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Isolation and Quantification of 14-Deoxy-11,12-didehydroandrographolide in *Andrographis paniculata* by HPLC

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Present work focuses on the development and standardization of a chromatographic method for quantification of 14-deoxy-11,12-didehydroandrographolide (DDAP), from *Andrographis paniculata* by HPLC method. The structure of isolated marker was characterized and confirmed by various advanced spectroscopic methods. A simple, precise, accurate and rapid high pressure liquid chromatographic method has been developed and validated for the estimation of 14-deoxy-11,12-didehydroandrographolide in different extract of *A. paniculata*. Chromatographic separation was carried out on Phenomenex Gemini RP C-18 (5 µ, 250 × 4.6 mm) at room temperature using a (isocratic phase) mobile phase consisting of flow rate of 0.5 mL/min, with UV detection at 235 nm. The method has been studied for linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ). The proposed HPLC method was simple, accurate, precise and suitable for performing the quality control of crude extract and herbal formulation.

Key Words: 14-Deoxy-11,12-didehydroandrographolide, *Andrographis paniculata*, LOD, LOQ and HPLC.

INTRODUCTION

In recent years, focus on use of non-traditional approaches to treat diseases has been revived worldwide. The evidence collected till now shows immense potential of medicine plants used in traditional systems. Kalmegh (*A. paniculata* Nees), commonly known as 'king of bitter' belonging to family Acanthaceae, indigenous to China, India and south east Asia has been traditionally used in Asia for gastric disorders, colds, influenza and other infectious diseases[1]. Extracts of the plant and their constituents have been reported to exhibit a wide spectrum of biological activities of therapeutic importance including antibacterial, antiviral[2,3], antiinflammatory[4], anti-malarial[5], immuno-stimulant[6,7], hepatoprotective[8], antithrombotic[9], anticancer[10], hypoglycemic[11] and hypotensive[12] properties. The constituents found in the plants were diterpene lactones and their glycosides, *i.e.* andrographolide, deoxyandrographolide,11,12-didehydro-14-deoxyandrographolide, neoandrographolide. Flavonoids were also reported to be found in this plant.

Andrographolide is considered to be the most active and important constituent in this plant. However, each component possesses some different potency in pharmacological activities. For instance andrographolide shows high activity for anti-inflammation[13] and hepatoprotection against galactosamine and paracetamol intoxication[14], 14-deoxy-11,12-didehydro-andrographolide produces a potent hypotensive effect[15], whilst neoandrographolide has greater activity against malaria[16]. During crop improvement programme, a quick, sensitive and accurate analytical method was required for the analysis of large numbers of plant samples for andrographolid and its derivatives. Although a few methods[17-20] have been applied for the quantitative determination of the major compound andrographolid, many of these procedures are time consuming and lack precision. Moreover all methods are for only the one diterpenoid andrographolide. In this paper is presented the isolation of the pharmacologically important diterpenoid, development and standardization of a chromatographic method for quantification of 14-deoxy-11,12-didehydro-andrographolide, from Andrographis paniculata by HPLC method.

EXPERIMENTAL

Authenticated materials of *A. paniculata* were purchased from Arvindo herbs, Chennai and authenticated by Dr. P. Jayaraman, Director, Plant Anatomy Research Centre (PARC), Tambaram, Chennai 600 045. A specimen has been stored at the herbarium unit of Asthagiri Herbal Research Foundation (AHRF), perungudi, Chennai-600 096.

Extraction and isolation of 14-deoxy-11,12-didehydro-andrographolide: Powdered dried leaves of *A. paniculata* (200 g) successively extracted with methanol by Soxhlet

apparatus. Filtered the methanol and concentrated using rotary vacuum under reduced pressure. A charcoal treated methanolic extract (11 g) was subjected to column chromatography on silica gel (Merck, 60:120 mesh) eluted with mixtures of hexane and ethyl acetate of increasing polarity to obtain fractions 30 and 14-deoxy-11,12-didehydroandrographolide was eluted in the fractions 16-25 in the ratio of hexane:ethyl acetate (35:65). Collected the fractions and which is further re-crystallized in chloroform:methanol mixture. The isolated compound was identified has 14-deoxy-11,12-didehydroandrographolide using various spectroscopic techniques *viz.*, ^{1}H, ^{13}C NMR, MS and compared with literature values[21] (Fig. 1).

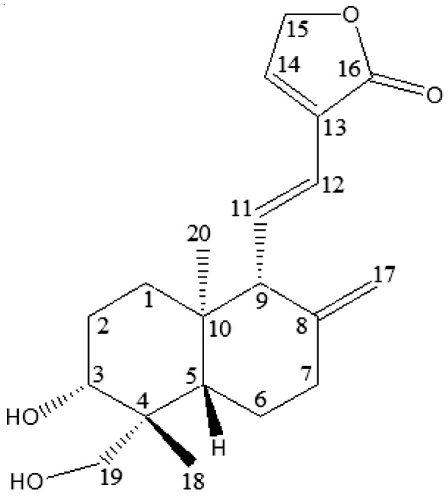

Fig 1. Structure of 14-deoxy-11,12- didehydroandrographolide

Melting point, 196-197 ºC (un-corrected); ($C_{20}H_{28}O_4$) MS, $[M+H]^+$. 333; ^{13}C NMR (deuterated methanol) δ (ppm), 38.11 (C1), 27.48 (C2), 79.80 (C3), 42.37 (C4), 54.41 (C5), 23.03 (C6), 36.36 (C7), 148.71 (C8), 61.43 (C9), 38.24 (C10), 135.08 (C11), 121.11 (C12), 126.20 (C13), 145.30 (C14), 70.17 (C15), 173.41 (C16), 107.70 (C17), 21.94 (C18), 63.62 (C19) and 14.88 (C20).

Extraction of plant material for HPLC analysis: Each 10 g of finely powdered *A. paniculata* were taken in five different 100 mL conical flasks and extracted with 50 mL of different solvents with varying polarity like chloroform, ethyl acetate, acetone, ethanol and methanol. After 24 h the extract has filtered through Whatman No.1 filter paper and the extraction process was repeated thrice with the same volume of the solvent each time. Collected all the three extracts of individual solvents and evaporated under reduced pressure using rotary evaporator. The crude extract was dried and transferred in to a clean bottle. The final weight of the crude extract was weighed and calculated for the yield.

Chromatographic conditions: A Shimadzu HPLC system (model LC 20A) with UV-VIS spectrophotometric detector (model SPD 20A) was used. A Phenomenex Gemini C18 octadecyl silane (ODS) column (5 µ, 250 × 4.6 mm) was used. The mobile phase consists of 0.03 mole of potassium dihydrogen ortho phosphate adjusted pH to 3 with phosphoric acid and acetonitrile (40:60). Aliquots of 20 µL of supernatant of the extracts were injected into the HPLC system and eluted with the mobile phase at a flow rate of 0.5 mL/min. The elutes were monitored at 235 nm with the detector range setting fixed at 0.1.

Preparation of standard solution: 10 mg of isolated compound 14-deoxy-11,12-didehydroandrographolide was dissolved in 10 mL of methanol by sonicating the solution for 5 min and allowed to stand for 5 min at room temperature. 1 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 and filtered through 0.2 µ syringe filter before the injection.

Preparation of sample solution: Accurately weighed 10 mg of each extract of *A. paniculata* was taken in 10 mL volumetric flask and dissolved in methanol by sonicating the solution for 5 min and allowed to stand for 5 min at room temperature. Filtered through 0.2 µ syringe filter before the injection.

Validation of the method: Validation of the analytical method was done according to the International Conference on Harmonization guideline[22]. The method was validated for linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ).

Linearity: Linearity was determined by using 14-deoxy-11,12-didehydroandrographolide standard solution of 1000 µg mL^{-1} in methanol. 12.5 to 200 µg mL^{-1} of the standard solution was prepared (n = 5). The calibration graphs were obtained by plotting the peak area versus the concentration of the standard solutions.

Limit of detection (LOD) and limit of quantification (LOQ): According to the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use recommendations, the approach based on Standard deviation (SD) of the response and the slope were used for determining the detection and quantification limits.

RESULTS AND DISCUSSION

The isolated diterpenoid lactone compound was identified has 14-deoxy-11,12-didehydroandrographolide based on its physico-chemical properties, chromatographic and spectrophotometric aspects. A simple, precise, accurate and rapid high pressure liquid chromatographic method has been developed and validated for the estimation of 14-deoxy-11,12-didehydroandrographolide in different extract of *A. paniculata*. Comparative HPLC chromatogram shows that 14-deoxy-11,12-didehydroandrographolide very well separated from other constituents of *A. paniculata* (Fig. 2). Optimization of various solvent extracts of *A. paniculata* were analyzed by the proposed method and the data are recorded, in which methanolic extract was showing more content of 14-deoxy-11,12-didehydroandrographolide compare with the other extracts (Table-1 and Fig. 3). The limit of detection (LOD) was obtained by successively decreasing the concentration of 14-deoxy-11,12-didehydroandrographolide as long as a signal to noise ratio of 3:1 appeared. The LOD was found to be 0.864 ppm. The limit of quantification (LOQ) was found to be 2.617 ppm of 14-deoxy-11,12-didehydroandrographolide. The calibration graph for 14-deoxy-11,12-didehydroandrographolide

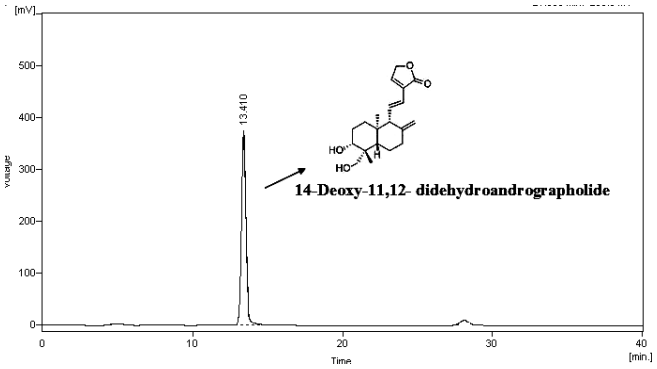

Fig. 2. HPLC Chromatogram of 14-deoxy-11,12- didehydroandrographolide

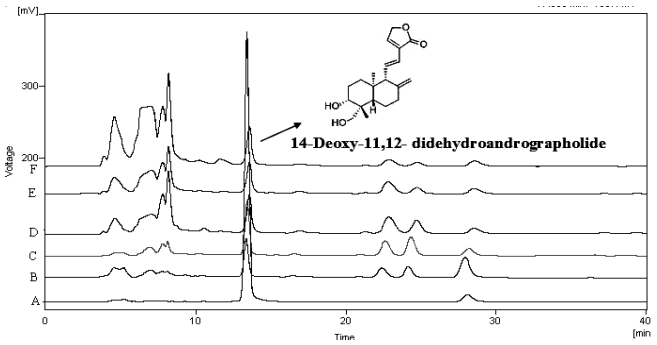

Fig. 3. Comparative HPLC chromatograms of *A. paniculata* (A = 14-Deoxy-11,12-didehydroandrographolide, B = Chloroform extract, C = Ethyl acetate extract, D = Acetone extract, E = Ethyl acetate: Methanol (1:1), F = Methanol extract)

TABLE-1
PERCENTAGE OF 14-DEOXY-11,12-DIDEHYDROANDRO-GRAPHOLIDE (DDAP) IN *Andrographis paniculata*

Name of the extract	Yield of crude extract (% w/w of dried powder)	DDAP content (% w/w)	
		In dried powder	In extract
Chloroform	7.71	1.575	20.43
Ethyl acetate	7.97	2.058	25.83
Acetone	6.92	1.508	21.80
Ethyl acetate: methanol (1:1)	10.15	3.210	31.63
Methanol	12.35	4.053	32.82

was within the concentration range of 12.5-200 µg mL^{-1}, with a correlation coefficient (r^2) of 0.998 (Table-2) and the calibration graph was obtained by plotting peak area *versus* the concentrations of the standard solution.

TABLE-2
LINEAR REGRESSION DATA FOR CALIBRATION CURVE (n = 5)

Parameter	DDAP
Retention time in minute (Mean± SD)	13.41 ± 0.05
Linearity range (µg/mL)	12.5-200
R^2	0.998
Regression equation	y = 56.72x + 1285
Limit of detection (µg/mL)	0.864
Limit of quantification (µg/mL)	2.617

DDAP = 14-Deoxy-11,12-didehydroandro-grapholide

Conclusion

Different solvents of varying polarity have been applied for the extraction and methanol was found suitable for the most efficient extraction of these 14-deoxy-11,12-didehydro-andrographolide. The method was found to be specific and suitable for routine analysis because of its simplicity and reproducibility. The method is particularly suitable for the analysis of a large number of plant samples for the improvement of *A. paniculata* drug for these major and biologically important diterpenoids. This proposed method will be useful for quantitative analysis in standardization and quality assessment of *A. paniculata* for pharmaceutical and cosmetic uses.

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