Fluorometric Determination of Sulfite with N-(9-Acrindinyl)-maleimide for High Performance Liquid Chromatography

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The fluorometric reaction of sulfite with N-(9-acridinyl)maleimide (NAM) was applied to high performance liquid chromatography (HPLC). It improved the sensitivity and accuracy for the determination of sulfite to 0.01 nmol/ml or 0.1 pmol on the column. Three NAM-sulfite adducts were isolated, and their structures were identified as N-(9'-acridinyl)-2-sulfonyl succinimide (I) and its two isomeric hydrolysates, N-(9'-acridinyl)-2 or -3-sulfonyl succinamides (II and II'). Two-step reactions, involving the addition of sulfite to the maleimide ring of NAM and subsequent hydrolysis of the resulting succinimide ring to two amide-form adducts, were established.

Sulfurous acid or sulfite is one of common oxidized forms of sulfur. It occurs widely in our environment through the oxidation of sulfur by industrial and volcanic activities, and by the biochemical reduction of sulfate in water and soil under anaerobic conditions. In foods, it is used as an additive for antioxidation, bleaching and preservation by taking advantage of its strong reducing power. Generally, two difficulties exist in analyses for sulfite: its extremely low concentration in samples and the instability of sulfite which can be easily oxidized to sulfate with dissolved oxygen, even at room temperature. Although many sulfite determination methods have been reported, they are not satisfactory in their sensitivity and/or specificity. In a preliminary communication, we reported a new fluorometric reaction of sulfite with N-(9-acridinyl)maleimide (NAM), which was originally developed as a thiol reagent. The ordinary fluorometric technique could determine a trace amount of sulfite as well as thiols in aqueous solutions.

However, it was difficult to differentiate the sulfite from thiols by the ordinary fluorometric method and, in addition, the background fluorescence or quenching limited the sensitivity and accuracy of the method. An application of HPLC was expected to solve these problems. The preliminary use of HPLC for the determination of sulfite in beer and blood serum showed two or three peaks.

In this report, we describe the application to two columns to separate three reaction products, their isolation and structural elucidation, the establishment of a reaction mechanism on the basis of a time-course study of the reaction, and the development of the prelabelled HPLC method applicable to a wide range of sample concentrations.

MATERIALS AND METHODS

Reagents and instruments. Sodium bisulfite was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). NAM was synthesized in our laboratory and used as a freshly prepared acetone solution. The other reagents used were of analytical grade and were purchased from Wako Pure Chemicals Co. (Osaka, Japan). Acetonitrile was distilled and filtered using a Toyo TM-2P membrane filter (0.45 μm) before use, the other reagents being used without further purification.

† Part III of a series for the fluorometric determination of sulfite.
The HPLC apparatus used was a JASCO (Japan Spectroscopic Co., Tokyo, Japan) model FLCL-100 equipped with a loop-type sample injector, JASCO model VL-611. For separation, three types of stainless steel columns were used: (a) LiChrosorb RP-8 (10 μm in particle size, 4.0 mm i.d. × 250 mm, Merck A.G., Darmstadt, G.F.R.), (b) Fine SIL NH2 (10 μm, 4.0 mm × 250 mm, JASCO), and (b') LiChrosorb NH2 (5 μm, 4.0 mm i.d. × 250 mm, Merck A.G.). A PELL ODS column (30 ~ 38 μm, 4.6 mm × 50 mm, Whatman Co., Kent, England) was used as a guard column. Fluorescence was monitored by a JASCO model FP-100 photometer, and all piping used 0.25 mm i.d. stainless steel tube.

A TC-1 isothermal bath (Toyo Sci. Ind. Co., Tokyo, Japan) and a Hitachi-Horiba model F-7 pH meter were used throughout.

Infra-red spectra were obtained by a JASCO model A-202 as KBr disks. NMR-spectra were recorded by a JNM-FX-100 spectrometer (99.5 MHz on proton and 25.0 MHz on carbon, Japan Electron Optics Laboratories, Tokyo, Japan) as pyridine-d5 or dimethylsulfoxide-d6 (DMSO-d6) solutions. Tetramethylsilane was used as an internal standard.

Preparation of the NAM-reaction buffer. Boric acid (30.92 g) and potassium chloride (37.28 g) were made up to 1 liter with distilled water (solution A). Sodium carbonate (53.00 g) was made up to 1 liter with distilled water (solution B). The pH values of buffers were adjusted to 6.0, 7.0, 8.0, 8.8, 9.0 and 10.0, respectively, by the addition of solution A to solution B. The pH 5.0 solution was a 0.05 M phosphate buffer.

Labelling reactions of NAM with sulfite for the determination. For the calibration and determination of sulfite in the sample solutions, 100 μl of the sample was added to a mixture of 100 μl of 1.2 mM NAM-acetone solution and 800 μl of NAM-reaction buffer (pH 10.0). After the mixture had been allowed to stand for more than 2 hr at 15°C, a 10 μl aliquot was injected into the HPLC system.

Isolation of compound A, N-(9'-acridinyl)-2-sulfonyl succinimide (I). N-(9'-acridinyl)-2-sulfonyl succinimide (I), which corresponds to peak A on the HPLC trace in Fig. 1, was prepared by the reaction of NAM with sulfite in acetone according to our previous method.1) mp, 300°C; UV λmax (in 0.1 M potassium phosphate buffer at pH 6.0) nm (ε): 251 (154000), 362 (12200); IR νmax cm⁻¹: 1790 and 1730 (succinimide ring), 1640 (dipolar ion form), 1200 and 1030 (sulfonic acid or its salt); NMR δ(dmso-d6): 33.973, 61.544, 121.393, 126.424, 126.717, 135.902, 148.722, 156.300, 171.120, 196.920. (Some resonances of the acridine ring were overlapped.)

Isolation of compound B, C, N-(9'-acridinyl)-2 or -3-sulfonyl succinamides (II and III). (50 mg) was hydrolyzed with 100 mM Tris-HCl buffer (pH 9.3, 50 ml) for 24 hr. The reaction mixture was desalted by passing through a short cation exchange column (Dowex 50 W × 8, H form, 10 mm i.d. × 15 cm). The eluant was evaporated in vacuo and dried over P2O5 until a hygroscopic powder (about 20 mg) was obtained. This gave the peaks B and C on HPLC. Anal. Found: C, 45.26; H, 3.62; N, 6.07. Caled. for C17H15N2O4S·HCl: C, 45.37; H, 3.42; N, 6.07.

The powder (20 mg) was further treated on a preparative-scale ODS column (9.0 mm i.d. × 300 mm) developed with 0.1 M KCl containing 2.5% acetonitrile to give 10 mg each of the compounds B and C, which correspond to the peaks B and C, respectively. Compound B was too hygroscopic to give a good PMR spectrum. Compound C, mp, 256 ~ 273°C (dec.); UV λmax (0.1 M potassium phosphate buffer at pH 6.0) nm: 252, 359; IR νmax cm⁻¹: 3000-2500, 1730 (carboxylic acid), 1705 (amide I), 1640 (dipolar ion form), 1560 (amide II), 1200 and 1030 (sulfonic acid or its salt); NMR δ(dmso-d6): 7 ~ 8.5 (8H, m), 11.5 (1H, s). In the region between 3 ~ 6 ppm, signals could not be assigned because of the overlapping HOD signal. 13C-NMR δ(dmsso-d6): 33.973, 61.544, 121.393, 124.242, 126.717, 135.902, 148.722, 171.120, 196.920. (Some resonances of the acridine ring were overlapped.)

Compound C, mp, 270 ~ 273°C (dec.); UV λmax (0.1 M potassium phosphate buffer at pH 6.0) nm: 251.5, 359; IR νmax cm⁻¹: 3000 ~ 2500, 1730 (carboxylic acid), 1705 (amide I), 1640 (dipolar ion form), 1560 (amide II), 1200 and 1030 (sulfonic acid or its salt); NMR δ(dmso-d6): 3.38 (1H, double doublet, J = 13.9 and 7.3), 3.42 (1H, double doublet, J = 7.3 and 14.2), 4.0 ~ 6.0 (1H, s, broad), 7.0 ~ 8.5 (8H, m), 11.5 (1H, s). 13C-NMR δ(dmsso-d6): 35.920, 61.544, 121.451, 126.424, 126.717, 135.902, 148.653, 169.775, 171.120. (Some resonances of the acridine ring were overlapped.)

(a) Reversed-phase column. Three reaction products (A, B and C) and an unidentified degradation product of NAM (D) were separated on a LiChrosorb RP-8 column eluted with 16% acetonitrile-0.2N ammonium acetate (1 ml/min) as shown in Fig. 1. Their fluorescence intensities were monitored with an excitation at 360 nm and an emission at 455 nm.

(b) Aminopropyl column. Two commercially available columns, (a) Fine SIL NH2 and (b') LiChrosorb NH2, were used. The elution order of the peaks was similar on both columns. The best separation was made with 14% or 16% acetonitrile-0.01 M potassium phosphate buffer (pH 6.9) at a flow rate of 1.0 ml/min.

RESULTS AND DISCUSSION

Figure 1 presents the chromatograms of the reaction of sulfite with NAM at the early
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Fig. 1. Chromatograms of NAM–Sulfite Adducts on a Reversed-Phase Column.

Column, LiChrosorb RP-8; mobile phase, 16% acetonitrile–0.2 N ammonium acetate (1 ml/min). A 300 µl sample of 0.3 mM sulfite aqueous solution was added to a mixture of 100 µl of 1.2 mM NAM–acetone solution and 600 µl of NAM-reaction buffer (pH 8.8), and followed by incubation at 30°C. A 10 µl aliquot was injected for HPLC after 15 sec (left) and 25 min (right) of the reaction.

Stage (after 15 sec) and at the advanced stage (after 25 min). The fluorescence quickly developed, and HPLC gave peak A at first. Peak A then gradually decreased and two more peaks, B and C, increased. Finally, peak A disappeared completely and the peak heights of B and C reached a constant level. The fluorescence intensities of peaks B and C were stable for more than 48 hr. Peak D is considered as representing the degradation products of NAM since it was always observed in the blank solution. The compounds A, B and C corresponding to peaks A, B and C were prepared, and their IR, PMR and CMR spectra showed the compound A to be a sulfite-succinimide adduct, and the compounds B and C as their hydrolyzed products as will be described later.

On the reversed phase column, their retention time could be shortened by increasing the concentration of acetonitrile to 20% in 0.2 N ammonium acetate. A slight modification was designed to make peaks B and C form one pseudo-peak. Their fluorescence intensities were monitored with an excitation at 360 nm and an emission at 455 nm.

**Structures of reaction products corresponding to peaks A, B and C**

In the reaction between the maleimide and thiols, the following mechanism is known: a Michael-type addition of thiol to the maleimide double bond forms a succinimide compound, and subsequent hydrolysis of the imide ring forms an isomeric mixture of two succinimides.\(^4\) Previously, we reported a similar Michael addition of sulfite to NAM in acetone to yield crystals of the succinimide.\(^1\) This imide gave peak A on HPLC and was shown to have the structure I shown in Fig. 2, based on the spectral data described in MATERIALS AND METHODS. In order to follow the second step of the reaction, I was hydrolyzed under moderate conditions which were almost the same as the labelling reaction for determination (pH 9.0). This gave an isomeric mixture of the two succinamides, B and C, as a hygroscopic powder. The mixture was separated in a large scale by liquid chromatography to give compounds B and C, which correspond to peaks B and C on HPLC, respectively. Their IR, PMR and CMR spectral data support the structure II and/or II' shown in Fig. 2. However, an unequivocal assignment of the isomers to the structures given in Fig. 2 has not yet been made.

In conclusion, a two-step reaction similar to the thiol–maleimide reaction is presented.

**Fluorescence spectra of the reaction products**

The fluorescence spectra of compounds A, B and C were measured in 0.1 M phosphate
Figure 3. Fluorescence Spectra of Compounds A, B and C.

Fluorescence spectra of compounds A, B and C were measured in 0.1 M phosphate buffer (pH 6.0). The excitation maxima of compounds A, B and C were observed at 362, 360 and 360 nm, respectively. The corresponding emission maxima were at 428, 455 and 455 nm, respectively. No hydrolysis of compound A was observed during the measurement. The fluorescence intensity was expected as a relative strength against the absorption of UV maxima at 362 nm in compound A and 360 nm in compounds B and C, which correspond to the excitation maxima. Their relative quantum yields to quinine in a 0.5 M sulfuric acid solution were measured at an excitation wavelength of 365 nm on the same apparatus at the same time using the data that the quantum yield of quinine in 0.5 M sulfuric acid solution was 0.55. Those of compounds A, B and C were 0.26, 0.50 and 0.40, respectively. These results suggest that the succinimide structure gave a lower fluorescence intensity than the amide-structure products, and account for the rapid increase of fluorescence intensity in the early stage and the gradual increase to reach a plateau in our ordinary fluorometer.

The excitation and emission maxima of compounds B and C were also measured to select the wavelength of the fluorescence detector in two ways by HPLC. The reversed-phase column with solvent of 16~20% acetonitrile in 0.2 N ammonium acetate gave an excitation maximum at 360 nm and an emission maximum at 455 nm. The aminopropyl column with solvent of 14~16% acetonitrile in 0.05 M phosphate buffer (pH 6.0) gave an excitation maximum at 360 nm and an emission maximum at 438 nm. The addition of acetonitrile caused some blue shift in the latter. However, in practice the excitation of 360~365 nm and emission of 430~460 nm caused few errors, although the sensitivity might have been slightly affected.

**Time course of the reaction at various pH values and temperatures**

In order to establish the optimum reaction conditions, the effects of pH and temperature were studied. Figure 4 shows the time course of the reaction at various pH values. At the beginning of the reaction, peak A was formed very promptly at pH 8~9, reached a maximum and then gradually decreased. The reaction rate was slower on the acidic side and it took more than 20 hr to reach a plateau at pH 5.

While peak A gradually decreased, the hydrolyzed peaks B and C increased. The rate increased markedly at higher pH values (8~10). It took one to two hours at pH 10, eight hours at pH 8 and twelve hours at pH 7 to reach a plateau. Figure 5 shows the influence of the reaction temperatures at pH 8.8. The formation of peaks B and C was accelerated by elevating the temperature. For example, at 0°C, the hydrolysis was quite slow, but became very fast at 60°C. The side reactions, mainly the degradation of NAM, were also accelerated at temperatures above 30°C. Compound C was yielded in a relatively greater amount than compound B with increasing pH values and higher temperatures. The ratios of the products (C/B) varied from 2/3 to 3/2. Since the hydrolysis products B and C were stable for a long period (more than 48 hr), it is possible to conduct labelling reactions of many samples at once and to apply the detection one by one based on the machine cycle time.

These observations lead us to design a sulfite determination method by monitoring peaks B and/or C instead of peak A. The reaction was conducted at pH 10.0 and above 15°C to complete the hydrolysis with minimum reac-
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Fig. 4. Effect of pH on Reaction Time (at 30°C).

---, compound A; ----, compounds B and C. Reaction conditions, see footnote to Fig. 1 except for the pH of the buffer (pH 5.0, 7.0, 8.0, 9.0 and 10.0).

Time (h)

Fig. 5. Effect of Temperature on Reaction Time (at pH 8.8).

A 300 μl sample of 0.2 mM sulfite solution was added to a mixture of 100 μl of 3.6 mM NAM-acetone solution and 600 μl of NAM-reaction buffer (pH 8.8), and followed by incubation at 0, 30, 45 and 60°C, respectively.

Retention time

Fig. 6. Chromatogram of Wine on an Aminopropyl Column.

Column, LiChrosorb NH2; mobile phase, 14% acetonitrile-0.01 M potassium sulfate-0.05 M potassium phosphate buffer (pH 6.0, 0.7 ml/min). Monitored with an excitation at 360 nm and an emission at 440 nm. Peak D' represents the mixture of decomposed products of NAM and possible thiol adducts of NAM in wine.

HPLC greatly improved the sensitivity and accuracy up to 0.1 pmol of sulfite on the column by monitoring peaks B and/or C. This sensitivity is 100 times greater than that of our ordinary method and 10,000 times greater than the Rankine method, which is used as the official method for wine and other foodstuffs. The sensitivity could be more improved by increasing the power of the light source. A linear relationship between peak height and sulfite concentration in the range of 0.13~130 pmol was obtained with correlation constants for the calibration curves of more than 0.999. The ordinary components in biological materials did not interfere with the labelling reaction, because the recoveries of spiked sulfite within this range in wine, human sera and...
other biological samples were almost quantitative. Details of these results will be reported elsewhere.

Care needed to be taken to use water which was free from metal ions and dissolved oxygen in preparing the sample solutions and standard solutions, since most of the errors were from loss of sulfite by oxidation, especially at high dilutions.

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**REFERENCES**


