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Pharmacokinetic analysis by high-performance liquid chromatography of intravenous nordihydroguaiaretic acid in the mouse

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Abstract

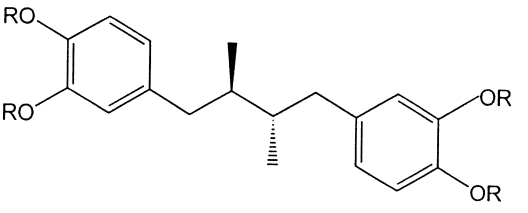
Nordihydroguaiaretic acid (NDGA) has been shown to inhibit both 5-lipoxygenase and ornithine decarboxylase and is active against several cancer cell lines and at least one mouse tumor model. Despite these findings, there have been no reports on the pharmacokinetics of NDGA. A reverse-phase high-performance liquid chromatography (HPLC) method was developed to detect NDGA in mouse plasma. The limit of detection of this method was 0.5 μg/ml. Administration of NDGA (50 mg/kg, i.v.) to mice resulted in a peak plasma concentration of 14.7 μg/ml. The terminal half-life of NDGA was 135.0 min with a clearance of 201.9 ml/min·kg.

Keywords: Nordihydroguaiaretic acid

1. Introduction

Larrea tridentata (Sessé & Moc.) Coville (Zygophyllaceae), a shrub common to the southwestern United States and northern Mexico, is commonly known as creosote bush, greasewood, or chaparral. It has been used traditionally for treatment of rheumatoid arthritis, relief of menstrual cramps, treatment of infections, and cancer [1].

Nordihydoguaiaretic acid (NDGA) is a lignan found in high amounts in the genus *Larrea* (Fig. 1). This compound has been reported to inhibit both 5-lipoxygenase and ornithine decarboxylase [2,3].



NDGA: R = H

M_4N: R = CH_3

Fig. 1. Chemical structure of NDGA and M_4N.

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Recent reports suggest that NDGA possesses anticancer activity both in vitro and in vivo. NDGA inhibits the incorporation of [^{3}H]-thymidine by K562 chronic myelogenous leukemia cells in culture and reversibly inhibits DNA synthesis by human glioma cells at 230 μM and 40 μM, respectively [2,4]. Both non-small and small cell lung cancer cell lines are inhibited by NDGA with an IC_{50} of 5–7 μM [5]. Moreover, 0.1% NDGA in the drinking water of athymic mice bearing non-small cell lung cancer tumors significantly inhibits tumor growth relative to controls. Histological analysis shows increased numbers of apoptotic bodies in treated tumors [6]. NDGA was also shown to modulate tumor cell sensitivity to vinblastine by a mechanism independent of interference with the MDR_1 gene product [7]. When topical NDGA (masoprocol) was used in the treatment of actinic keratoses, it was shown to be superior to 5-fluorouracil [8,9].

Given the anticancer potential of NDGA, we developed a high-performance liquid chromatography (HPLC) assay in order to detect this compound in biological matrices as part of an effort to define its pharmacokinetics and metabolite profile. Here we present a quantitative, HPLC assay for detecting NDGA in plasma. We have used this assay to characterize the pharmacokinetics of intravenously-administered NDGA in the mouse.

2. Experimental

2.1. Reagents and materials

NDGA (99.5% purity), dimethylsulfate, and potassium carbonate were purchased from Aldrich Chemical Co (Aldrich, Milwaukee, WI, USA). A co-solvent system used to solubilize the anticancer agent etoposide (Vepesid™, Bristol-Myers Squibb Co., Princeton, NJ, USA) was used to solubilize NDGA for injection into mice. This co-solvent system contains (per ml): citric acid, 2 mg; benzyl alcohol, 30 mg; polysorbate 80, 80 mg; polyethylene glycol 300, 650 mg and absolute alcohol, q.s. 1.0 ml [10]. Whole mouse serum was purchased from ICN Biochemicals (Costa Mesa, CA, USA). All other chemicals used in this study were of the highest grade available.

2.2. Animals

Female CD1 mice (~25 g) were purchased from Harlan Sprague Dawley, (Indianapolis, IN, USA), and allowed to acclimatize for at least 1 week before initiation of studies. Animals were housed under normal conditions according to animal care guidelines established by The University of Arizona Institutional Animal Care and Use Committee (Tucson, AZ, USA).

2.3. Equipment

Analysis was performed on a Perkin-Elmer Series 4 liquid chromatograph (Perkin-Elmer, Norwalk, CT, USA) equipped with a Hewlett-Packard Series 1100 diode array detector (Hewlett-Packard, San Fernando, CA, USA). Extracted plasma was injected with a Perkin-Elmer ISS-100 auto injector equipped with a 100 μl loop. An Adsorbosphere™ C_{18} reverse phase column (250 mm×4.6 mm) with a 5 μm pore size (Alltech, Deerfield, IL, USA) was used for all applications. The mobile phase was a 15 min gradient from H_2O and acetonitrile (70:30, v/v) to H_2O and acetonitrile (30:70, v/v). This was followed by a 15 min period of isocratic H_2O and acetonitrile (30:70, v/v). All mobile phase solvents contained 0.1% trifluoroacetic acid (v/v) and were degassed with helium. The flow-rate was 1.5 ml/min.

2.4. Synthesis of an internal standard: Tetra-O-methylnordihydroguaiaretic acid

Tetra-*O*-methylnordihydroguaiaretic acid (M_4N, Fig. 1) was synthesized using the method of Hwu et al. [11]. NDGA (1 eq.) and potassium carbonate (6 eq.) were dissolved in acetone. Dimethyl sulfate (4 eq.) was added and the reaction mixture was refluxed for 8 h. The reaction was stopped with HCl (1 N, 6 eq.) and the acetone was evaporated under vacuum. The resultant M_4N was extracted three times with 100 ml ethyl acetate. The organic phases were combined and washed three times with 100 ml HCl (0.1 N) and three times with 100 ml NaCl (1 *M*). The organic phase was dried completely under vacuum and the product was recrystallized from methylene chloride. HPLC analysis showed a single

peak with a retention time of 21–22 min. The structure was confirmed using mass spectrometry.

2.5. Standardization of assay

Known amounts of NDGA (0.05–5 μg) dissolved in acetonitrile were added to 0.5 ml mouse serum. M_4N (2.5 μg) was added to each sample as an internal standard. Serum was applied to Varian Bond Elut C_{18} solid-phase sample preparation columns (Varian, Harbor City, CA, USA) that had been conditioned by washing three times with 3 ml methanol and three times with 3 ml H_2O. Columns were washed with 3 ml of H_2O. Analytes were eluted with 300 μl of methanolic–HCl (0.1 N). Duplicate, 100 μl samples were injected onto the HPLC. A ratio of the area of the NDGA peak to the area of the M_4N peak was plotted as a function of the amount of NDGA injected. This served as a standard curve for pharmacokinetic analysis. Daily calibrators were prepared as above from separate stock solutions of NDGA and M_4N. These calibrators were used to determine the repeatability of the assay within the same day and over the course of three consecutive days.

2.6. Determination of assay extraction efficiency

Known amounts of NDGA (0.05–5 μg) were dissolved in 300 μl of methanolic–HCl (0.1 N). Duplicate, 100 μl samples were injected onto the HPLC. A ratio of the area of the NDGA peak to the area of the M_4N peak was calculated and compared to the ratio for each amount of NDGA derived from samples extracted from serum to obtain the extraction efficiency.

2.7. Determination of NDGA protein binding

NDGA was dissolved in 5 ml mouse serum (2.5 μg/ml in acetonitrile) and equilibrated with the dialysis chamber for 4 h at 37°C. A 0.5 ml sample was removed to determine the degree to which NDGA binds the dialysis chamber. The remaining serum was then dialyzed against 150 m*M* Tris–HCl (pH 7.5) and 50 m*M* NaCl at 37°C for 24 h. The molecular mass cut-off of the dialysis membrane (VWR Scientific, So. Plainfield, NJ, USA) was 12 kDa. NDGA was extracted from the pre-dialyzed serum, the dialyzed serum and the Tris–HCl/NaCl by solid-phase extraction and eluted with 300 μl of 0.1 N methanolic–HCl. Duplicate 100 μl samples of each were analyzed by HPLC as described above.

2.8. Pharmacokinetic analysis of NDGA in CD1 mice

Female CD1 mice (25–30 g) were injected with NDGA (50 mg/kg, i.v.) dissolved in the etoposide diluent and water (30:70, v/v). Blood was collected 5, 10, 20, 40, 60, 120, 240, and 360 min after injection by cardiac puncture following ether anesthesia. Plasma was separated out by centrifugation at (250 *g*) and 2.5 μg M_4N was added as an internal standard. NDGA and M_4N were extracted and analyzed as previously described. Plasma NDGA levels were determined by comparison of the peak ratios of mouse samples with the standard curve.

A plot of plasma NDGA concentration as a function of time was prepared. From this plot, peak plasma concentration (C_{max}) was determined by back extrapolation to time zero. The area under the curve (systemic exposure, *AUC*) was determined by integration of the plasma concentration–time plot using Microcal Origin (Microcal Software, Northampton, MA, USA). Clearance (*CL*) was determined by dividing the i.v. dose (μg/kg) by the systemic exposure. The volume of distribution (*Vd*) was calculated by dividing the dose by C_{max}; half-life ($t_{1/2}$) was determined graphically.

3. Results

3.1. Synthesis of Tetra-O-methylnordihydroguaiaretic acid

M_4N was synthesized by the method of Hwu et al. [11] with a yield greater than 90%. The product appeared as a light beige, amorphous powder. The structure was confirmed by mass spectrometry. Fragments of *m*/*z*: 359, 344, 221, 179, 165, 151, and 136 were observed, corresponding to the fragments previously reported for this compound [11].

3.2. Standardization of assay

The retention time of NDGA varied between 8 and 9 min under the assay conditions described. The retention time of M_4N was approximately 19 to 20 min. A representative chromatogram of NDGA and M_4N extracted from mouse serum is shown in Fig. 2.

The standard curve of NDGA extracted from mouse serum was linear from 0.5 μg/ml to 10 μg/ml with a slope = 0.3979 (n = 3, S.E. = 0.049), a y-intercept = −0.0251 (n = 3, S.E. = 0.024), and $r^2$0.998. The limit of detection was 0.5 μg/ml(n = 3, S.E. = 0.052 μg/ml) at a signal-to-noise ratio of 5:1. The assay was reproducible with an average within day coefficient of variation less than 10% and an average between day coefficient of variation less than 15% (Table 1).

The extraction efficiency was determined by preparing a standard curve of NDGA dissolved in 0.1 N methanolic–HCl. The average percent recovery of NDGA from mouse serum was 64.67% (n = 3, S.E. = 3.65%).

To determine if the incomplete recovery of NDGA was due to its irreversible binding to the C_{18} sample preparation columns, NDGA was dissolved in methanolic–HCl and eluted through a column. HPLC analysis of the eluant showed that no NDGA was retained on the sample preparation column.

Equilibrium dialysis was then used to determine if the loss of NDGA was due to protein binding. After 4 h and 24 h, 50% and 82% of NDGA bound the dialysis apparatus, respectively. Of the 18% that was remained free after 24 h, 97% was retained on the serum side, while 3% dialyzed to the Tris–HCl/NaCl side.

Table 1
Within day and between day coefficient of variation

Concentration (μg/ml)	Within day variation (%)	Between day variation (%)
0.5	10.0	14.8
1.0	ND	8.2
2.0	11.0	9.9
5.0	ND	2.4
10.0	7.8	0.88
Average	9.6	7.2

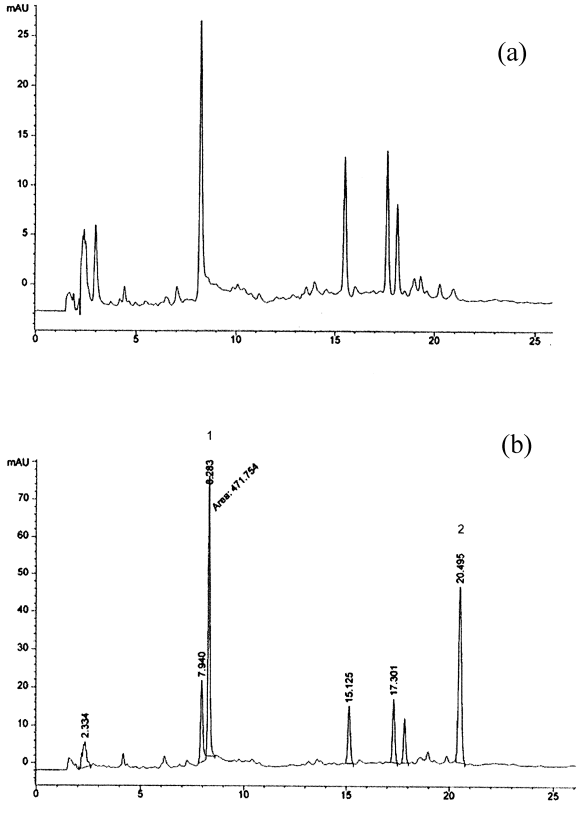

Fig. 2. (a) Chromatogram of blank mouse serum. (b) Representative chromatogram of NDGA and M_4N extracted from mouse serum. Peaks: 1 = NDGA, 2 = M_4N.

3.3. Pharmacokinetic analysis of NDGA in the mouse

The pharmacokinetic characteristics of intravenously-administered NDGA were determined in female CD1 mice. Mice were injected with a single dose of 50 mg/kg. The plasma concentration of NDGA was determined at 5, 10, 20, 40, 60, 120, 240, 360 min after injection. Fig. 3 shows the plasma concentration of NDGA as a function of time. This compound appears to follow a two-compartment model of pharmacokinetics. The level of NDGA in the mouse falls below the limit of detection for our assay by 360 min. Based on the concentration–time plot, we determined C_{max}, exposure, distribution half-life ($t_{1/2}\alpha$), terminal half-life ($t_{1/2}\beta$), volume of distribution, and clearance (Table 2).

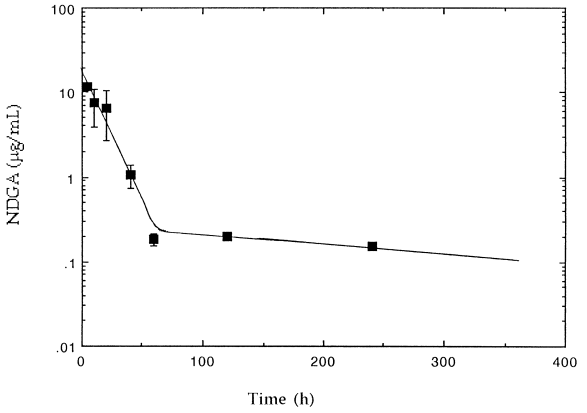

Fig. 3. Plasma concentration–time profile of NDGA following 50 mg/kg (i.v.) dose to female CD1 mice ($n=4$).

4. Discussion

We have developed a quantitative and reproducible method of detecting NDGA in biological materials. This assay was used to determine the pharmacokinetic parameters of intravenous NDGA in the mouse. Our assay reproducibly detected this compound in plasma and serum with a limit of detection of 500 ng/ml. The extraction method used appears to release NDGA from serum proteins thus allowing the easy detection of this highly protein–bound compound.

While the high degree of protein binding may limit the bioavailability of free NDGA in vivo, the relatively long half-life may allow plasma levels of the free drug to reach effective levels in tissues. Prior studies in tumor cells suggested that cytotoxic levels of NDGA range from 20–40 μ*M* in acute myeloid leukemia cells [12] and human glioma cells [4] and from 2–7 μ*M* in human non-small cell lung cancer cell lines [6]. These values compare to the peak plasma level of 48.6 μ*M* (14.7 μg/ml) achieved with the 50 mg/kg i.v. dose in CD1 mice in the current study. Thus, it appears that cytotoxic NDGA concentrations can be achieved acutely in mice. Long term toxicologic consequences of these doses are currently under investigation.

The high clearance of NDGA, approximately double that of creatinine, suggests that the compound may be cleared by non-renal means. One possibility is that it may be extracted by the liver and metabolized to as yet unknown derivatives. Currently, we are attempting to quantify and identify metabolites of NDGA both in primary isolated mouse hepatocytes and in whole animals.

Table 2
Pharmacokinetic parameters of NDGA (i.v.) in female CD1 mice

Parameter	Value
C_{max}	14.7 μg/ml
Exposure (*AUC*)	247.7 (μg/ml)·min
CL	201.9 ml/(min·kg)
Vd	3.4 l/kg
$t_{1/2}\alpha$	30.0 min
$t_{1/2}\beta$	135.0 min

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