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A SENSITIVE METHOD TO SCREEN FOR HYDROXYL RADICAL SCAVENGING ACTIVITY IN NATURAL FOOD EXTRACTS USING COMPETITIVE INHIBITION ELISA FOR 8-HYDROXY DEOXYGUANOSINE

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SUMMARY

Representative endogenous antioxidants and natural food extracts were screened for hydroxyl radical scavenging activity by an ELISA. Whereas conventional assays for hydroxyl radical scavenging activity use either spin traps following the induction of Fenton reaction or measure thiobarbituric acid-reactive substances, this assay measures 8-hydroxy deoxyguanosine liberated from the hydroxylation of deoxyguanosine by Cu^{2+}/ascorbate system.

INTRODUCTION

Experimental evidence accumulated during the past 25 years show that oxidative DNA damage is a prominent cause of aging (Holmes et al. 1992). 8-hydroxy deoxyguanosine (8-OH dG) is one of the sensitive and stable biomarkers for in vivo oxidative DNA damage (Floyd et al., 1988). The in vitro system described by Kohen et al. (1988) to quantitate 8-OH dG consisted of two steps. In the first step, deoxyguanosine was hydroxylated by a Cu (II)/ascorbic acid system to release 8-OH dG. This is based on the production of hydroxy radical from Cu(II) and ascorbate interaction (Chiou, 1983). In the second step, the released 8-OH dG was measured by HPLC-electrochemical detection (ECD) method.

We report here a competitive inhibition ELISA based on a monoclonal antibody specific to 8-OH dG, which in our opinion is a better alternative to the tedious and time consuming HPLC-ECD method to measure 8-OH dG.

MATERIALS AND METHODS

Materials: Unless otherwise stated all chemicals used in this study were purchased from Sigma Chemical Co. and used without further purification. The 8-OH dG standard was synthesized according to Kasai and Nishimura (1984). Representative natural food extracts from beef, bonito, chicken meat, garlic and onion, with total solid content (as expressed by Brix value) ranging between 60 and 75, were obtained from Nikken Foods

(Fukuroi City, Japan). The eel muscle extract was prepared from *Anguilla japonica* in the laboratory.

Optimal Release of 8-OH dG from deoxyguanosine: Though Kohen et al.(1988) had used Cu(II)/ascorbic acid model system to release 8-OH dG from deoxyguanosine, we independently examined the two optimization parameters: (a) the reactivity of various transition metal salts with ascorbic acid, and (b) the reactivity of Cu(II) with different organic acids. All reactions were carried out in phosphate buffer/saline (8 mM $Na_2HPO_4.12H_2O$, 1.5 mM KH_2PO_4, containing 140 mM NaCl), pH 7.4, in a final volume of 1.0 ml. The specific components which constituted the reaction mixture are shown in the foot-notes of Tables 1 and 2. The reaction mixture was incubated at 37°C for 30 min and 50 µl of resulting product was subjected to ELISA for 8-OH dG.

ELISA for 8-OH dG: Competitive inhibition ELISA was conducted on polystyrene 96-well flat bottom plates (Nunc-Immunoplate Maxisorb) monoclonal IgG (T45.1 clone) specific for 8-OH dG, as described previously (Sri Kantha et al. 1996). The 8-OH dG standards used for the assay ranged between 0.64 and 2000 ng/ml. The concentration of 8-OH dG in the test samples were interpolated from the standard curve using log transformation.

RESULTS AND DISCUSSION

The Fenton reaction involving a Fe(II) salt and H_2O_2 has been studied in depth relating to the potential of hydroxyl radical in oxidizing a wide variety of organic acid substrates (Walling, 1975). Data presented in Table 1 validates the efficacy of the Cu(II)/ascorbic acid system studied by us in the release of 8-OH dG from deoxyguanosine.

Table 1: Reactivity of Various Transition Metal Ions with Ascorbic Acid in the release of 8-OH dG from deoxyguanosine

Transition metal salt[a]	Released 8-OH dG[b] (ng/ml)
$CuSO_4.5H_2O$/ascorbic acid	87.6
$FeSO_4.7H_2O$/ascorbic acid	18.1
$CoSO_4.7H_2O$/ascorbic acid	12.5
$NiSO_4.6H_2O$/ascorbic acid	9.4
$MnSO_4.4H_2O$/ascorbic acid	7.5

[a] The reaction mixture (1ml) contained 25 µM salt, 1 mM ascorbic acid, 10 µM deoxyguanosine and phosphate saline buffer.
[b] Each value is an average of duplicate determinations

Table 1 shows that for the five transition metal salts examined, the efficacy in the release of 8-OH dG from deoxyguanosine decreased in the following order:
Cu(II) > Fe (II) > CO(II) > Ni(II) > Mn(II). The amount of 8-OH dG released from deoxyguanosine in the Cu(II)/ascorbic acid system was almost five fold higher in comparison to Fe(II)/ascorbic acid system. The observed difference in the efficacy between Ni(II) and Mn(II) salts is marginal at best.

Chiou (1983) had inferred that only Cu(I), Cu(II), Fe(II) and Fe(III) salts exhibit a significant DNA cleavage activity in the presence of ascorbate. He reported that the DNA-scission efficacy of different metal ions decreased in the following order;
Cu(I) and Cu(II) > Fe(II) > Fe(III) > Co(II) > Mn(II) and Ni(II).

The apparent resemblance of this DNA-scission efficacy profile of different metal ions with ascorbic acid presented by Chiou (1983) and our data on the 8-OH dG release efficacy profile of different metal ions with ascorbic acid establishes the potency of the Cu(II)/ascorbic acid system as a useful tool for evaluating the hydroxy radical scavenging activity.

Table 2: Screening of representative antioxidants and natural food extracts for hydroxyl radical scavenging activity

Test compound	% inhibition of 8-OH dG formed[c]	hydroxyl radical scavenging scale[d]
Antioxidants		
bovine serum albumin[a]	97 ± 1	++++
L-carnosine[b]	89 ± 3	++++
L-cysteine $HCl.H_20$[b]	97 ± 1	++++
glutathione (GSH)[b]	99 ± 1	++++
Natural Food Extracts		
defatted beef[a]	64 ± 2	+++
bonito (*katsuo*)[a]	54 ± 1	++
chicken meat[a]	73 ± 6	+++
eel muscle[a]	75 ± 6	+++
garlic[a]	88 ± 4	++++
onion[a]	89 ± 4	++++

[a] 1% solution; [b] 1 mM solution

[c] calibrated by the degree of hydroxylation initiated by Cu(II)ascorbic acid on releasing ~50 ng/ml of 8-OH dG after 30 min at 37°C from standard 10 μM deoxyguanosine is equated to 100%. Each value is a mean of four determinations (mean ± SEM).

[d] modified from the scale used by Kohen et al.(1988) and marked as ++++(>75% inhibition), +++(61-75% inhibition), ++ (45-60% inhibition), + (30-44% inhibition) and - (<30% inhibition).

The hydroxyl radical scavenging activity of representative antioxidants and five natural food extracts are shown in Table 2. Based on percent inhibition of 8-OH dG formed, each test substance was graded in an arbitrary five point scale. Representative antioxidants screened in this study, such as bovine serum albumin, cysteine and glutathione (GSH) scored a 4+ grade in hydroxyl radical scavenging activity. L-carnosine (β-alanine-histidine) also exhibited 89% inhibition of 8-OH dG formed. This result is in agreement with the findings of Kohen et al. (1988). Among the six natural food extracts screened by this method, all exhibited hydroxyl radical scavenging activity at varying degrees.

The hydroxyl radical scavenging activity is routinely examined by the use of spin traps such as 5,5-dimethyl pyrroline N-oxide (DMPO) and α-phenyl-N-tert-butyl nitrone (PBN) following induction of Fenton reaction by the Fe(II)/H_2O_2 system or by the release of thiobarbituric acid-reactive substance from deoxyribose. Whereas the use of spin traps requires electron spin resonance spectrometer which is expensive in terms of cost, the measure of thiobarbituric reactive substances has been reported as lacking specificity (Hoyland and Taylor, 1991). Therefore, we believe that the sensitive method described in this paper is a better alternative in terms of cost and specificity to screen for hydroxyl radical scavenging activity in natural food extracts.

The merits of this ELISA method over the HPLC-ECD method for estimation of 8-OH dG (Shigenaga et al., 1990; Floyd et al., 1990) also include the following: (a) absence of a tedious pre-treatment step for test samples; (b) nearly 20-fold increase in the sensitivity limit, and (c) simultaneous analysis of multiple samples.

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