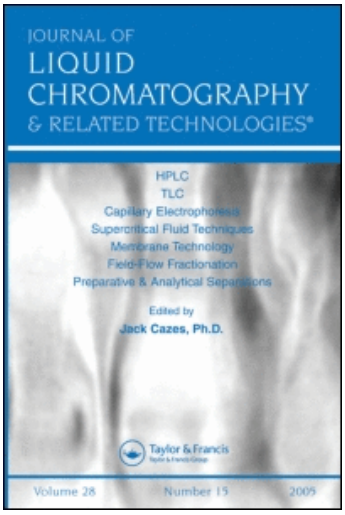

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DETERMINATION OF HYDROXYL RADICAL FORMATION IN THE TESTES OF CADMIUM-TREATED MICE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

An application of high performance liquid chromatography (HPLC) with electrochemical detection to investigate the hydroxyl radical production in the testes of cadmium-treated mice is described. Salicylate was used as a free radical trapping agent to trap hydroxyl radicals in vivo in the testes of cadmium-treated mice. Using this HPLC method, the products formed by hydroxyl radical addition to salicylate, 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihyroxybenzoic acid (2,5-DHBA), were separated and quantitated. It was found that the concentrations of both 2,3-DHBA and 2,5-DHBA in the testes of cadmium-treated mice were significantly higher than that without the treatment of cadmium. This study demonstrated that the tissue damage induced by cadmium was the result of the increase in the production of hydroxyl radicals.

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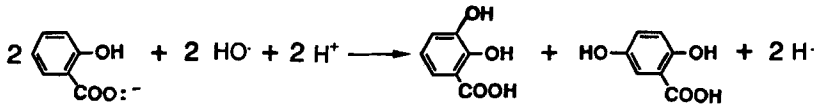
INTRODUCTION

Hydroxyl radicals ($HO^{\cdot}$) and superoxide are two oxygen radicals which have been implicated as possible etiological agents in the development of several disease or pathological conditions such as in aging, [1] arthritis, [2] carcinogenesis, [3] tumor promotion, [4] hyperbaric oxygen toxicity, [5] radiation injury, [6] ischemic injury to heart, brain and other tissues and the toxic action of certain chemicals. [7, 8] Superoxide radicals are not as reactive as hydroxyl radicals in aqueous solution. In comparison, hydroxyl radicals are highly reactive and short lived in both chemical [9, 10] and biological systems, [11] and present at very low concentrations in tissues in normal conditions. Hydroxyl radicals can cause tissue injury through a variety of pathways including damaging DNA, inactivating specific proteins or via lipid peroxidation of cell membrane components, and disrupting the interstitial matrix by degradation of hyaluronic acid and collagen. [12]

Cadmium is a toxic trace metal. Occupational and environmental pollutants are the main sources of cadmium exposure. Cadmium is toxic to virtually every system in the animal body, whether ingested, injected, or inhaled. The toxic effects of exposure to cadmium include anemia, dermatitis, testicular degeneration or atrophy, reduced growth rate, liver and kidney damage, cardiovascular disorders, pulmonary edema and emphysema, teratogenic malformations and increased mortality. [13, 14] Cadmium induced lipid peroxidation has been observed in numerous tissues either in vivo or in vitro. [15, 16] The relationship between cadmium toxicity and $HO^{\cdot}$ is not clear because $HO^{\cdot}$ is difficult to detect and quantitate in vivo. Salicylate is non-toxic at a low concentration and has been used to trap $HO^{\cdot}$ in animals. [12, 17] Two main products

are 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihyroxybenzoic acid (2,5-DHBA) formed by hydroxyl addition to salicylate. [16]



2,3-DHBA 2,5-DHBA (1)

This study was to use salicylate as a radical trapping agent to detect HO· in vivo and provided a direct evidence for pathogenetic role of the hydroxyl radical in the testis toxicity caused by cadmium.

MATERIALS AND METHODS

The HPLC instrumentation was from Waters (Waters, Division of Millipore, Milford, MA, USA), which included a 501 HPLC pump, an U6K injector with a 500 µl sample loop, a 460 electrochemical detector with a glassy carbon working electrode, an auxiliary electrode and a Ag/AgCl reference electrode. Data acquisition and processing was accomplished by a Waters Baseline 810 chromatographic work station which included a NEC PowerMate SX/16 computer and a system interface. The column used was a Hibar RT Lichrosorb-RP-18 (10 µm, 250 x 4 mm) (E. Merck, Gibbstown, NJ, USA). The voltage in the electrochemical detector was set at 0.8 V. The injection volume was 50 µl and the flow rate of the mobile phase was 0.8 ml/min. The mobile phase was 0.03 M citric acid and 0.03 M acetic acid, and prepared by titration with sodium hydroxide to pH 3.0 followed by titration with sodium acetate to a final pH of 3.6.

The statistic analysis of the experimental date of 2,3-DHBA and 2,5-DHBA in testes were carried out using Student "t" test. The results were considered significant at $P < 0.05$ and very significant at $P < 0.01$.

All the reagents were of analytical grade. Cadmium chloride ($CdCl_2$), trichloroacetic acid (TCA), sodium salicylate and its monohydroxylated products, 2,3-DHBA and 2,5-DHBA were obtained from Sigma Chemical Company (St. Louis, MO). Water was from a Millipore Milli-Q system (Millipore, Milford, MA, USA).

The calibration curves of the DHBAs were built with 2,5-DHBA and 2,3-DHBA standards in 10% TCA solution. The concentrations of the two DHBAs in the tissue extracts were determined by comparison of the peak areas of the extracts with that of the calibration curves of 2,5-DHBA and 2,3-DHBA.

Animal Preparation

Male CD-1 mice with body weights of 30-35 g were used in all experiments. They were housed in groups of 5 mice/cage, kept at a constant room temperature and maintained on a controlled environment with a 12 h light : 12 h dark cycle. Food and water were provided *ad libitum*. After one week adaption, the mice were divided into two groups of 5 mice each. Cadmium chloride solution (2 mg/kg) was injected to cadmium treatment group in s.c. once a day for 7 days. Control group was given equal volume of saline.

Sample Preparation

After 7 days cadmium treatment, the mice were given i. p. sodium salicylate solution (100 mg/kg), 30 min prior to killing mice by cervical dislocation. The testes

were removed immediately, weighted and placed in ice-cooled 10% TCA solution (1.5 ml/200 mg tissue). The testes were homogenized in a polytron homogenizer (Polytron model # PT 10/35, Brinkman Instruments, Switzerland) 20 seconds. The homogenate was centrifuged at 2,000 RPM at room temperature for 10 minutes. The supernatants were filtered with a 0.22 μm Millipore filter, and then 50 μl of this solution was injected into the HPLC system.

Recovery Assay

2,5-DHBA and 2,3-DHBA solutions at various concentrations were spiked into the blank tissues and then extracted with the same procedure described above. The concentrations of the DHBAs in the tissue extracts were determined by comparison of the HPLC peak areas of the extracts with that of the calibration curves of 2,5-DHBA and 2,3-DHBA. The recoveries of 2,5-DHBA and 2,3-DHBA were 92.1% and 91.4%.

RESULTS AND DISCUSSION

As shown in Figure 1a, 2,5-DHBA and 2,3-DHBA were not found in the blank extracts of mice testes which were not treated with salicylate and cadmium. Figure 1b shows the chromatogram of the control extract, which was from the mouse given only salicylate (100 mg/kg). Figure 1c shows the chromatogram of the extract of cadmium-treated mouse. The peaks of 2,5-DHBA and 2,3-DHBA in the control testes (Figure 1b) are much smaller than that in the cadmium-treated testes (Figure 1c). Figure 1d shows the chromatogram of the 1 μM standard extract of 2,5-DHBA and 2,3-DHBA, which was obtained by spiking standard of 2,5-DHBA and 2,3-DHBA into a testis tissue and then extracted.

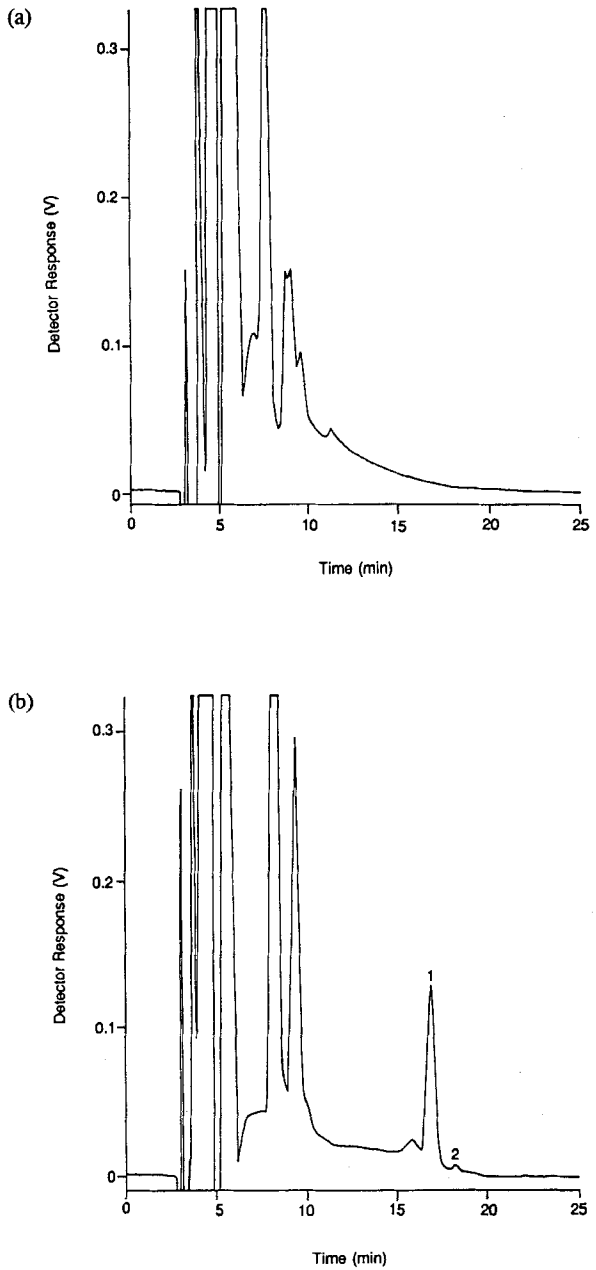


Figure 1 Chromatograms of the testes extracts in mice (a) a blank mouse; (b) a control mouse (treated with salicylate); (c) a salicylate- and cadmium-treated mouse; (d) 1 μM standard of 2,5-DHBA and 2,3-DHBA in testis tissue. Principal peaks, 1: 2,5-DHBA; 2: 2,3-DHBA

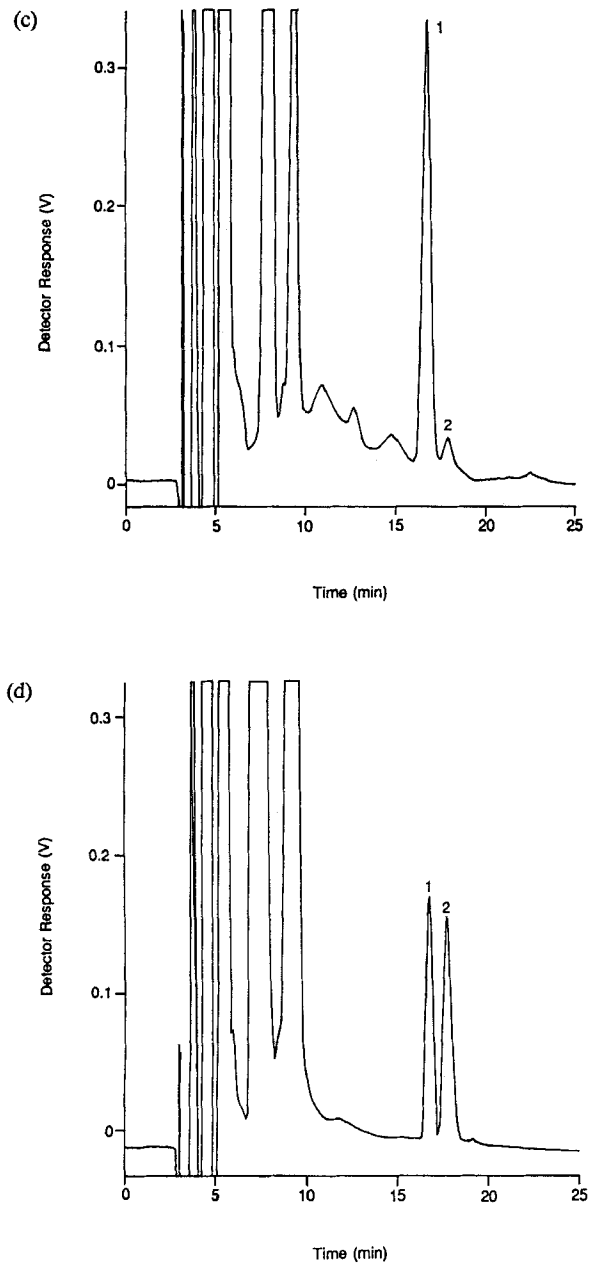


Figure 1 (continued)

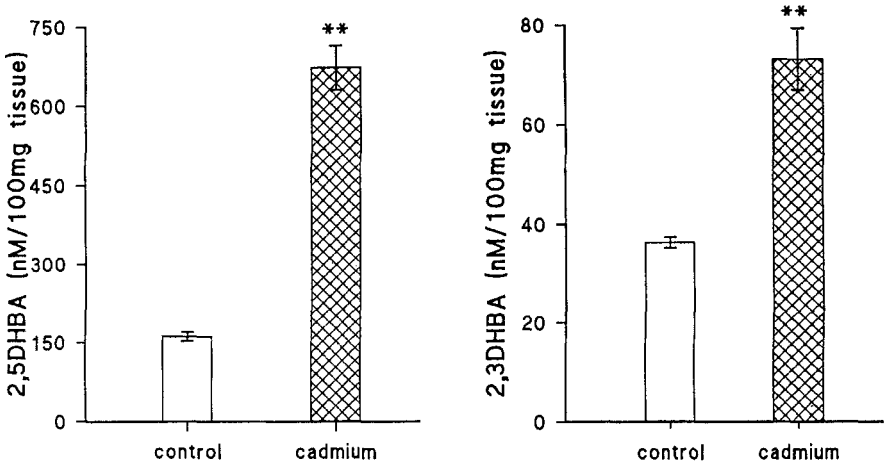


Figure 2 Mean (± SE) level of 2,5-DHBA and 2,3-DHBA in the testes of cadmium-treated mice (n=5) and control mice (n=5).

Figure 2 shows the mean ± SE (SE, standard error) concentrations of 2,3-DHBA and 2,5-DHBA in the testes of the control mice and and cadmium-treated mice. The mean ± SE of 2,3-DHBA and 2,5-DHBA in the control group was 36.34 ± 1.11 and 161.4 ± 68.33 nM/100 mg tissue. The mean ± SE of 2,3-DHBA and 2,5-DHBA in the cadmium treated group were 73.02 ± 6.25 and 671.32 ± 42.12 nM/100 mg tissue. The changes in the concentrations of 2,3-DHBA and 2,5-DHBA in the cadmium treated group in comparison with that in the control were 2.01 and 4.16 times, indicating significant increases.

The experiments were carried out to determine the possibility of further metabolism of the adducts, 2,5-DHBA and 2,3-DHBA, in testes. Testis tissues were spiked with 2,5-DHBA and 2,3-DHBA standard and extracted, and then stored at 4 °C for one week. The concentrations of 2,5-DHBA and 2,3-DHBA in this sample was

TABLE 1 The Level of 2,5-DHBA and 2,3-DHBA in Mouse Testes Homogenates During One Week at 4°C

day	2,5-DHBA Level (nM/100mg tissue)	2,5-DHBA change (%)	2,3-DHBA Level (nM/100mg tissue)	2,3-DHBA change (%)
1	161.46	0.00	36.34	0.00
2	159.97	0.92	35.31	2.83
3	155.67	3.58	32.97	9.27
4	149.50	7.41	32.52	10.51
5	146.51	9.26	31.05	14.56
6	134.56	16.66	30.20	16.90
7	132.32	18.25	29.76	18.11

determined every day by HPLC. As shown in Table 1, there is no significant change in the concentrations of DHBAs in the testes homogenate in 24 hours.

2,5-DHBA was the only product formed In an enzymic salicylate metabolism in vivo. [19] A previous study of the reaction of hydroxyl radicals with salicylate in vitro indicated that both 2,5-DHBA and 2,3-DHBA were formed in almost equal amounts. [19] Our results indicated that the ratio of 2,5-DHBA and 2,3-DHBA was 9.19 and the increases of 2,5-DHBA and 2,3-DHBA were 4 and 2 folds in the testes of cadmium treated mice. This conclusively demonstrates that the testis toxicity caused by cadmium is related to the formation of the hydroxyl radicals. While the detail mechanism of cadmium-mediated hydroxyl radical generation remains to be investigated, one possibility may involve the interaction of cadmium with ferritin and

other iron-containing proteins, resulting in the release of iron. The released iron reacts to form hydroxyl radicals via the Fenton reaction as suggested previously. [20]

Cadmium exerts its toxic effects on testicular vascular endothelium which could lead to ischemia, hypoxia, lipid peroxidation followed by generation of highly reactive hydroxyl radicals in the testicular tissues. [21], [22] The high content of polyunsaturated fatty acids in a testis rendered this organ particularly susceptible to peroxidative damage. It was found that the lipid peroxidation increased in homogenates of the testes after cadmium treatment of male rats. [23] Since thiol agents protect testis toxicity caused by cadmium, the testis damage is apparently oxidative in nature. It is quite possible that cadmium toxicity in testes is mediated by oxygen free radicals.

Salicylate as a specific hydroxyl radical trap is used and it produces stable hydroxylation products. Our results show that there was a 4-fold and 2-fold increase in 2,5-DHBA and 2,3-DHBA, demonstrating direct evidence for the involvement of hydroxyl radicals in cadmium toxicity in vivo. Hydroxyl radicals are extremely reactive with a number of compounds, including lipids and proteins. The presence of $HO^{\cdot}$ in the testes provides an important information for further study of the mechanism of testis toxicity caused by cadmium.

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