Journal of
APPLIED
BIOMEDICINE

J. Appl. Biomed.
5: 81–84, 2007
ISSN 1214-0287

ORIGINAL ARTICLE

Assay of radical scavenging activity of antidotes against chemical warfare agents by DPPH test using sequential injection technique

Vít Kolečkář[1,2], **Daniel Jun**[2,3], **Luboš Opletal**[1], **Luděk Jahodář**[1], **Kamil Kuča**[2,3]

[1]Department of Pharmaceutical Botany and Ecology, Faculty of Pharmacy, Charles University, Hradec Králové, Czech Republic
[2]Center of Advanced Studies, Faculty of Military Health Sciences, University of Defence, Hradec Králové, Czech Republic
[3]Department of Toxicology, Faculty of Military Health Sciences, University of Defence, Hradec Králové, Czech Republic

Received 2nd November 2006.
Revised 3rd January 2007.
Published online 19th March 2007.

Summary
Free radicals are believed to play an important role in many pathological states. Consequently antioxidants are receiving increased attention in medicinal research. As part of studies of the biological effects of the antidotes against chemical warfare agents currently used in the Czech Armed Forces, eleven compounds were assayed for their free radical scavenging activity. An optimized PC-controlled sequential injection analysis (SIA) system with spectrophotometric detection was used for evaluation of the antioxidation activity of the antidotes. The automated method is based on the known reaction of 2,2´-diphenyl-1-picrylhydrazyl radical (DPPH) with antioxidants. Only the radical scavenging activity of the tested compound 2,3-dimercaptopropanol (BAL) was found to be weaker compared to the reference antioxidant acidum ascorbicum. Other tested antidotes did not possess any DPPH radical scavenging activity. The results obtained show that a correlation between the required biological activity (prophylaxis or treatment of intoxications by warfare agents) and possible antioxidation activity of the tested antidotes is doubtful.

Keywords: antioxidant – radical – analysis – antidote – warfare agent

✉ Kamil Kuča, Department of Toxicology, Faculty of Military Health Sciences, University of Defence, Třebešská 1575, 500 01, Hradec Králové, Czech Republic
kucakam@pmfhk.cz
☎ +420 973 251 523
+420 495 518 094

INTRODUCTION

In recent years there has been an increased interest in the application of antioxidants to medical treatment as information is constantly gathered linking the development of human diseases to oxidative stress. Free radicals play a role in the pathogenesis of chronic degenerative diseases including cancer, autoimmune, inflammatory, cardiovascular and neurodegenerative diseases and

aging generally (Cantuti-Castelvetri et al. 2000, Surh et al. 2001, Vaya and Aviram 2001, Aruoma 2003). It is also known that oxidative stress can be induced by a wide range of environmental factors including UV stress, pathogen invasion, pesticide action and oxygen shortage (Blokhina et al. 2003). Owing to these facts, synthetic and natural compounds with potential antioxidation activity are receiving increased attention in biological research, medicine and pharmacy (Aruoma 1994, Hollman et al. 1999).

There are many methods to evaluate the free radical scavenging activity of tested compounds (Paulova et al. 2004). One of the widely used detection procedures, which facilitates analysis of various antioxidants is based on 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH) bleaching (Bondet et al. 1997).

The present paper deals with the application of an automated DPPH method, based on the sequential injection analysis (SIA) technique (Ruzicka and Marshall 1990, Polášek et al. 2004). In this assay we performed antioxidation screening of eleven antidotes against chemical warfare agents currently used in the Czech Armed Forces. These compounds are employed in the prophylaxis and treatment of intoxications with organophosphorus acetylcholinesterase (AChE) inhibitors including nerve agents (sarin, cyclosarin, tabun, agent VX) and pesticides (chlorpyrifos, diazinon etc.), vesicant drugs and incapacitating hallucinogenic chemical warfare agents (Bajgar 2004, Jun et al. 2006). The group of compounds consisted of acetylcholinesterase (AChE) reactivators: pralidoxime, obidoxime, HI-6, methoxime and trimedoxime. Other antidotes tested were as follows: atropine, benactyzine, trihexyphenidyl (anticholinergic drugs), dimercaptopropanol (BAL, antivesicant drug), 7-methoxytacrine (7-MEOTA), pyridostigmine (antidotes against incapacitating hallucinogenic chemical warfare agents). The DPPH radical scavenging activity of antidotes was compared to known antioxidant acid ascorbic.

MATERIAL AND METHODS

Apparatus

All SIA experiments were carried out by PC-controlled (through FIAlab for Windows software) FIAlab 3000 analyser (FIAlab Instruments Inc., Bellevue, USA) equipped with a 2.5 ml syringe pump, six-port selector valve, USB2000-UV/VIS spectrophotometer with LS-1 light source (Ocean Optics, USA) and SMA-Z flow cell (1 cm path length); volume of the holding coil was 0.6 ml and the connecting PTFE tubing (Watrex, Prague, Czech Republic) had i.d. 0.72 mm. Sonication was carried out with SONOREX Super 10P (Bandelin electronic, Berlin, Germany) ultrasonic bath.

Reagents

Ethanol (spectrophotometric grade), 2,2´-diphenyl-1-picrylhydrazyl (95%), and L-ascorbic acid (99%) were obtained from Sigma-Aldrich (Czech Republic). Pralidoxime, obidoxime, trimedoxime, HI-6 and methoxime were earlier synthesized at the Department of Toxicology of the Faculty of the Military Health Sciences (Czech Republic). A Millipore Milli-Q RG ultra-pure water was used throughout the experiment. Stock 0.5 mM solutions of the standard L-ascorbicum in ethanol-water 1:1 (v/v) and stock 10 mM solutions of antidotes were prepared. Less concentrated tested solutions were prepared by diluting the stock solutions with aqueous 50% (v/v) ethanol. Aqueous 50% (v/v) ethanol used for the dissolution of standard, antidotes samples and dilution of stock solutions, DPPH reagent (0.1 mM), and SIA carrier stream [aqueous 50% (v/v) ethanol] was degassed by 10 min sonication in Sonorex Super 10P (Bandelin electronic, Berlin, Germany) ultrasound bath (sonication level 10) (Polášek et al. 2004).

Method

All 11 antidotes were assayed for DPPH scavenging activity by the SIA method. The antioxidation activity of the tested samples was described by two parameters: DPPH quenching efficacy index (%Q) and parameter EC_{50} (concentration providing 50% inhibition of DPPH; the more efficient the antioxidant, the smaller the EC_{50}). The DPPH quenching efficacy index (%Q) value was defined as the relative decrease of the absorbance peak height of the aspirated DPPH solution caused by the injection of the antioxidant solution [$\%Q=(1-A_X/A_0)\times100$] where A_0 stands for the mean peak height value of DPPH with the blank solution (ethanol-water 1:1) aspirated instead of the antioxidant solution, and A_X is the mean peak height of DPPH with antioxidant solution aspirated. Under SIA conditions the dependences of the %Q values on the concentration of standard antioxidants were evaluated for 0.01–0.5 mM ascorbic acid and 0.01-10 mM antidotes with 0.1 mM DPPH as reagent. All SIA measurements were conducted in triplicate (Polášek et al. 2004). Statistical evaluation was carried out by GraphPad Prism (version 3.02) statistical software.

RESULTS

The results of the DPPH radical scavenging activity of BAL are shown in Fig. 1 and Table 1. These results are compared with the well-known antioxidant ascorbic acid. Decrease in the absorption of the DPPH solution (%Q) caused by acid ascorbic and BAL is shown in Fig. 1. Ascorbic acid exhibited a much higher decrease in absorption

of DPPH solution compared with BAL. EC_{50} values of ascorbic acid and BAL are shown in Table 1. EC_{50} of BAL is more then hundred times higher then that of ascorbic acid. These results suggested BAL as a weak to moderate free radical scavenging activity. Other tested antidotes did not possess any DPPH scavenging activity.

DISCUSSION

Organophosphates are widely used in agriculture as pesticides (chlorpyrifos, diazinon, malathion etc.) or were prepared as nerve chemical warfare agents (sarin, cyclosarin, soman, tabun, agent VX). These organophosphates cause irreversible inhibition of the AChE by phosphorylation or phosphonylation of serine hydroxyl group at the esteratic site of the enzyme active site (Bajgar 2004). It is also known that some pesticides can induce oxidative stress which can also contribute to the pathogenesis of organophosphates. On the other hand antioxidants are a group of substances which, when present at low concentrations in relation to oxidizable substrates, significantly inhibit or delay oxidative processes (Halliwell 1990, Vaya and Aviram 2001). Owing to these facts, synthetic and natural compounds including antidotes which can act as a radical scavengers are evaluated.

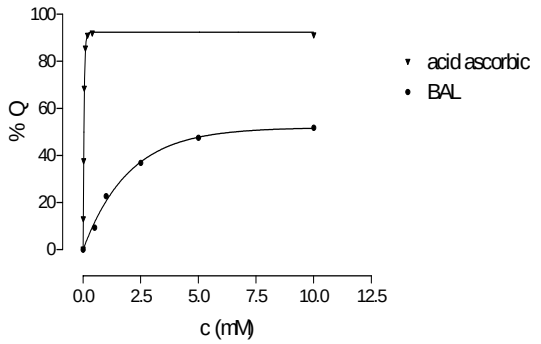

Fig. 1. **Dependence of DPPH quenching efficacy index (%Q) on the concentration of ascorbic acid and BAL**

In this assay the possible antioxidation effect of antidotes was evaluated. The DPPH radical scavenging screening of antidotes was performed with the automated method, based on the sequential injection analysis (SIA) technique, which facilitates fast and sensitive analysis. In this assay pralidoxime, obidoxime, HI-6, methoxime, trimedoxime, atropine, benactyzine, trihexyphenidyl, dimercaptopropanol (BAL), 7-methoxytacrine (7-MEOTA) and pyridostigmine were tested. Of the compounds tested, only BAL was found to be active against DPPH radical. Even though all tested compounds did not scavenge the DPPH radical, the possibility of these compounds scavenging free radicals in another specific antioxidation assay system cannot be ruled out. However a correlation between the required biological activity used in the prophylaxis and treatment of intoxications and free radical scavenging activity evaluated by BAL is doubtful.

Although the results of the antioxidation activities of the tested antidotes were not as high as was expected, the proposed method was verified as suitable for rapid and sensitive antioxidation screening of a large series of newly synthesized antidotes with a modified radical scavenging susceptible structure.

Table 1. **EC_{50} values of active samples**

Sample	EC_{50} (mM)
BAL	6.524
Ascorbic acid	0.034

ACKNOWLEDGEMENT

This work was supported by a grant from Charles University (Czech Republic) No. GAUK 124/2005/B-BIO/FaF.

REFERENCES

Aruoma OI: Nutrition and health aspects of free radicals and antioxidants. Food Chem. Toxicol. 32:671–683, 1994.

Aruoma OI: Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. Mutat. Res. 523–524:9–20, 2003.

Bajgar J: Organophosphates/nerve agents poisoning: mechanism of action, diagnosis, prophylaxis and treatment. Adv. Clin. Chem. 38:151–216, 2004.

Blokhina O, Virolainen E, Fagerstedt KV: Antioxidants, oxidative damage and oxygen deprivation stress: a review. Ann. Bot. (Lond.) 91:179–94, 2003.

Bondet V, Brand-Williams W, Berset C: Kinetics and Mechanisms of Antioxidant Activity using the DPPH Free radical method. Food Sci. Technol. 30:609–615, 1997.

Cantuti-Castelvetri I, Shukitt-Hale B, Joseph JA: Neurobehavioral aspects of antioxidants in aging. Int. J. Dev. Neurosci. 18:367–381, 2000.

Halliwell B: How to characterize a biological antioxidant. Free. Radic. Res. Commun. 9:1–32, 1990.

Hollman PCH, Katan MB: Dietary flavonoids: intake, health effects and bioavailability. Food Chem. Toxicol. 37:937–942, 1999.

Jun D, Kuca K, Hronek M, Opletal L: Effect of some acetylcholinesterase reactivators on human platelet aggregation *in vitro*. J. Appl. Toxicol. 26:258–61, 2006.

Paulova H, Bochorakova H, Taborska E: *In vitro* methods for estimation of the antioxidant activity of natural compounds. Chem. Listy. 98:174–179, 2004.

Polášek M, Skála P, Opletal L, Jahodář L: Rapid automated assay of anti-oxidation/radical-scavenging activity of natural substances by sequential injection technique (SIA) using spectrophotometric detection. Anal. Bioanal. Chem. 379:754–758, 2004.

Ruzicka J, Marshall GD: Sequential injection: A new concept for chemical sensors, process analysis and laboratory assays. Anal. Chim. Acta. 237:329–343, 1990.

Surh YJ, Chun KS, Cha HH, Keum YS, Park KK, Lee SS: Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-κB activation. Mutat. Res. 480–481:243–268, 2001.

Vaya J, Aviram M: Nutritional antioxidants mechanisms of action, analyses of activities and medical applications. Curr. Med. Chem. 1:99–117, 2001.