

## Rate Constants for Reaction of Hydroxyl Radicals with Sulfapyridine and Aminosalicyclic Acids

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Sulfasalazine, an anti-inflammatory drug, is metabolized to sulfapyridine and 5-aminosalicylic acid (5-ASA) which is therapeutically active. In the inflammation, active oxygen species have been suggested to play an important role. Among various active oxygen species, hydroxyl radical is known to be the most active radical. To elucidate the reactivity of sulfapyridine, 5-ASA, 4-ASA and 3-ASA with hydroxyl radicals, the rate constants were determined measuring the fluorescence of hydroxybenzoates induced by radiolysis hydroxylation of benzoate. The constant for 5-ASA, strongly fluorescent but insoluble in ether, was measured after ether extraction of hydroxybenzoates under acidic condition. For the other compounds, the rate constants were determined using a method which separates hydroxybenzoates by HPLC after the ether extraction, since the three compounds moved to the ether layer and interfered with the fluorescence of the hydroxybenzoates induced. Four rate constants were obtained in the range of  $6.4\text{--}7.0 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ , showing that they similarly scavenge hydroxyl radicals *in vitro*.

**Key words** sulfapyridine; 5-aminosalicylic acid; hydroxyl radical; rate constant;  $\gamma$ -irradiation; hydroxybenzoate

Sulfasalazine has long been used as an anti-inflammatory drug in the treatment of inflammatory bowel disease like ulcerative colitis, and recently in rheumatoid arthritis where reactive oxygen species such as hydroxyl radicals and superoxide anions are proposed to play an important role. When the drug is orally administered, sulfasalazine is metabolized to sulfapyridine and 5-aminosalicylic acid (5-ASA) by gut bacteria (Fig. 1).<sup>1)</sup> The 5-ASA exerts its therapeutic effect as the active moiety of sulfasalazine in intestine.<sup>2)</sup> Miles and Grisham have proposed<sup>3)</sup> that the anti-inflammatory activity *in vivo* of 5-ASA may be due to its antioxidant properties such as the ability to decompose neutrophilic oxidants (*e.g.*, HOCl) and to detoxify hemoprotein-associated oxidizing agents. Other workers have also considered<sup>4)</sup> that the radical scavenging activity is a putative mechanism of therapeutic action. Allgayer *et al.*<sup>5)</sup> measured the hydroxyl radical scavenging activities of sulfasalazine, sulfapyridine, 5-ASA and 4-ASA and described that only 5-ASA which is a therapeutically active compound inhibited hydroxyl radicals, whereas hydroxyl radicals are known to be an extremely reactive species, reacting immediately with most organic and biological substances at diffusion-limiting rates.<sup>6)</sup>

Thus, we measured the reaction rate constants of sulfapyridine, 5-ASA, 4-ASA and 3-ASA with hydroxyl radicals. Earlier we measured<sup>7)</sup> the rate constants for water-soluble, colorless compounds using the fluorescence intensity of hydroxybenzoates (HOBZ) produced through the reaction of benzoate with hydroxyl radicals generated by radiolysis of water. For colored water-soluble porphyrins and metalloporphyrins, the method was modified by extracting HOBZ induced into the ether layer.<sup>8)</sup> The modified method was applicable to 5-ASA which is water-soluble but strongly fluorescent itself, but was inapplicable to sulfapyridine, 4-ASA or 3-ASA which moves to the ether layer with HOBZ induced and interferes with the fluorescence of HOBZ. In this work, therefore, the method was further modified to determine HOBZ

finally using HPLC. Sulfasalazine, the parent drug of the two metabolites of sulfapyridine and 5-ASA, could not be measured because of its insolubility in water.

### Experimental

**Materials** Sodium benzoate, 2-hydroxybenzoate (2-HOBZ), and 4-aminosalicylic acid (4-ASA) were purchased from Nacalai Tesque Inc. (Kyoto, Japan). 3-ASA, 5-ASA and sulfapyridine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals used were of the highest purity available. Deionized water (18M  $\Omega$ ) generated from a Millipore Milli Q purification system was used in all experiments. All reaction mixtures were prepared in Chlex 100-treated Dulbecco phosphate-buffered saline solution without calcium or magnesium ions, pH 7.5 (PBS).<sup>9)</sup>

**Irradiation** One-ml volume of  $\text{N}_2\text{O}$ -saturated 0.2 mM benzoate solution containing various concentrations (0—0.24 mM) of ASA or sulfapyridine was irradiated for 60 min with a  $^{137}\text{Cs}$ -source (32 Gy/h) as done previously.<sup>7)</sup>

**Measurement of Hydroxybenzoates** After irradiation, 200  $\mu\text{l}$  of 0.1 M HCl was added to the benzoate solution. HOBZ in the acidic solution were extracted into ether and the ether was then completely removed as described previously.<sup>8)</sup> For 5-ASA, the residue was dissolved in 3.0 ml PBS and then the fluorescence intensity of hydroxybenzoates was

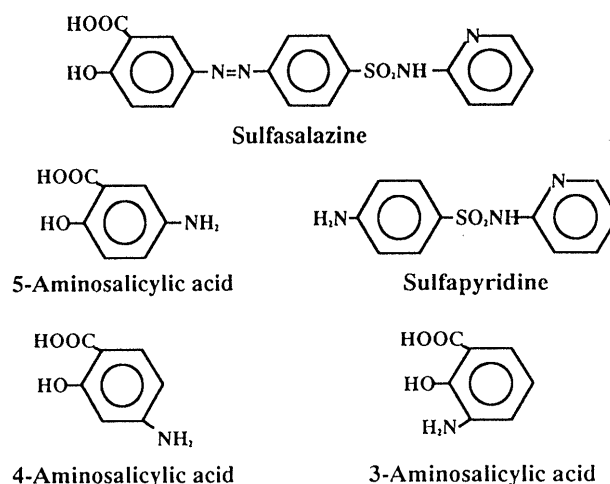


Fig. 1. Structure of Sulfasalazine, Sulfapyridine, 5-, 4- and 3-Aminosalicylic Acids

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measured at 407 nm emission ( $\lambda_{ex} = 305$  nm). For sulfapyridine, 3-ASA and 4-ASA, the residues were dissolved in 3.0 ml HPLC solvent. For 4-ASA, the solution was further diluted 10-fold. 2-HOBZ induced in the solution was determined by HPLC.

**Analysis by HPLC** Chromatography was carried out on a LC-6A HPLC instrument (Shimadzu, Kyoto, Japan) equipped with a RF-10A spectrofluorometer. 2-HOBZ induced was separated using a Lichrosphere 100RP-18 column (250 × 4 mm i.d.) and a guard column (4 × 4 mm i.d.) in a column oven at 40 °C. As solvent, 5% CH<sub>3</sub>CN or 15% CH<sub>3</sub>CN/10 mM phosphate buffer (pH 6.8)/10 mM *sec*-butylamine was used for 3-ASA and 4-ASA or for sulfapyridine, respectively. The sample solution of 20  $\mu$ l was injected into the column and the system was run at a flow rate of 1.0 ml/min. 2-HOBZ was detected at 407 nm emission ( $\lambda_{ex} = 305$  nm) and the amount was determined from the peak area. At least five separate experiments were performed in duplicate.

**Calculation of Rate Constants** Rate constants for reaction of hydroxyl radicals with sulfapyridine and ASA ( $k_s$ ) were calculated using the following equation.<sup>7)</sup>

$$k_s = \frac{mk_{BZ}[BZ]}{a} \quad (1)$$

In Eq. 1,  $m$  and  $a$  represent a slope of a straight line and an intercept on the  $y$ -axis, respectively. The straight line is given by a plot of reciprocal of the fluorescence intensity of HOBZ or peak area of 2-HOBZ induced against concentration of sulfapyridine or ASA. The concentration of benzoate, [BZ], was 0.2 mM, and the rate constant for reaction of hydroxyl radical with benzoate,  $k_{BZ}$ , was  $5.9 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ .<sup>6)</sup>

## Results and Discussion

The aqueous solutions of sulfapyridine and ASAs were colorless, but the solutions of 4-ASA and 5-ASA fluoresced in 350 to 600 nm when they were excited at 305 nm (Fig. 2). These compounds decreased or increased the fluorescence intensity at 407 nm of authentic 2-HOBZ (Table 1). Accordingly, to accurately measure the amount of 2-HOBZ induced, the compounds tested must be removed from the irradiated solution. For measurement of the rate constants for porphyrins and metalloporphyrins which show characteristic absorption in the visible region, we earlier introduced the modified  $\gamma$ -irradiation method which includes the extraction pathway.<sup>8)</sup> In this method,

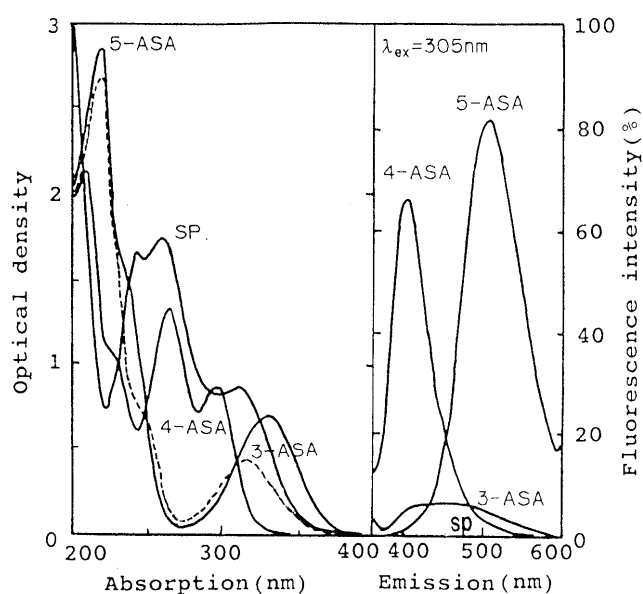


Fig. 2. Absorption and Fluorescence Spectra of Sulfapyridine (SP), 5-, 4- and 3-ASA

For absorption spectra, SP was measured at 100  $\mu$ M, 5-ASA 20  $\mu$ M, 4-ASA 10  $\mu$ M and 3-ASA 20  $\mu$ M. The fluorescence spectra were measured using 500  $\mu$ M SP, 5  $\mu$ M 5-ASA, 2.5  $\mu$ M 4-ASA and 50  $\mu$ M 3-ASA.

HOBZ induced were extracted into ether under an acidic condition, and then separated from the colored solute. When the acidic aqueous solutions of ASA were treated with ether, about 25% of 3-ASA was extracted into ether, about 40% 4-ASA and 5-ASA remained in aqueous layer (Table 2). All the sulfapyridine thoroughly was moved to the ether layer (data not shown). From these results, the rate constant for 5-ASA could be measured using the modified method including the extraction pathway as described previously.<sup>8)</sup> On the other hand, sulfapyridine, 100% of which was extracted into ether and the coexistence of which quenched the fluorescence of 2-HOBZ, and 4-ASA, of which more than 60% was moved to the ether layer and which itself is strongly fluorescent, disturbed the fluorescence of 2-HOBZ even after the treatment with ether. The 2-HOBZ induced, therefore, was separated from sulfapyridine and 4-ASA by HPLC. Their residues after the ether extraction were redissolved in each HPLC solvent. The sulfapyridine solution was directly injected into the HPLC system. The 4-ASA solution was diluted 10-fold before HPLC because the strong fluorescence of the 4-ASA peak influenced that of the 2-HOBZ peak. We confirmed previously<sup>8)</sup> that authentic 2-HOBZ was completely extracted into the ether layer from the acidic aqueous layer. Accordingly, the solution of authentic 2-HOBZ was used at various concentrations for calibration by HPLC. As shown in Fig. 3, the peak area of 2-HOBZ linearly increased against the concentration, showing that 2-HOBZ induced could be determined by HPLC. The rate constant for 3-ASA was also analyzed using HPLC since about 25% was moved to the ether layer and the coexistence quenched about 20% of the fluorescence intensity of 2-HOBZ. Figure 4 shows chro-

Table 1. Effects of Sulfapyridine and ASA on Fluorescence Intensity of 2-HOBZ

Compound	Concentration ( $\mu$ M)	Fluorescence	
		Intensity (%)	Ratio
2-HOBZ	1	79.8	1
+ Sulfapyridine	80	43.6	0.55
+ 3-ASA	80	64.9	0.81
+ 4-ASA	80	> 100	> 1.2
+ 5-ASA	80	85.2	1.07

Table 2. Extraction of ASA into Ether Layer

Compound	Conc. (mM)	FI (peak area)		% Extraction
		Direct <sup>a)</sup>	Extract <sup>b)</sup>	
3-ASA	0.16	16850	3757	22.3
	0.24	20111	5409	26.9
4-ASA	0.16	1361602	851970	62.6
	0.24	2119781	1362124	64.3
5-ASA	0.16	118578	N.D.	—
	0.24	191868	N.D.	—

N.D., not detected; FI, fluorescence intensity. The fluorescence intensity of ASA was detected at 407 nm emission ( $\lambda_{ex} = 305$  nm) under the HPLC condition described in Experimental. *a)* ASA was chromatographed without treatment. *b)* ASA extracted into ether layer was analyzed by HPLC.

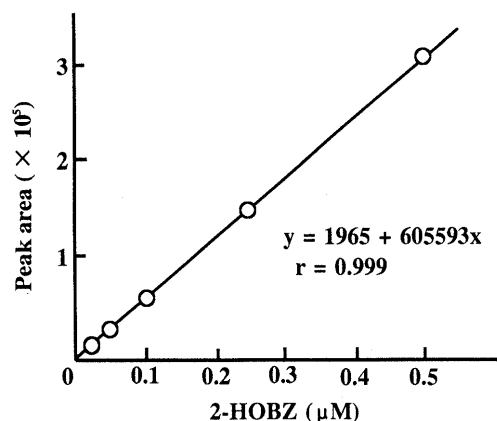


Fig. 3. Calibration Curve of Authentic 2-HOBZ by HPLC  
2-HOBZ was detected at 407 nm emission ( $\lambda_{ex}$  = 305 nm).

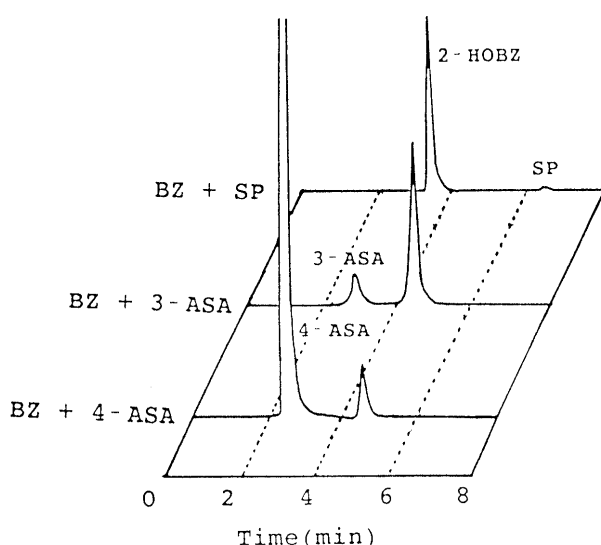


Fig. 4. Chromatograms of Irradiated Benzoate (BZ) Solution (0.2 mM) Containing 0.24 mM Sulfapyridine (SP), 3- or 4-ASA

The HPLC solvent was 5%  $\text{CH}_3\text{CN}$ /10 mM phosphate buffer (pH 6.8)/10 mM *sec*-butylamine for 3-ASA and 4-ASA, and for SP the concentration of  $\text{CH}_3\text{CN}$  was increased to 15%. The detection was performed at 407 nm emission ( $\lambda_{ex}$  = 305 nm).

matograms of irradiated benzoate solutions containing sulfapyridine, 3-ASA or 4-ASA. The 2-HOBZ was completely separated from these compounds.

Figure 5 shows the result for 5-ASA measured by the modified method including extraction pathway. The relationship between the concentration and reciprocal of the fluorescence intensity gave a straight line. The rate constant for 5-ASA was obtained by substituting two values of the slope and intercept on the  $y$ -axis in Eq. 1. A further modified method using HPLC after extraction was also tested for 5-ASA to compare the rate constant with that measured by the extraction method; the two constants showed similar values (data not shown). Therefore, the further modified method is believed to be equivalent to the extraction method<sup>8)</sup> and the original non-modified method<sup>7)</sup> since the extraction method and the original method have given similar rate constants for potassium iodide.<sup>8)</sup>

The rate constants for sulfapyridine, 3-ASA and 4-ASA were measured using the further modified method including HPLC after extraction. Figure 6 shows a plot

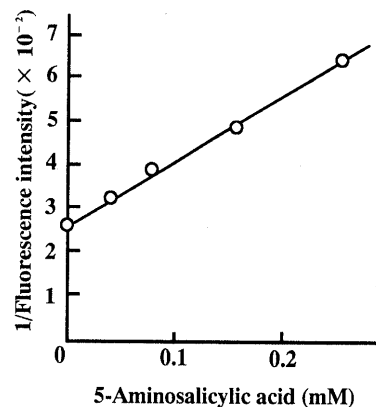


Fig. 5. Effect of 5-Aminosalicylic Acid on Benzoate Hydroxylation Determined using Ether Extraction

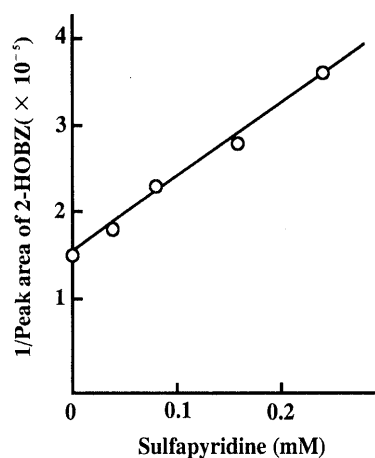


Fig. 6. Effect of Sulfapyridine on Benzoate Hydroxylation Measured by HPLC

Table 3. Rate Constants of Sulfapyridine, 3-, 4- and 5-ASA Determined by Benzoate Hydroxylation

Compound	Rate constant ( $\times 10^{-9} \text{ M}^{-1} \text{ s}^{-1}$ )
Sulfapyridine	$6.7 \pm 0.2$
3-ASA	$7.0 \pm 0.1$
4-ASA	$6.4 \pm 0.2$
5-ASA	$6.5 \pm 0.2$

of reciprocal of the peak area against sulfapyridine concentration. The slope and intercept on the  $y$ -axis of the straight line were used to calculate the rate constant for sulfapyridine. The rate constants for 3-ASA and 4-ASA were obtained in the same manner. Table 3 summarizes the results obtained. The four rate constants were in the range of  $6.4\text{--}7.0 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ , showing that the four compounds react similarly with hydroxyl radicals. Allgayer *et al.*<sup>5)</sup> investigated the reaction of 4-ASA and the sulfasalazine metabolites, 5-ASA and sulfapyridine, with hydroxyl radicals using ESR spectroscopy. Only 5-ASA which is therapeutically active inhibited hydroxyl radicals. This study showed that 4-ASA, 5-ASA and sulfapyridine react with hydroxyl radicals at the similar rate constants of  $6.4$ ,  $6.5$  and  $6.7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ , respectively. Hydroxyl radical is known to react rapidly with

most organic and biological substances at almost a diffusion controlled rate.<sup>6)</sup> Thus, it is probable that 4-ASA would react with hydroxyl radicals at a similar rate as 5-ASA. The concentrations of ASAs and sulfapyridine used in the ESR measurement (*ca.* 10 mM) were higher than those in this study (*ca.* 0.24 mM). In the higher concentrations, 5-ASA is soluble in water, but 4-ASA and sulfapyridine have only low solubility in water (1 g per about 500 ml for 4-ASA and 1 g per about 3500 ml for sulfapyridine<sup>10)</sup> and do not dissolve completely). Possible explanations for the discrepancy in rate constants may be different concentrations of tested compounds.

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