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Colorimetric Assay for Methanesulfinic Acid in Biological Samples

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ABSTRACT

We describe a simple colorimetric method to measure 30 to 300 μM concentrations of sulfinic acids in biologic samples. The procedure employs the coupling reaction of an aromatic diazonium salt ($Ar\text{-}N{=}N^{+}$) with the sulfinic acids (RSOOH) to produce a colored diazosulfone derivative (Ar-N=N-SOOR), which can be selectively extracted into an organic solvent. Linearity and noninterference by liver homogenate, phenols, amines, and thousand-fold or greater excesses of sulfate, thiol, and dimethyl sulfoxide are demonstrated. Sensitivity of the method is about 10 nmol per sample. Because methanesulfinic acid is the principal product of the action of hydroxyl radicals upon dimethyl sulfoxide, and because intact animals can tolerate dimethyl sulfoxide in millimolar concentrations, the method may prove widely useful for detecting the involvement of hydroxyl radicals in pathologic processes in vivo.

Key words: diazonium salts; diazosulfones; Fast Blue BB; hydroxyl radical trap; sulfinic acids.

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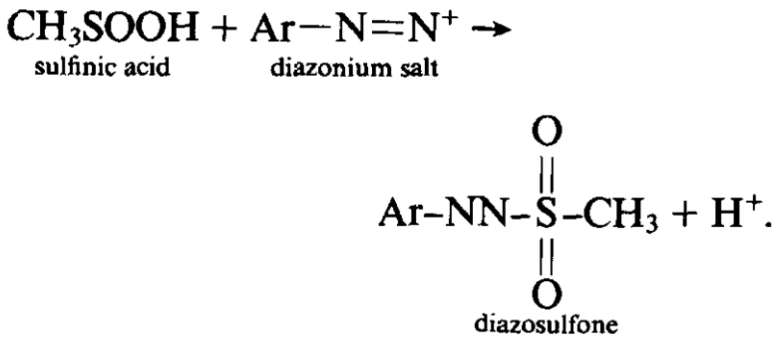
Sulfinic acids (RSOOH) are distinct from sulfonic acids (RSO_3H), sulfenic acids (RSOH), sulfones (RSOOR′), and sulfoxides (RSOR′). A sensitive and specific assay for sulfinic acids in the presence of other sulfur-based functions that is suitable for biological samples has not heretofore been reported. The measurement of methanesulfinic acid (CH_3SOOH) in biological samples is of particular interest to us because this compound is the primary product of the trapping reaction of dimethyl sulfoxide with hydroxyl radicals (HO*), as proposed by Dixon, Lagercrantz, Cohen, and their respective coworkers (l-3)

$$CH_3\text{-}SO\text{-}CH_3 + HO^* \rightarrow CH_3SOOH + CH_3^* \qquad [1]$$

The existence of the methyl radical as a product has been confirmed by electron spin resonance studies (1, 2), and the rate constant of reaction [1] has been estimated as $7 \times 10^9\ M^{-1}sec^{-1}$ (4).

A variety of authors have proposed that hydroxyl radicals are formed in vivo during phagocytosis and bacterial cell killing by leukocytes (5, 6) and also during reperfusion after tissue ischemia in brain, heart, kidney, and other tissues (7-11). The assay of methanesulfinic acid in tissues of dimethyl sulfoxide-treated animals would provide direct evidence to confirm or deny this hypothesis.

The present approach involves extraction of nondissociated methanesulfinic acid, $pK_a \approx 2$ (12, 13), into n-butanol at pH 0, followed by back extraction of sulfinate ions into aqueous buffer at pH 4 to 5. Potentially interfering species, including proteins and amines, are precipitated or remain in the original aqueous phase. Color development is based upon the reaction of organosulfinates with highly colored diazonium salts, first described by Ritchie et al. (14), to form diazosulfones that are selectively extractable into an organic solvent



[2]

The molar extinction coefficients range from about 50 to 10,000 $M^{-1}cm^{-1}$, depending upon the particular diazonium salt employed for the coupling reaction. In practice, we have found Fast Blue BB salt to be the most satisfactory coupling reagent of the 22 diazonium salts tested (Table 1). The following procedure is optimized for Fast Blue BB salt.

TABLE 1 COLOR REACTION OF DIAZONIUM SALTS WITH METHANESULFINATE

Reaction conditions: aqueous volume 4 ml, methanesulfinate concentration 0.25 mM, dye concentration 10 to 20 mg/ml, reaction time 10 min, product extracted into toluene/butanol (1/1), absorbance read against toluene/butanol blank.

Diazonium salt	λ_{max} (nm)	Approx molar extinction $(M\ cm)^{-1} \times 10^{-3}$
Fast Blue BB Salt	420	11
Fast Black K Salt	360	7
Fast Red RC Salt	380	4
Rubine B Salt	360	4
Fast Red Violet LB	375	4
Fast Blue RR Salt	420	4
Fast Red PDC	350	4
Fast Violet B Salt	380	2
Fast Yellow GC Salt	≤340	2
Fast Blue B Salt	360	1.5
Fast Orange RD Salt	≤340	1.3
Fast Red TR Salt	330	1.2
Diazo Red	380	1.2
Bordeaux GP Salt	350	1.0
Fast Red B Salt	375	1.0
Fast Red AL Salt	335	0.9
Scarlet 2G Salt	≤340	0.8
Red B Salt	420	0.4
Fast Red KL Salt	360	0.3
p-Diazo-*N,N* diethyl-*m*-toluidine	440	0.3
Fast Scarlet G Salt	440	0.1
Orange GR Salt	440	0.05

MATERIALS AND METHODS

Materials

Sulfuric acid, n-butanol, acetic acid, toluene, and pyridine were obtained from Fisher Scientific Co. (Fair Lawn, NJ). Diazonium salts, and specifically Fast Blue BB salt, were obtained from Aldrich Chemical Co. (Milwaukee, WI). Some of the diazonium salts in Table 1 were obtained from Pfister Chemical Inc. (Ridgefield, NJ). Standard curves were prepared using authentic methanesulfinic acid, sodium salt, obtained from Fairfield Chemical Co. (Blythewood, SC) or authentic benzenesulfinic acid, sodium salt, obtained from Aldrich. Note that dry diazonium salts must be considered as potentially explosive (15, 16), since they decompose when heated, causing emission of toxic fumes. These compounds also react with tissue proteins, and they are skin and eye irritants. Inhalation of powdered diazonium salts and sulfinates should be avoided (17).

Methods

Tissue extraction. Tissue is homogenized in a chilled Teflon-glass homogenizer in 2 vol of water. A 1.0-ml aliquot of the aqueous sample, expected to contain 10 to 300 μM sulfinate, is placed in a test tube containing 1.0 ml of 2 M sulfuric acid. After mixing, 8 ml of butanol, previously saturated with 1 M sulfuric acid, is added and mixed thoroughly with the aqueous phase for 30 sec on a Vortex mixer. The upper butanol phase is removed with a Pasteur pipet to a second clean test tube containing 4.0 ml of sodium acetate buffer (pH 4.5) and is mixed vigorously. The phases are allowed to separate under l g for 3 min.

Color reaction. The lower, aqueous phase is transferred with a Pasteur pipet to a third test tube containing 2.0 ml of 0.015 M Fast Blue BB salt (freshly prepared and kept in the dark). This solution is stable for at least 8 h at room temperature. (We found no detectable diminution in color development when 0.1 mM Fast Blue BB solutions, aged for up to 8 h, were reacted with 1 mM methanesulfinate.) Ten minutes are allowed for product development at room temperature in the dark. Then 3.0 ml of toluene/butanol is added and mixed thoroughly with the aqueous phase for 60 sec on a Vortex mixer. The lower phase, containing unreacted diazonium salt, is removed by aspiration and discarded. The toluene/butanol phase is washed with 5 ml of butanol-saturated water to remove the remaining unreacted diazonium salt. The tubes are centrifuged at 500g for 3 min, and the upper phase, containing the diazosulfones, is transferred to a cuvette. One milliliter of pyridine is added to stabilize the color, which otherwise fades gradually at acid pH. The bright yellow color is reasonably stable after pyridine addition, fading about 6% per day at room temperature. The absorbance is determined at 420 nm against a blank carried through the same procedure.

RESULTS

Figure 1 illustrates visible spectra for diazosulfone derivatives of Fast Blue BB and sodium methanesulfinate standard or sodium benzenesulfinate standard extracted into toluene/butanol. Reaction conditions are given in the figure legend. Both aryl and alkyl sulfinates react to form diazosulfones with absorption maxima at 420 nm. Color reactions carried out with sodium methanesulfinate and sodium benzenesulfinate solutions were linear up to concentrations of 75 μM ($r > 0.998$). Molar extinction coefficients are nearly identical: 14,400 $M^{-1}cm^{-1}$ for methanesulfinate and 14,800 $M^{-1}cm^{-1}$ for benzenesulfinate. The dependence of color development upon the molar ratio of Fast Blue BB to sulfinate standard is illustrated in Fig. 2.

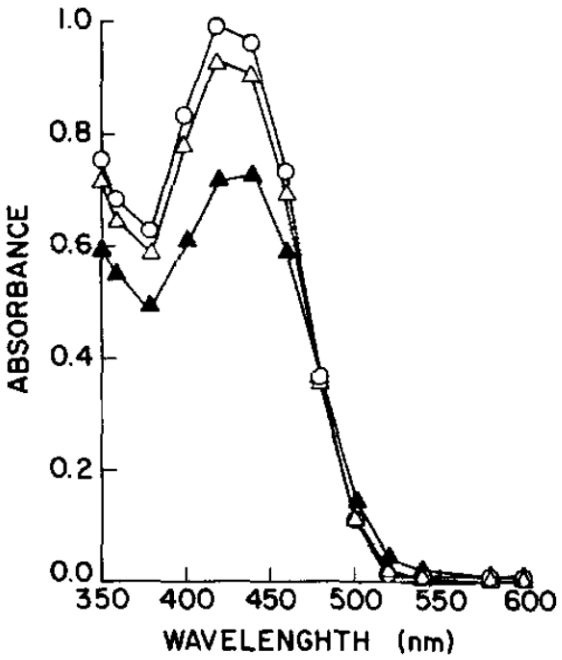


FIG. 1. Visible spectra for diazosulfone derivatives of Fast Blue BB extracted into toluene/butanol (1/1). Methanesulfinate derivative; reaction at pH 2 (open triangles) vs. pH 4 (open circles); benzenesulfinate derivative; reaction at pH 2 (solid triangles). Reaction conditions: 75 μM sodium methanesulfinate, 50 μM sodium benzenesulfinate in 1 mM acetate/l mM phosphate buffer; development time 10 min. Other details as described under Materials and Methods for color reaction.

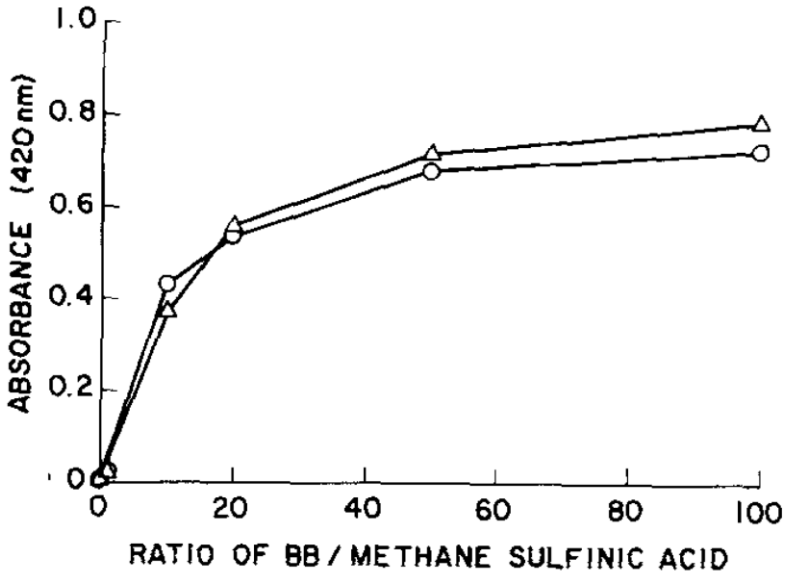


FIG. 2. Dependence of color development upon the molar ratio of Fast Blue BB to methanesulfinate standard with (circles) vs. without (triangles) addition of 0.5 ml 1.0 M acetic acid. Sulfinate concentration in aqueous phase during color reaction was 33 μM. Diazosulfone concentration in toluene-butanol extract (assuming 100% yield) was 50 μM. Color reaction performed as described under Materials and Methods. Data points represent means of triplicate determinations. Maximal color development occurs with molar ratios > 50.

Similar color reactions can be obtained with a variety of diazonium salts (Table 1). The typical molar absorptivities of about 1000 for absorbance peaks in the visible range were similar to those reported previously for diazosulfones by Freeman and co-workers (18). However, in preliminary studies standard curves obtained using Fast Blue BB salt had the greatest molar absorbtivity and reproducibility, and so Fast Blue BB was used exclusively in subsequent experiments.

The extraction of diazosulfone product from the aqueous phase of the color reaction depends upon the ratio of toluene to butanol in the organic phase. Table 2 presents results of single extractions of Fast Blue BB-diazomethylsulfone into toluene/butanol in various ratios by volume. We have found toluene/butanol 1/1, 2/1, or 3/1 to be satisfactory for extraction of the chromophores derived from either methanesulfinic acid or benzenesulfinic acid. The specificity of the color reaction is illustrated in Table 3. Solutions of various potentially interfering compounds including phenol, sulfate, sulfoxide, thiol, and amine functions produced no significant color reaction.

TABLE 2 EXTRACTION OF DIAZOMETHYLSULFONE INTO TOLUENE/BUTANOL

Reaction conditions: aqueous volume 4.5 ml, 300 nmol methanesulfinate, 15 μM Fast Blue BB, pH adjusted to 2.0 with addition of glacial acetic acid. Colored product extracted into 3.0 ml organic phase; 1.0 ml pyridine added to stabilize color. Blanks contained Fast Blue BB without sulfinate. Data represent means of triplicate determinations ± 1 standard deviation.

Percentage toluene by volume	$\Delta A(420)$
0	0.06 ± 0.042
33	0.83 ± 0.019
50	0.84 ± 0.033
66	0.95 ± 0.021
75	0.97 ± 0.002
100	0.05 ± 0.035

The interference from phenols and amines, which abound in biological samples, appears to be substantially reduced by performing the coupling reaction at acid pH. If high blanks are encountered at the pH of standard acetic acid/acetate buffer (4-5), the color of blanks can often be reduced, at the price of about 6% loss in sensitivity, by adjusting the pH of the aqueous color reaction to 2.0 with the addition of sulfuric acid.

Freedom of the complete assay, including the tissue extraction and color reaction, from interference from selected biological compounds is demonstrated in Fig. 3. Standard curves were generated by adding graded amounts of authentic methanesulfinate to distilled water and to fresh rat liver homogenate (500 mg/ml); the resulting test samples were extracted with acidic butanol and assayed, as described under Materials and Methods. The standard curves are similar in the presence or absence of liver homogenate.

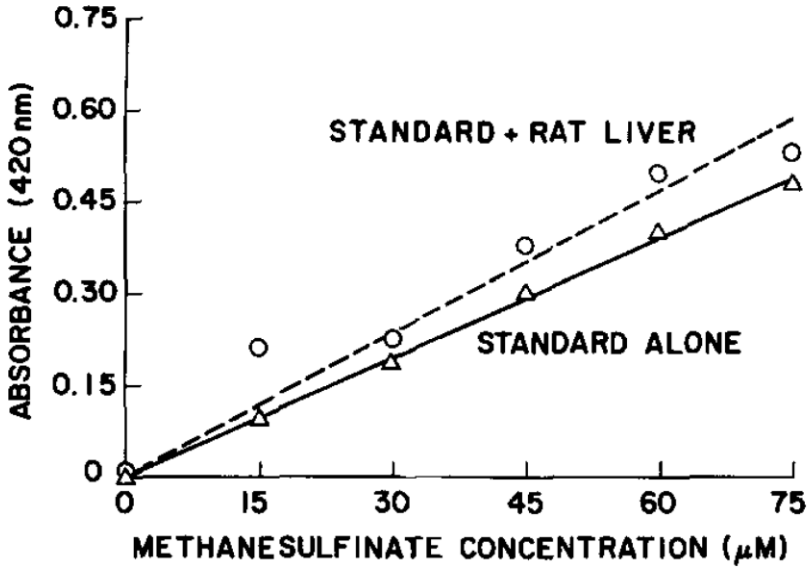


FIG. 3. Standard curves for methanesulfinic acid added to rat liver homogenate. Data points represent means of triplicate determinations. One half milliliter of 500 mg/ml liver homogenate (or distilled water) was added to reaction media containing various amounts of methanesulfinate standard.

TABLE 3 COLOR REACTIONS OF VARIOUS LABARATORY REAGENTS WITH FAST BLUE BB SALT

Reaction conditions: 2.0 ml 1 mM acetate/1 mM phosphate buffer at indicated pH, plus 1.0 ml of test reagent, plus 1.0 ml 15 mM Fast Blue BB. Reaction time 10 min. Colored product extracted into 3.0 ml toluene/butanol (1/1). Color stabilized by addition of 1.0 ml pyridine. Blanks contained buffer at indicated pH, Fast Blue BB, water. Values indicate means of three replications.

CH_3SO_2H (75 μM final)	2	0.927	13,100	100
	4	0.989	14,000	100
Na_2SO_4 (125 mM final)	4	−0.001	nil	nil
Phenylephrine (5 mM final)	4	0.001	13.8	0.09
Phenol (2.5 mM final)	4	0.299	127	0.90
Glycine (50 mM final)	4	−0.023	nil	nil
Glutathione (1.25 mM final)	2	0.402	341	2.0
	4	0.092	78	0.6
Thiodiglycol (25 mM final)	2	−0.002	nil	nil
	4	0.000		
CH_3SO_3H, methanesulfonic acid (5 mM final)	4	0.007	15	0.11
Sodium pentobarbitol	2	−0.013	nil	nil
(20 mM final)	4	−0.008	nil	nil
Thiopental (25 mM final)	2	0.001	2.5	0.02
	4	0.015	3.35	0.02
Dimethyl sulfoxide (3.50 M final)	4	0.026	0.008	0.0
NaCl (0.25 M final)	2	0.089	0.40	0.003
KCl (0.25 M final)	2	0.103	0.44	0.004
H_20_2 (7.5 mM final)	4	0.216	31.0	0.22
$FeSO_4$ (2.5 mM final)	4	0.086	36.0	0.26

TABLE 4 DEGRADATION OF STANDARD METHANE SULFINIC ACID, SODIUM SALT UNDER VARIOUSCONDITIONS

All reactions run at room temperature for 60 min. All tubes contained 300 nmol sodium methanesulfinate standard, plus or minus 0.5 ml liver homogenate (500 mg/ml). Sulfuric acid (2 M) was added either before or after 60-min incubation period to denature liver enzymes. Then 30 μmol Fast Blue BB was added for color reaction. Color was stabilized with 1.0 ml pyridine (final organic phase volume 4.0 ml). Values indicate means of three trials vs. toluene/butanol blanks ± 1 SD. Values for condition D, in which there was opportunity for liver enzymes to degrade sulfinate standard, were little different from those of controls A, B, and C.

Condition[a]	Absorbance (420 nm)
A Std + H_2SO_4 + H_2O; wait 60 min; assay	0.64 ± 0.01
B Std + H_2O; wait 60 min; +H_2SO_4; assay	0.63 ± 0.03
C Liver + H_2SO_4 + std; wait 60 min; assay	0.66 ± 0.12
D Liver + std; wait 60 min; +H_2SO_4; assay	0.63 ± 0.06

DISCUSSION

The present method provides a simple and inexpensive assay for sulfinic acids in biological materials. As little as 10 nmol of sulfinate can be detected, and interference from diverse biological compounds is minimal. Additionally, there is no interference from a large excess of dimethyl sulfoxide, which is necessary if the assay is to be used as a probe for evolution of hydroxyl radicals in tissues pretreated with dimethyl sulfoxide, and there is minimal interference from the other sulfur-containing compounds tested.

Previous chemical methods for isolating and assaying sulfinic acids include precipitation with ferric iron from an acidic solution, as described by Thomas (19), Krishna and Singh (20), and others (21-23), and complexation with palladium(II) ions in 1 N HCl, as described by Akerfeldt (24). The precipitation of sulfinato-iron salts from acidic solutions is an effective purification step when the sulfinates exist in molar concentrations.

We tried to adapt this approach to a colorimetric complexometric assay for millimolar concentrations of sulfinate without success. Although the sulfinato-iron salts do absorb at 430 nm--a peak distinctly different from that of free Fe^{3+} ions--the reaction $Fe^{3+} + 3\ RSOO^- \rightarrow Fe(RS00)_3$ does not appear to be driven to completion at sulfinate concentrations of 10 mM or less, even at the optimal pH of 2.5. (At lower pH formation of the

free acid, $RSOO^- + H^+ \rightarrow RSOOH$, competes with iron salt formation, and at higher pH Fe^{3+} ions precipitate as $Fe(OH)_3$.) Complexation of sulfinates with palladium ions does occur at millimolar concentrations (24); however, thiols and sulfoxides (in particular, unreacted dimethyl sulfoxide) also give strong color reactions, and the molar extinction coefficient is only on the order of 1000 (24).

We have found the diazo coupling reaction with sulfinic acids, described by Ritchie et al. (14) and mentioned briefly by Gringras and Sjostedt (23), to be more suitable. The colored products are diazosulfones, which are stable at near neutral pH (5 to 9), and are easily extracted from the aqueous phase into organic solvents. The necessary reagents are inexpensive and available, and the results have been quite satisfactory.

The possibility that sulfinic acids might be degraded in biological samples before they can be measured is worthy of mention. Bergeret and Chatagner (26) described enzymes in rabbit liver capable of degrading cysteine sulfinic acid. In addition, sulfinic acids spontaneously disproportionate (27). However, the rate of disproportionation is quite slow ($k \sim 10^{-}3\ M^{-1}sec^{-}1$ at 70 °C) (28). Moreover, we have found that solutions of 1 mM methanesulfinate at 20 °C show no measurable degradation after several days, as measured by the present assay, in which methanesulfonic acid does not react. Sulfinates can react with H_2O_2, bromine, and other strong oxidants (13), but these are not prominent in biological materials. Indeed, standard solutions mixed with liver homogenate show little, if any, deterioration compared to standard solutions in distilled water (Table 4). We conclude that the diazonium coupling reaction can be exploited to provide an efficient and inexpensive means of detecting sulfinic acids in biological materials.

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