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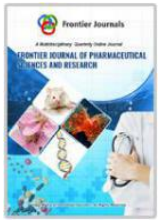

Original Research Paper

VALIDATION OF A HPTLC- DENSITOMETRY METHOD FOR THE QUANTITATIVE DETERMINATION OF ANXIOLYTIC CONSTITUENTS IN *STELLARIA MEDIA* LINN.

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ABSTRACT

A quantitative TLC-densitometry method was developed for the determination of anxiolytic constituents of the plant — *Stellaria media* Linn. (Caryophyllaceae) traditionally used for the treatment of mental tension and inflammations of the digestive, renal, respiratory and reproductive tracts.

Objective: To determine anxiolytic constituents of *Stellaria media* Linn. by quantitative TLC-densitometry.

Methods: The separation was achieved on precoated TLC silica gel $60F_{254}$ using toluene-chloroform-methanol (6.8:2.2:1, v/v/v) as mobile phase. The plates were developed vertically up to a distance of 80 mm. TLC-densitometry method was successfully validated for linearity, precision, accuracy, robustness, limit of detection (LOD) and quantitation (LOQ).

Results: The investigation carried out revealed that contents of the two bioactive markers A1 (6-methylheptyl-3′-hydroxy-2′-methylpropanoate) and A2 (2,2,4-trimethyloctan-3-one) in the plant, were found to be 0.180 and 0.078 % w/w respectively.

Conclusion: The proposed method was found to be simple, precise, specific, sensitive, and accurate.

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1. INTRODUCTION

S. media Linn. (Chickweed) is a winter annual herb,[1] found throughout the Himalayas up to altitudes of 4,300 m.[2] It is well known as an invasive weed in gardens, fields and grounds.[3] The plant is reported to be very useful in the treatment of mental tension and inflammations of the digestive and reproductive tracts. The plant is employed in plasters used for broken bones and swellings.[4] It also possesses anxiolytic,[5] diuretic, expectorant and antiasthmatic properties.[6] The plant contains phenolic acids, flavonoids,[7] C-glycosyl flavones,[8] triterpenoid saponins,[9]pentasaccharide,[10]lipids[6] and aqueous constituents.[11]

Different chromatographic methods are used to analyze the marker compounds in herbs with the help of modern sophisticated tools and HPTLC is one such best technique.[12-15] HPTLC is the method commonly applied for the identification assay and stability study of herbal raw materials and formulations. HPTLC has several advantages over HPLC[16-17] such as: (i) samples and standards both are simultaneously processed under the same conditions leading to better analytical precision and accuracy, (ii) mobile phase consumption per sample is extremely low, (iii) thus reducing the acquisition and disposal cost, (iv) there is no possibility of interference from previous analysis as fresh stationary phase is used for each analysis, (v) instrumentation is simple, inexpensive and easy to handle and (vi) many samples can be analyzed at the same time. Therefore, HPTLC is rapidly gaining importance in pharmaceutical analysis, biochemistry and pharmacokinetics studies, but to the best of our knowledge there is no report on the quantitative determination of bio-constituents of *S. media*.

HPTLC analysis of many plants used in Indian system of medicine has been performed.[18-19] Hence, in the present investigation an attempt has been made to develop a validated rapid, simple, precise and accurate method for simultaneous quantification of the two bioactive markers — A1 (6-methylheptyl-3′-hydroxy-2′-methylpropanoate) and A2 (2,2,4-trimethyloctan-3-one) in *S. media.*

2.MATERIAL AND METHODS

Plant material

Aerial parts of *S. media* were collected from around the U.I.P.S. building, Panjab University, Chandigarh in February 2009. The identity of the plant was confirmed by Dr. H.B. Singh, Head, Raw Materials, Herbarium & Museum at the National Institute of Science Communication and Information Resources, (NISCAIR, CSIR), New Delhi 110 067. A voucher specimen no: NISCAIR/RHMD/Consult/2008-09/1170/202 is deposited in the same herbarium.

Solvents, reference standards and instrumentation

Markers A1 and A2 were isolated in the laboratory from *S. media* and identified by various spectral techniques.[20] All other solvents used for chromatographic analysis were of analytical grade (E. Merck Ltd., Mumbai, India). TLC scanner, winCATS-III, twin trough chamber, TLC plate heater and Linomat-IV applicator (Camag, Switzerland) were used for digital image scanning, method development and validation.

Standard stock solutions and sample preparation

Preparation of standard stock solution of A1

A1 (10 mg) was dissolved in 1 ml of methanol in a 2 ml volumetric flask, and the volume was made up to 2 ml to obtain 5 mg/ml stock solution. From this stock solution, four working standard solutions were obtained by appropriate dilution with methanol. The concentrations of working standard solutions were 1, 2, 3 or 4 mg/ml.

Preparation of standard stock solution of A2

A2 (6 mg) was dissolved in 3 ml of methanol to obtain 2 mg/ml stock solution. From this stock solution, four working standard solutions were obtained by appropriate dilution with methanol. The concentrations of working standard solutions were 0.25, 0.5, 1 or 1.5 mg/ml.

Preparation of test sample

Accurately weighed dried aerial parts of *S. media* (1 g each), packed in filter paper sachets, were placed in three separate 100 ml round bottom flasks and allowed to macerate for 30 min with methanol (50 ml each) followed by refluxing (30 min) on a boiling water bath. The methanol extracts were pooled, and solvent was recovered under vacuum. Dried methanol extract was reconstituted in methanol, in a volumetric flask, and its volume was made up to 10 ml.

Chromatographic procedures

Chromatography was performed on a precoated TLC plates (10 cm×10 cm or 20 cm×10 cm). The plates were washed with methanol and activated at 120 °C for 20 min. Standards (A1 and A2) and sample solution (2 µl each) were applied. Linear ascending development was carried out in pre-saturated (optimized to 5 min for better resolution) vertical twin trough glass chambers (10 cm×10 cm or 20 cm ×10 cm) saturated with the mobile phase. The mobile phase selection and optimization was carried out wherein different compositions consisting of different ratios of solvents of varying polarity were tried. Finally, a mobile phase consisting of toluene-chloroform-methanol (6.8:2.2:1, v/v/v) was used as mobile phase and the separation of the markers was achieved without interference of sample matrix components under laboratory conditions (25 ± 3 °C). The plates were scanned and quantified densitometrically at 246 and 243 nm. TLC Scanner-III controlled by winCATS 1.4.2 software (Camag) was used for quantitative evaluation. The densitometry scanning was performed in the reflectance/absorbance mode.

Method validation

Method validation was performed on the parameters such as linearity, precision, accuracy, robustness, limit of detection and limit of quantitation as per ICH[21] and IUPAC[22] guidelines. For providing linearity, a series of points of A1 (1000-5000 ng/spot) and A2 (250-2000 ng/spot) were analyzed. For each point three measurements were made.

Preparation of standard curve of A1

Two microlitres each of the stock solution and the four working standard solutions were applied, in triplicate, on 20×20 cm precoated TLC plates. The TLC plates were developed in TLC chamber (Merck) saturated (10 min) with toluene: chloroform: methanol (6.8:2.2:1) as the mobile phase. The developed plate was air-dried and scanned at 243 nm using Camag TLC scanner. Fig. 1 shows the standard plot of sample/marker/alkane in TLC-densitometric analysis. A standard graph was plotted against mean area under curve and the amount of the standard/marker (µg).

Preparation of standard curve of A2

Two microlitres each of the stock solution and the four working standard solutions were applied, in triplicate, on 20×20 cm precoated TLC plates. The TLC plates were developed in TLC chamber (Merck) saturated (10 min) with toluene: chloroform: methanol (6.8:2.2:1) as the mobile phase. The developed plate was air-dried and scanned at 246 nm using Camag TLC scanner. Fig. 2 shows the TLC-densitometric chromatogram of standard sample/marker/ester. A standard graph was plotted against mean area under curve and the amount of the standard/marker (µg).

Intra and inter day variation and repeatability were chosen to determine the precision of developed method. Variability of the method was studied by analyzing standard solution containing 1, 2, 3, 4 or 5 µg/spot of A1 and 0.25, 0.5, 1, 1.5 or 2 µg/spot of A2 on the same day (intra-day precision) and on different days (inter-day precision). The repeatability of measurement for peak area of the two markers (A1 and A2) has been expressed in terms of percent coefficient of variation (RSD %). The % RSD was taken as a measure of precision and repeatability.[23]

The accuracy of quantitation in terms of recovery was also assessed at 50, 100 and 150 per cent level. Known amount of investigated compounds A1 (0.18, 0.36, 0.54 mg) and A2 (0.21, 0.42, 0.63 mg) were added to accurately weighed portion of *S. media* (1 g) and analyzed. The recovery percentage was calculated by using the formula: recovery (%) = (amount found − original amount)/amount spiked × 100%.[24] To test robustness of the method, small changes in the chromatographic parameters were deliberately made, which may affect the performance of the method such as mobile phase composition, mobile phase volume, chamber saturation time.

At a time only one parameter was varied while the rest were kept constant. The effects on the results i.e. peak areas were examined. LOD and LOQ were determined using equation LOD = 3.3 × N/B and LOQ = 10 × N/B,

where "N" is standard deviation of the peak areas of the both A1 and A2 (n=3), taken as measure of noise, and "B" is the slope of the corresponding calibration curve.

3.RESULTS

In the present study, we quantified two marker compounds A1 and A2 in aerial parts of *S. media* by TLC densitometric method. Preliminary TLC studies revealed that A1 and A2 were well resolved using toluene-chloroform-methanol (6.8:2.2:1) as the mobile phase. The spots of A1 and A2 on the chromatograms were scanned under UV at 246 and 243 nm respectively. Linearity of the calibration curve for A1 was achieved between 1-5 µg (r^2 = 0.996, Table 1 and Fig. 1). For A2, linearity was achieved between 0.25-2 µg (r^2 = 0.998, Table 2 and Fig. 2). The per cent recovery of A1 and A2 was calculated, and found to be 99.38 and 98.06 respectively.

TABLE 1: Method validation studies of A1 in TLC-densitometric analysis of *S. media*

S. No.	Parameter	Result
1.	Linearity (coefficient of correlation)	0.996
2.	Range	1-5 µg
3.	Repeatability (% RSD, n=9)	0.068
4.	Intra-day precision (% RSD, n=9)	0.56
5.	Inter-day precision (% RSD, n=9)	1.5
6.	Accuracy (average % recovery)	99.38 %
7.	Limit of detection (ng/spot)	260
8.	Limit of quantification (ng/spot)	800

TABLE 2: Method validation studies of A2 in TLC-densitometric analysis of *S. media*

S. No.	Parameter	Result
1.	Linearity (coefficient of correlation)	0.998
2.	Range	0.25- 2 µg
3.	Repeatability (% RSD, n=9)	0.053
4.	Intra-day precision (% RSD, n=9)	0.67
5.	Inter-day precision (% RSD, n=9)	1.78
6.	Accuracy (average % recovery)	98.06 %
7.	Limit of detection (ng/spot)	60
8.	Limit of quantification (ng/spot)	180

Fig. 1: Standard plot of alkane/marker (A1) in TLC: Densitometric analysis

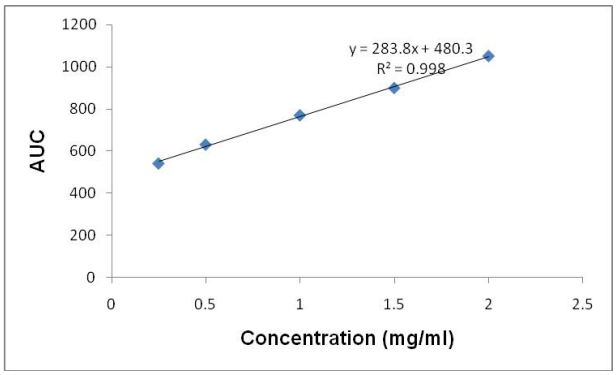


Fig. 2: Standard plot of alkane/marker (A2) in TLC-densitometric analysis

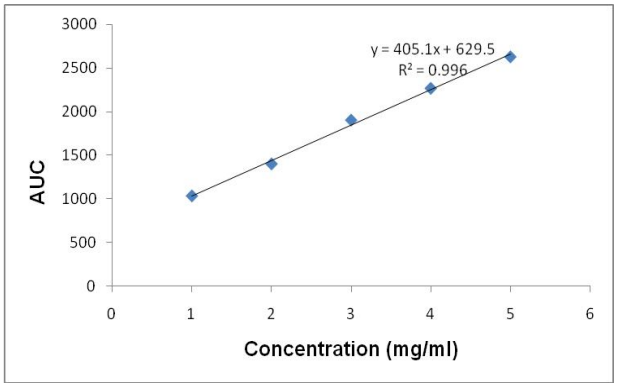


TABLE 3: Robustness of the TLC-densitometric method for analyzing A1 and A2

Parameter		A1		A2	
		S.D.	R.S.D. (%)	S.D.	R.S.D. (%)
Mobile phase composition (Toluene: Chloroform: Methanol)	6.8:2.0:1.2	0.95	0.17	1.44	0.30
	6.8:2.2:2:1.0	1.02	0.21	1.56	0.25
	6.6:2.4:1.0	0.99	0.19	1.27	0.16
Mobile phase volume	8 ml	0.72	0.12	1.25	0.19
	10 ml	0.75	0.16	1.32	0.24
	12 ml	0.70	0.14	1.34	0.27
Duration of saturation	8 min	1.08	0.20	1.20	0.18
	10 min	1.09	0.28	1.12	0.21
	12 min	1.05	0.24	1.23	0.17

4. DISCUSSION

The measurement of the peak area at five different concentration levels showed low values of % RSD (< 2 %) for inter and intraday variations for both A1 and A2 (Tables 1 and 2), which suggested an excellent precision of the method. The low values of % RSD of A1 and A2 obtained after

introducing small deliberate changes in the developed TLC-densitometric method indicated the robustness of the method (Table 3). The limits of detection (LOD) and quantification (LOQ) for both A1 and A2 (Tables 1 and 2) indicate the adequate sensitivity of the method. The per cent recovery of A1 and A2 indicates that the accuracy of the method was good.

Quantitative estimation of A1 and A2

The content of A1 and A2 in aerial parts of *S. media* was determined from the regression equation of the standard plot. In the present investigation, A1 and A2 were found to be present to the extent of 0.180 and 0.078 % w/w respectively in the aerial parts of the plant. Typical TLC-densitometric chromatograms of reference compounds with methanol extract of *S. media* are shown in Fig. 3 and 4.

Fig. 3: TLC-densitometric chromatograms of A1 and the methanol extract of *S. media*

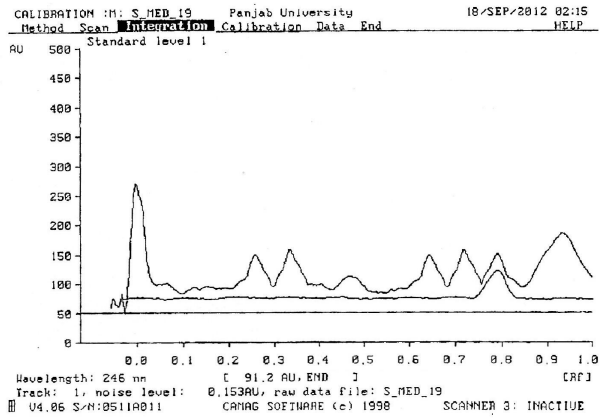
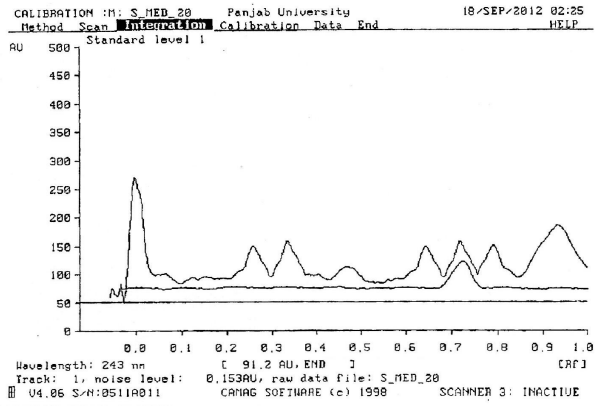


Fig. 4: TLC-densitometric chromatograms of A2 and the methanol extract of *S. media*



5. CONCLUSION

For the first time, a simple, accurate, and rapid TLC method was developed for the simultaneous determination of the two bioactive compounds from aerial parts of *S. media*. The assay is proved to be accurate, reproducible, and sensitive. It can be also used in routine quality control of herbal materials as well as formulations containing any or all of these compounds

6.CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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