



## A QUANTITATIVE ESTIMATION OF 14-DEOXY-11, 12 -DIDEHYDROANDROGRAPHOLIDE IN ANDROGRAPHIS PANICULATA BY HPTLC

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### ABSTRACT

The present paper deals with development and standardization of HPTLC method used for quantification of 14-Deoxy-11,12-didehydroandrographolide in *Andrographis paniculata* commonly known as Kalmegh, a plant of the family Acanthaceae. It is an Andrographolide derivative, isolated from the whole plant of *Andrographis paniculata*, which possesses immunostimulatory activity. Present work focuses on the development and standardization of a chromatographic method for quantification of 14-Deoxy-11,12-didehydroandrographolide, for the standardization of *A. paniculata* by HPTLC method. A simple, precise, accurate and rapid high performance thin layer chromatographic method has been developed and validated for the estimation of 14-Deoxy-11,12-didehydroandrographolide. The lowest detectable limit of 14-deoxy-11,12-didehydroandrographolide was found up to 40 ng with a good resolution and separation of the marker from the other constituents of *A. paniculata*. Further, the recovery value of 14-deoxy-11,12-didehydroandrographolide was found to be about 94.02 -96.34 %, which shows the reliability and suitability of the method. The structure of isolated marker was characterized and confirmed by various advanced spectroscopic methods

**Keywords** - HPTLC, *A. paniculata*, 14-deoxy-11,12-didehydroandrographolide

### INTRODUCTION

*Andrographis paniculata* Nees belonging to the family of Acanthaceae popularly known as "Kalmegh" is a well known drug in the Indian system of medicine. *A. paniculata* possesses antihepatotoxic<sup>[1-4]</sup>, antibacterial<sup>[5]</sup>, anti-malarial<sup>[6]</sup>, antithrombogenic<sup>[7-8]</sup>, anti-inflammatory<sup>[9]</sup>, anti-pyretic<sup>[10]</sup>, analgesic<sup>[11]</sup>, vermifugal<sup>[12]</sup> properties and has been used for the treatment of snake bites<sup>[13]</sup>. Since it has a broad range of pharmacological effects and high therapeutic value, it is used as a wonder drug in traditional Siddha and Ayurvedic system of medicine as well as in tribal medicine in India for multiple clinical applications<sup>[14]</sup>. This plant also has a reputable use in treating diabetes and hypertension<sup>[15-17]</sup>.

Many of the isolated compounds from *A. paniculata* have shown anti-viral properties against bird flu, ebola and human immunodeficiency viruses which appear to inhibit glycoproteins in the virus<sup>[18]</sup>. Three major diterpene compounds have been isolated viz. andrographolide, 14-deoxyandrographolide and 14-deoxy-11,12-didehydroandrographolide (DIAP). 14-deoxy-11,12-didehydroandrographolide (DIAP) possesses antipyretic, analgesic, immunostimulatory activity and anti-inflammatory activities<sup>[19]</sup>. It is a potent compound for inducing vaso-relaxation and decreasing heart rate<sup>[20]</sup>. It also enhances the proliferation and interleukin-2 (IL-2) induction in HPBLs<sup>[21]</sup>.

Different chromatographic methods such as HPTLC, HPLC are available for quantification of 14-Deoxy-11, 12-didehydroandrographolide in *Andrographis paniculata*<sup>[22-24]</sup>. The reported HPTLC methods are less sensitive in terms of its linearity range as well as limit of detection (LOD), which may give erroneous results during analysis / quantification of DIAP in *A. paniculata* samples especially its formulations.

The present work focuses on development and validation of high-performance thin layer chromatographic method for the determination of isolated major constituent in *A. paniculata* extract with an advantage of sensitive method with lower LOD and LOQ as well as precise even at lower concentration of isolated major compound in various samples of *A. paniculata* such as raw materials, extracts and formulation. The proposed HPTLC method was attempted for fast, precise, sensitive and reproducible method with good recoveries for standardization of extracts of *Andrographis paniculata*.

### MATERIALS AND METHODS

*Andrographis paniculata* Nees. (Acanthaceae) was purchased from the approved herb dealer from local market of Mumbai. Authentication of the plant was performed at the Botany Department of Piramal Life Sciences Ltd, Mumbai. The plant material was dried under shade and grounded in to # 18 powder.

#### Isolation of 14-Deoxy-11,12-didehydroandrographolide

Powdered dried leaves of *A. paniculata* (0.3 kg) successively extracted with petroleum ether, chloroform and methanol by soxhlet apparatus. A charcoal treated methanolic extract (15 g) was subjected to column chromatography on silica gel (Merck) eluted with mixtures of chloroform and methanol of increasing polarity to obtain fractions 16 and 14-Deoxy-11,12 didehydroandrographolide was eluted in the fractions 1-12 from the silica gel column eluted with chloroform : methanol (98:2) as an amorphous powder. The isolated compound using different chromatographic techniques was then subjected to various spectroscopic techniques viz. <sup>1</sup>H, <sup>13</sup>C NMR, MS etc. to elucidate the structure<sup>[23]</sup>.

#### Equipment

A Camag HPTLC system equipped with a sample applicator Linomat V, twin trough plate development chamber, TLC Scanner 3, Wincats and integration software 4.02 (Switzerland).

#### Procedure 1 - Preparation of standard

10 milligram of isolated compound (DIAP) was dissolved in 50 mL of methanol by sonicating the solution for 5 minutes and allowed to stand for 5 minutes at room temperature. The methanol extract was then filtered through Whatmann no.42 filter paper. 5.0 mL of this solution was added in 10-mL volumetric flask and volume made up with methanol to obtain 0.1 mg per mL standard solution of the isolated marker (DIAP).

#### Procedure 2 - Preparation of the sample

Accurately weighed 100 mg of methanolic extract of *A. paniculata* was extracted with methanol (50 mL) by sonicating the solution for 5 min. and allowed to stand for 5 minutes at room temperature. The methanol extract was then filtered through Whatmann no.42 filter paper. The final volume was made to 100 mL with methanol in volumetric flask. 14-Deoxy-11, 12-didehydroandrographolide (DIAP) content were then analyzed by subjecting it to HPTLC.

### Procedure 3 - Preparation of extract spiked with DIAP

The content of DIAP in aqueous and methanolic extracts was determined by comparing with the calibration curve of the working standard of DIAP. The aqueous extract, which showed lowest content of DIAP, was then used as blank. This blank was then used to spike extract with the working standard of DIAP. Different samples with varying amount of standard DIAP in range of 500 ng to 1500 ng per spot was spiked separately in 10 mg of blank extract with pre-determined DIAP. Procedure for sample preparation was followed as mentioned above. In each sample preparation, 10  $\mu$ L of spiked solution was then subjected to HPTLC with 10  $\mu$ L of blank solution for comparison. The percent recovery of DIAP standard was calculated.

### HPTLC method

Silica gel 60 F<sub>254</sub> pre-coated plates (20 x 10 cm; 0.25 mm layer thickness) were used with Toluene: Ethyl acetate (50:50) as a solvent system. Composition of the mobile phase was optimized by testing different solvent compositions of varying polarities. 10  $\mu$ L of working standard of 14-Deoxy-11,12-didehydroandrographolide (DIAP) as well as test samples were spotted on pre-coated HPTLC plates as 6 mm wide bands, positioned 10 mm from the bottom of the plate, using an automated TLC applicator. The sample applicator consisted of Linnomat V (Camag, Muttenz, Switzerland) with a nitrogen flow providing delivery from the syringe at a speed of 10s/mL. Ascending mode was used for the development of thin layer chromatography. Saturation time was 20 min along with humidity level - 65%  $\pm$  5% RH and room temperature - 25°C  $\pm$  2°C. TLC plates were developed up to 8 cm. The TLC plates were scanned at 263 nm for quantification purpose. These critical parameters were maintained for all analyses performed.

### Linearity along with limit of detection and limit of quantification of the marker

For a long-term use of the analytical method, a rigorous validation is indicated and requires the following procedures. For the preparation of calibration curve, the stock solution was diluted freshly with methanol to obtain a set of 5 calibration standards (100 ng, 500 ng, 1000 ng, 1500 ng and 2000 ng). These standards were measured and the integrated peak areas were plotted against the corresponding concentrations of the standards. The complete

procedure was repeated on three consecutive days. The so obtained three calibration curves were used to calculate a mean calibration graph. The limit of detection was obtained by analyzing signal to noise ratio and limit of quantification was defined as the lowest concentration of linear range.

### Accuracy by recovery

The method was applied to determine the concentration of spiked 14-Deoxy-11, 12-didehydroandrographolide test sample in the range of 500 to 1500 ng for assessing the accuracy of the procedure by recovery.

### Precision

Precision was reported in the terms of relative standard deviation (RSD) over the range of quantification for a single concentration in which standards and samples were assayed in replicates. Accuracy of the method was determined by the low values of RSD.

### Robustness and ruggedness studies

Robustness and ruggedness parameters were applied by making small deliberate changes of the conditions (mobile phase composition, scanning wavelength, time from chromatography to scanning and analyst) to validate the method.

### Stability

DIAP (1 mg) and *A. paniculata* methanolic extract (10 mg) were extracted in 10 mL methanol as per the method described in sample preparation. The sample solutions were kept at 25°C in dark and analyzed on consecutive days (6, 12, 18, 24 and 30 hrs) to observe the stability of standard as well as sample solution.

### RESULTS AND DISCUSSION

Working standard 14-Deoxy-11, 12-didehydroandrographolide showed a single peak in HPTLC chromatogram along with its UV spectrum (Figure 1). The spot of 14-Deoxy-11, 12-didehydroandrographolide is visible with  $R_f$  0.24 - 0.29 at 263 nm. Various solvent extracts of *Andrographis paniculata* were analyzed by the proposed method and the data are recorded. (Table 1) Comparative HPTLC photograph shows that DAIP very well separated from other constituents of *Andrographis paniculata*. (Photograph 1).

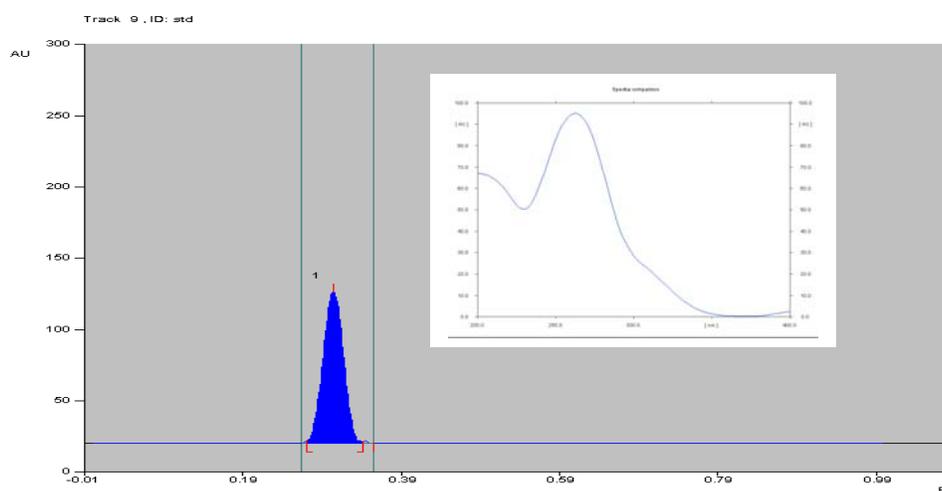
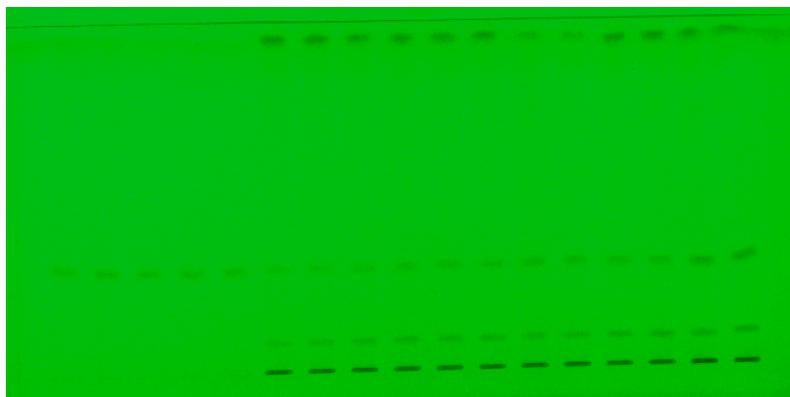


Fig. 1: It shows HPTLC Chromatogram of standard 14 - Deoxy - 11, 12 - didehydroandrographolide along with its UV spectra.

Table 1: Table shows content of DIAP in various extracts of *Andrographis paniculata*

Sr.	Extract	DIAP content (in %)
1.	Pet ether ext	1.740438
2.	Chloroform ext	14.34762
3.	Methanol ext	7.007782
4.	Water ext	Nil



Photograph 1: It shows HPTLC chromatogram of the standard 14-Deoxy-11,12-didehydroandrographolide along with the methanolic extract

### Specificity

It was observed that the other phytoconstituents present in the extracts did not interfere with the peak of the marker. Therefore the method was specific and helps in separation of 14-Deoxy-11, 12-didehydroandrographolide from the other constituents of herbal extract and hence, helps to get the exact content of the same. Test extract of *A. paniculata* showed a separated peak of 14-Deoxy-11,12-didehydroandrographolide along with other phytoconstituents as obtained in HPTLC chromatogram (Fig. 2)

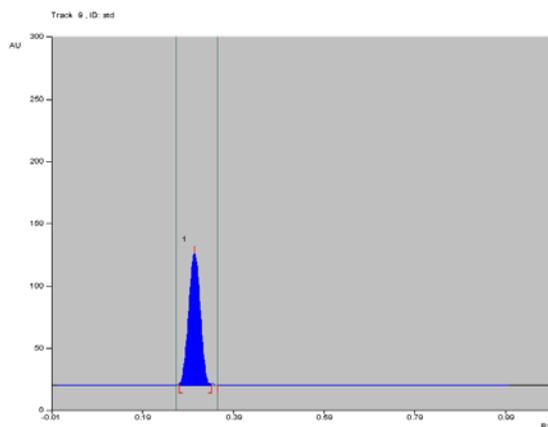


Fig. 2: HPTLC chromatogram of methanolic extract of *Andrographis paniculata*:

### Limit of detection

By applying the proposed method, the minimum detectable limit of 14-Deoxy-11,12-didehydroandrographolide was found to be 40 ng per spot at 263 nm.

### Limit of quantification

By applying the proposed method, the minimum quantification limit of 14-Deoxy-11,12-didehydroandrographolide was found to be 100 ng per spot at 263 nm.

### Linearity

The linearity of the method was checked with working standard of DIAP with the calibration curve in the concentration range of 100 - 2000 ng / spot based on 10 $\mu$ L sample volume. The regression equation was  $Y=228.872 + 3.732 \times X$  and correlation coefficient were obtained with 6 replicate analysis for each concentration. Correlation coefficients were obtained in the range of 0.9986 - 0.9992 indicated excellent linearity of the procedure for working standard DIAP analyzed. Calibration curve of the working standard of 14-Deoxy-11,12-didehydroandrographolide is shown in Figure 3.

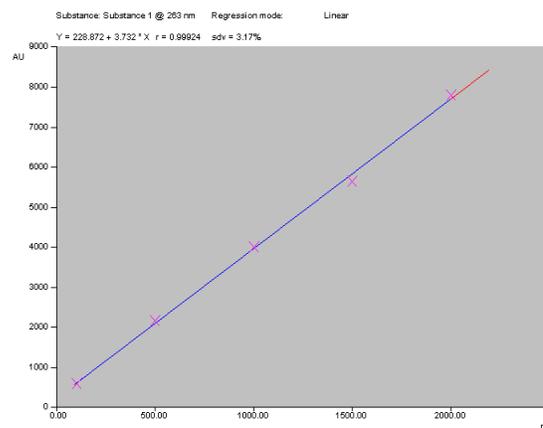


Fig. 3: Calibration curve of standard 14-Deoxy-11,12-didehydroandrographolide:

### Accuracy

The recovery value of 14-deoxy-11,12-didehydroandrographolide was found to be about 94.02 -96.34 %, which shows the reliability and suitability of the method. The results are given in Table 2.

Table 2: Table shows accuracy by percentage recovery of the standard

Sr. No.	Sample	Area	% Recovery
1.	Aqueous extract (A)	-	-
2.	1000 ng of standard	4006.33	-
3.	1500 ng of standard	5584.00	-
4.	Extract (A) + 1000 ng of standard	3860.00	96.34
5.	Extract (A) + 1500 ng of standard	5250.00	94.02

### Precision

Furthermore, the precision of the intraday and interday analyses was investigated on the basis of the lower values of RSD which were obtained in the range of (1.5558-6.5180).

### Robustness and ruggedness studies

The method was found to be re-producible from one analyst to another. The low values of R.S.D. (2.4401% - 2.9497%) obtained after small deliberate changes of the conditions (mobile phase composition, scanning wavelength used for quantification of DIAP and time from chromatography to scanning) used for the method indicated its robustness.

### Stability

In the current assay, analyses of stability samples in methanol on consecutive days (0, 6, 12, 18, 24 and 30 hrs) revealed that the constituent, 14-Deoxy-11,12-didehydroandrographolide, either in standard solution or in the methanolic extract of *A. paniculata* are stable in solution form till 24 hrs. The results are shown in Table 3.

**Table 3: Table shows stability of DIAP content and its reproducibility (in % when compared with 0 hr) in methanolic extract**

Sr.	Time (Hrs)	% content in MeOH ext	Reproducibility (in %)
1.	0	7.820845	100.00
2.	6	7.612493	97.33
3.	12	7.521374	96.17
4.	18	7.335738	93.79
5.	24	7.260785	92.83
6.	30	5.835625	74.61

### CONCLUSION

The lowest detectable limit of 14-Deoxy-11,12-didehydroandrographolide was found upto 40 ng provides good resolution and separation of the marker from other constituents of *Andrographis paniculata*. Further, recovery values of 14-Deoxy-11,12-didehydroandrographolide were found to be about 94.02 - 96.34 %, which shows the reliability and suitability of the method. The proposed HPTLC method is simple, rapid, reproducible, accurate and precise for quantitative monitoring of 14-Deoxy-11,12-didehydroandrographolide in *Andrographis paniculata* samples.

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