Studies on the Reaction of *p*-Hydroxybenzyl Alcohol and Hydroxyl Radicals

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Abstract

The reaction of *p*-hydroxybenzyl alcohol and hydroxyl radicals generated by the Fenton reaction is studied. The products of the reaction are separated and identified by high-performance liquid chromatography (HPLC)–diode-array detection and HPLC–mass spectrometry. According to the structures of the products, a mechanism of the reaction is proposed.

Introduction

Free radicals play an important role in food oxidation and can induce many diseases (1,2). Hydroxyl radicals, being extremely reactive species, serve as both a primary toxicant and a source of secondary toxicant. It can result in the strand breakage of DNA and contribute to significant biological effects such as carcinogenesis, mutagenesis, and cytotoxicity. The hydroxyl radical can be produced in cells by a variety of processes such as phagocytosis, antineoplastic agents, fungal phytotoxins, ionizing radiation, and others (3,4). It is generally proposed that such a radical could originate from a Fenton-type reaction (5,6): $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$. Damage caused by the toxic effects of the hydroxyl radicals is often decreased by antioxidants such as phenol, mannitol, and flavonoids (7–10). Much attention has been paid to the selection of inexpensive antioxidants with low toxicity. Studies on searching for active antioxidants in natural plants have attracted more and more attention.

Gastrodia elata Blume (GE) is a common traditional Chinese medicine that has been used for many years for the treatment of convulsion and epilepsy (11,12). The main components of the GE rhizome are *p*-hydroxybenzyl alcohol (HBA) and *p*-hydroxymethyl-phenyl-β-D-glucopyranoside (gastrodin) (13–16). Gastrodin is the most abundant and important biologically active component of GE. A pharmacodynamic study has shown that gastrodin exists in blood mainly as HBA, which is the aglycone of gastrodin (17). Some studies have reported that rats can live longer in anoxic conditions after the injection of GE extract (18). Liu et al. (19) reported that the extract of GE inhibits an increase in lipid peroxide levels in the ipsilateral cortex and induces an early increase of superoxide dismutase activity. They further confirmed that HBA is a powerful scavenger of superoxide and hydroxyl radicals using electron spin resonance spectroscopy (ESR) (20). Recent studies on antioxidant mechanisms have indicated that the chain reaction is controlled mainly through free-radical scavenging by the phenolic hydroxyls of antioxidants (21–24). In this study, we first systematically studied the reaction of HBA with the ${}^{\cdot}OH$ radical and then separated and identified the products of the reaction by high-performance liquid chromatography (HPLC)–diode-array detection (DAD) and HPLC–mass spectrometry (MS). Detailed studies of the products of the reaction of HBA with the ${}^{\cdot}OH$ radical should help clarify the individual mechanisms.

Experimental

Reagents

HBA and *p*-hydroxybenzaldehyde (HD) were of analytical grade and purchased from Sigma. *p*-Hydroxybenzoic acid (HA) was of analytical grade and purchased from Beijing Chemical Reagents Company (Beijing, China). Methanol was of HPLC grade, and all other chemicals were of analytical grade.

Instruments

The HPLC system (HP1100 chromatograph) consisted of a quaternary pump, a DAD, and an HP ChemStation data system. The HPLC–MS consisted of an HP1050 Chromatograph and an England VG platform. The MS was equipped with an atmosphere pressure ionization (API) source using the APCI inlet Shimadzu (Kyoto, Japan) UV 2100S.

Chromatographic conditions

HPLC–DAD conditions

A Zorbax SB-C_{18} column (250- × 4.5-mm i.d., 5 µm) (Agilent, Wilmington, DE) was used for separation. Methanol–water–isopropyl alcohol (35:55:10, v/v/v) was used as the mobile phase with a flow rate of 0.5 mL/min. The mobile phase was filtered by a 0.45-µm membrane filter before use. The UV detection wavelength was

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Table I. Peak Assignments for the Analysis of the Reaction

Peak number	t_R (min)	$(M-H)^-$	UV λ_{max} (nm)	Compound
1	7.76	121	278	HD
2	5.43	137	275	3,4-dihydroxybenzaldehyde
3	4.65	123	275	HBA
4	4.01	139	270	3,4-dihydroxybenzyl alcohol
5	4.05	137	250	HA

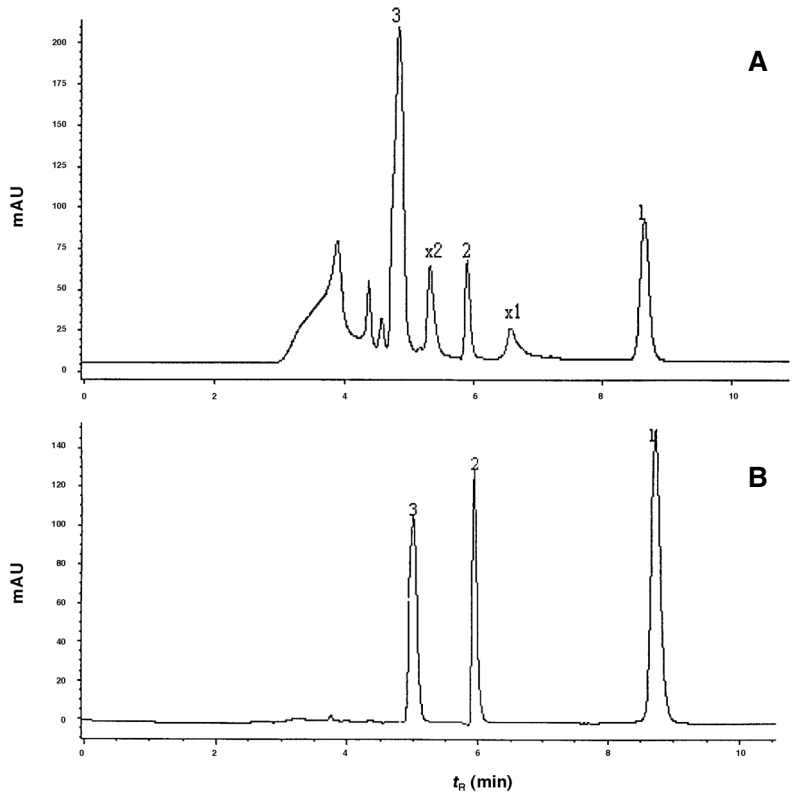

Figure 1. HPLC chromatograms of (A) the reaction mixture of HBA and ·OH and (B) standards: HD, 1; HBA, 2; and HA, 3.

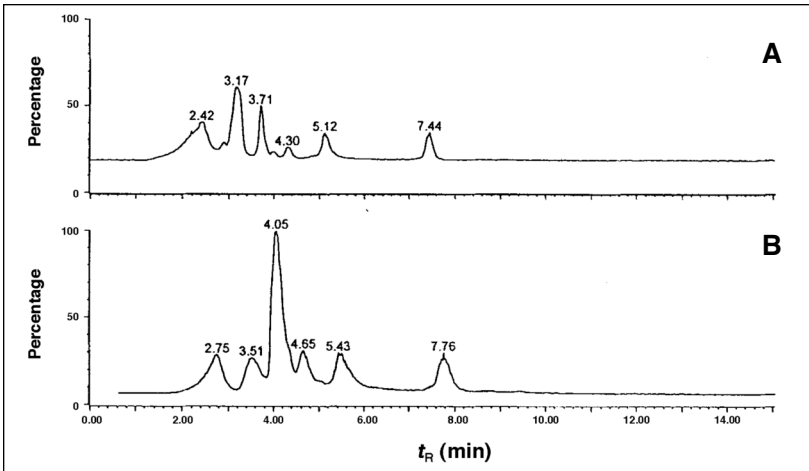

Figure 2. HPLC chromatograms of the compounds separated and detected by HPLC–APCI-MS: (A) HPLC–UV chromatogram and (B) HPLC–MS chromatogram.

270 nm and the column temperature 30°C. The inject volume of samples was 10 µL.

HPLC–MS conditions

The HPLC conditions were the same as described previously. The probe and ion source temperature were 550°C and 150°C, respectively. The cone voltage was 14 V and the corona discharge was set at 3.36 kV. Negative ion mass spectra of the analytes were acquired from 100 to 300 amu at a scan rate of 1 scan/s. The HPLC was directly connected to the MS without stream splitting.

Experimental methods

The reaction mixture contained 5.0 mL of $FeSO_4$ (1.0 mmol/L), 2.5 mL of H_2O_2 (30%), and 40 mL of HBA (10^{-2} mol/L). The mixture was incubated at 37°C in a water bath for 10 min. Then, the reaction products were analyzed by HPLC–DAD and HPLC–MS after the mixture was filtrated with a 0.45-µm membrane filter.

Results and Discussion

Separation and identification of the products in the reaction system of HBA and the ·OH radical by HPLC–DAD

Separation was performed by HPLC–DAD, which can provide the UV spectrum of each chromatographic peak. The HPLC conditions were as described previously. Baseline separation was achieved within 10 min, as shown in Figure 1A. When comparing the retention times (t_R) of the sample and the standard as shown in Figure 1B, the peaks with t_R values of 4.8, 5.9, and 8.7 min corresponded with HA, HBA, and HD, respectively. By changing the constitutes and concentrations of the mobile phase, the t_R values of the analytes were still corresponding well. The UV spectra of the sample were very similar to those of the standards (λ_{max} data of each peak are shown in Table I). The further identification of these three compounds and others was made by HPLC–MS.

Separation and identification of the reaction products by HPLC–MS

Both the HPLC–UV and total ion chromatogram (TIC) of the sample are shown in Figure 2. The peaks in TIC were slightly retarded because of the dead volume of the apparatus. Peaks were identified by negative ion mass, which presented the molecular ion $(M-H)^-$. The chromatograms of HPLC–UV (Figure 2A) and HPLC–DAD (Figure 1A) were different even though the chromatographic conditions were identical. The reasons were: (*a*) the chromatograms of HPLC–DAD and HPLC–MS were

performed on different apparatuses; (*b*) the column efficiency of HPLC–MS, which was interfaced by MS, was lower than that of HPLC; and (*c*) in the chromatograms of HPLC–DAD and HPLC–MS, the t_R values of individual compounds were different, and the relative positions of each peak in the chromatograms were similar. The identification of the compounds in Figure 2B is shown as follows.

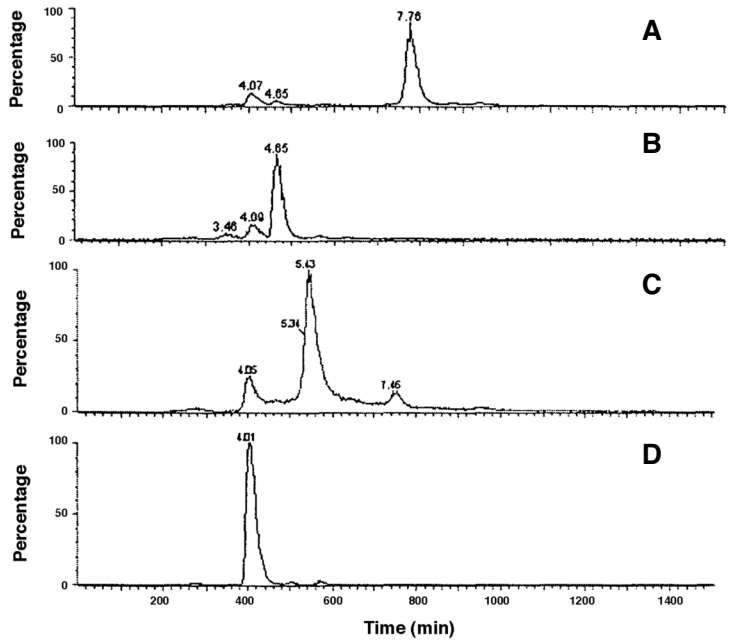

Figure 3. Mass chromatograms of the compounds separated and detected by HPLC–APCI-MS: (A) *m/z* 121, (B) *m/z* 123, (C) *m/z* 137, and (D) *m/z* 139.

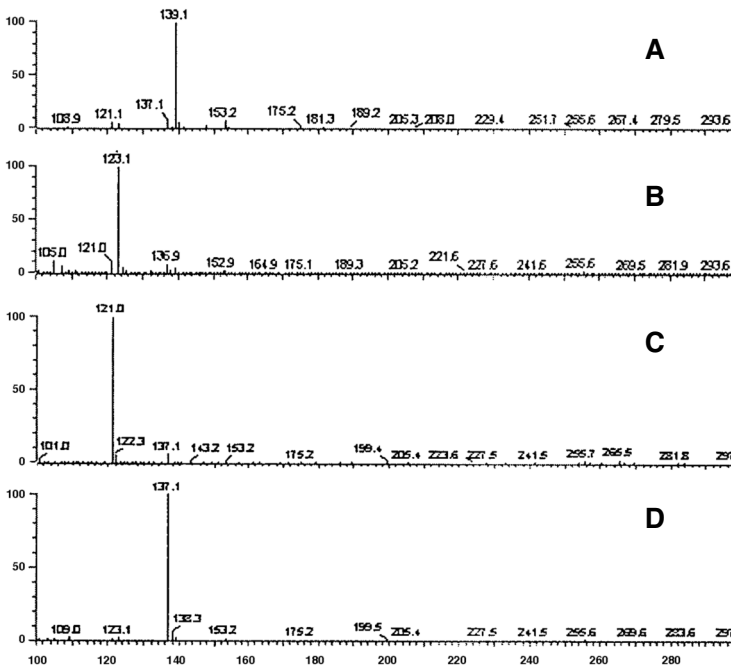

Figure 4. Mass spectra of the compounds separated and detected by HPLC–APCI-MS: (A) *m/z* 139 and 137 (t_R = 4.05 min), (B) *m/z* 123 (t_R = 4.65 min), (C) *m/z* 121 (t_R = 7.76 min), and (D) *m/z* (t_R = 5.43 min).

Identification of HBA

When using negative ion mass in order to detect, the *m/z* of HBA was 123, corresponding with M-1 (M, the molecular weight of HBA, 124). From the mass chromatogram and mass spectrum of peak 3 (Figures 3B and 4B), it can be seen that the compound (peak 3) had relatively high intensity and purity. According to the t_R characteristics, UV spectrum, and mass spectrum, peak 3 with a t_R at 4.65 min was identified as HBA.

Identification of HD

According to the mass chromatogram (*m/z* 121, Figure 3A), there was a compound with a molecular weight of 122 at 7.76 min, which had a relatively high signal-to-noise ratio. The mass spectrum displayed a relative high intensity (Figure 4C). Using MS, UV, and t_R, peak 1 was identified as HD (Figure 5).

Identification of HA

According to HPLC–DAD, the peak at 4.05 min should be HA. Obtained from HPLC–MS, the mass chromatogram (*m/z* 137, Figure 3C) and mass spectrum (Figure 4A) showed that the compound with a molecular weight of 138 was one of the products. Using MS, UV, and t_R, HA was identified as one of the products (Figure 6).

Two other products of the reaction

According to the mass chromatogram (*m/z* 137, Figure 3C) and mass spectrum (Figure 4D), there was a compound with a molecular weight of 138 at 5.43 min in TIC. From the mass chromatogram and mass spectrum (*m/z* 139, Figures 3D and 4A), there was a compound with a molecular weight of 140 at 4.01 min, which coeluted with HA. Using MS and UV data, peaks with t_R values at 5.43 min and 4.01 min were identified as 3,4-dihydroxybenzaldehyde and 3,4-dihydroxybenzyl alcohol, respectively. These two products will be further discussed. The peak identification results are summarized in Table I.

The mechanism of the reaction of HBA and hydroxyl radicals

Chimi et al. (25) reported that some natural phenolic compounds can scavenge a hydroxyl radical by the ESR method. The ·OH radical scavenging ability of phenolic compounds seems directly correlated with the number of hydroxyl groups substituted at the aromatic ring and to the nature of the substituent at the *p*-position. Polar substituents at the *p*-position were correlated with a higher hydroxyl radical quenching ability. In the molecular structure of HBA, there were phenolic hydroxy and methylic hydroxy, which were the main factors of the reaction of HBA and the ·OH radical.

The α-C hydrogen that was directly connected

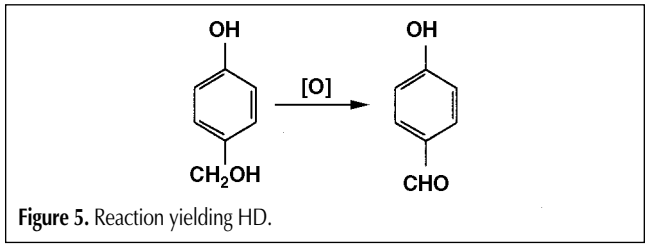

Figure 5. Reaction yielding HD.

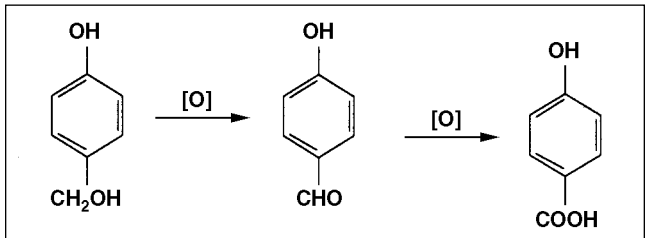

Figure 6. Reaction yielding HA.

with methylic hydroxy was very active and affected by the hydroxy group. The hydrogen was prone to be oxidized or extracted (21–25). The methylic hydroxy of HBA can be oxidized into aldehyde or carboxyl when HBA reacts with the oxidative ·OH radical. The reaction is shown in Figure 6.

Phenolic hydroxy has been regarded as the main factor of the free radicals scavenging activities of antioxidants. The hydrogen atoms of phenol at the neighboring or para position of OH became very active and were prone to be substituted by other atoms or atom groups. Including phenolic substances substituted with a hydroxyl group in the para position in a free radical generating system will result without equivocation in a hydroxyl radical attack at the meta position (7,21–26). Therefore, the products with M^+ 138 and 140 were probably 3,4-dihydroxybenzyl alcohol and 3,4-dihydroxybenzaldehyde. The identification of these two compounds needed more proof for confirmation because no standards were available.

Conclusion

The products of the reaction of HBA and the hydroxyl radical were separated and identified by HPLC–DAD and HPLC–MS. A mechanism of the reaction was proposed according to the structures of the products.

Acknowledgments

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