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STUDIES ON METHYLGLYOXAL 1. FLUOROMETRIC DETERMINATION OF METHYLGLYOXAL USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Toshiki Matsuura,[a] Kyoji Yoshino,[a] Eishi Ooki,[a] Shin-ichi Saito,[b] Etsuo Ooishi,[a] and Isao Tomita*,[a]

Shizuoka College of Pharmaceutical Sciences,[a] 2-2-1, Oshika, Shizuoka-shi Shizuoka 422, Japan and Shizuoka Women's College,[b] 3-2-3, Nunohashi, Hamamatsu-shi, Shizuoka 432, Japan

A high-performance liquid chromatographic (HPLC) method has been developed for the determination of methylglyoxal (MG). When MG was treated with o-phenylenediamine and trichloroacetic acid, it was converted to a highly fluorescent new compound, 2-(2-benzimidazolyl)-3-methylquinoxaline (BIMQ) the chemical structure of which was elucidated by NMR and MS. BIMQ was analyzed by HPLC using a 4 mm x 25 mm column packed with LiChrosorb Si-60 (10 μm).

This method is highly sensitive and was satisfactorily applied to the measurement of MG in biological materials.

KEYWORDS —— methylglyoxal determination; fluorometric HPLC; 2-(2-benzimidazolyl)-3-methylquinoxaline; mouse liver; mouse blood

INTRODUCTION

Methylglyoxal (MG) is formed from dihydroxyacetone phosphate by MG synthase[1-3] or from glycine or threonine through aminoacetone by monoamine oxidase[4] in mammalian tissues. Though the physiological role of MG is not yet known, much interest in it developed after it was found that intraperitoneally injected MG in mice inhibited the growth of Ehrlich ascites carcinoma, L1210 and L4946 leukemia, TA_3 mammary carcinoma, 6C3HED lymphosarcoma and sarcoma 180.[5] The mutagenicity of MG to S. typhimurium TA 100[6,7] and the tumor promoting property of MG in F344 rats[8] are other recent discoveries of interest.

In the present investigation, we undertook to establish a reliable and sensitive method to measure MG in order to determine the fluctuations of the amount of MG in animal tissues.

MG has been measured by enzymatic,[9] fluorometric,[10] spectrophotometric,[11-16] radioisotopic,[17] polarographic[18-20] and gas chromatographic[21,22] methods. A method using high-performance liquid chromatography (HPLC) after MG is converted to MG-isopropylidene guanosine adduct has recently been introduced[23] but even this method is not satisfactory in the presence of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate since both of these substances give the

same retention time as MG.

Our method reported in this paper is based on the conversion of MG to a fluorescent compound by treating MG with o-phenylenediamine (o-PD) and trichloroacetic acid (TCA) which is then subjected to HPLC analysis.

MATERIALS AND METHODS

Apparatus- A Spectraphysics Model SP-8770 HPLC connected with a Hitachi Model F-1000 fluorescence spectrophotometer was used for HPLC determinations. The fluorescence was recorded at an excitation wavelength of 370 nm and an emission wavelength of 405 nm. Separation of the fluorescent compound was performed on a 4 mm x 250 mm stainless-steel column packed with LiChrosorb Si-60 (10 μm) using a balanced density slurry packing method. HPLC was performed using n-hexane:ethanol (49:0.6, v/v) at a flow rate of 2.0 ml/min. ^{1}H-NMR and ^{13}C-NMR spectra were recorded with a JEOL JNM-Fx90Q, employing tetramethylsilane as an internal standard. Mass spectra were recorded with a JEOL JMS-D100.

Reagents- Pyruvaldehyde dimethylacetal purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) was hydrolyzed by H_2SO_4 to prepare MG. o-PD was obtained from Nakarai Chemicals Co. (Kyoto, Japan) and was recrystallized from a mixture (1:1, v/v) of aqueous 1% sulfuric acid and ethanol. Other reagent and organic solvents used in this experiment were of reagent grade.

Preparation of the sample solution for HPLC- Mouse liver (0.2 g) or blood (0.2 ml) was homogenized at 0°C with a glass homogenizer after adding distilled water (0.8 ml) and MeOH (4 ml). The homogenate was centrifuged at 1,500 x g for 5 min. Four percent o-PD in EtOH (50 μl) was added to the supernatant (3.0 ml) which was incubated at 37°C for 30 min. It was then extracted 2 times with $CHCl_3$ (5 ml) after adding 2% NaCl solution (10 ml). The organic phase was collected and evaporated to dryness. To the residue which was dissolved in MeOH (1.0 ml) was added 30% TCA in EtOH (100 μl), 4% o-PD in EtOH (50 μl) and nitrobenzene (50 μl). The mixture was heated at 150°C for 1 h and $CHCl_3$ (10 ml) was added. The $CHCl_3$ layer was washed 2 times with 5% NaOH solution (5 ml each). It was then dried over sodium sulfate and evaporated to dryness. The residue was dissolved in $CHCl_3$-MeOH (9:1, v/v) mixture and made up to 0.5 ml. The resulting solution (30 μl) was subjected to HPLC.

RESULTS AND DISCUSSION

The reaction of MG with o-PD and TCA produced a new fluorescent compound. The compound was purified by a silica-gel column chromatography (3 cm x 30 cm) and recrystallized from a benzene, petroleum benzine mixture to give slightly yellow crystals, mp 187°C. The data of elementary analysis (Calcd for $C_{16}H_{12}N_4$: C, 73.83; H, 4.65; N, 21.52. Found: C, 74.12; H, 4.74; N, 20.68.), ^{1}H-NMR (in $CDCl_3$) δ :3.35(3H, CH_3), 7.20-8.00(8H, CH), 10.90(1H, NH), ^{13}C-NMR (in $CDCl_3$) δ :25.4(q), 111.0(d), 121.0(d), 122.7(d), 124.7(d), 128.3(d), 128.6(d), 129.5 (d), 130.5(d), 133.4(s), 140.1(s), 141.8(s), 142.2(s), 144.9(s), 149.8(s), 153.8 (s) and Mass spectra(m/e: 260(M^+), 143(M^+-$C_7H_5N_2$(benzimidazole)) were consistent

with the structure of 2-(2-benzimidazolyl)-3-methylquinoxaline (BIMQ). The structure of BIMQ was confirmed by its synthesis from 2-methylquinoxaline-3-carboxylic acid with o-PD.

In the course of studying the optimum conditions for MG to react with o-PD and TCA, it was found that the reaction proceeded in 2 successive steps, i.e. MG first reacted with o-PD (1) to give 2-methylquinoxaline (MQ), (2) (Reaction 1 in Fig. 1) which then reacted with another mol of o-PD with TCA (3) to produce BIMQ (4) (Reaction 2 in Fig. 1). The formation of MQ was maximal over 30°C with incubation time over 30 min, while that of BIMQ was maximal over 150°C and 1 h. Reaction 2 depended on the amounts of TCA and nitrobenzene added. Their optimum amounts were 184 and 490 nmol respectively. BIMQ showed excitation and fluorescence spectra with maxima of 370 nm and 405 nm, respectively, with a fluorescence quantum yield of 0.28.

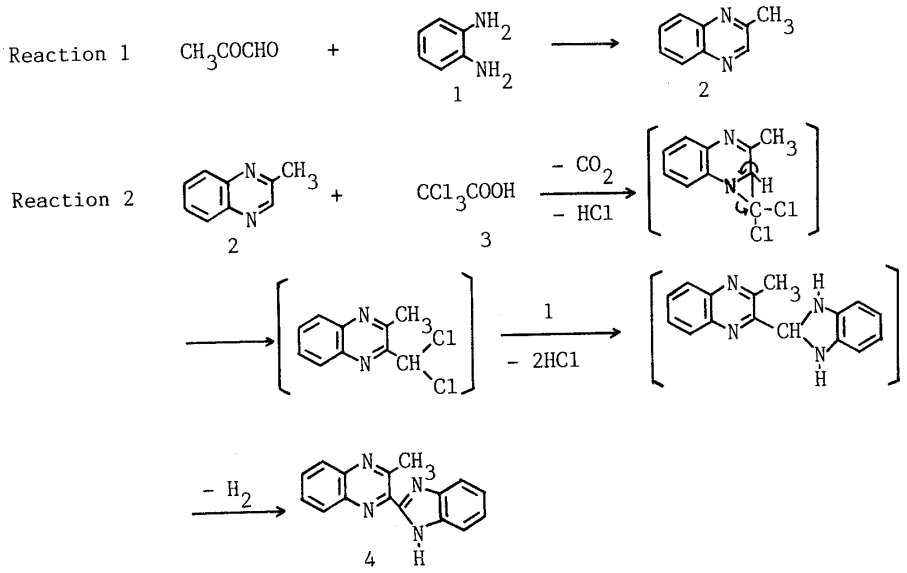

Fig. 1

Based on the above findings, the analytical procedure for MG was developed. To determine the amounts of MG in biological materials (liver, blood etc.), the tissues were pretreated as described in "preparation of the sample solution for HPLC."

The retention time of BIMQ in HPLC analysis was 17 min. The calibration curve was linear up to 8.0 mmol of MG and the detection limit was 48.4 pmol/30 µl of HPLC sample with S/N = 5. Repeated injections (n = 10) of the fluorophore from MG (5 µg) gave an average relative standard deviation of 2.4% for peak area and 1.0% for retention time.

This method is very specific to MG and no interference by pyruvic acid, α-ketoglutaric acid, glyceraldehyde-3-phosphate and glyoxal was observed.

The amounts of MG in the liver and blood of mice (5 weeks old female), determined by the present method, were 3.69 µg/g and 2.15 µg/ml respectively.

The present analytical method is very sensitive and it needs only 0.1-0.2 g of liver and 0.1-0.2 ml of blood from mice. This method could be applied effectively to study the fluctuation of MG in mice inoculated intraperitoneally with

sarcoma 180 ascites tumor. The details of these results will appear in another paper.

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