ISSN 0975 - 6299 Vol 2 / Issue 4 / Oct - Dec 2011

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE

ANALYTICAL CHEMISTRY

SIMULTANEOUS QUANTIFICATION OF B-AMYRIN AND STIGMASTEROL IN *PUTRANJIVA ROXBURGHII* WALL. BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

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ABSTRACT

HPTLC method for simultaneous determination of β-amyrin and stigmasterol in *Putranjiva roxburghii* wall. has been developed and validated. The analytes were separated on silica gel $60F_{254}$ HPTLC plates with n - hexane: chloroform: methanol (3: 6.5:0.5 v/v/v) as mobile phase after chamber saturation for 10 min. The development distance was 80mm. The derivatization was done by using anisaldehyde – sulphuric acid reagent. Detection and quantification were performed by densitometry, with a tungsten lamp, at 580 nm. The response to β-amyrin and stigmasterol was linear in the concentration range 0.045 to 0.360 µg per band and 0.041 to 0.328 µg per band respectively. The validated method was used for quantitative analysis of β-amyrin and stigmasterol in *Putranjiva roxburghii* wall. and can be used for routine quality-control analysis of leaf powder of *Putranjiva roxburghii* wall.

ISSN 0975 - 6299 Vol 2 / Issue 4 / Oct - Dec 2011

KEYWORDS

Putranjiva roxburghii wall., β-amyrin, stigmasterol, HPTLC, Densitometry.

INTRODUCTION

Putranjiva roxburghii wall. is a medicinal plant found throughout India. Leaves are bitter, astringent, refrigerant and procreant. The leaves are useful in the treatment of catarrh, skin disease, fever and sterility. The leaves are given in decoction for cold and fever, and are also used in rheumatism.[1] *Putranjiva roxburghii* wall. is one of the constituents of "Y-Spur" an ayurvedic formulation, prepared by Vilco Laboratories, which is found to be very effective in male infertility.[2] *Putranjiva roxburghii* wall. contains bioconstituents like β-amyrin, stigmasterol, putrol, putrone, putranjivic acid, putraflavone, amentoflavone, saponins- A, B, C, D.[3] B-amyrin has anti-inflammatory[4], antifungal activity [5]. It has protective effect against acetaminophen-induced liver injury of mice[6]. Stigmasterol has potential anti-osteoarthritic property[7], antihypercholesterolemic activity [8], and antimutegenic activity [9], hence in the present research work, simultaneous quantitation of β-amyrin and stigmasterol has been carried out.

Column chromatography of *Putranjiva roxburghii* wall. is reported in literature and the solvents used for β-amyrin and stigmasterol were light petroleum-benzene (1:3) and benzene respectively[10]. The HPTLC method for determination of amentoflavone in *Putranjiva roxburghii* wall. has been reported in literature.[11] However HPTLC method for simultaneous quantitation of β-amyrin and stigmasterol, has not been reported in literature. Densitometric HPTLC has been widely used for the phytochemical evaluation of the herbal drugs, due to its simplicity and minimum sample clean up required. Hence densitometric HPTLC method has been developed in the present research work for simultaneous qauntitation of β-amyrin and stigmasterol from methanol extract of dry leaf powder of *Putranjiva roxburghii* wall.

EXPERIMENTAL

Reagents, Chemicals and Standards

Analytical grade chloroform (purity 99.5%), hexane (purity 99.0%), methanol (purity 99.9%) were obtained from Qualigens Fine Chemicals (Mumbai, India). Standard β-amyrin (98.5%), and Stigmasterol (95.0%) was procured from Sigma-Aldrich Chemie GmbH (Aldrich Division, Steinbeim, Germany).

Plant material

Putranjiva roxburghii wall. was collected from Keshav shrushti, Mumbai, India and authenticated by Botanical Survey of India, Pune. The leaves of *Putranjiva roxburghii* wall. were washed with water to remove soil particles, dried in the shade, and finely powered. The powder was passed through an 85 – mesh sieve and stored in an airtight container at room temperature (28 ± 2^0 C).

Preparation of stock and working standard solution of β-amyrin and stigmasterol

About 1.5 mg of β-amyrin was accurately weighed and transferred to 10.0 cm^3 standard volumetric flask. 5.0 cm^3 of methanol was added and the contents were sonicated for 5 min. and then diluted with methanol to give a solution of β-amyrin containing 0.15 mg/ cm^3 of β-amyrin. 1.0 cm^3 of β-amyrin was then transferred to 10.0 cm^3 volumetric flask and content was diluted up to 10.0 cm^3 by methanol to give stock solution of β-amyrin containing 0.015 mg/ cm^3 of β-amyrin. 3.0µl, 6.0µl, 9.0µl, 12.0µl, 15.0µl, 18.0µl, 21.0µl and 24µl were applied on TLC plate to obtain standard solution of 0.045 µg per band to 0.360 µg per band.

About 12.5 mg of stigmasterol was accurately weighed and transferred to 50.0 cm^3 standard

volumetric flask. 10.0 cm^3 of methanol was added and the contents were sonicated for 5 min. and then diluted with methanol to give a solution of stigmasterol containing 0.25 mg/cm^3 of stigmasterol. 5.5cm^3 of stigmasterol was then transferred to 100.0 cm^3 volumetric flask and content was diluted up to 100.0 cm^3 by methanol to give stock solution of stigmasterol containing 0.0137 mg/ cm^3 of stigmasterol. 3.0µl, 6.0µl, 9.0µl, 12.0µl, 15.0µl, 18.0µl, 21.0µl and 24µl were applied on TLC plate to obtain standard solution of 0.041µg per band to 0.328 µg per band.

Sample preparation

The powder of the leaves of *Putranjiva roxburghii* wall. was accurately weighed (1000mg) and vortex mixed with 10.0 cm^3 of methanol for 15 minutes. The extract was filtered through a Whatman no. 1 qualitative filter paper, pore size 11 µm. The contents were then evaporated to dryness and final volume was adjusted to 5.0 cm^3 with methanol in a volumetric flask.

CHROMATOGRAPHY

Chromatography was performed on 20.0 x 10.0 cm HPTLC plate coated with 200 µm layers of silica gel $60F_{254}$ (E. Merck). Standard and sample solutions were applied to the plates as 7mm bands, 6mm from each other and 10 mm from bottom edge of the plate by means of a CAMAG Automatic TLC sampler 4 (ATS4). The plates were developed to a distance of 80 mm from the bottom edge of the plates with n-hexane-chloroform-methanol 3.0:6.5:0.5(v/v/v) as a mobile phase, in a Camag glass twin – trough chamber, equilibrated with mobile phase for 10 min. The derivatization was done by using anisaldehyde – sulphuric acid reagent. Densitometric scanning was performed at 580 nm with CAMAG TLC scanner using tungsten lamp. The scanned data were processed using win CATS software version 1.4.4.

METHOD VALIDATION

Liner Working Range for β-amyrin and Stigmasterol

Working standard solutions of β-amyrin and stigmasterol in the concentration range of 0.045 to 0.360 µg per band and 0.041 to 0.328 µg per band respectively were applied, in triplicate, to three different plates and developed and scanned using the optimized conditions described above. The densitograms were then acquired and the peak areas were recorded for each concentration of β-amyrin and stigmasterol. Percent relative standard deviation of β-amyrin and stigmasterol peak area for solutions of the same concentration were less than 2, indicating there was no statistically significant variation. The calibration plot for β-amyrin and stigmasterol were linear in this concentration range with correlation coefficient (r) of 0.9994 and 0.9998 respectively.

System Suitability

System Suitability test was conducted to determine whether the method gave accurate results. B-amyrin and stigmasterol standard solution of (0.225 µg per band and 0.205 µg per band) was applied six times to same TLC plate and analysed using the optimized conditions. The RSD of β-amyrin and stigmasterol were 0.29% and 0.46% respectively, hence the system is suitable for the analysis.

Limit of Detection (LOD) and Quantification (LOQ)

The limit of detection (LOD) and limit quantification (LOQ) were determined at signal to noise ratios of 3:1 and 10:1, respectively. These values obtained for β-amyrin were 0.045 and 0.09 µg per band and stigmasterol were 0.041 and 0.082 µg per band respectively.

Precision

The method was validated for instrumental precision, intra assay precision, and intermediate precision. Instrumental precision was studied by repeated analysis (n =10) of β-amyrin and stigmasterol standard solution (0.225 µg per band and 0.205 µg per band

respectively) on the same day. The values of relative standard deviation for instrumental precision were 0.57% and 0.50 % respectively. Intra assay precision (repeatability) was measured on one day by analyzing sample solution by accurately weighing approximately 1000.0 mg dried leaf powder six times and extracting with methanol as described above. Each sample solution was applied in triplicate, on the same plate and analysed under the conditions described above. Values of Intra-assay precision, expressed as relative standard deviation of β-amyrin and stigmasterol peak area were 0.81% and 0.80% respectively. Intermediate precision was determined in the same way as intra- assay precision, but on three successive days. Intermediate precision as relative standard deviation for β-amyrin peak areas, on three different days, was 0.52, 0.70, and 0.80% respectively and for stigmasterol 0.52, 0.70 and 0.97% respectively.

Specificity

The specificity of the proposed HPTLC method was ascertained by overlaying uv spectra of β-amyrin and stigmasterol standard and sample. The β-amyrin and stigmasterol bands of both were compared at three different positions, the peak start, peak centre and peak end. There was good correlation between both spectra obtained at each of the three positions on the β-amyrin and stigmasterol bands. The β-amyrin, stigmasterol peak was therefore, not masked by any peak of other components present in the sample (fig 1, 2), which was indicative of their peak purities.

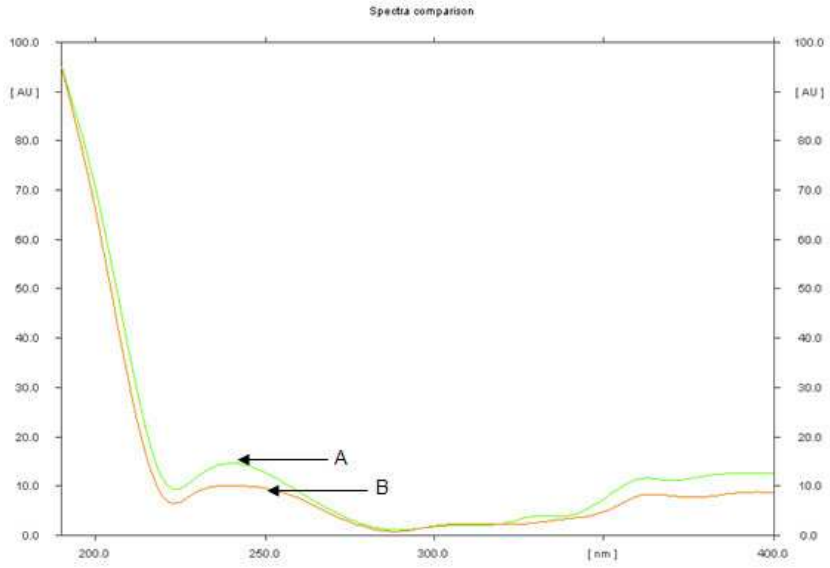

Figure 1

Overlay of spectra of β-amyrin standard solution (A) and β-amyrin in extract of Putranjiva roxburghii wall. leaf powder (B).

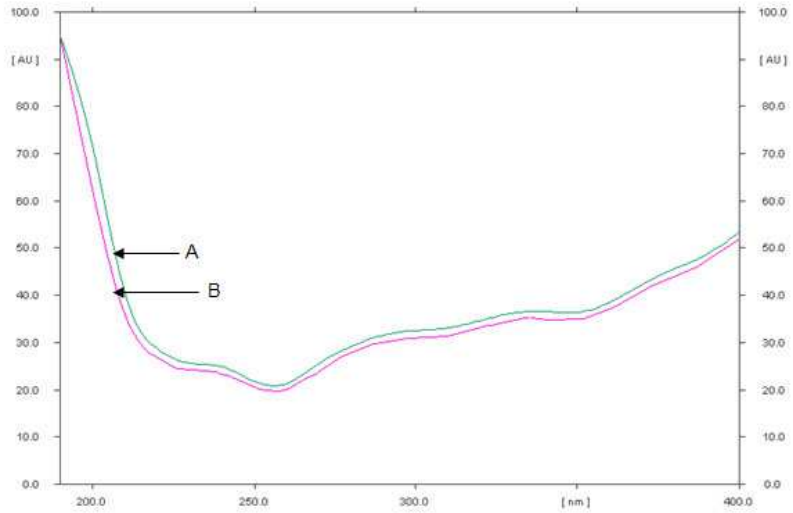

Figure 2
Overlay of spectra of stigmasterol standard solution (A) and stigmasterol in extract of Putranjiva roxburghii wall. leaf powder (B).

Table 1
Recovery of β-amyrin and stigmasterol in Putranjiva roxburghii wall. leaf powder by the proposed method

Level	0	1	2	**3**
Mass of sample (mg)	1000.2	1000.4	1000.6	1000.1
Mass of β-amyrin added to Sample (mg)	0.0	0.073	0.091	0.109
Mass of stigmasterol added to sample (mg)	0.0	0.067	0.083	0.1
Mean amount of β-amyrin found in sample (mg)	0.073	0.143	0.162	0.180
Mean amount of Stigmasterol found in sample (mg)	0.067	0.132	0.149	0.165
Mean Mean Recovery of β-amyrin (%)			97.6 %	
Mean Recovery of Stigmasterol (%)			98 %	

Accuracy

The accuracy of the method was established by measurement of recovery at three different levels, using the standard addition method. *Putranjiva roxburghii* wall. leaf powder (approx. 1000.0 mg) was accurately weighed in four different 10.0 cm^3 standard volumetric flask. 0.0 mg, 0.073 mg, 0.091 mg, 0.109 mg of β-amyrin and 0.0 mg, 0.067 mg, 0.083 mg, 0.1 mg of stigmasterol was added to respective flasks. The samples were extracted as described in section 2.4. Seven replicate analyses were performed on each extract and the amount of β-amyrin and stigmasterol recovered from the sample was determined for each level. Percentage recovery was then determined. The results are listed in table1

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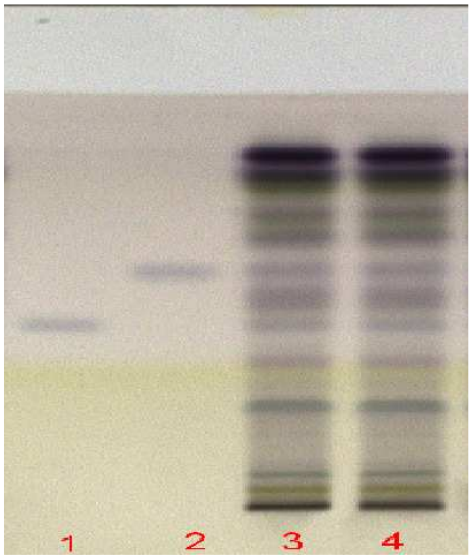

Figure 3

Photograph of developed TLC plate showing chromatography of stigmasterol (1), β-amyrin (2) and methanol extract of leaf powder of Putranjiva roxburghii wall. (3, 4)

Estimation of β-amyrin and stigmasterol in Dried Leaf powder of Putranjiva roxburghii wall.

Seven replicate of *Putranjiva roxburghii* wall. sample solution (prepared as described in section 2.4, 10 µl each) were analyzed by the HPTLC method and peak areas and mean peak areas for β-amyrin and stigmasterol were recorded. The identity of β-amyrin and stigmasterol band from sample solution was confirmed by comparison of their R_f with that from β-amyrin and stigmasterol standard. Fig-3 shows a developed TLC plate illustrating chromatography of β-amyrin and stigmasterol standard and separation of an extract of dried leaf powder of *Putranjiva roxburghii* wall. From the calibration plot, the mean amount of β-amyrin and stigmasterol present in dried leaf powder of *Putranjiva roxburghii* wall. were found to be 0.072 mg/g and 0.066 mg/g respectively.

RESULTS AND DISCUSSION

Different mobile phases were investigated for simultaneous HPTLC separation of β-amyrin and stigmasterol from other components of the dried leaf powder of *Putranjiva roxburghii* wall. Use of Hexane: Chloroform: Methanol (3:6.5:0.5 v/v/v) resulted in good separation of β-amyrin and stigmasterol from other phyotochemicals present in the extract. When the method was validated for instrumental precision, intra-assay precision, and intermediate precision, percentage relative standard deviation was less than 2, indicating the proposed method is precise and

repeatable. To achieve quantitative extraction, conditions used in extraction procedure, like nature and volume of extracting solvent and time of extraction, were optimized by use of the HPTLC method. The identity of the β-amyrin and stigmasterol band from the sample solution was confirmed by overlaying the chromatogram obtained from sample and reference standard, and by comparing the Rf of β-amyrin and stigmasterol in the sample with that of β-amyrin and stigmasterol standard (fig 1 and 2). The response to β-amyrin and stigmasterol were found to be a linear function of concentration in the range 0.045 to 0.360 µg per band and 0.041 to 0.328 µg per band respectively. The correlation coefficient for β-amyrin and stigmasterol were 0.9994 and 0.9998 respectively. The average β-amyrin and stigmasterol content were found to be 0.072 mg/g and 0.066 mg/g respectively.

CONCLUSION

The method used in this research work is accurate, sensitive, and can be used as a routine quality-control method for simultaneous quantification of β-amyrin and stigmasterol in the leaf powder of *Putranjiva roxburghii* wall.

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