sample (*Pueraria tuberosa* DC.)

Assay: 10 µL of the solution of standard puerarin (50 µg mL^{-1}) and sample solutions were spotted on a HPTLC plate. The amount of puerarin present in this solution was calculated by comparison of area measured for the sample to that for the standard. Similarly, 10 µL of the solution of standard β-sitosterol (30 µg mL^{-1})

TABLE-1
LINEARITY DATA FOR PUERARIN AND β-SITOSTEROL

Data	Puerarin	β-Sitosterol
Linearity range (μg mL^{-1})	20 to 80	10 to 60
Slope (m)*	15.34	27.64
Intercept (c)*	19.78	4.39
Correlation coefficient (R)	0.9988	0.9999
LOD (μg mL^{-1})	10	5
LOQ (μg mL^{-1})	20	10
Instrument precision (RSD [%], n = 10)	0.21	0.05
Intraday precision (RSD [%], n = 3)	0.17	0.06
Interday precision (RSD [%], n = 3)	0.18	0.06

*Of the equation y = mx + c, where y is peak area, m is the slope, x is the concentration and c is the intercept.

and sample solutions were spotted on a HPTLC plate. The amount of β-sitosterol present in this solution was calculated by comparison of area measured for the sample to that for the standard. The assay procedures described earlier were repeated seven times starting from weighing of the whole plant powder. The amount of puerarin and β-sitosterol found in root powder of *Pueraria tuberosa* was 2.45 and 0.03 %, respectively.

Accuracy: The accuracy of the methods was established by performing recovery experiments by the standard addition method. The recovery of the standard puerarin and β-sitosterol added to *Pueraria tuberosa* DC. root powder individually, was studied at 2 different levels, each being analyzed in a manner similar to that described for the assay. The contents of puerarin and β-sitosterol were quantified by the proposed methods and the percentage recovery was calculated. The results are listed in Table-2A and in Table-2B for puerarin and β-sitosterol respectively. The recovery obtained for both the standards was from 99 to 103 %, showing the reproducibility of the methods was good. The average recovery was found to be 102.10 % for puerarin and 100.51 % for β-sitosterol.

RESULTS AND DISCUSSION

Of the different mobile phases investigated, ethyl acetate:methanol:distilled water 80:10:10 (v/v/v), resolved puerarin (R_f = 0.40) very efficiently from the other components of the methanolic extract of *Pueraria tuberosa* DC. The response to puerarin was found to be linearly dependent on concentration in the range 20 to 80 μg mL^{-1}, with correlation coeffient of 0.9988.

Ethyl acetate:toluene 90:10 (v/v), resolved β-sitosterol (R_f = 0.35) efficiently from the other components of the methanolic extract of *Pueraria tuberosa* DC. The response to β-sitosterol was found to be linearly dependent on concentration in the range 10 to 60 μg mL^{-1}, with correlation coefficient of 0.9999.

TABLE-2A
RESULTS FROM RECOVERY ANALYSIS FOR PUERARIN

Amount of puerarin in preanalyzed sample (mg)	Amount of puerarin added to preanalyzed sample (mg)	Total amount of puerarin found (mg)	SD	RSD [%] n = 7	Recovery (%)
0.49	0.00	0.50	0.0019	0.38	102.25
0.49	0.05	0.55	0.0014	0.25	101.62
0.49	0.10	0.61	0.0020	0.34	102.42
Mean	–	–	–	–	102.10

TABLE-2B
RESULTS FROM RECOVERY ANALYSIS FOR β-SITOSTEROL

Amount of β-sitosterol in preanalyzed sample (mg)	Amount of β-sitosterol added to preanalyzed sample (mg)	Total amount of β-sitosterol found (mg)	SD	RSD [%], n = 7	Recovery (%)
0.30	0.00	0.2996	0.0777	0.26	99.86
0.30	0.15	0.4510	0.0387	0.09	100.21
0.30	0.30	0.6088	0.0707	0.12	101.47
Mean	–	–	–	–	100.51

The variability of the methods was studied by analyzing aliquots of the different concentrations of puerarin and β-sitosterol solutions on the same day (intra-day precision) and on different days (inter-day precision) and by instrument precision. The results were expressed as % RSD. The % RSD values were found to be less than 2 %, indicating that the selected methods are precise and reproducible. The mean recovery of puerarin was found to 102.10 % and of β-sitosterol was found to 100.51 % which indicates the accuracy of the methods.

The robustness of the methods was studied, during method development, by determining the effects of small variation, of mobile phase composition (± 2 %), duration of plate pre-washing, chamber saturation period, development distance and scanning time (10 % variation of each). No significant change in R_f or in response of puerarin and β-sitosterol was observed, indicating the robustness of the methods.

Conclusion

The proposed method is simple, rapid, precise, accurate and economic and can be used for routine quality-control analysis of *Pueraria tuberosa* DC. root powder and for quantitative determination of puerarin and β-sitosterol from root powder.

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