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High-performance liquid chromatographic determination of methanesulphinic acid as a method for the determination of hydroxyl radicals

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ABSTRACT

For the determination of hydroxyl radicals, dimethyl sulphoxide was used as a molecular probe and the methanesulphinic acid produced was determined by high-performance liquid chromatography of its Fast Yellow GC salt derivative. The results for hydroxyl radicals formed using the Fenton and hypoxanthine-xanthine oxidase systems agreed well with the theoretical values. Interferences from phenols, aromatic amines and amino acids, which give coloured substances by reaction with the diazonium salt, could be avoided. The recovery of methanesulphinic acid added to liver homogenates and incubated for 1 h at 37°C was 70.2 ± 2.1%. The detection limit for methanesulphinic acid in a sample solution was *ca.* 8 ng/ml.

INTRODUCTION

Oxygen-derived free radicals are thought to be involved in the pathogenesis of many toxicological and disease states [1–3]. Further, the free radicals are involved in both the initiation and promotion of multi-stage carcinogenesis [4–6]. Among the various radicals, the hydroxyl radical (‘OH) is presumed to play a central role owing to its strong activity [7].

A number of methods have been developed for the dermination of ‘OH, e.g., spin-trapping electron paramagnetic resonance spectroscopy [8–10], gas chromatography (GC) of ethylene produced from methional by the reaction of ‘OH [11] and GC or high-performance liquid chromatography (HPLC) of hydroxylated substances obtained by the reactions of ‘OH with aromatic compounds such as phenol, benzoic acid or salicylic acid [12,13].

Dimethyl sulphoxide (DMSO) has become of interest as a probe for ‘OH formation in recent years. As pointed out by Babbs and Griffin [14], DMSO must be an ideal molecular probe for ‘OH owing to its unique chemcial and biological properties such as its benign biological effects and the product of its reaction with ‘OH, methanesulphinic acid (MSA), is stable and non-metabolized. DMSO interacts readily with ‘OH to produce MSA and a methyl radical ($^{\cdot}CH_3$) [14]. Subsequently, $^{\cdot}CH_3$ is converted into methane [15] and formaldehyde [16], but the reactions of formation of formaldehyde will occur very sparingly in biological system, because of alternative radical scavengers [14].

$$(CH_3)_2SO + {}^{\cdot}OH \rightarrow CH_3SO_2H + {}^{\cdot}CH_3$$
$$^{\cdot}CH_3 + O_2 \rightarrow CH_3OO^{\cdot}$$
$$2CH_3OO^{\cdot} \rightarrow HCHO + CH_3OH + O_2$$
$$^{\cdot}CH_3 + R \rightarrow CH_4 + R^{\cdot}$$

Among the above products, MSA, a stable non-radical compound and normally absent in biological samples, was successfully adopted for spectphotometric measurement of ‘OH by Babbs and coworkers [14,17,18] by a method based on azo dye

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formation through the reaction of MSA and a diazonium salt (Fast Blue BB salt). In this paper, we describe HPLC for the determination of MSA formed by the reaction of 'OH and DMSO. MSA was allowed to react with Fast Yellow GC salt and the resulting o-chlorobenzene diazomethyl sulphone was determined by HPLC. This procedure offered a quantitative determination of 'OH generated by the Fenton reaction system and the hypoxanthine-xanthine oxidase system. Interferences due to coloured samples or the production of coloured substances by the reaction with diazonium salts (*e.g.*, phenols or aromatic amines) were successfully avoided.

EXPERIMENTAL

Reagents

Fast Yellow GC salt (FY-GC, salt content 25%) was obtained from Sigma. FY-GC reagent was freshly prepared by dissolving 1.0 g of the salt in 100 ml of water and filtering.

Hypoxanthine, bovine milk xanthine oxidase, horse heart superoxide dismutase and catalase were obtained from Sigma and were used as received. Sodium methanesulphinate (MSA) was obtained from Fairfield Chemical. Other reagents were of special grade from Nacalai Tesque.

Equipment

A Shimadzu Model HLC-10AS high-performance liquid chromatograph equipped with an SPD-10AV UV-Vis spectrophotometric detector and a C-R6A Chromatopac integrator was used. A Capcell-Pak NH_2 column, (150 mm × 4.6 m m I.D.) was obtained from Shiseido.

Chromatographic conditions

The stationary phase was Capcell-Pak NH_2 packed in a stainless-steel column (150 mm × 4.6 mm I.D.) (Shiseido) and the column temperature was ambient. The mobile phase was an isocratic mixture of ethanol (specific gravity adjusted to 0.800 with water) and n-hexane (3:100, v/v) maintained at a flow-rate of 1 ml/min. A Shimadzu SPD-10AV UV-Vis detector was used at a wavelength of 285 nm. A 20-μl portion of sample was injected each time.

Determination of methanesulphinic acid (MSA)

To 5 ml of sample solution, 1 ml of 0.5 *M* phosphate buffer (pH 4.0) and 1 ml of FY-GC reagent were added and the mixture was shaken and allowed to stand for 10 min, then 2 ml of ethyl acetate were added and the mixture was shaken well for 5 min. The ethyl acetate layer was separated by centrifugation at 1000 g for 5 min and filtered through a Millipore filter (pore size 5 μm), then analysed under the above chromatographic conditions.

Fenton system

The procedure described by Steiner and Babbs [19] was used. To 2.5 ml of freshly prepared 1 m*M* $FeSO_4$ in 50 m*M* DMSO solution, O-2.5 ml of 200 μM H_2O_2 were added dropwise. The volumes of the mixtures were made up to 5.0 ml with 50 m*M* DMSO solution and allowed to stand for 10 min. MSA in the mjxtures was determined as described above.

Hypoxanthine-xanthine oxidase system

The procedure described by Babbs and Griffin [14] was used with a slight modification. To a solution of 2.0 ml of 250 μM hypoxanthine in 150 m*M* phosphate buffer (pH 7.4), 1 .O ml of 50 m*M* DMSO solution, 0.3 ml of 2 m*M* $FeSO_4$ solution, 0.3 ml of 2 m*M* EDTA solution, 1 ml of water or 'OH scavenger solution and 0.4 ml of 0.60 U/ml xanthine oxidase solution were added. The mixtures were allowed to stand for 12 min at 37°C. The total volume of the mixture was 5.0 ml, hence the final concentrations were 100 μM hypoxanhine and 48 mU/ml xanthine oxidase. MSA in the mixtures was determined as described above.

Recovery of MSA from liver homogenate

A bovine liver was collected on ice immediately after killing the animal and was minced with scissors. A 1.0-g amount of the minced liver was blended for 1 min in a blender with 1.0 ml of ice-cold water and then homogenized in a motor-driven PTFE glass homogenizer with six downward strokes. The homogenate was centrifuged at 1000 g for 5 min, 2.5 ml of MSA standard solution were added to the supernatant and the volume was made up to 5.0 ml with water. The mixture was incubated at 37°C for 1 h, then extracted with 10 ml of *n*-hexane for defatting, and the aqueous layer was

passed through a Sep-Pak C_{18} cartridge (Waters Assoc.). MSA in the eluate was determined as described above.

RESULTS AND DISCUSSION

Normal-phase HPLC using Capcell-pak NH_2 was used for the determination of the diazosulphone derived from MSA. The mobile phase was *n*-hexane–ethanol (100:3, v/v). It was necessary with this solvent system to add a trace amount of water to either solvent in order to obtain better resolution. Therefore, ethanol of specific gravity adjusted to 0.800 with water at 20°C was used.

Fast Blue BB salt was recommended by Babbs and Gale [17] as a satisfactory diazonium salt for the spectra-photometric determination of MSA. We examined several diazonium salts from the viewpoint of applicability to HPLC and the results are given in Table I. Fast Yellow GC salt gave a diazosulphone showing a single sharp peak on reaction with MSA, as shown in Fig. 1. Fast Red TR salt also gave a single sharp peak, but the peak height was lowered owing to the presence of Fe^{2+} ion, which did not occur with Fast Yellow GC. Fast Yellow GC was therefore selected as a satisfactory diazonium salt for the HPLC detection of MSA.

The dependence of the formation of the diazosulphone on the concentration of FY-GC reagent is shown in Fig. 2. When the concentration of FY-GC was higher than 1 .0%, the peak height of the diazosulphone remained constant.

TABLE I

REACTIONS OF DIAZONIUM SALTS WITH METHANESULPHINIC ACID

Reaction conditions: to 3.0 ml of 1 .O m*M* methanesulphinic acid 1.0 ml of 1.0% diazonium salt was added and the product was extracted into 2.0 ml of ethyl acetate.

Diazonium salt	λ_{max} (nm)	Peak height (cm)
Fast Yellow GC salt	285	8.97
Fast Red TR salt	310	10.51
Fast Blue BB salt	430	5.00
Fast Red AL salt	330	3.34
Fast Black K salt	–[a]	–
Fast Blue RR salt	–	–
Fast Red ITR salt	–	–

[a] Visible reaction did not occur.

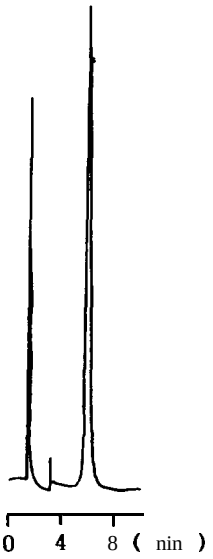

Fig. 1. High-performance liquid chromatogram of the diazosulphone derivative. For HPLC conditions, see Experimental.

For the extraction of the diazosulphone from the aqueous phase, ethyl acetate was successfully used. Table II presents the results of single extractions of the diazosulphone into several solvents.

Fig. 3 illustrates the relationship between pH during the MSA-FY-GC reaction and the peak height of the resulting diazosulphone. Under acidic conditions (pH 2–6) a constant peak height was obtained.

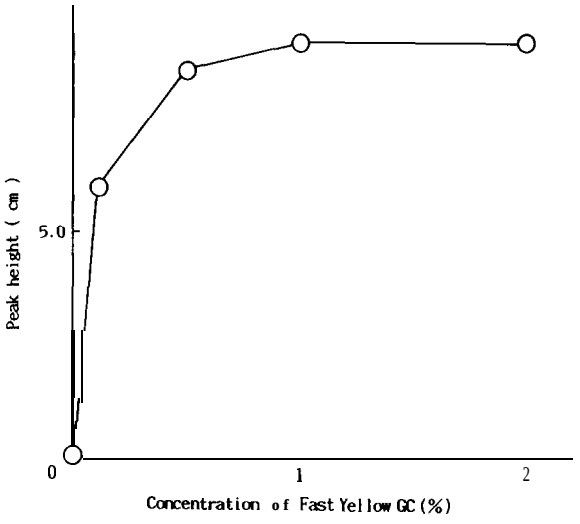

Fig. 2. Relationship between formation of the diazolsulphone derivative and concentration of Fast Yellow GC.

TABLE II

EXTRACTION OF THE DIAZOSULPHONE DERIVATIVE INTO ORGANIC SOLVENTS

Reaction conditions: to 3.0 ml of 0.1 m*M* methanesulphinate 1 .0 ml of 1 .0% Fast Yellow GC salt and 0.5 ml of phosphate buffer (pH 4.4) were added and the product was extracted into 2.0 ml of organic solvent.

Solvent	Peak height (cm)
Ethyl acetate	8.35
Methyl isobutyl ketone	4.22
Hexane-butanol (2: 1)	3.46
Hexane–ethanol (2: I)	2.46
Dichloromethane	0.92

The reaction of DMSO and 'OH generated by the Fenton system may occur via the following reactions:

$$H_2O_2 + Fe^{2+} \rightarrow {}^{\cdot}OH + OH^- + Fe^{3+}$$
$$^{\cdot}OH + CH_3SOCH_3 \rightarrow CH_3SO_2H + {}^{\cdot}CH_3$$

Fig. 4 shows that the two calibration graphs prepared from equimolecular amounts of H_2O_2 and MSA were in good agreement with each other. This indicates that the formation of MSA from DMSO in the Fenton reaction is quantitative. A linear calibration graph was obtained in the range 0.1-100.0 μM 'OH radical generated by the Fenton reaction.

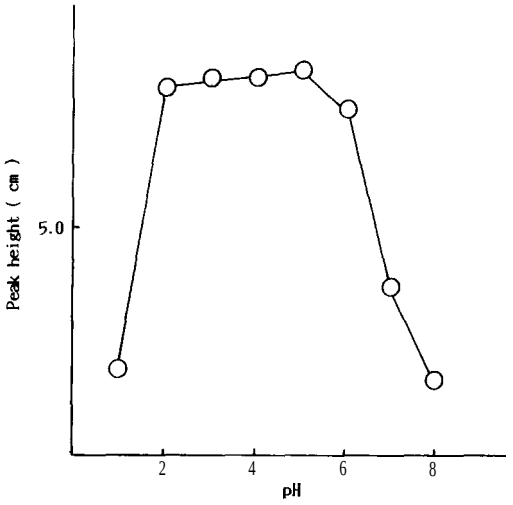

Fig. 3. Relationship between formation of the diazosulphone derivative and pH.

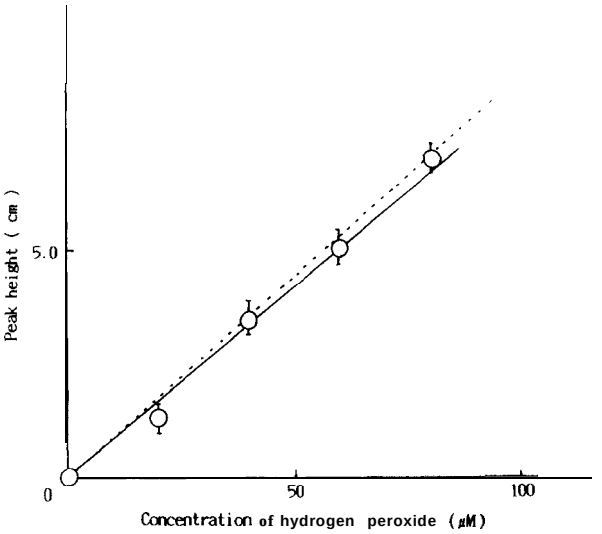

Fig. 4. Relationship between formation of the diazosulphone derivative and concentration of hydrogen peroxide in the Fenton system. Calibration graphs obtained from (solid line) H_2O_2 and (dashed line) methanesulphinic acid.

The detection limit was 0.1 μM 'OH at 0.005 a.u.f.s. in a simple reagent system.

Babbs and Grifin [14] calculated the MSA production in the hypoxanthine-xanthine oxidase oxidation of DMSO by the use of a kinetic model of 50 relevant enzymatic and free radical reactions. They concluded that the concentration of MSA produced with the enzymatic system given under Experimental should be ca. 35 μM. The present method indicated that the MSA formed in this system was close to 35 μM, as shown in Fig. 5. The xanthineexanthine oxidase enzyme system is known to generate several reactive oxygen compounds, including superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ('OH). The formation of MSA by the oxidation of DMSO is dependent on both O_2^- and H_2O_2 (Haber-Weiss reaction), as indicated by the inhibitory effects of both superoxide dismutase (SOD) and catalase (Table III). In addition, the formation of MSA was inhibited by known scavengers of 'OH, e.g., ethanol, mannitol, benzoate and formate.

Diazonium salts can couple with diverse compounds, especially phenols and aromatic amines, to give coloured azo compounds. The reactions are fa-

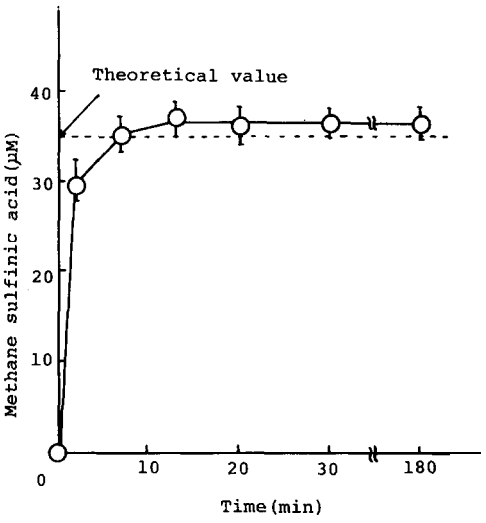

Fig. 5. Production of methanesulphinic acid from DMSO in the hypoxanthine-xanthine oxidase system: 2.0 ml of 250 μM hypoxanthime, 1.0 ml of 50 mM DMSO, 0.3 ml of 2 mM $FeSO_4$, 0.3 ml of 2 mM EDTA and 0.4 ml of 0.6 U/ml xanthine oxidase.

voured at neutral and alkaline pH, while the reaction of diazonium salts with MSA is favoured at acidic pH, as shown in Fig. 3. Table IV illustrates the colour reaction of diverse compounds, including phenol, aniline, aromatic amino acids, methylsulphonate, Fe^{3+} or EDTA with FY-GC reagent, and the influence of chromatographic peaks of the reactants on that of the diazosulphone. Phenol and aromatic amino acids yielded coloured substances, but the chromatographic peaks were well resolved from that of the diazosulphone. Among the substances tested, only aniline gave a broad peak partially overlapping that of the diazosulphone, but the influence on the determination of MSA was negligible when the concentration of aniline was up to 100 μM.

Bovine liver homogenate spiked with MSA was incubated for 1 h at 37°C and MSA was determined by the proposed procedure. The results are given in Table V. The mean recovery for 3 runs was 70.2 ±

TABLE III

EFFECT OF SCAVENGERS OF HYDROXYL RADICALS AND SUPEROXIDE ANIONS ON FORMATION OF METHANESULPHINIC ACID BY THE REACTION OF DMSO AND HYDROXYL RADICAL IN THE HYPOXANTHINE–XANTHINE OXIDASE SYSTEM

Reaction conditions: 1.0 ml of 50 mM DMSO, 2.0 ml of 250 μM hypoxanthine, 0.3 ml of 2 mM $FeSO_4$, 0.3 ml of 2 mM EDTA and 1.0 ml of scavenger solution were mixed, 0.4 ml of xanthine oxidase (0.6 U/ml) was added and the mixture was allowed to stand for 12 min at 37°C.

Scavenger	Concentration		MSA detected (nmol/ml)	Formation ratio of MSA (%)[b]
	U/ml	mM		
None			34.4	100.0
Superoxide dismutase	20		16.0 ± 0.9	46.5
	2		23.1 ± 0.4	67.1
	0.5		28.6 ± 0.9	83.0
Catalase	20		18.9 ± 0.8	54.9
	2		28.0 ± 2.0	81.5
	0.5		31.2 ± 1.2	90.7
Mannitol		20	14.0 ± 0.01	40.7
		10	18.2 ± 0.1	53.0
		2	31.3 ± 1.8	90.3
Ethanol		20	23.3 ± 0.7	67.8
		10	24.6 ± 3.1	71.4
		2	32.5 ± 0.8	94.5
Formate		5	17.3 ± 1.7	50.2
		3	24.4 ± 0.9	71.0
		1	32.7 ± 2.3	95.1
Benzoate		5	18.4 ± 0.1	53.4
		3	26.2 ± 0.1	76.3
		1	34.2 ± 2.3	99.4

[a] Mean ± S.D. (n = 3).
[b] (With scavenger/without scavenger) × 100.

TABLE IV

COLOUR REACTIONS OF DIVERSE COMPOUNDS WITH FAST YELLOW GC SALT AND INFLUENCE OF THE REACTANTS ON HPLC OF THE DIAZOSULPHONE

Compound	Concentration (m*M*)	Absorbance at 285 nm	Interference in HPLC
Methanesulphonate	10	*0.259*	None
Phenol	10	*0.604*	None
Phenylalanine	10	*0.263*	None
Tryptophan	10	*0.670*	None
Tyrosine	10	*0.258*	None
Aniline	10	>> *2.000*	Partial[a]
	0.1	*0.708*	None
Fe^{3+}	10	*0.453*	None
EDTA	10	*0.259*	None

[a] Showed a broad peak partially overlapping with the peak of the diazosulphone.

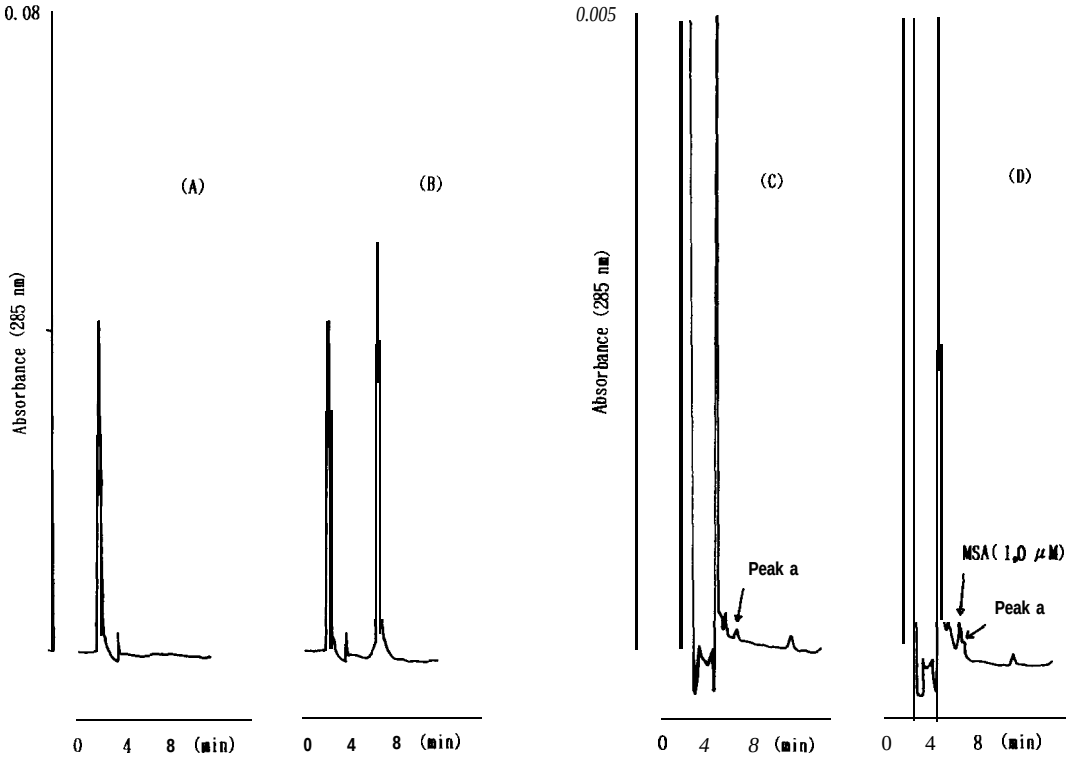

Fig. 6. High-performance liquid chromatogram of the diazosulphone derivative extracted from bovine liver homogenate. (A) Liver blank (0.08 a.u.f.s.); (B) 100 μM MSA-spiked homogenate (0.08 a.u.f.s.); (C) liver blank (0.005 a.u.f.s.); (D) 1.0 μM MSA-spiked liver homogenate (0.005 a.u.f.s.).

TABLE V

RECOVERY OF METHANESULPHINIC ACID FROM BOVINE LIVER HOMOGENATE

Methanesulphinic acid added (μmol/g)	Determined (μmol/g)	Recovery (%)[b]
1.0	0.83 ± 0.03	82.9 ± 2.7
0.6	0.47 ± 0.03	78.4 ± 1.6
0.4	0.31 ± 0.003	76.1 ± 1.9
0.2	0.14 ± 0.02	70.2 ± 2.1

[a] Mean ± S.D. (n = 3).
[b] Recovery (%) + C.V. (%).

2.1%, indicating that MSA is fairly resistant to enzymatic degradation in liver.

When the concentration of MSA in liver was at the μmol/g level, MSA could be determined without any interference from other peaks, as shown in Fig. 6A and B. When the instrumental sensitivity was set to the highest possible range (0.005 a.u.f.s.), the liver blank showed a small peak (peak a) having a retention time close by that of MSA, as shown in Fig. 6C and D. The peak-height ratio of peak a to MSA was about 1:1 when the concentration of MSA in liver was 5 nmol/g (1 μM in the homogenate). Hence the practical detection limit is considered to be l-2 μM MSA in liver homogenate.

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