Original Research Article

Development of a novel, rapid and validated HPTLC protocol for the quantitative estimation of marrubiin (a furan labdane diterpene) from the extract of *Marrubium vulgare* Linn.

ABSTRACT

Aim: To develop a novel, simple, precise and rapid HPTLC protocol for the analysis of marrubiin in herbal extracts and formulations.

Methodology: The marrubiin can be quantified by performing the HPTLC on silica gel F_{254} plates using toluene: ethyl acetate: acetic acid (5:4:1) as mobile phase. The developed method was validated as per ICH guidelines.

Results: The protocol was found to be linear in the concentration range of 40-400ng/spot and simultaneous comparison of R_f (0.47 ± 0.05) and overlapping UV spectra of samples confirm the specificity of the method. The LOD and LOQ of biomarker were found to be 15 and 40 ng by the selected method. The low value of %RSD (less than 2) in peak area on analyzing the sample on same and different days ensures the precision of the developed method. Further the recovery of more than 95% of the marrubiin affirms the accuracy of developed analytical method.

Conclusion: It can be concluded that the developed protocol could be beneficial for the qualitative and quantitative analysis of the marrubiin in herbal extracts and formulations.

Key words: *Analysis, chromatography, fingerprint, TLC, Lamiaceae*

1. INTRODUCTION

The herbal extracts are the complex mixture of chemical constituents and separation of these entities is a quite challenging task. The quantitative and qualitative analysis of the different compounds in plant extracts with acute accuracy is need of the hour. The various chromatographic techniques such as high pressure liquid chromatography (HPLC), gas chromatography (GC), high performance thin layer chromatography (HPTLC) have been employed by large number of researchers for the analysis of herbal extracts ([1-4]. Of these the HPTLC was considered to be quite advantageous in terms of time saving, high output and cost effectiveness for the analysis of multi component samples. Moreover the analysis of plant based mixtures with HPTLC having mass spectrum (MS) interface not only separate the various constituents but the proportion and identity of biomarkers/compounds can also be recognized. [5-8].

Chemically the marrubiin (Figure 1) is a furan labdane diterpenoid, isolated from the various species of Marrubium genus (family- Lamiaceae). Generally in India, the species *Marrubium vulgare* Linn is found at an altitude of 5000 – 6000 ft in Kashmir region. This perennial herb is conventionally used as diuretic, bitter tonic, expectorant, antipyretic and also useful in the treatment of bronchitis, joint pain and various complications related to spleen, lever and uterus [9]. The pharmacological significance of the marrubiin was approved by many scientists as it was found to possess the vasorelaxant, hypotensive, cardioprotective, analgesic, antinociceptive, antidiabetic, gastroprotective, antioxidant and antioedematogenic properties [10-18]. Due to its vast therapeutic potential, marrubiin could be included in various herbal formulations. The multicomponent nature of polyherbal formulations necessitates the requirement of a cost effective and specific analytical method to evaluate the marrubiin. The HPTLC has its own inherent advantage as compared to gas chromatography (GC) and high performance liquid chromatography (HPLC). Nevertheless the analytical strategies such as high pressure liquid chromatography (HPLC) and gas chromatography (GC) can not be replaced but development of optimized novel methods for phytopharmaceuticals by HPTLC were always proved to be quite useful. Therefore an approach to develop a validated process for the qualitative and quantitative analysis of marrubiin from plant extract by HPTLC has been planned and executed in the present research work.

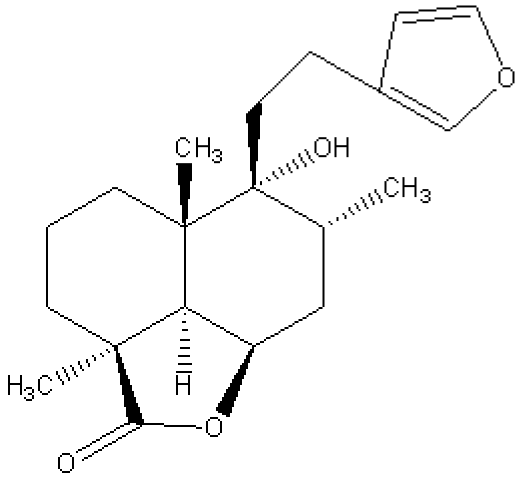

Figure 1

2. METHODOLGY

2.1 Plant sample and reagents

The plant, *Marrubium vulgare* Linn was collected from the Pulwama district of Jammu and Kashmir state, India in April 2013. The herb was identified by, Dr. Sunita Garg, taxonomist from National Institute of Science Communication and Information Research, New Delhi, vide reference no NISCAIR/RHMD/Consult/2013/2336-116 dated- 19-11-2013. A voucher specimen was kept in the department for future reference. The bio marker marrubiin (HPLC grade) was procured from Extrasynthese, France. The HPLC grade solvents were used in the study and obtained from Spectrochem Pvt. Ltd., Mumbai, India.

2.2 Preparation of test samples and reference solution

The selected medicinal plant was dried and grounded using a household mixer grinder. The powder of the drug (10 g) was sieved and extracted with ethanol (500 ml) in a soxhlet apparatus. After exhaustive extraction the solution was filtered and concentrated through rotary evaporator. The extract was dried in lyophillizer and kept in dessicator before further use. For analysis of the extract by HPTLC, the dried extract was dissolved in methanol to prepare a solution of 5 mg/ml concentration. The solution (test sample) was filtered through a membrane filter (0.45µm) before applying on the stationary phase (Silica gel plate). The solution of the standard compound was formed by dissolving the biomarker (marrubiin) in methanol to get a concentration of 1 mg/ml. This solution was further diluted to reduce the concentration upto 10µg/ml and termed as standard stock solution or reference solution.

2.3 HPTLC analysis of different samples

A HPTLC system (CAMAG, Muttenz, Switzerland) was used to analyze the test and reference samples for the quantification of marrubiin. The precoated and preactivated plates of silica gel 60 F_{254} (E. Merck) supported on aluminum sheet were used as stationary phase. The various samples were applied using CAMAG automatic sample applicator (Linomat V) with the help of micro syringe (100 µl). The mobile phase consisting of toluene, ethyl acetate and acetic acid in a ratio of 5:4:1 was used to develop the chromatogram. The chromatogram was developed upto 80% height of plates by ascending technique in a presaturated twin trough chamber with 20 ml of solvent system. The developed plates were dried at room temperature and then heated at 110℃ for 15 minutes on CAMAG TLC plate heater. The spots were visualized in UV light of CAMAG TLC visualizer system at wavelength of 254 nm. The images of the developed plates were captured using winCATS software. For the quantification of biomarker in the samples, the plates were scanned in CAMAG TLC densitometric scanner at wavelength of 254 nm and analyzed using winCATS 1.4.8 software. All the experiments were carried in laboratory conditions with temperature of 26 ± 2℃ and relative humidity was 50 ± 5%. The resolved spots were used to determine the retention factor (R_f).

2.3.1 Development of calibration curve

The marrubiin reference solution (10µg/ml) was used to prepare the calibration curve which was used to quantify the marrubiin in samples with unknown concentration. The ten different spots (4mm wide) of reference solution with a volume of 4, 8, 12, 16, 20, 24, 28, 32, 36, 40µl were applied on the stationary phase with the help of a micro syringe under the flow of N_2 gas. The spots were applied on the plate at a distance of 10mm from bottom and side with a 4mm space between them. The each consecutive spot was supposed to have 40, 80,120, 160, 200,240, 280, 320, 360, 400ng of the marrubiin. The chromatogram was developed by the method described in the previous section and R_f was calculated for isolated spot (marrubiin). The plates were scanned and analyzed with winCATS computer software to observe the area under curve (AUC) for each resolved spot. The mean peak area for each sample with known concentration was used to plot the calibration curve. The equation of straight line was generated by linear regression of the data and used to determine the concentration of biomarker (marrubiin) in test sample.

2.4 Validation of HPTLC protocol

The HPTLC protocol for the quantification of marrubiin was validated as per the guidelines described by International Conference on Harmonization (ICH) [19, 20].

2.4.1 Linearity

The linearity of the method was ascertained by plotting calibration graph between the various concentrations of marker (40-400 ng/spot) compound to the corresponding area under curve (AUC). The data was linearly analyzed to develop equation of straight line and coefficient of correlation (R^2) was determined.

2.4.2 Specificity and sensitivity

The specificity of developed analytical procedure was confirmed by the simultaneously comparing the R_f of marker compound in reference solution and test sample. Further it was ascertained by superimposing the UV spectra of extract and standard solution. The limit of detection (LOD) and limit of quantification (LOQ) are the measure of sensitivity for selected protocol. These were estimated by analyzing different concentrations of marrubiin till the mean AUC was about three (for LOD) ten (for LOQ) times the standard deviation (n=6).

2.4.3 Precision

The precision of the instrument and developed method was affirmed by repeated analysis of reference sample (100 ng/spot) for six times. Further intraday and interday variability of procedure was determined by analyzing the standard solution at three different concentration (60, 120, 180 ng/spot) in triplicate on same day and different day. The results were communicated as % relative standard deviation (% RSD).

2.4.5 Accuracy/Recovery

The accuracy of the protocol was assessed by recovery after addition of 50,100,150% of marrubiin to a previously analyzed standard solution (100ng/band). The experiment was conducted in triplicate at each three level and percentage recovery and average recovery (%) was calculated.

2.4.6 Robustness

The robustness of the selected strategy was also evaluated by analyzing the chromatogram, for test sample (300 ng/spot), developed by slightly fluctuating the development distance (8 ± 0.5 cm), volume of mobile phase (20 ± 2 ml), composition of solvent system (±10%) and time taken for saturation (30 ± 5 min). The results were expressed as % RSD for each deliberate change in chromatographic conditions.

2.4.7 Suitability of system

To ensure the reproducibility of the results the suitability of the system was assured by performing the HPTLC profile of the biomarker at concentration of 200 ng/spot (n=6). The chromatogram was developed with selected mobile phase and plates were scanned to note the AUC and R_f for each concentration of marrubiin. The standard deviation (SD), % RSD, and mean peak area was observed and reported.

2.4.8 Quantification of marrubiin in herbal extract

The *Marrubium vulgare* Linn extract was dissolved in ethanol to get a concentration of 5 mg/ml and subjected to HPTLC evaluation for the quantification of marrubiin by developed method (n=3).

3. Results and Discussion

The development of chromatographic fingerprint for the selected extract could convey the necessary information to determine the proportion of dynamic constituents in a quite short span. The fingerprint patterns also guarantee about the consistency of extract and make it assessable to confirm the quantity of all discernible analytes [21].The various solvents in different proportion were tried to analyze the marrubiin in the extract and reference solution. After conducting the large number of experiments the mobile phase consisting of toluene: ethyl acetate: glacial acetic acid (5:4:1) was selected. The different samples were applied with a band width of 4mm on the plates to enhance the response of detector and accuracy in scanning results was also improved. The simultaneous development of chromatogram for standard and test sample confirmed that the marrubiin was well resolved at R_f of about 0.47 (Figure 2).

The HPTLC chromatogram developed with the selected mobile phase for standard and extract solution confirmed that the marrubiin was present in the test sample (Figure 3).

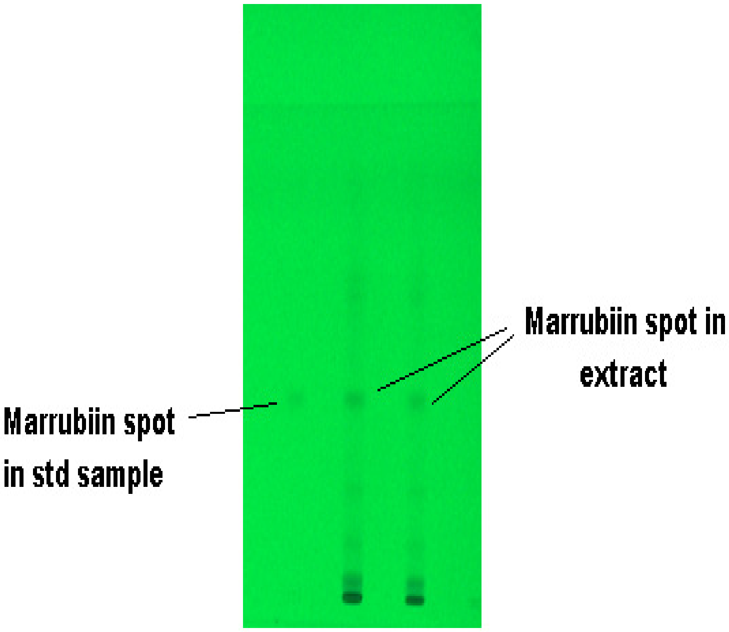

Figure 2

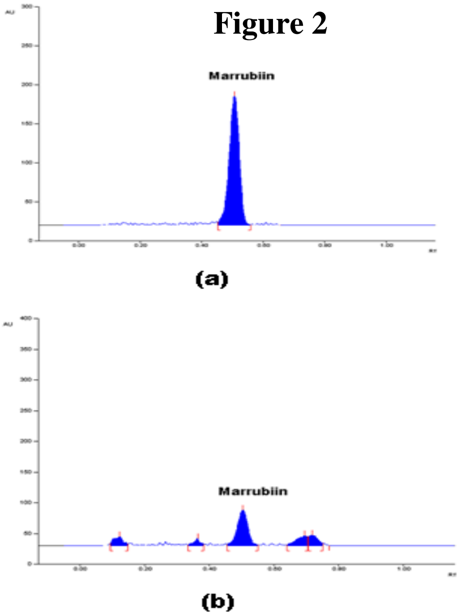

Figure 3

3.1 Validation of HPTLC protocol

3.1.1 Linearity

The calibration curve plotted in the selected range and was found to be linear (Figure 4). The linearity equation generated by regression analysis of data was Y= 8.705 X + 445.98 where Y is the mean peak area (AUC) and X represents the concentration of particular sample. The 3 D diagram representing the AUC for the different concentration of marker compound in reference and test samples was depicted in figure 5. A high R^2 value (0.996) signifies how close the data fits the regression line.

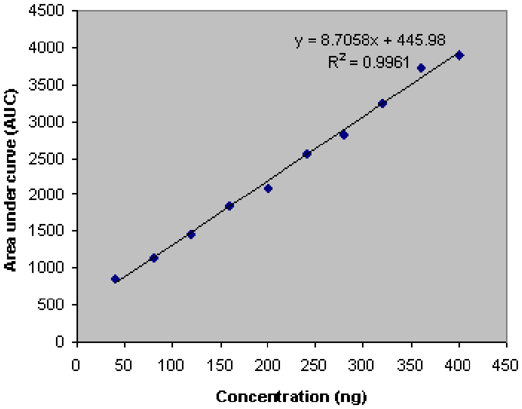

Figure 4

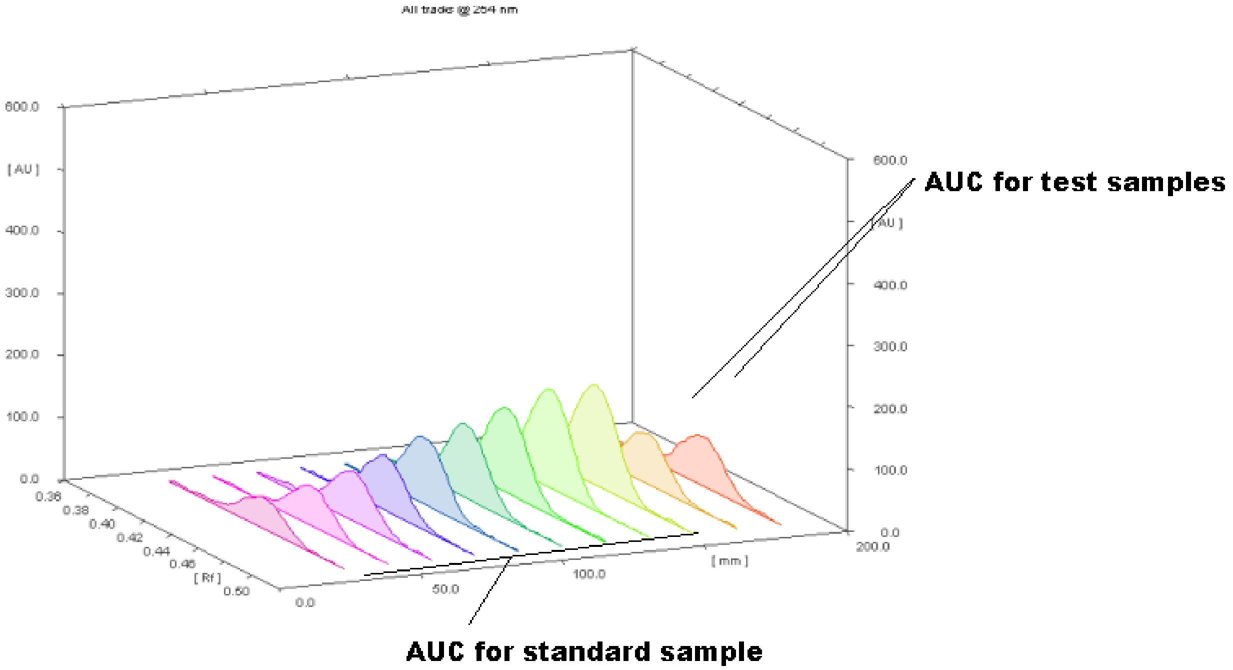

Figure 5

3.1.2 Specificity and sensitivity

The specificity of the developed method was confirmed by absence of interference in detection of marrubiin from test samples with R_f – 0.47±0.05. Moreover the UV spectra of two samples was overlapped in the region of 254 nm which further assured the specificity of the method for determination of marrubiin in extract (Figure 6). The method sensitivity was evaluated by analyzing the different concentration of marker compound (5 – 50 ng/spot). The observed LOD and LOQ for marrubiin in the selected protocol was 15 and 40 ng respectively (Table 1).

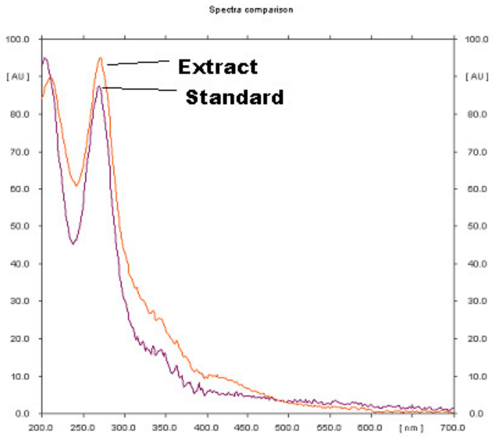

Figure 6

Table 1: Summary of validation parameters for analysis of marrubiin by HPTLC

Validation Parameter	Value
Linear regression equation	Y=8.705 X + 445.98
Regression coefficient (R^2)	0.996
Linearity range	40-400 ng
Retention factor (R_f)	0.47 ± 0.05
Instrument precision at 100 ng/band; n=6 (%RSD)	0.70
Limit of detection (LOD)	15 ng
Limit of quantification (LOQ)	40 ng
Repeatability at 200 ng/band; n=6 (%RSD)	0.27
Marrubiin in test sample at 5 mg/ml; n=3 (%)	0.69 ± 0.04
Robustness	Robust
Specificity	Specific

3.1.3 Precision

The random investigation of the precision of instrument and protocol on same day (intraday) and different day (interday) established the validity of analytical procedure. The low value of % relative standard deviation (<2%) for different parameters confirms that the developed method was precise and reproducible (Table 2).

Table 2: Validation of precision parameter for marrubiin by HPTLC

Marker Concentration (ng/band)	Inter-day precision*		Intra-day precision*	
	Mean peak area ± SD	%RSD	Mean peak area ± SD	%RSD
60	1006.6 ± 6.18	0.61	1009.3 ± 17.55	0.41
120	1475.6 ± 3.68	0.24	1471.3 ± 11.57	0.78
180	1968.3 ± 30.64	1.51	1971.0 ± 7.3	0.37

* n = 3 for each concentration; values are expressed as mean ± Standard deviation

3.1.4 Accuracy/Recovery

The recovery of more than 95% of marrubiin in preanalyzed samples after spiking with known concentration of standard sample confirmed the accuracy of the method. The recovery results are depicted in table 3.

Table 3: Recovery studies to validate the accuracy of HPTLC protocol for marrubiin

Amount of marker in Preanalyzed sample (ng/spot)	Amount of marker spiked (%)	Mean peak area ± SD (Preanalyzed sample + standard)	Total area obtained ± SD	Recovery (%) ± SD	% RSD
100	50	1923.26 ± 2.23	1901.5 ± 3.22	98.8 ± 0.95	0.96
	100	2498.9 ± 3.81	2425.5 ± 12.27	97.06 ± 0.54	0.56

150	3151.4 ± 7.51	3071.7 ± 22.33	97.45 ± 0.61	0.63
Average Recovery			97.77 ± 1.25	

Analysis in triplicate at each level (n=3); values are expressed as mean ± Standard deviation

3.1.5 Robustness

The slight deviation in the selected parameters did not represent any huge fluctuation in %RSD (less than 2%) which clearly justify the robustness of the derived protocol (Table 4).

Table 4: Robustness of developed HPTLC method for marrubiin analysis

Test sample (ng/spot)	Chromatogram development conditions	Mean peak area ± SD	RSD (%)
300	Development distance (8 ± 0.5 cm),	2996.63 ± 24.12	0.80
	Volume of mobile phase (20 ± 2 ml)	2963.13 ± 36.46	1.23
	Composition of solvent system (±10%)	3032.9 ± 51.79	1.71
	Time taken for saturation (30 ± 5 min)	3021.86 ± 8.36	0.27

n=3 for each conditions; values are expressed as mean ± Standard deviation

3.1.6 System suitability

The mean peak area for selected concentration of standard sample (200 ng/spot) was found to be 2084.1 ± 5.75 with %RSD of 0.27. Moreover the R_f for the marrubiin was confirmed at 0.47 ± 0.05. The small value of %RSD confirms the suitability for the analysis of marrubiin in extract.

3.1.7 Quantification of marrubiin:

The prepared extract of *Marrubium vulgare* Linn was analyzed by developed method in triplicate and the percentage of marrubiin was quantified to be 0.69 ± 0.04.

4. CONCLUSION

The marrubiin is considered to be the chemotaxonomic marker for the plants related to Marrubium genus of Lamiaceae family. Hence the developed and validated chromatographic fingerprint pattern for marrubiin by HPTLC could be beneficial for the evaluation of particular plant species. Moreover the developed pattern clearly speaks about the various chemical entities of the extract and could be employed for the qualitative and quantitative assessment of marrubiin in the herbal formulations.

CONSENT

It is not applicable

ETHICAL APPROVAL

It is not applicable

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CAPTIONS FOR FIGURES

Figure 1: Chemical structure of marrubiin

Figure 2: Photomicrograph of TLC plate with the spot of (i) Standard sample (ii) & (ii) test sample

Figure 3: HPTLC chromatogram showing area under curve for marrubiin (a) standard sample (ii) test sample

Figure 4: Calibration curve of marrubiin

Figure 5: 3D diagram with AUC for various standard samples and test samples

Figure 6: UV overlay spectra confirming presence of marrubiin in extract