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HPTLC Method for Estimation of Ursolic Acid in *Ocimum sanctum* Extract

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A quantitative HPTLC method was developed for the estimation of ursolic acid in *Ocimum sanctum* extracts. The ursolic acid was separated from plant extracts on a silica gel 60 F_{254} pre coated plate using a saturated mixture of toluene:ethyl acetate:glacial acetic acid in the ratio of 5.5:4.5:0.2 was used as mobile phase for estimation ursolic acid. Validation of the method was done in terms of LOD, LOQ, linearity, precision and accuracy. The repeatability of methods was excellent and average recovery was in the range of 100.43-102.12 %. The result of all parameter was within the limit. The methods were found to sensitive, simple and accurate. In the present study, the samples of *Ocimum sanctum* were collected from various geographical sources that is from Kerala, Karnataka and Kolkata. The method could satisfactorily quantify aqueous and aqueous methanolic extracts of samples from various geographical sources. The sample collected from Kerala was found to contain more amounts of ursolic acid.

Key Words: Ursolic acid, HPTLC, *Ocimum sanctum* extract.

INTRODUCTION

Plants are one of the most important sources of medicines. The medicinal plants are rich in secondary metabolite and essential oils of therapeutic importance. The biosynthesis of the secondary metabolite although controlled genetically, is affected by environmental influence[1]. As a result there are fluctuation in the concentration of the secondary metabolites and essential oils. There is a need for standardization of herbal crude drugs and their bio constituents in justifying their acceptability in modern system of medicine. With the advent of new analytical tools and sophisticated instrument technology, it is possible to suggest a practicable standardization profile for herbal drugs. Development of HPTLC method for identification and quantification for chemical markers is one of the popular methods for standardization. High performance thin layer chromatography[2-4] has gained importance in recent years as it allows reliable quantification of analyses at micro and nanograms levels[5-8]. *Ocimum sanctum* known as Tulsi in Hindi and holy basil in English is commonly used as medicinal plants in day-to-day practice in Indian homes for various ailments. Different parts of the plant like stem, flower, seed, leaves, root, *etc.* are known to possess

therapeutic potential and have been used, by traditional medical practitioners, as expectorant, analgesic, anticancer, antiasthmatic, antiemetic, diaphoretic, antidiabetic, antifertility, hepatoprotective, hypotensive and antistress agent[9,10]. Tulsi has also been used in treatment of fever, bronchitis, arthritis, convulsion[11], *etc*. The chemical constituents of *Ocimum sanctum* include volatile oil and triterpene. Tulsi has been standardized only on the basis of amount of volatile oil and eugenol[12]. Ursolic acid and rosmarinic acid have been isolated and characterized from the leaves of *Ocimum sanctum* and many other plants. These compounds can be used as marker compounds to evaluate and standardize extracts and formulations containing Tulsi.

EXPERIMENTAL

The instrument used in present study was CAMAG-HPTLC system comprises CAMAG linomat V automatic sample applicator, CAMAG TLC scanner III with Wincat software and CAMAG twin trough glass chamber for chromatographic development.

Ursolic acid pure drug was obtained as a gift sample from Natural Remedies Private Ltd. The *Ocimum sanctum* was collected from three different places like Kerala, Karnataka and West Bengal. Toluene, ethyl acetate, formic acid and glacial acetic acid were purchased from Merck Limited. Whole plant of *Ocimum sanctum* was collected from various geographical sources that are from Kerala, Karnataka and West Bengal. Then the sample was authentified in RRI Bangalore and the specimen number is RRCBI-12475.

Preparation of extract: 100 g of each powdered sample were refluxed with water for 8 h. The extracts were concentrated with rotary evaporator at 70 ºC. The same way 70 % methanolic extracts are also prepared.

Preparation of standard stock solution: 2.5 mg of ursolic acid was dissolved in 50 mL methanol to give a concentration of 50 ng/µL. The linearity of method was determined at five-concentration level ranging from 50-400 ng per spot. Plates were developed by linear ascending development using HPLC grade solvents of toluene, methanol, ethyl acetate, formic acid, *etc*. After several trials mixture of toluene:ethyl acetate:acetic acid in the ratio of 5.5:4.5:0.2 was found to be ideal for analysis of ursolic acid.

Sample preparation

Development of HPTLC method for estimation of ursolic acid: 1 g of aqueous extract of *Ocimum sanctum* were dissolved in 100 mL of methanol and filtered through Whatman No. 1 filter paper. 10 µL of each extracts obtained from different geographical sources were spotted along with 3 µL of standard solution chromatogram and derivatized with Libermann Burchard's reagent. Spectrum analysis was done for the determination of λ_{max} of ursolic acid in the range of 200-700 nm using D_2 and tungsten lamp. The wavelength at which the peak obtained maximum height and area was considered as λ_{max}. The λ_{max} for ursolic acid was found to be 550 nm. The image of the derivatized plates were recorded using CAMAG Reprostar 3 (Fig. 1).

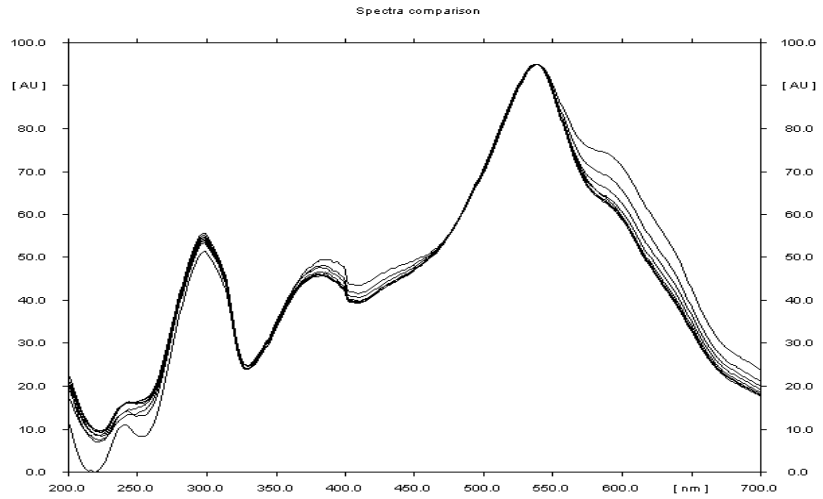

Fig. 1. Spectrum showing λ_{max} at 550 nm for ursolic acid

Validation of developed method: Validation of developed method was done in terms of limit of detection, limit of quantification, linearity, precision and accuracy.

Limit of detection (LOD): The limit of detection is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated. 1 mg of standard ursolic acid was dissolved in 100 mL of methanol. This gives a concentration 10 ng/µL. From the standard stock solution 1-5 µL were spotted in duplicates. The chromatogram were developed and scanned at 550nm after derivatization with Libermann Burchard's reagent.

Limit of quantification (LOQ): The limit of quantification is the lowest amount of analyte in a sample that can be quantitated with suitable precision and accuracy. 2.5 mg of standard ursolic acid was dissolved in 50 mL of methanol. This gives a concentration of 50 ng/µL. From this 1-5 µL were spotted in duplicate. The chromatogram was developed and scanned at 550 nm after derivatization with Libermann Burchard's reagent.

Linearity: Linearity of an analytical method is its ability to elicit test result that are directly or by a well defined mathematical transformation proportional to the concentration of analyte in the sample within a given range. Linearity is determined by a series of three to six application of standard. 2.5 mg of standard ursolic acid were dissolved in 50 mL of methanol. This gives a concentration of 50 ng/µL. From this standard stock solution 1-6 µL were spotted. The chromatogram was developed and scanned at 550 nm after derivatization with Libermann Burchard's reagent. Linear graph was plotted with concentration against area under curve (Fig. 2).

Precision: Precision is the degree of similarity among the individual test result when the procedure is applied repeatedly to multiple samples. Precision is a measure of repeatability or reproducibility. It can be calculated by relative standard deviation.

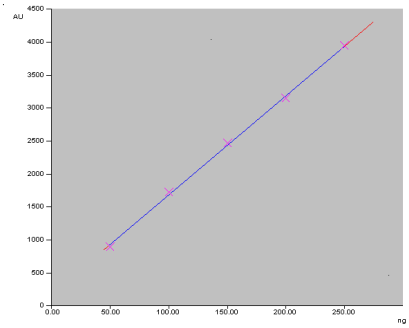

Fig. 2. Linear graph of ursolic acid at 550 nm

Intra day variation: 2.5 mg of standard ursolic acid was dissolved in 50 mL of methanol, which gives a concentration of 50 ng/µL. From this stock solution 2 µL of standard ursolic acid was spotted 6 times in the morning, developed and scanned at 550 nm after derivatization with Libermann Burchard's reagent. The same stock solution was spotted on a fresh plate on the same day in the after noon. The plate was developed and scanned in the same way.

Inter day variation: The same method was followed but plates were spotted on two consecutive days to study inter day variations. The chromatogram was developed and scanned at 550 nm after derivatization with Libermann Burchard's reagent. The results of intra- and inter-day variations are given in Tables 1 and 2.

TABLE-1
PEAK AREAS OF INTRA DAY VARIATIONS OBTAINED
DURING PRECISION STUDIES OF URSOLIC ACID

Concentration of ursolic acid (ng)	R_f value	Peak area 1	R_f value	Peak area 2
100	0.83	1254.25	0.71	1246.45
100	0.82	1324.28	0.71	1321.74
100	0.82	1298.39	0.72	1257.36
100	0.82	1297.62	0.72	1287.32
100	0.82	1318.78	0.72	1299.01
100	0.82	1287.47	0.72	1308.41
Relative standard deviation		1.932 %		2.287 %

TABLE-2
PEAK AREAS OF INTER-DAY VARIATIONS OBTAINED
DURING PRECISION STUDIES OF URSOLIC ACID

Concentration of ursolic acid (ng)	R_f value	Peak area 1	R_f value	Peak area 2
100	0.82	1279.01	0.72	1298.82
100	0.82	1269.72	0.72	1363.51
100	0.82	1318.39	0.72	1323.75
100	0.82	1316.92	0.72	1287.77
100	0.82	1249.75	0.72	1298.14
100	0.81	1256.34	0.72	1271.25
Relative standard deviation		2.314 %		2.482 %

Accuracy: The accuracy of an analytical method is established over its range. In case of quantitative determination of actives in extracts accuracy is determined by adding known amount of analyte to the extract as appropriate and calculating the spiked recovery.

Preparation of standard solution: 2.5 mg of ursolic acid was dissolved in 50 mL of methanol to give a concentration of 50 ng/µL.

Preparation of sample solution: 100 mg of methanolic extract of *Ocimum sanctum* from Kerala was dissolved in 100 mL of methanol and filtered through Whatman No. 1 filter paper.

Standard and sample application: 5 µL of sample solution, 1 µL, 3 µL, 4 µL of standard ursolic acid and 5 µL of sample spiked with 1 µL, 3 µL, 4 µL of standard ursolic acid were spotted on the same plate. Chromatogram is developed and scanned at 550 nm after derivatization with Libermann Burchard's reagent. Areas of sample, standard and spiked samples were recorded and % recovery was calculated using the following formula:

$$\% \text{ Recovery} = \frac{A + B}{C} \times 100$$

where, area of sample = A, area of standard = B, area of sample + area of standard (spiked) = C.

The results of accuracy studies are given in Table-3.

TABLE-3
% RECOVERY OBTAINED DURING ACCURACY STUDIES OF URSOLIC ACID

Vol. of ursolic acid (µL)	Peak area of Std	Vol. of sample solution (µL)	Peak area of Sp	Actual peak area of spiked Sp	Obtained peak area of spiked Sp	Recovery (%)
1	1740.31	5	2171.55	3911.86	3984.5	98.1
3	2486.32	5	2171.55	4657.87	4593.7	99.2
4	3212.50	5	2171.55	5384.05	5162.5	104.4
				Mean % recovery		100.43

RESULTS AND DISCUSSION

Whole plant of *Ocimum sanctum* belonging to the family lamiaceae was collected from various geographical sources to study the phytochemical characters. The sample were dried, powdered and extracted with water and 70 % methanol by reflux method. HPTLC methods were developed for estimation of ursolic acid in *Ocimum sanctum* using different solvent system. The proper solvent system selected for estimation of ursolic acid was toluene:ethyl acetate:glacial acetic acid in the ratio of 5.5:4.5:0.2. The spectrum analysis was done to find the λ_{max}. The λ_{max} for ursolic acid was found to be 550 nm after derivatization with Libermann Burchard's reagent. Validation of the HPTLC method was done in terms of LOD, LOQ, linearity, precision and accuracy. Limit of detection of ursolic acid was found

to be 20 ng. The limit of quantification was found to be 50 ng. The linearity range of ursolic acid was found to be 100-400 ng. The regressive value was 0.99. The method was precise with low standard deviation. The recovery value for ursolic acid was 100.43 %. The validated method were applied to quantify the markers in aqueous and aqueous methanolic extracts of *Ocimum sanctum* samples collected from different geographical sources. The aqueous methanolic extracts of all samples were found to have more amounts of active constituents compared to aqueous extracts. The amount of ursolic acid in aqueous methanolic extract was in the range of 0.37-0.23 %. The sample collected from Kerala showed more amount of ursolic acid that is 0.37 % (Table-4). The method developed was specific, sensitive, precise and accurate. Therefore this validated method can be used for routine quality control analysis for *Ocimum sanctum* extracts and formulations.

TABLE-4
AMOUNT OF URSOLIC ACID PRESENT IN *Ocimum sanctum* EXTRACTS OBTAINED BY HPTLC

Sample identity	70 % Methanolic		Aqueous	
	Amount of ursolic acid in total extracts (mg)	% of ursolic acid	Amount of ursolic acid in total extracts (mg)	% of ursolic acid
Kerala	29.71	0.37	11.73	0.15
Karnataka	24.10	0.28	10.71	0.13
Kolkata	18.14	0.23	8.67	0.12

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