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Isolation, Characterization and Determination of Nordihydroguaiaretic Acid in Nutrition Supplement by Using Reversed Phase-High Performance Liquid Chromatography

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Nordihydroguaiaretic acid (NDGA) is a lignan found in large amounts in the ethnobotanically important plant, *Larrea tridentata* (Moc. & Sess.) Cov. (Zygophyllaceae). So far NDGA was not isolated and estimated in *larrea tridentata* (moc. & sess.) cov. In this study isolation of Nordihydroguaiaretic acid was achieved by preparative TLC and the compound thus isolated was characterised by Ultraviolet and Mass spectral analysis. Mass spectral data shows molecular ion peak of m/z=302.2. An isocratic RP-HPLC method was developed for the estimation of Nordihydroguaiaretic acid in marketed formulation. Retention time of Nordihydroguaiaretic acid was found as 4.173 min. This method has obeyed linearity over the concentration range of 10-50µg/ml and the regression coefficient obtained from linearity plot for Nordihydroguaiaretic acid was found as 0.999. RP-HPLC method was validated in pursuance of International conference on Harmonization (ICH) guidelines. This method is simple and economic one and it could be used for the estimatimation and routine analysis of the NDGA in any crude drug extract and in any formulation.

Keywords: nordihydroguaiaretic acid, isocratic RP-HPLC, UV detection, validation

INTRODUCTION

Nordihydroguaiaretic acid (NDGA), 2,3-dimethyl-l,4-bis (3, 4-dihydroxyphenyl) butane (Figure 1) is a plant lignan derived from the leaves and twigs of shrub creosote bush, *Larrea tridentata* (Sesse and Moc. ex DC) Coville; family Zygophyllaceae. Creosote bush dominates areas of the desert southwest in the USA and Northern Mexico, as well as some areas of Argentina [1-3]. The concentration of

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NDGA in *L. tridentata* leaves is in the range of 5%–10% of dry weight. Research on this compound and its natural and synthetic derivatives has shown them to be potentially useful in the treatment of cancer, diabetes, viral and bacterial infections, and as regulators of fertility [4, 5]. NDGA is a recognized inhibitor of lipoxygenase (LOX) and has antioxidant and free radical scavenging properties NDGA can also inhibit the platelet derived growth factor receptor and the protein kinase C intracellular signalling family, which both plays an important role in proliferation and survival of cancers. Moreover, NDGA induces apoptosis in tumour xenografts. Nordihydroguaiaretic acid (NDGA) has a unique and strong inhibitory effect on various TGF-beta activities [6-8]. Besides its antioxidant activity, NDGA has several other properties, which are of potential use for humans such as it acts as an enzyme inhibitor antimicrobial agent, potential vaso- and branchodilating agent and antimutagenic agent [9-11].

Though a very few methods have been developed to determine Nordihydroguaiaretic acid in plant extracts by HPLC/TOF-MS, there were no reported methods developed for the estimation of NDGA in commercial formulations [12-15]. This emolliates the author to develop an accurate, suitable isocratic and specific reversed phase high performance liquid chromatography method for determination of Nordihydroguaiaretic acid content in Chaparral tablets with good linearity, and less solvent consumption resulted from the less run time set in the method. This method has been validated according to ICH guidelines [16-17].

EXPERIMENTAL

Instruments

Chromatographic separations were achieved by using Shimadzu LC-20AT Prominence Liquid chromatograph comprising a LC-20AT VP pump, Shimadzu SPD-20A Prominence UV-Vis detector and Welchrom C18 column (4.6 mm i.d. X 250 mm, 5 micron particle size). 20 µL of sample was introduced into the HPLC system. The HPLC data acquisition was performed with Spinchrom" software. Double beam UV-Visible Spectrophotometer (Systronics model 2203) with matched cuvettes was used in this study. In addition, an electronic balance (Shimadzu TX223L), digital pH meter (Systronics model 802), and an ultrasonic bath sonicator (spectra lab, model UCB 40).

Chemicals and reagents

The Nordihydroguaiaretic acid was provided from Sigma Aldrich. Methanol, Ethyl acetate, etc, were purchased from Merck Pvt. Ltd. Mumbai, India. All the other chemicals used to include the solvents were of analytical grade. HPLC grade Methanol and water from Merck specialities Pvt. Ltd. Mumbai, India, Chaparral tablets of 500mg shipped from New York, Arizona natural products, Phoenix, Arizona**.**

Preparation of reagents and standards

Mobile phase (Methanol: Water 3:1v/v) was prepared by taking 750 parts of methanol 250 parts of water were mixed to get one litre of the mobile phase. The mobile phase was then filtered through 0.22 µm nylon membrane vacuum filtration and degassed by sonication. A standard stock solution was prepared by dissolving 100 mg of Nordihydroguaiaretic acid in 100 ml volumetric flask containing 60 ml mobile phase, then sonicated for about 10 minutes and made upto 100 mL with mobile phase to get the primary standard stock solution containing 1000 µg/mL of

Nordihydroguaiaretic acid. Working standard solutions were prepared by further dilution with mobile phase. Sample solution was prepared by dissolving one tablet of 500 mg in 100 mL volumetric flask containing 60mL mobile phase, then sonicated for 10 min and made upto 100 mL with mobile phase.

Isolation and Purification of Active Compounds

Analytical TLC

Analytical TLC was carried out on preparative TLC plates (5 × 5 cm with 0.2mm thickness, silica gel GF_{254}, Merck, Darmstadt, Germany). An aliquot of standard solution of Nordihydroguaiaretic acid and a sample solution of chaparral tablets was spotted onto the silica gel plate and allowed to dry for a few minutes. Afterwards, the chromatoplate was developed with Methanol: Ethyl acetate (70:30v/v) as mobile phase in a previously saturated glass chamber with eluting solvents for some time at room temperature. The developed plate was dried under normal air and the spots were visualised by spraying with a solution of 0.5% (w/v) ferric chloride and dried under oven. The Rf (retention factor) values of isolated compounds and standard were calculated and compared. [18]

Preparative TLC for purification

A streak of crude extract was applied manually on a preparative TLC glass plate (20 cm × 20 cm; 1500 μm thickness) with inorganic fluorescent indicator binder (Analtech, Sigma-Aldrich, Steinheim, Germany). The scratched sample was dissolved in HPLC grade methanol and centrifuged at 12000 rpm for 15 min in order to remove silica. Further, all the dried samples were passed under nitrogen gas for 5min and then dissolved in methanol for further characterization and quantitative HPLC analysis. The entire purification process was carried out under dark or dim light conditions. [19]

Characterization of purified compound

The UV spectrum of the purified compound was recorded from 190 to 600 nm on a *ELICO* double beam spectrophotometer UV-visible spectrophotometer.

Recommended procedure for determination of nordihydroguaiaretic acid in chaparral tablets by RP-HPLC

Simple RP-HPLC method was not reported for determination of NDGA and so many formulations with the species *larrea tridentate,* thus this this study was initiated and as for as the system suitability. The chromatograph was stabilised for about 45minutes with mobile phase at the required flow rate to get a steady base line. System suitability was ascertained by six replicate analyses of the drugs at concentrations of 10 µg/mL of Nordihydroguaiaretic acid.

In this study, the chromatograph was stabilized for about 45minutes with mobile consisting of methanol: water (30:10v/v). The flow rate was 1.0 mL/min phase at the required flow rate to get a steady base line. Aliquots of standard solution containing Nordihydroguaiaretic acid (1.0-5.0 mL, 100 µg/mL) were transferred to a series of 10ml capacity volumetric flasks to get the concentrations ranging from 10-50 µg/mL. accurately injected about 20µL of each calibration standard into the chromatograph. Peak areas of each solution were recorded. A calibration curve was plotted between concentration and peak area response. 20 µL of sample solution prepared was injected and the area of peak was recorded duly maintaining the

ambient experimental conditions as followed for the standard drug solutions. The amount of Nordihydroguaiaretic acid present in the sample was computed from its calibration graph.

RESULTS AND DISCUSSION

The calibration curve was obtained and the data of regression analysis of the method is depicted in Table 1. The regression coefficient obtained from linearity plot for Nordihydroguaiaretic acid was found as 0.999, which indicates this method had good linearity and the linearity data was given in Table 2. The developed method was applied to the determination of concentration of sample and results are shown in Table 3. The amount of Nordihydroguaiaretic acid was found as 7.53mg/100ml extract it is comparatively simple method than the reported by (Julianne et al., 1986).The method validation parameters were established in this work, LOD and LOQ of the Nordihydroguaiaretic acid were found as 0.78µg/ml and 2.38µg/ml and the proposed method was found to be precise for the determination. The %RSD for the proposed method was found to be less than 2.0 which indicate the method's precision. Results of the precision study are shown in the Table 4 and 5. Isolation of Nordihydroguaiaretic acid from the sample (Chaparral tablets) was achieved by preparative thin layer chromatography using the same chromatographic conditions followed for identification of active constituent. Characterisation of isolated compound was done by studying ultraviolet and ESI mass spectra. Mass spectral data shows molecular ion peak m/z=302.2 which has a moderate abundance (Figure 3). Nordihydroguaiaretic acid shows UV absorption at about at 281.6 nm in methanol: water (3:1) indicates the presence of conjugation and hydroxyl auxochrome which shifts the absorption maximum towards visible side of the spectrum and it was represented in Figure 2. Chromatograms showed a peak of Nordihydroguaiaretic acid at retention time of 4.173 min. The representative chromatograms of this method were given in Figure 4 and Figure 5 for calibration of standard and sample respectively. The proposed method was found to be robust as there were no marked changes in the performance characteristics of the method according to the ICH guidelines.

Table 1: Regression Analysis of the proposed method

Parameter	Nordihydroguaiaretic acid
Detection wavelength(nm)	UV at 281nm
Linearity range (µg/mL)	10-50 µg/ml
Regression equation (Y = aX + b)	y = 12.08x + 1.198 R^2 = 0.999
Slope(a)	12.08
Intercept(b)	1.198
Standard error of slope (S_a)	0.095056404
Standard error of intercept (S_b)	2.877975541
Standard error of estimation (S_y)	3.976494659
Regression coefficient (R^2)	0.999
Limit of detection(µg/mL)	0.785698694
Percentage range of errors (Confidence limits) 0.005 significance level 0.001 significance level	0.014721 0.017256

Table 2: Linearity analysis of HPLC method

S. No.	Conc.(µg/ml)	Area of standard Nordihydroguaiaretic acid
1	0	0
2	10	120.23
3	20	244.422
4	30	365.89
5	40	489.56
6	50	600.25

Table 3: Determination of concentration of Nordihydroguaiaretic acid in tablet formulation from its Precision study

Trail no	Nordihydroguaiaretic acid		
	Area	Concentration (µg/ml)	Amount (mg/ 100ml extract)
1	365.89	30.1897351	7.547435
2	364.95	30.11192053	7.52798
3	365.89	30.1897351	7.547435
4	366.45	30.0705298	7.5176325
5	365.98	30.19718543	7.5492975
6	365.54	30.16076159	7.54019
Result		Mean	7.538328333
		S.D	0.012845674
		%RSD	0.170404808

Table 4: Interday and Intraday Precision for tablet formulation

Trail	Interday precision			Intraday Precision		
	Area	conc	Amt (mg/ml)	Area	conc	Amt (mg/ml)
1	365.89	30.18974	754.7435	365.89	30.18974	754.7435
2	364.95	30.11192	752.798	364.95	30.11192	752.798
3	365.89	30.18974	754.7435	364.78	30.11077	752.76925
4	366.45	30.07053	751.76325	364.78	30.11077	752.76925
5	365.98	30.19719	754.92975	364.78	30.11077	752.76925
6	365.54	30.16076	754.019	364.78	30.11077	752.76925
Mean	365.7833	30.15331	753.8328333	364.9933	30.12412	753.1030833
SD	0.501903	0.051381	1.284567395	0.444507	0.032149	0.803719038
%RSD	0.137213	0.170398	0.170404808	0.121785	0.106721	0.106720986

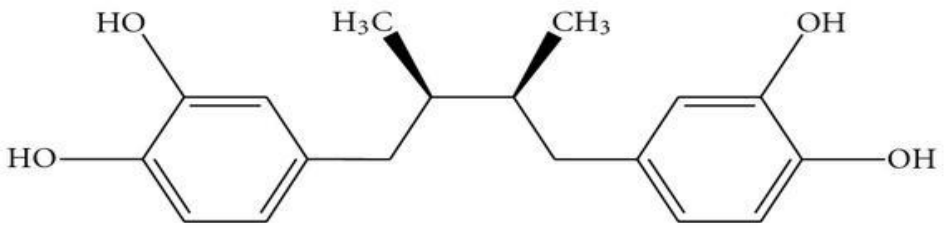

Figure 1: Structure of NDGA [20}

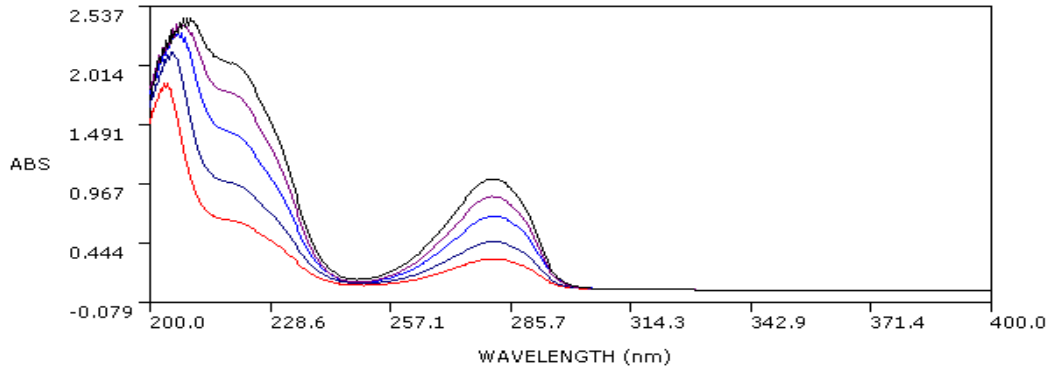

Figure 2: UV absorption spectrum of Nordihydroguaiaretic acid in methanol:water (3:1)

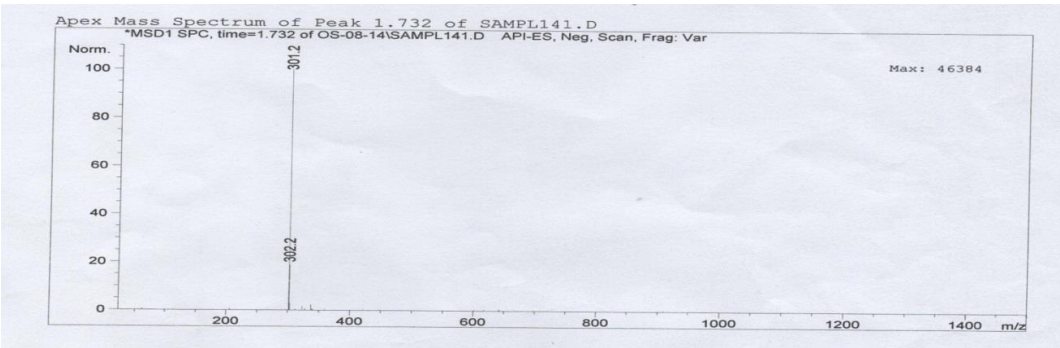

Figure 3: Mass spectra of Nordihydroguaiaretic acid isolated from tablets

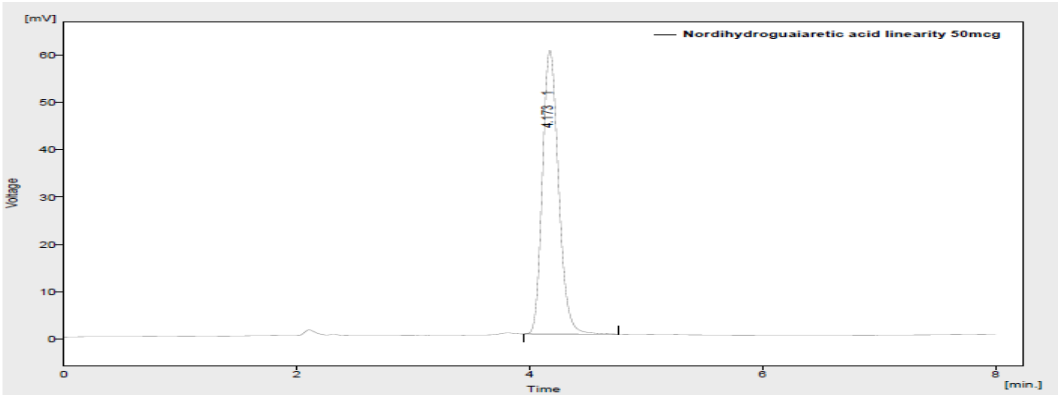

Figure 4: Chromatogram of NDGA standard

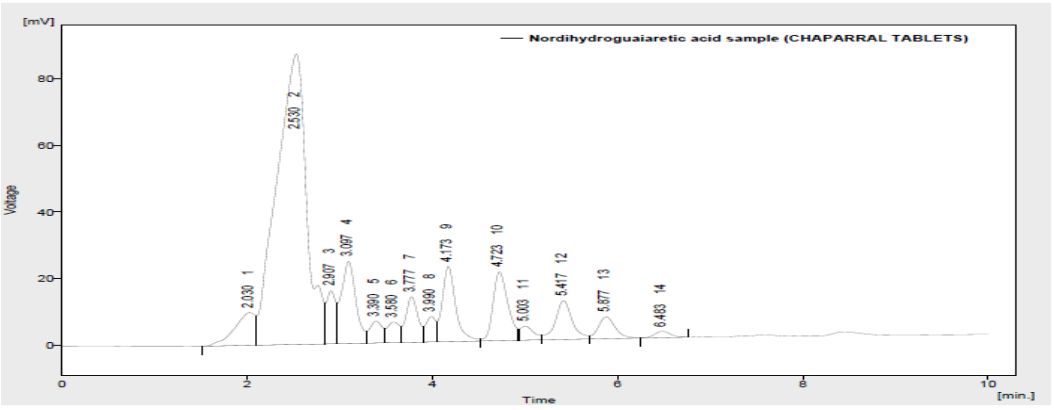

Figure 5: Chromatogram of NDGA in tablet formulation

CONCLUSION

To determine the concentration of Nordihydroguaiaretic acid in Chaparral tablet formulation and for the standardisation of herbal formulations containing active constituent (Nordihydroguaiaretic acid) the proposed HPLC method is linear, sensitive, accurate and precise, which can be adopted with shorter run time and good efficiency.

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