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HPLC Analysis of Free Malonaldehyde in Nine Ginseng Polyacetylene-Treated Liver Microsome

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Abstract□ Free malonaldehyde was determined in nine kinds of ginseng polyacetylene-treated microsome by HPLC analysis. Antioxidant activities of some phenolic compounds and ginseng saponin were also determined both by a new HPLC method and by TBA method. A new HPLC system separated malonaldehyde at a retention time of 5.6 min and showed a linear relationship between the peak are a and malonaldehyde concentration. Panaxynol showed the strongest activity among nine polyacetylenes, and the addition of either chlorine or acetyl group reduced polyacetylene's own activity. Since C_{14}-polyacetylenes such as panaxyne and panaxyne-epoxide had little or no antioxidant activities, C_{17}-structure should be preserved to exert a radical-scavenging or trapping activity. The antioxidant activities of chlorogenic acid, ferulic acid and catechol were much weaker than those of C_{17}-polyacetylenes. Ginseng saponin showed no antioxidant activity. Since TBA reactive substances and malonaldehyde contents were almost the same in peroxidized microsome, TBA value seems a good indicator for lipid peroxidation in this particular Fe^{+3}-ADP/NADPH system.

Keywords□ *Panax ginseng* C.A. Meyer, polyacetylene, free malonaldehyde, HPLC.

Introduction

Polyacetylenes are distributed in several species of Araliacea as C_{17}-or C_{18}-Structures and only in very small amounts[1]. The first polyacetylene compound isolated from *Panax ginseng* C. A. Meyer was panaxynol named as falcarionl in 1964[2]. Later, series of C_{17} or C_{14}-polyacetylenes were isolated from ginseng root and callus, and some have chlorine or acetyl group[3-9]. Cytotoxic activities of panaxydol, panaxynol and panaxytriol against cultured murine L1210 leukemic cells[10] and cytotoxicity of panaxytriol on B16 mouse melanoma and fibroblast-derived tumor L929[11] made polyacetylenes of interest to biochemists, chemists and pharmacologists. Specific cytotoxic mechanisms of these polyacetylenes on cancer cells were investigated[12,13]. Even though antiinflammatory effect of panaxynol was proposed in early 1980[14], cytotoxic effect against cancer cells was the main biological action of polyacetylenes until 1987. In 1988, antioxidant activities of panaxydol, panaxynol and panaxytriol were identified by suppressing CCl_4-induced lipid peroxidation *in vivo* and *in vitro* hepatic microsomal lipid peroxidation, which supported the earlier studies on antioxidant activities of panaxyne A and C whose structures were unidentified[17]. Recently, it was clarified that antioxidant activity of panaxytriol may prevent a plethora of biochemical events caused by tumor promoter, croton oil in mouse skin tumorigenesis[18].

Malonaldehyde(MA) is one of the most studied products of lipid peroxidation and has been implicated in aging, mutagenesis and carcinogenesis. The toxicity of MA is believed to be the result of its reactivity with biological nucleophiles such as

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amino acids and thiols and its ability to induce cross-linking of proteins and nucleic acids[19]. Several methods have been developed to estimate MA formed by lipid peroxidation. The most widely used method is thiobarbituric acid(TBA) assay. However, TBA method is not specific for MA and often overestimate MA levels because some chemicals, such as aldehydic compounds, react with TBA to produce absorbance spectra similar to those of the TBA-MA complexes. Besides the lack of specificity, artificial production of MA could be occurred by heat and acid in the TBA reaction itself [20-21].

In present study, a modified HPLC method for measuring MA content in lipid peroxidation system was used by simply changing the earlier developed HPLC analysis for MA[22] and had a shorter retention time. Antioxidant activities of nine polyacetylenes and total saponin from Panax ginseng C. A. Meyer and some phenolic compounds were determined both by a new HPLC analysis and by TBA value.

Materials and Methods

Materials

The materials used in the study were obtained from the following sources : nicotinamide adenine dinucleotide phosphate(NADPH), adenosine 5'-diphosphate(ADP), DL-α-tocopherol, catechol, chlorogenic acid, ferulic acid, bovine serum albumin (BSA), 2-thiobarbituric acid(TBA) and 1,1,3,3,-tetraethoxypropane from Sigma chemical Co.(St. Louis, MO): Tris(hydroxymethyl) aminomethane, Kanto chemical Co.(Tokyo, Japan): Trichloroacetic acid, Junsei chemical Co.(Tokyo, Japan): ferric chloride from Fluka chemical Co.(Phillipsburg, NJ) and membrane filter(GA-8, TF-200; 0.2 μm) from Gelman sciences Inc. (Ann Arbor, MI). All other reagents were of guaranteed reagent grade commercially available. Nine polyacetylenes were isolated from Panax ginseng C. A. Meyer by the method of Kim[9] and ginseng saponin was prepared by the procedure described by Ando *et al* [23]. The structures of nine polyacetylenes used in the study are shown in Table 1.

Table 1. Structures of polyacetylene compounds from *Panax ginseng* root

C_7H_{15}-(X)-$(C\equiv C)_2$-CH(OR)-CH=CH_2

Compounds	R	X
Panaxydol	H	-CH-CH-CH_2 (O)
Panaxynol	H	-CH=CH-CH_2
Panaxytriol	H	-CH(OH)-CH(OH)-CH_2
Heptadeca-1,8-t-dien-4, 6-diyn-3,10-diol	H	-CH(OH)-CH=CH-
Acetyl panaxydol	$COCH_3$	-CH-CH-CH_2 (O)
Panaxydolchlorhydrin	H	-CH(Cl)-CH(OH)-CH_2
10-Acethy panaxytriol	H	-CH($OCOCH_3$)-CH(OH)-CH_2
Panaxyne		H-$(C\equiv C)_2$-CH_2-CH(OH)-CH(OH)-$(CH_2)_5$-CH=CH_2
Panaxyne epoxide		H-$(C\equiv C)_2$-CH_2-CH-CH- (O) -$(CH_2)_5$-CH=CH_2

Calibration and preparation of the malonaldehyde standard

A malonaldehyde stock solution(10 mM) was prepared from malonaldehyde tetraethyl acetal (1,1,3,3-tetraethoxypropane). 1 mmol(220 mg) of the acetal was dissolved in 100 m*l* of 1%(v/v) sulfuric acid. After 2 hr standing at ambient temperature, 1 m*l* of the solution was brought to a volume of 100 m*l* with 1%(v/v) sulfuric acid and the malonaldehyde concentration was checked by measuring the UV absorbance in 1-cm cuvettes at 245 nm(ε = 13,700). For HPLC calibration curve aliquots of the 10 mM stock solution was diluted with 0.1 M Tris buffer (pH 7.4) to give a final malonaldehyde concentration in the range of that present in the peroxidized microsomal suspension, preferably 20.0×10^{-6} M.

Table 2. HPLC conditions

Condition	Malonaldehyde analysis
Column	Merck Lichrosorb NH_2 column (10 μm, 25 cm×4.0 mm)
Mobile phase	0.1 M pH 7.4 Tris buffer/ acetonitrile (9:1, v /v)
Flow rate	1 ml/min
U.V. detection	270 nm
Pressure	900 psi
Termperature	ambient

HPLC system and condition

Dionex basic chromatography module(Dionex Co.. Sunnyvale, CA) connected with SP4270 computing integrator(Spectra-physics Co., San Jose, CA) and Applied Biosystems 1000S diode array detector (Ramsey Analytical Div., San Jose, CA) was used. The column was a Lichrosorb NH_2 column(10 μm particle size; E. Merck, Darmstadt, F.R. Germany). A sample of microsomal suspension or malonaldehyde standard solution was serially filtered through membrane filter(0.45 μm and 0.2 μm) and injected into HPLC with 50 μl Hamilton syringe (Hamilton Co., Reno, Nevada). HPLC chromatographic condition is shown in Table 2.

In vitro microsomal lipid peroxidation

Livers from male Sprague-Dawley rats(200-250g) were homogenized and centrifuged at 1,500× g for 20 min in a refrigerated centrifuge. The supernatant was further centrifuged at 20,000×g for 10 min and 120,000×g for 60 min to harvest microsomal fraction. The pellet was suspended in 0.1 M Tris-HCl buffer, pH 7.4[24]. Protein concentration in microsomal suspension was measured by Lowry *et al.*[25] NADPH-dependent microsomal lipid peroxidation reaction mixture contained microsomal protein (1 mg/ml), 1.7 mM ADP, 0.1 mM $FeCl_3$, 0.1 mM NADPH and 100 μM polyacetylenes or phenolic compounds and/or 100 μg of ginseng saponin dissolved in absolute ethanol in 0.1 M Tris-HCl buffer, pH 7.4. Incubation was carried out at 25 °C for 30 min under an air atmosphere in a water bath. Lipid peroxidation was determined by

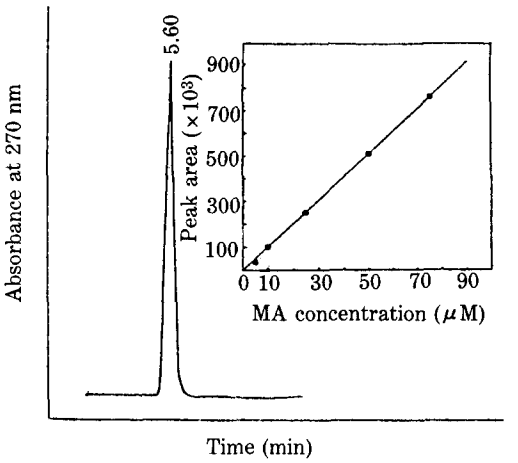

Fig. 1. HPLC elution profile for standard malonaldehyde (MA), which was prepared from tetraethoxypropane. Inset shows a standard curve for HPLC of malonaldehyde on a Lichrosorb NH_2 column: peak area vs injected amount in μm.

the formation of the TBA-reactive material, malondialdehyde(MDA) using a method of Fairhurst *et al.*[24]. For malonaldehyde(MA) determination by HPLC, microsomal suspensions after incubation were filtered and directly injected to HPLC system.

Results and Discussion

HPLC procedure for standard curve of malonaldehyde

The spectrum of malonaldehyde(MA) stock solution showed a maximum absorption at 270 nm with a retention time of 5.6 min (Fig. 1). This system is a modification of that developed by Esterbauer *et al.*[22] by simply changing the molar concentration of eluent and earned a shorter retention time as compared with a retention time of 7 min. The stock solution of MA was diluted with 0.1 M Tris-HCl buffer, pH 7.4 to achieve a final concentration in the range 5.0×10^{-6} to 75.0×10^{-6}M for the preparation of the standard curve. There was a linear relationship between peak area and MA concentration. Recovery of standard MA in this procedure, after addition to the known concentrations similar to those present in microsomal suspension, was above 96%(Data are not shown). Csallany *et al.*[26]

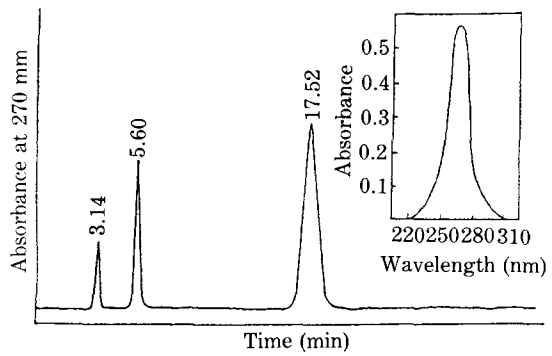

Fig. 2. HPLC separation of malonaldehyde in peroxidized microsome incubated in Fe^{+3}-ADP/NADPH system at 37°C for 30 min. Retention times are given above the peaks in minutes; nicotinamide (3.14), malonaldehyde (5.60), unknown peak (17.52). Inset shows the on-line spectrum of malonaldehyde as taken during its elution from HPLC column; the absorption maximum is indicated at 270 nm.

reviewed HPLC methods for the quantification of MA. They found that none of these methods satisfied the need for a simple, sensitive and specific method for quantification of bona fide MA in tissues because of requirement of a large amount of tissue, low recovery or unidentification of the purity of the questioned peak. They separated free MA in biological systems specifically but retention time was too long as 49.9 min. Recently TBA-MDA(malondialdehyde) adduct was quantitated with a fluorescence detector and picomole quantities of MDA in plasma and liver samples were detected within 4.9 min[27]. Even though the lower detection limit of MA in the present system has not been checked, present HPLC method can be easily applied for the detection of MA in biological systems when fluorescence detection is unavailable.

Free malonaldehyde in peroxidized microsome

After 30 min incubation in Fe^{+3}-ADP/NADPH system, liver microsomes were peroxidized and peak area of MA was increased at a retention time of 5.60 min(Fig. 2.). Earlier peak was identified as nicotinamide(3.14 min) present both in normal microsome and reaction mixture in different amounts. Later peak was not identified but surmised as a reaction product of lipid peroxidation other than MA because it was not appeared in normal microsome.

Table 3. Comparison of antioxidant activities of polyacetylene compounds by TBA value and HPLC analysis

Treatment	TBARS	%	Malonaldehyde	%
None	0.740±0.049	100	0.701±0.033	100
Panaxydol	0.444±0.058	60	0.449±0.020	64
Acetylpanaxydol	0.570±0.053	77	0.652±0.050	93
Panaxydol chlorhydrin	0.577±0.070	78	0.603±0.048	86
Panaxynol	0.407±0.073	55	0.365±0.019	52
Panaxytriol	0.525±0.089	71	0.505±0.027	72
10-acetyl panaxytriol	0.555±0.071	75	0.582±0.037	83
Heptadeca-1.8-diene-4,6-diyn-3,10-diol	0.525±0.059	71	0.603±0.051	86
Panaxyne	0.651±0.061	88	0.687±0.042	98
Panaxyne epoxide	0.681±0.099	92	0.694±0.048	99

Rat liver microsome (1 mg protein/ml) was incubated with 100 μM polyacetylenes in Fe^{+3}-ADP/NADPH lipid peroxidation system. Values represent mean ± SD of five determinations. TBARS (TBA reactive subtances) and malonaldehyde are expressed as nmol/mg protein/min.

Comparison of antioxidant activities of nine polyacetylenes by TBA value and HPLC analysis

The most extensively employed method for the detection and analysis of peroxidation is the TBA test, predicated upon the reactivity of a colorless aldehyde end-product of lipid peroxidation, malondialdehyde(MDA), with TBA to produce a red adduct. The reaction mechanism, by which MDA is derived from is considered as lipid peroxidation of polyunsaturated fatty acids with three or more double bonds[28]. In spite of nonspecificity of TBAs chemical reactivity and the low efficiency of fatty acid hydroperoxide breakdown to MDA, this method is still widely used by its convenience, ease and rapidity. Antioxidant activities of nine kinds of polyacetylenes from Panax ginseng C. A. Meyer showed similar trends both by TBA value and by MA contents(Table 3). Panaxynol and panaxydol suppressed microsomal lipid peroxidation very

Table 4. Antioxidant activities of some phenolic compounds and ginseng saponin

Treatment	TBARS	%	Malonaldehyde	%
None	0.740±0.049	100	0.701±0.033	100
Catechol	0.659±0.061	89	0.617±0.045	88
Chlorogenic acid	0.688±0.075	93	0.582±0.040	83
Ferulic acid	0.725±0.094	98	0.694±0.058	99
DL-α-tocopherol	0.518±0.093	70	0.519±0.022	74
Ginseng saponin (GS)	0.743±0.053	100	0.702±0.052	100
GS+chlorogenic acid	0.709±0.071	96	0.652±0.049	93
GS+DL-α-tocopherol	0.607±0.065	82	0.588±0.042	84

Liver microsomes of male Sprague-Dawley rats (1 mg protein/ml) were incubated with 100μM phenolic compounds or 100μg ginseng saponin in Fe^{+3}-ADP/NADPH lipid peroxidation systems *in vitro*. % indictes percentages of TBARS (TBA reactive substances) or malonaldehyde produced by control with none treatment. Values (nmol/mg protein/min) represent mean±SD of five separate determinations.

strongly and panaxytriol took a third place among nine polyacetylenes for antioxidant activity. Addition of either chlorine or acetyl group reduced its own antioxidant activity because acetylpanaxydol and panaxydol chlorhydrin prevented the formation of lipid peroxides less than panaxydol did. 10-Acetylpanaxytriol also had a weak radical-scavenging activity as compared with panaxytriol. However, the numbers of hydroxyl group present in the structure showed a different antioxidant activity when measured by TBA-reactive substances and by malonaldyhyde contents. This fact was observed by comparison of activities between panaxytriol and heptadeca-1, 8-t-dien-4,6-diyn-3,10-diol. Since hydroxyl group may donate its hydrogen ion to free radicals including reactive oxygen species, it should be further investigated whether more hydroxyl functional group has stronger radical-scavenging activity or not. Panaxyne and panaxyne epoxide which are C_{14}-polyacetylene compounds seem to have little or no antioxidant activity. This emphasized the importance of existence of C_{17}-structure for radical-scavenging or trapping activity even though polyene chain may be broken when they interact with radical species.

Antioxidant activities of some phenolic compounds and ginseng saponin

Most phenolic compounds have antioxidant activities by reducing free redicals and converting their own structures into a stable radical, phenoxy radical[29]. Isolated phenolic compounds from Panax ginseng C. A. Meyer were maltol, salicylic acid, vanillic acid[30], gentisic acid, ferulic acid and caffeic acid[31]. Han *et al.*[30] reported that antioxidant activities of those phenolic compounds are different depending on the numbers as well as the position of hydroxyl group. As shown in Table 4, chlorogenic acid and catechol showed better antioxidant activities than ferulic acid. However, these activities were very weak as compared with those of C_{17}-polyacetylene compounds. DL-α-tocopherol had a similar radical-quenching activity as panaxytriol. Ginseng saponin had no antioxidant activity and even caused the reduction in the activities of chlorogenic acid or DL-α-tocopherol. This result is different from the paper by Hong and Koo[32] but in agreement with the report by Han *et al.*[30] Since radical-quenching activity is related to antifatigue effect[33], our result about ginseng saponin also agree with a recent presentation by Chang and Chang who observed that antifatigue component of ginseng is neither saponin nor polysaccharide[34].

After 30 min incubation in Fe^{+3}-ADP/NADPH environment, liver microsome was peroxidized and TBA reactive substances and malonaldyhyde were formed in amounts of 0.721 and 0.701 nmole/mg/min respectively (Table 3,4). This result demonstrates that lipid peroxidation system stimulated by Fe^{+3}, ADP and NADPH forms only free malonaldehyde and no significant amounts of other malonaldehyde-like substances. From these results TBA value seems a good indicator for lipid peroxidation in this particular system.

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