Available online at www.sciencedirect.com

SCIENCE DIRECT

JOURNAL OF CHROMATOGRAPHY A

Journal of Chromatography A, 1016 (2003) 123–128

www.elsevier.com/locate/chroma

Determination of hydroxyl radical by capillary zone electrophoresis with amperometric detection

Qingjiang Wang [a], Fei Ding [a], Ningning Zhu [b], Hui Li [c], Pingang He [a], Yuzhi Fang [a,*]

[a] *Department of Chemistry, East China Normal University, Shanghai 200062, China*
[b] *Department of Chemistry, Shanghai Teachers University, Shanghai 200234, China*
[c] *School of Chemistry and Chemical Engineering, Shanghai Jiaotong University, Shanghai 200240, China*

Received 28 February 2003; received in revised form 10 July 2003; accepted 16 July 2003

Abstract

Hydroxyl radical ($OH^{\bullet}$) can cause severe damage to cells and tissues. However, its analysis is very difficult for its high reactivity and very short half-life. In this paper, a simple and highly sensitive method, capillary zone electrophoresis with amperometric detection (CZE-AD) was introduced indirectly to determine $OH^{\bullet}$ by determining its reaction products with salicylic acid (SAL), 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA). The optimum conditions of CZE-AD for the determination of 2,3-DHBA and 2,5-DHBA were explored. Under the optimum conditions, SAL, 2,3-DHBA and 2,5-DHBA could be perfectly separated within 15 min, and the linearity ranges of 2,3-DHBA and 2,5-DHBA were between 1.0×10^{-7} and 1.0×10^{-4} mol l^{-1}. Their detection limits were as low as 2×10^{-8} mol l^{-1}, which were much lower than that in CE-UV method. The method was also applied to study the free $OH^{\bullet}$ scavenging activity of angelica polysaccharide. The experimental results showed that this CZE-AD method was very sensitive and practical in both the determination of free $OH^{\bullet}$ and the evaluation of free $OH^{\bullet}$ scavenging activities of antioxidants.
© 2003 Elsevier B.V. All rights reserved.

Keywords: Radical scavengers; Angelica polysaccharide; Hydroxyl radical; Polysaccharides

1. Introduction

It is well known that reactive oxygen radicals can attack biomacromolecules including proteins, nucleic acids, polyunsaturated fatty acids, and then destroy the structures and functions of cells. There is a certain relationship between the reactive oxygen radicals and some diseases, such as cancer, cardiovascular diseases, Alzheimer's disease and Parkinson's disease [1,2]. $OH^{\bullet}$ is the most aggressive one among these reactive oxygen radicals and causes cell injury when it is generated in excess or the cellular antioxidant defense is impaired. However, $OH^{\bullet}$ is very difficult to be detected for its high reactivity and very short half-life [3–5].

Because of the high reactivity of $OH^{\bullet}$, indirect methods are usually used to determine its content and damage to cells and tissues. For these indirect

* Corresponding author. Tel.: +86-21-62232627; fax: +86-21-62451921.
E-mail address: yuzhi@online.sh.cn (Y. Fang).

0021-9673/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0021-9673(03)01294-9

methods, some endogenous markers and free radical traps such as pentane, butane, 8-hydroxydeoxyguanosine, salicylate (SAL), dimethyl sulfoxide (DMSO) and dimethyl thiourea are usually used [6].

Many analytical methods have already been used to detect $OH^{\bullet}$, such as electron spin resonance (ESR)/ electron paramaganetic resonance (EPR) [7,8], high-performance liquid chromatography (HPLC) with ultraviolet (UV)/electrochemical detection (ED)/mass spectrometry (MS) [9–13], gas chromatography (GC) with MS [14,15], chemiluminescence [16–18], etc. ESP method has been proved to be a good method for the measurement of $OH^{\bullet}$, but it also needs spin traps such as nitrone, nitroso compounds, 5,5-dimethyl-L-pyrroline-*N*-oxide (DMPO), etc. Other disadvantages of ESP are the complicated and high-cost instruments, and the restriction in sensitivity and quenching in vivo. For the analysis of $OH^{\bullet}$, HPLC is very effective when coupled with other detection methods, in which SAL is usually used as free radical trap and the two main hydroxylated derivatives (2,3-DHBA and 2,5-DHBA) are determined. When HPLC is coupled with AD, the sensitivity is about 1000 higher than that of UV detection. However, HPLC also needs high-cost column and large consumable agents. Chemiluminescence method has some advantages in sensitivity and speed for $OH^{\bullet}$ analysis, but the used lucigenin also reacts with superoxide, hydrogen peroxide, or single oxygen and cause errors.

In recent years, capillary electrophoresis (CE) has been extensively studied and applied as a highly effective analytical method in chemical and biological areas. Compared to HPLC, CE has many advantages such as much lower sampling volume, cheaper instrumentation and higher separation efficiency [19–21]. But the analysis of free $OH^{\bullet}$ by CE is rarely studied. Gokoren and Tuncel firstly used micellar electrokinetic capillary chromatography (MECC) to determine free $OH^{\bullet}$ by analyzing 2,5-DHBA and 2,3-DHBA [22]. Coolen et al. used CZE and MECC with UV detection to determine free $OH^{\bullet}$ by assaying 2,3- and 2,5-DHBAs, the calibration curves for both CZE and MECC were recorded in the range of 10^{-6} to 10^{-4} mol l^{-1} and the detection limit was determined as 2×10^{-7} mol l^{-1} [23].

In this paper, capillary zone electrophoresis with amperometric detection (CZE-AD) was used to indirectly determine the content of free $OH^{\bullet}$ which was produced by Fenton reaction. Fenton reaction is the most used system to produce free $OH^{\bullet}$ in vitro. When SAL is added to this system, it can trap $OH^{\bullet}$ and produce electroactive 2,3-DHBA and 2,5-DHBA, which can be detected by AD [24]. The optimum conditions of CZE-AD for the determination of 2,3-DHBA and 2,5-DHBA were explored. Under the optimum conditions, SAL, 2,3-DHBA and 2,5-DHBA could be perfectly separated within 15 min, and the linearity ranges of 2,3-DHBA and 2,5-DHBA were between 1.0×10^{-7} and 1.0×10^{-4} mol l^{-1}. Their detection limits were as low as 2×10^{-8} mol l^{-1}, which were much better than that in CE-UV method. The method was also applied to study the free $OH^{\bullet}$ scavenging activity of angelica polysaccharide. When angelica polysaccharide was added into the Fenton reaction system, part of $OH^{\bullet}$ was removed and the contents of 2,3-DHBA and 2,5-DHBA were reduced. The experimental results showed that the CZE-AD method introduced was very sensitive and practical in both the determination of free $OH^{\bullet}$ and the evaluation of the free $OH^{\bullet}$ scavenging activity of antioxidants.

2. Experimental

2.1. Apparatus

CZE-AD system was laboratory-built [25,26]. Electrophoresis was driven by a high-voltage supplier (±30 kV, Shanghai Institute of Nuclear Research, China). Separations were performed in a fused-silica capillary (25 μm i.d., 360 μm o.d., 60 cm long, Polymicro. Tech. Ltd., USA). Potential control and current output were employed by a BAS LC-3D amperometric detector (Bioanalytical System, West Lafayette, IN, USA). Electropherograms were recorded by a chart recorder (Model XWT-204, Shanghai Dahua Instrument Factory, China). Electrochemical experiments were carried out by a CHI 630 electrochemical analyzer (CHI Instruments, USA). A three-electrode system, which consisted of a carbon disk working electrode (Ø 300 μm), a saturated calomel reference electrode (SCE) and a platinum wire counter electrode, was used in both electrochemistry and detection experiments.

2.2. Reagents

All reagents were of analytical-reagent grade. 2,3-DHBA and 2,5-DHBA were purchased from Sigma Chemical Company. The stock solutions of 2,3-DHBA, 2,5-DHBA and SAL with a concentration of 1.0×10^{-3} mol l^{-1} were prepared with doubly distilled water and diluted with running buffer to the needed concentrations in CZE experiments.

Angelica was purchased from Chinese Traditional Medicine Division of Shanghai Fuxing Medicine Company.

Before CZE experiments, all used solutions were filtered through 0.45 μm polypropylene acrodisc syringe filter and sonicated for 5 min to remove bubbles.

2.3. Procedure

2.3.1. Preparation of carbon working electrode

The used carbon electrode was prepared according to the introduced procedure in our former paper [27]. Prior to use, the surface of the carbon electrode was polished with emery paper and alumina powder, respectively, and then it was sonicated in doubly distilled water for 3 min to get enough cleanness.

2.3.2. CZE separation

Before CZE experiments, the three-electrode system was fixed in the corresponding holes of the electrochemical cell and the carbon disk electrode was positioned straightly opposite the capillary outlet as close as possible by a three-dimension positioner.

Before each run in CE experiments, the capillary was sequentially rinsed with 0.5 mol l^{-1} hydrochloric acid, doubly distilled water, 0.2 mol l^{-1} sodium hydroxide, 3 min for each and with running buffer till the inside current of the capillary reached stability. This was important to get a reproducible electroosmotic flow.

The optimum conditions of CZE-AD applied in this experiment were 0.9 V as detection potential, 30 mmol l^{-1} $Na_2B_4O_7$–HCl solution (pH 7.4) as running buffer, 15 kV as separation voltage and 6 s (at 15 kV) as electrokinetic sampling time.

2.3.3. Extraction of angelica polysaccharide

An accurate weight of angelica powder was firstly refluxed with acetone and 1:1 ethanol–ether, respectively, under a boiled-water bath for 2 h to remove pigments. Then, the residue was leached by stirring in water at 90–100 °C three times each for 6 h. Next, the leached solution from the above operation was concentrated and precipitated by adding non-aqueous ethanol. The precipitate was separated from the above solution by centrifugation and purged with 4:1 chloroform:3-methylbutanol to remove heteroproteins. After freezing and drying, the angelica polysaccharide was obtained. The coarse polysaccharide was further purified by cellulose column chromatography and the pure polysaccharide was obtained.

2.3.4. Reaction between SAL and hydroxyl radical from Fenton system

Hydroxyl radical was produced through Fenton reaction by incubating for 60 min at 37 °C in the presence of 1.0 mmol l^{-1} Fe^{2+}, 2.0 mmol l^{-1} H_2O_2, 1.0 mmol l^{-1} EDTA, 1.0 mmol l^{-1} sodium salicylate and 20 mmol l^{-1} NaH_2PO_4–Na_2HPO_4 buffer (pH 7.4) in a final volume of 5 ml. Solutions of H_2O_2 and Fe^{2+} were made up immediately before use in deaerated water and the iron salt was mixed with the EDTA before addition. After 1 h of reaction, this solution was diluted by running the buffer to 50 times of volume and injected into capillary by electrokinetic sampling. For free radical scavenging activity experiment of angelica polysaccharide, different concentrations of test material were present in above Fenton system.

3. Results and discussion

3.1. Conditions of amperometric detection

As there are electroactive hydroxyl groups in their molecular structures, 2,3-DHBA, 2,5-DHBA and SAL molecules can be oxidized at a carbon electrode and produce current responses. Fig. 1 shows the hydrodynamic voltammograms (HDVs) of the above analytes, which were obtained by monitoring their current responses after CZE separations at the applied potential range from 0.0 to 1.0 V. It was found that the current responses of these analytes increased with the increase of the applied potential. For determining 2,3-DHBA, 2,5-DHBA and SAL together and the best signal-to-noise ratio, 0.90 V was selected as the detection potential in this experiment.

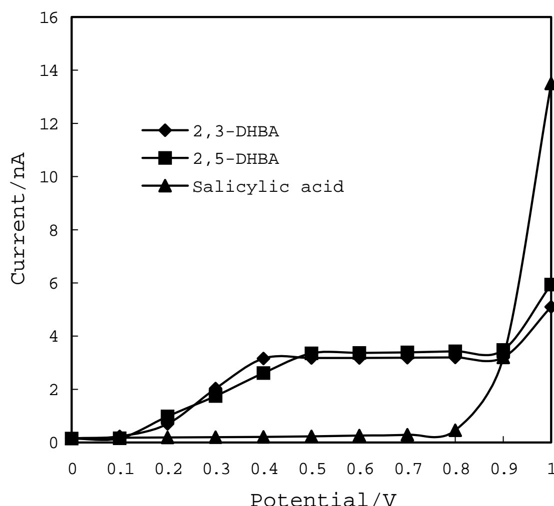

Fig. 1. Hydrodynamic voltammograms of SAL, 2,3-DHBA and 2,5-DHBA with same concentration of 1.0×10^{-6} mol l^{-1} in CZE under different detection voltages from 0 to +1.0 V. Other conditions were as the optimum conditions.

3.2. Separation conditions in CZE

Because 2,3-DHBA and 2,5-DHBA are isomers and have very similar ionization constants ($pK_{a1(2,3\text{-DHBA})} = 2.94$, $pK_{a1(2,5\text{-DHBA})} = 2.98$), they perform very similar electrophoretic mobility (μ_e) in CZE and cannot be separated under the neutral or basic running buffer condition. But in borate buffer solution, because 2,3-DHBA has *vic*-hydroxyl groups, it can form complex with boric acid and the product has a bigger ionization constant, while 2,5-DHBA does not perform this reaction. Thus, 2,3-DHBA and 2,5-DHBA can be perfectly separated in the introduced borate–HCl running buffer (pH 7.4) in CZE in this paper. The effect of the concentration of $Na_2B_4O_7$ was investigated. When the running buffer concentration was increased, the electroosmotic flow was reduced and the migration time was prolonged. So, lower concentration of running buffer is suitable to reduce the migration time and improve sensitivity. In this experiment, 30 mmol l^{-1} $Na_2B_4O_7$ was used as running buffer.

The pH value of the running buffer was the most important factor affecting the resolution in this experiment. The pH effect was investigated within the pH range from 4.0 to 11.0. In the pH range from 4.0 to 7.0, the current response peaks of these three analytes could not be separated in baseline. But in the pH range from 7.0 to 9.0, the three current response peaks were separated in the baseline. However, their current responses reduced and the migration time was prolonged obviously with the increase of the pH value. So, the pH value selected should be as low as possible to reduce the separation time. In this experiment, pH 7.4 was selected as an optimum pH, which was obtained by gradually adding hydrochloric acid into 30 mmol l^{-1} borate solution.

The separation efficiency of CZE was investigated within the separation voltage range from 10 to 22 kV. The migration time of the analytes was significantly shortened and their corresponding current peaks were sharpened when the separation voltage was increased. However, if the separation voltage was too high, more Joule heat was produced because of the higher current inside the capillary, which caused peak-broadening and reduced separation efficiency. For a comprehensive thought, 15 kV was selected as the optimum separation voltage in this experiment.

Electrokinetic sampling was used in the CZE experiment. It was found that when the sampling time was prolonged, the peak currents increased correspondingly. However, the current response peaks of the analytes were obviously broadened if the sampling time was more than 10 s. So, 6 s was selected as the sampling time in this experiment and satisfactory results were obtained under this condition.

Under the optimum conditions, the electropherograms of standard 2,5-DHBA, SAL and 2,3-DHBA with same concentration of 1.0×10^{-6} mol l^{-1} are shown in Fig. 2(a).

3.3. Linearity, repeatability and detection limits

A series of standard solutions of 2,3-DHBA, 2,5-DHBA and SAL with a concentration range from 1.0×10^{-8} to 5.0×10^{-3} mol l^{-1} were analyzed under the optimum conditions and the results are shown in Table 1. Their linear ranges were from 1.0×10^{-7} to 1.0×10^{-4} mol l^{-1} and their detection limits were 2.0×10^{-8} mol l^{-1} (S/N = 3). The above results showed that this method was very sensitive.

The relative standard deviations (R.S.D.) of both the migration time and peak currents of the analytes were less than 2% when the analysis was repeated for

Table 1
Regression equation and detection limit[a]

Analyte	Regression equation I (nA); C (mol l^{-1})	Linear range (mol l^{-1})	R^2	Detection limit (mol l^{-1})
2,3-DHBA	$I = 3.12 \times 10^6 C + 0.08$	1.0×10^{-7} to 1.0×10^{-4}	0.9989	2.0×10^{-8}
2,5-DHBA	$I = 3.42 \times 10^6 C + 0.06$	1.0×10^{-7} to 1.0×10^{-4}	0.9975	2.0×10^{-8}
SAL	$I = 3.10 \times 10^6 C + 0.10$	1.0×10^{-7} to 1.0×10^{-4}	0.9988	2.0×10^{-8}

[a] Detection limit was estimated according to three times of signal-to-noise ratio.

six times. These results demonstrated that this method was of good repeatability.

3.4. Determination of scavenging activities of angelica polysaccharide

According to the method in Section 2, different concentrations of angelica polysaccharide were present in Fenton system. Fig. 2(b) shows the electropherograms of Fenton reaction system, and Fig. 2(c) shows the electropherograms of Fenton reaction system when

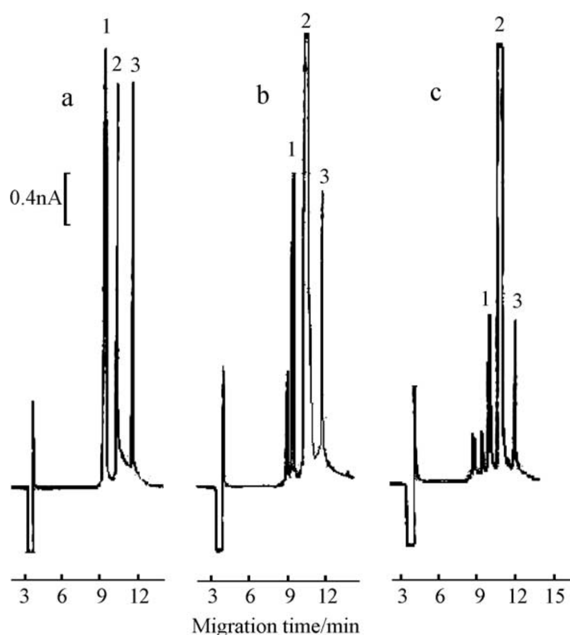

Fig. 2. Electropherograms of standard SAL, 2,3-DHBA and 2,5-DHBA with same concentration of 1.0×10^{-6} mol l^{-1} (a), products of Fenton reaction (b), and products of Fenton reaction when 1.0 mg/ml angelica polysaccharide was present (c) under the optimum conditions of CZE-ED. (1) 2,5-DHBA, (2) SAL, (3) 2,3-DHBA.

1.0 mg ml^{-1} angelica polysaccharide was present. By comparing Fig. 2(b) and (c), it was found that the current peaks of 2,5-DHBA and 2,3-DHBA were reduced. Therefore, the $OH^{\bullet}$ scavenging percentage of angelica polysaccharide could be calculated by the differences between the original currents of 2,3-DHBA or 2,5-DHBA in Fenton reaction system and those when different concentrations of angelica polysaccharide were present. It was found by this experiment that the free $OH^{\bullet}$ scavenging percentage were 13.6, 24.2, 32.1, 38.4 and 44.9%, respectively, corresponding to the angelica polysaccharide concentration added as 0.2, 0.4, 0.6, 0.8 and 1.0 mg ml^{-1}.

4. Conclusions

The experiment showed that CZE-AD was a convenient and sensitive method to indirectly determine the free $OH^{\bullet}$. Under the introduced optimum conditions of CZE-AD, SAL, 2,3-DHBA and 2,5-DHBA could be perfectly separated within 15 min, and the linear ranges of 2,3-DHBA and 2,5-DHBA were between 1.0×10^{-7} and 1.0×10^{-4} mol l^{-1}. Their detection limits were 2×10^{-8} mol l^{-1}, which were much better than that in the CE-UV method. The method was also applied to study the free $OH^{\bullet}$ scavenging activity of angelica polysaccharide. The experimental results showed that the introduced CZE-AD method was very sensitive and practical in both the determination of free $OH^{\bullet}$ and the evaluation of free $OH^{\bullet}$ scavenging activities of antioxidants.

References

[1] B. Halliwell, J.M. Gutteridge, Methods Enzymol. 186 (1990) 1.
[2] B. Halliwell, J. Br, Exp. Pathol. 70 (1989) 737.

[3] H.B. Michaels, J.W. Hunt, Radiat. Res. 56 (1973) 57.
[4] J.E. Repine, R.B. Fox, E.M. Berger, J. Biol. Chem. 256 (1981) 7094.
[5] L. Sarma, T.P. Devasagayam, H. Mohan, J.P. Mittal, P.C. Kesavan, Int. J. Radiat. Biol. 69 (1996) 633.
[6] J.J. Hageman, A. Bast, N.P.E. Vermeulen, Chem. Biol. Int. 82 (1992) 243.
[7] N.J. Stokes, et al., Chemosphere 28 (1994) 999.
[8] S. Stolc, et al., Free Radic. Biol. Med. 20 (1996) 89.
[9] D.R. McCabe, T.J. Maher, I.N. Acworth, J. Chromatogr. B 691 (1997) 23.
[10] V. Bergh, I. Vanhees, R. De-Boer, F. Compernolle, C. Vinckier, J. Chromatogr. A 896 (2000) 135.
[11] T.H. Tsai, F.C. Cheng, L.C. Hung, C.F. Chen, J. Chromatogr. B 734 (1999) 277.
[12] J.M. Duine, F. Floch, C. Cann-Moisan, P. Mialon, J. Caroff, J. Chromatogr. B 716 (1998) 350.
[13] K. Hiramoto, A. Nasuhara, K. Michikoshi, T. Kato, K. Kikugawa, Mutat. Res. Gen. Toxicol. Environ. Mutag. 395 (1997) 47.
[14] F. Vargas, I. Martinez-Volkmar, J. Sequera, H. Mendez, J. Rojas, G. Fraile, M. Velasquez, R. Medina, J. Photochem. Photobiol. B 42 (1998) 219.
[15] B. Ferger, S. Rose, A. Jenner, B. Halliwell, P. Jenner, NeuroReport 12 (2001) 1155.
[16] G. Yildiz, A.T. Demiryurek, J. Pharmacol. Toxicol. Methods 39 (1998) 179.
[17] K.I. Ohno, H. Arakawa, R. Yoda, M. Maeda, Luminescence 14 (1999) 355.
[18] Y. Yoshiki, T. Kahara, K. Okubo, K. Igarashi, K. Yotsuhashi, J. Biolumin. Chemilumin. 11 (1996) 131.
[19] A. Wang, Y. Fang, Electrophoresis 21 (2000) 1281.
[20] R.P. Baldwin, Electrophoresis 21 (2000) 4017.
[21] F.M. Matysik, Electroanalysis 12 (2000) 1349.
[22] N. Gokoren, M. Tuncel, Pharmazie 52 (1997) 726.
[23] S.A.J. Coolen, F.A. Huf, J.C. Reijenga, J. Chromatogr. B 717 (1998) 119.
[24] M.J. Sanz, M. Ferrándiz, M. Cejudo, M.C. Terencio, B. Gil, G. Bustos, A. Úbeda, R. Gunasegaran, M.J. Alcaraz, Xenobiotica 24 (1994) 689.
[25] Y. Fang, X. Fang, J. Ye, Chem. J. Chin. Univ. 16 (1995) 1514.
[26] C. Fu, L. Song, Y. Fang, Anal. Chim. Acta 399 (1999) 259.
[27] Q. Wang, F. Ding, H. Li, P. He, Y. Fang, J. Pharm. Biomed. Anal. 30 (2003) 1507.