A Selective Preparation of Highly Deuterated Hydroxybenzoic Acids

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Abstract: A practical method to prepare selected deuterated hydroxybenzoic acids was developed. A hydroxybenzoic acid was dissolved or suspended in deuterium oxide (D_2O), then deuterium chloride (DCl) was added to adjust the pD to 0.32, and the mixture was refluxed under nitrogen for one to six hours. After removing D_2O and DCl by lyophilization, 2,6-dihydroxybenzoic-3,5-d_2 acid, 3,5-dihydroxybenzoic-2,4,6-d_3 acid, 3,5-dimethoxy-4-hydroxybezoic-2,6-d_2 acid, and 3,4,5-trihydroxybenzoic-2,6-d_2 acid were obtained quantitatively. Aromatic carbons with double substituent effects of hydroxy or methoxy groups were selectively deuterated. It was shown that the deuterium exchange reactions, which were electrophilic substitution reactions of deuterium cations to the aromatic carbons on the hydroxybenzoic acids, were regulated by the substituent effects. Deuteriums on all aromatic carbons were stable under a physiological condition and were detected in plasma by mass spectrometry analysis after oral administration to rats.

Keywords: Hydroxybenzoic acid, Deuteration, Selective preparation, Substituent effect.

Hydroxybenzoic acids are classified as a category of plant phenols. They are contained in common foods and have dietary functionality such as antioxidant activity [1], antiobesity effects [2], and antitumoral activity [3]. Mechanisms of most hydroxbenzoic acids are still not clear because they are altered by a variety of chemicals by metabolic biotransformation *in vivo*. In addition, it is impossible to distinguish the same metabolite originating from foreign substrates or *in vivo*. Therefore, kinetic analysis of hydroxybenzoic acids has been extremely difficult. Using isotope-labeled compounds for kinetic analysis is an efficient method. An isotope-labeled compound has been utilized as a kinetic reagent because it is metabolized in the same way as unlabeled natural compounds. Two types of isotopes exist: a radio isotope and a stable isotope. A radioisotope-labeled compound has high sensitivity and exceptionally small quantities of a sample are detectable, however the biological activity of radioisotope-labeled compounds is fraught with peril. On the other hand, a stable isotope-labeled compound has relatively lower sensitivity but is much safer. Stable isotopes exist at absolute ratio in nature and are treated as general reagents. Kinetic analyses of natural products and drugs can be accomplished by using stable isotope-labeled compounds [4] and can be put to practical use in the diagnosis of infection by *Helicobacter pylori* [5]. Deuterium is a type of stable isotope of hydrogen composed of a proton and a neutron. A compound labeled with deuterium was prepared by exchange of hydrogen with deuterium. Phenols were partially deuterated with treatment of Raney alloy catalyst in the presence of alkali or alkaline-earth metal carbonate in deuterium oxide (D_2O) [6]. Flavonoids were deuterated by using D_3PO_4/BF_3 as the deuteration reagent in D_2O [7]. In sub- and supercritical water, an aromatic ring containing phenols was deuterated without using a catalyst [8]. Preparation of deuterated phenols in efficient H-D exchange with Pt/C was reported by Sajiki *et al.* [9]. A deuterated phenol was utilized as a reagent for kinetic analysis and the effectiveness of this method was shown [10]. However, a selective deuteration method for hydroxybenzoic acids has not been reported. We developed a simple and efficient selective preparation method for highly deuterated hydroxybenzoic acids without using an expensive reagent. Stability of the deuterated hydroxybenzoic acids was investigated under a physiological condition and a single oral administration test in rats was performed to validate evidence of deuterated hydroxybenzoic acids as reagents for kinetic analysis of functional food ingredients. The deuteration mechanism was considered based on substituent effects by functional groups and estimated electron densities of the hydroxybenzoic acids.

Deuteration of ten hydroxybenzoic acids was investigated, including **1**; 4-hydroxybenzoic acid, **2**; 2,3-dihydroxybenzoic acid (pyrocatechuic acid), **3**; 2,4-dihydroxybenzoic acid (β-resorcylic acid), **4**; 2,5-dihydroxybenzoic acid (gentisic acid), **5**; 2,6-dihydroxybenzoic acid (γ-resorcylic acid), **6**; 3,4-dihydroxybenzoic acid (protocatechuic acid), **7**; 3,5-dihydroxybenzoic acid (α-resorcylic acid), **8**; 3-methoxy-4-hydroxybenzoic acid (vanillic acid), **9**; 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid), and **10**; 3,4,5-trihydroxybenzoic acid (gallic acid). Each hydroxybenzoic acid was dissolved or suspended in D_2O and the mixture was refluxed under nitrogen for 48 hours. After removing D_2O, all partially deuterated hydroxybenzoic acids except for compound **3** (β-resorcylic acid) were obtained [11]. It seems that **3** was first converted to resorcinol by decarbonation and then decomposed to CO_2 and H_2O in the reflux process [12]. Hydrogen on 2,6 positions of **10** were relatively highly exchanged by deuterium, with an exchange ratio of 79.3% by NMR determination [13, 14]. The deuteration, which is an acid-catalized hydrogen-exchange reaction, was caused by electrophilic substitution reaction of the deuterium cation to the aromatic carbons on the hydroxybenzoic acids [6]. To

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determine a more efficient deuteration condition, deuteration of **10** was investigated under several concentrations of deuterium cation. In this report, the concentration of deuterium cation is shown as pD (power of deuterium) in the same sense as pH [15]. By adding deuterium chloride (DCl) or sodium deuteroxide (NaOD) to the D_2O solvent of **10**, which was originally pD 3.5, the pD was adjusted to 7.0, 2.0, 1.0, 0.56, and 0.32, respectively. And the deuterated ratio on 2,6 positions of **10** was determined after the reflux condition during 24 hours in each pD [16]. The results are shown in Fig. (**1**). At the original pD of 3.5, the deuterated ratio was increased linearly depending on reaction time and the ratio was 61.4% after 24-hour reaction. At pD 7.0, the color of the reaction mixture turned brown immediately after starting the reflux. Deuteration ratio of the brown product could not be determined by NMR because of degradation of **10**. In contrast, in the reaction at low pD, the deuterated ratios increased dramatically. An exchange ratio of over 97% was achieved in 24, 12, 2, and 0.5 hours at pD 2, 1, 0.56, and 0.32, respectively. Compound **10** was stable in the strongest acidic condition and the product was obtained quantitatively. Reactions at lower pD achieved a more efficient deuterated exchange reaction. At pD 0.32, 3,4,5-trihydroxybenzoic-2,6-d_2 acid (**11**), whose deuterated ratio at 2,6 positions was 99.3 %, was obtained quantitatively in 1 hour as white powder. This is the ideal condition in which to prepare **11** from **10** in lab-scale production.

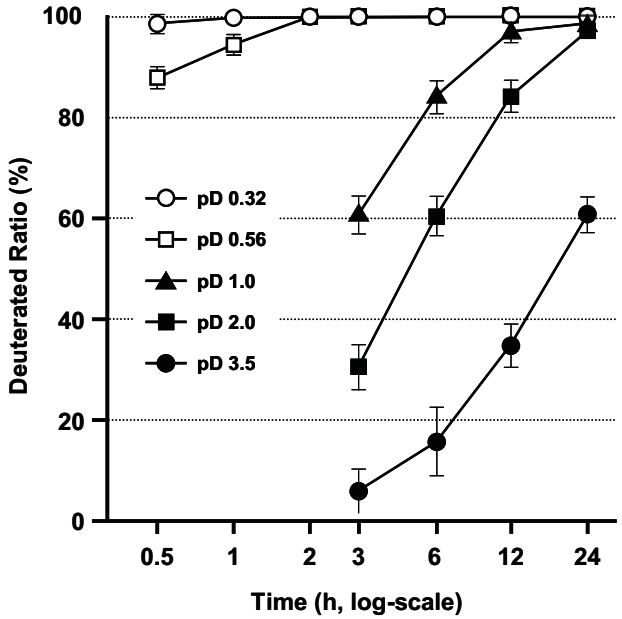

Fig. (1). Time Course of Deuterated Ratio at 2,6 Positions of **10** in D_2O at Various pD Values. ○: pD 0.32, □: pD 0.56, ▲: pD 1.0, ■: pD 2.0, ●: pD 3.5.

Subsequently, compounds **1**, **2**, **4**-**9** were deuterated by the reflux condition at pD 0.32. Preparation of highly deuterated hydroxybenzoic acids in this study is summarized in Table **1**. Additionally, 3,5-dihydroxybenzoic-2,4,6-d_3 acid (**12**) and 2,6-dihydroxybenzoic-3,5-d_2 acid (**13**) were prepared quantitatively in the same ideal condition as preparation of compound **11**. Deuterated ratio at 2,6 and 4 positions of **12**, and 3,5 positions of **13** were 99.0%, 94.3%, and 95.5%, respectively. Elongating the reaction times, 3,5-dimethoxy-4-hydroxybezoic-2,6-d_2 acid (**14**) with 92.6% of deuterated ratio was quantitatively obtained in 6-hour reaction. It was estimated that the deuterated ratio at 4 position of **12** decreased slightly by re-hydrogenation after the almost complete deuteration. As determined later, 15.8% of the deuterium at the 4 position on **12** was exchanged with hydrogen under a physiological condition in 7 days and this actual example supported the re-hydrogenation. At pD 0.32, compounds **1**, **4**, **6**-**8** were only scarcely deuterated in 24 hours, and **2** was partially deuterated in 1 hour but half of it decomposed. It was impossible to obtain highly deuterated compounds by elongating the reaction time.

To examine stability of the deuterated hydroxybenzoic acids under a physical condition, changes of the deuterated ratio on **11** and **12** were determined during 7 days [17]. As shown in Table **2**, deuterium on 2,6 positions of **11** and **12** were just barely re-hydrogenated and 94.8% and 99.9% of deuterium were maintained in saline for 7 days. The deuterated ratio of the 4 position on **12** was 84.2% after 7 days and the deuterium was slightly re-hydrogenated. After oral administration of compound **11** to rats, an mass spectrometry (MS) peak of protonated **11** at 173.1 *m/z* was detected in the plasma [18]. Compounds **11** and **12** were stable under the physiological condition during 7 days and deuterium isotopes on both were maintained sufficiently. In addition, they were absorbed through the digestive system of living bodies and were detected from analysis of body tissue. The conclusion is that deuterated hydroxybenzoic acids are able to be utilized as reagents for kinetic analysis.

The deuterium exchange reaction in this study was an electrophilic substitution reaction of deuterium cations to the aromatic carbons on hydroxybenzoic acids. Aromatic carbons with a high density of electrons with π-bonds generally react with an electrophilic reagent. The reactivity was directly affected by substituent effect. Hydroxybenzoic acids in this research possess hydroxy group(s), methoxy group(s) and a carboxyl group as substituted groups in the molecule. Because negative charges on ortho/para positions to the hydroxy group increase in the resonance forms of phenol, electrophilic substitution reactions have an ortho/para directing effect. A methoxy group also has a similar, and weaker, effect. On the other hand, because positive charges on ortho/para positions to the carboxyl group increase in the resonance forms of benzoic acid, electrophilic substitution reaction have a meta directing effect. Electrophilic sensitivities of aromatic carbons on hydroxybenzoic acids were estimated to be integrated in both directing effects. We calculated electron densities of the aromatic carbons as an indicator of the electrophilic sensitivities using CAChe work system [19]. It was estimated that a carbon atom with a greater negative charge was more easily attacked by deuterium cations.

Table **3** shows deuterium exchange ratios in the same reaction conditions (pD 0.32, reflux, 1 hour); substituent effects with hydroxy, methoxy and carboxyl groups; and electron densities of the aromatic carbons on **1**, **2**, **4**-**10**. Only aromatic carbons with double substituent effects of the hydroxy groups were highly deuterated. Deuterium exchange ratios of 2,6 positions on **10**, 2,6 and 4 positions on **7**, and

Table 1. Preparation of Highly Deuterated Hydroxybenzoic Acids with DCl/D_2O

Deuterated hydroxybenzoic acid	Reaction time (h)	D ratio on aromatic carbon (%)					Yield (%)
		2	3	4	5	6	
3,4,5-Trihydroxybenzoic-2,6-d_2 acid (**11**)	1	99.3	-	-	-	99.3	99
3,5-Dihydroxybenzoic-2,4,6-d_3 acid (**12**)	1	99.0	-	94.3	-	99.0	99
2,6-Dihydroxybenzoic-3,5-d_2 acid (**13**)	1	-	95.5	7.4	95.5	-	99
3,5-Dimethoxy-4-hydroxybenzoic-2,6-d_2 acid (**14**)	6	92.6	-	-	-	92.6	99

Table 2. Stabilities of D on Compounds 11 and 12 in a Physiological Condition

Compound	C* No.	Days				
		0	1	3	5	7
3,4,5-Trihydroxybenzoic-2,6-d_2 acid (**11**)	2,6	99.3 (100)**	96.6 (97.5)	95.8 (96.5)	94.7 (95.4)	94.1 (94.8)
3,5-Dihydroxybenzoic-2,4,6-d_3 acid (**12**)	4	94.3 (100)	88.8 (94.2)	84.1 (89.2)	82.1 (87.1)	79.4 (84.2)
	2,6	99.0 (100)	99.0 (100)	99.0 (100)	99.0 (100)	98.9 (99.9)

*Position of carbon atom on the deuterated hydroxybenzoic acid. **% of deuterated ratio (relative ratio) on the corresponding carbon(s).

Table 3. Deuterium Exchange Ratios (pD 0.32, reflux, 1 hour), Substituent Effects* and Calculated Electron Densities of 1, 2, 4-10

	Carbon No.	2	3	4	5	6
4-Hydroxybenzoic acid (**1**)	D ratio (%)	0	2.7	-	2.7	0
	substituent effects	– / ×	○ / –	-	○ / –	– / ×
	electron density	-0.035	-0.230	-	-0.197	-0.036
2,3-Dihydroxybenzoic acid (**2**)	D ratio (%)	-	-	19.1	20.8	18.2
	substituent effects	-	-	○/×	○/–	○/×
	electron density	-	-	-0.103	-0.105	-0.075
2,5-Dihydroxybenzoic acid (**4**)	D ratio (%)	-	2.5	0	-	2.9
	substituent effects	-	○/–	○/×	-	○/×
	electron density	-	-0.160	-0.089	-	-0.122
2,6-Dihydroxybenzoic acid (**5**)	D ratio (%)	-	95.5	7.4	95.5	-
	substituent effects	-	○○/–	–/×	○○/–	-
	electron density	-	-0.266	-0.025	-0.232	-
3,4-Dihydroxybenzoic acid (**6**)	D ratio (%)	0	-	-	0	0
	substituent effects	○/×	-	-	○/–	○/×
	electron density	-0.108	-	-	-0.167	-0.069
3,5-Dihydroxybenzoic acid (**7**)	D ratio (%)	99.0	-	94.3	-	99.0
	substituent effects	○○/×	-	○○/×	-	○○/×
	electron density	-0.194	-	-0.216	-	-0.151
3-Methoxy-4-hydroxybenzoic acid (**8**)	D ratio (%)	0	-	-	0	0
	substituent effects	●/×	-	-	○/–	●/×
	electron density	-0.131	-	-	-0.166	-0.070
3,5-Dimethoxy-4-hydroxybezoic acid (**9**)	D ratio (%)	42.4	-	-	-	42.4
	substituent effects	●●/×	-	-	-	●●/×
	electron density	-0.107	-	-	-	-0.107
3,4,5-Trihydroxybenzoic acid (**10**)	D ratio (%)	99.3	-	-	-	99.3
	substituent effects	○○/×	-	-	-	○○/×
	electron density	-0.144	-	-	-	-0.144

*○, ●, and × means substituent effects of hydroxy, methoxy and carboxyl groups, respectively.

3,5 positions on **5** were 99.3%, 99.0%, 94.3%, and 95.5%, respectively. Secondly, deuterium exchange ratios of 2,6 positions on **9**, with double substituent effects of the methoxy groups, was 42.4%. All carbons with single substituent effect of a hydroxy or a methoxy group were just barely deuterated, except for compound **2**. Carbons on **2** were deuterated in relatively large portions compared to others with single substituent effects. Compound **2** was unstable in the reaction condition and another excitation effect, not the substituent effects, could accrete the reaction. Suppressive substituent effects with a carboxyl group did not affect all of the deuteration reactions. Results of the semi-empirical molecular orbital method showed that all aromatic carbons had negative charges. However, the intensity of the negative charge did not correlate with the deuterated ratio. The reason for this may be that the extremely low pD was not considered in the calculation results. At pD 0.32, every hydroxy and carboxyl group was almost completely undissociated. Substituent effects by the functional groups were supposed to be reduced under the strong acidic conditions. According to this supposition, under strong acidic conditions, single substituent effects by hydroxy, methoxy or carboxyl groups were hardly able to work and double substituent effects by hydroxy or methoxy groups were effective in the deuterated reaction of hydroxybenzoic acids. In acidic conditions, suppression of the substituent effects decreased the reactivity of aromatic carbons and was expected to increase the stability of some hydroxybenzoic acids.

As described above, highly deuterated hydroxybenzoic acids, **11**, **12**, **13**, and **14** were selectively and quantitatively prepared only by refluxing hydroxybenzoic acids in acidic D_2O. These deuterated hydroxybenzoic acids were stable under physiological conditions and were detected by MS analysis *in vivo*. Our results showed that these deuterated compounds can be utilized as reagents for kinetic analysis. The deuterium exchange reactions on the hydroxybenzoic acids were induced by double substituent effects of hydroxy or methoxy groups.

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[11] Each hydroxybenzoic acid (100 mg) was dissolved or suspended in D_2O (20 ml, D 99.9%, Cambridge Isotope laboratories, Inc., Andover, USA) and the mixture was refluxed on an oil bath at 120 ˚C under nitrogen for 48 hours. After removal of D_2O by lyophilization, partially deuterated hydroxybenzoic acids except for **3** (β-resorcylic acid) were quantitatively obtained. The products were analyzed by NMR and MS to confirm deuterated products. NMR spectroscopic data were recorded on a Bruker DRX500 spectrometer (Bruker BioSpin Corp., Billerica, MA, USA) at 500 MHz for ^{1}H and 125 MHz for ^{13}C NMR at 25 ˚C. Chemical shifts were referenced to the signal of trimethylsilane as an internal standard. LC-MS analysis was performed with a Waters 2695 (LC) and Quattro micro API (MS) system (Waters, Milford, MA, USA). The HPLC separations were performed using a Cadenza HS C-18 reversed phase column (150 mm × 4.6 mm i.d., Imtakt, Kyoto, Japan). Elution was performed with 0.1 % (v/v) folic acid in purified water (Solvent A) and acetonitrile with 0.1 (v/v) folic acid (Solvent B) as mobile phase: 0-20 min, isocratic Solvent A; 20-30 min, gradient 0-70% Solvent B; 30-40 min, isocratic 70% Solvent B. Chromatography was performed at 35 ˚C with a flow rate of 0.8 mL/min (LC) and 0.3 mL/min (MS), injection volume of 10 μL, and detection at 280 nm. Mass spectra were acquired in electrospray ionization (ESI) mode using 3500 V capillary voltage, 20 V cone voltage, desolvation gas (N_2) flow of 350 L/h, cone gas (N_2) flow of 50 L/h, source temperature of 100 °C, and desolvation temperature of 350 °C. The mass spectrometer was operated in positive mode as 80 eV, target *m/z* = 200 and scanning range *m/z* 50-1000.

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[14] Deuteration ratios in this research were determined by a modified method of Yoshino *et al.* [13]. A well-dried deuterated product (10.0 mg) was dissolved in D_2O or CD_3OD (1.00 ml each, D 100%, Cambridge Isotope Laboratories, Inc.) and 3-(trimethylsilyl)propionic-2,2,3,3-d_4 Acid sodium salt (TSP, 50.0 μl of 10.0 mg/ml in D_2O) was added as an internal standard. And 800 μl of the mixture was put into an NMR tube and ^{1}H NMR spectra was obtained as described previously. The peak area of ^{1}H-NMR spectrum (Δ^1H) of deuterated product and standard compound were obtained based on that of TSP. A deuteration ration was determined by the following formula:

Deuteration ratio (%) = (Δ^1H of product/Δ^1H of standard) x 100

In this calculation, increases of molecular weight of deuterated products were not considered. So, the maximum error in the

deuteration ratio was estimated to be 0.72%, in the case of 50% deuteration of **1**, which was composed of **1** (exact mass: 138.03) and 4-hydroxybenzoic-2,3,5,6-d_4 acid (exact mass: 142.06) in the ratio 1:1. Deuterated ratios of the products from **1**-**9** were as follows. compound, position of carbon atom (D%): **1**, 2,6 (7.2%), 3,5 (9.7%); **2**, 4 (1.8%), 5 (41.2%), 6 (4.6%); **3**, N.D.; **4**, 3 (11.9%), 4 (15.8%), 6 (10.3%); **5**, 3,5 (66.6%), 4 (21.4%); **6**, 2 (1.3%), 5 (18.4%), 6 (0.84%); **7**, 2,6 (83.8%), 4 (66.9%); **8**, 2 (1.3%), 5 (24.7%), 6 (1.3%); **9**, 2,6 (2.6%).

[15] A power of deuterium cation (pD) in this research was a measurement value determined with a commercially available pH meter (IM-22P, DKK-TOA Corporation, Tokyo, Japan) and a pH meter reading was not corrected.

[16] Original solution of **10** at pD 3.5 was prepared by dissolving **10** (100 mg) into D_2O (20 ml). By adding 40 µl of NaOD (40% w/w in D_2O) and 20 µl, 40 µl, 400 µl, and 1000 µl of DCl (35% w/w in D_2O) to the original solution, a series of solutions at pD 7.0, 2.0, 1.0, 0.56, 0.32 were prepared.

[17] **11** and **12** were dissolved in saline (10 ml each) adjusting pH to 7.0 using NaOD and the solutions were then incubated for 7 days at 37 °C. Changes in the deuterated ratio were determined by the method using NMR as described previously. The relative standard deviation for the nine determinations was 0.15% and the accuracy of the determination of **11** and **12** were excellent.

[18] Aqueous solution of **11** at pH 7 was orally administrated to rats (n =3) at the dose of 10.0 mg/kg. After 30 minutes, LC/MS analysis described previously detected the peak originating from the administered compound at m/z $[M+H]^+$ 173.1 in every plasma sample.

[19] CAChe ver. 6.01-MOPAC2002 (Fujitsu Ltd.) was used for the calculation. At the beginning, energy minimization of each molecule was performed by using molecular mechanics method (CONFLEX/MM2). Then the global minimum conformation in aqueous medium was obtained by the semi-empirical method (CONFLEX/PM5), in which the solvent effect was modeled with Conductor-like Screening Model (COSMO). Electron densities of the aromatic carbons were automatically displayed on the global minimum conformation.