Journal of Chromatography, 414 (1987) 149–155
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3433

Note

Determination of methylglyoxal as 2-methylquinoxaline by high-performance liquid chromatography and its application to biological samples

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(First received June 30th, 1986; revised manuscript received September 25th, 1986)

Methylglyoxal (MG) has been demonstrated to be present in a wide variety of organisms and is widely found in food [1,2], beer [3], coffee [4], cigarette smoke [5] and the atmosphere [6]. MG is a chemically reactive [7,8], cytotoxic [9,10] and mutagenic substance [4,11]. The biochemistry of MG has been a long-standing problem since early this century [12,13]. Even today the metabolism and biosynthesis of MG in animals are still uncertain. To study precisely these problems in our laboratory, a simple and sensitive method for determining MG was necessary. MG has been determined by spectrometry [14–16], high-performance liquid chromatography (HPLC) [3,5,17,18], gas chromatography (GC) [5], and enzymic analysis [19]. Moree-Testa and Saint-Jalm [5] determined MG in cigarette smoke as 2-methylquinoxaline (MQ) by GC and HPLC according to the reaction given in Fig. 1.

We applied this method to biological and food samples, but failed to determine MG because of problems with overlapping peaks in the chromatograms. In order to determine MG in biological samples we had to improve the method. This paper describes the best conditions for the derivatization to MQ, and the extraction method for MQ prior to determination by HPLC. The improved method was applied to biological samples.

EXPERIMENTAL

Reagents

MQ and *o*-phenylenediamine (o-PDA) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and Wako (Osaka, Japan), respectively. MG was pre-

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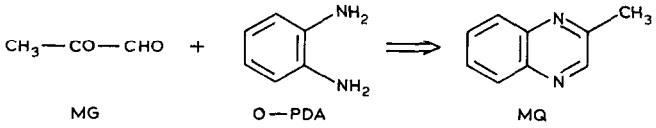


Fig. 1. Formation of MQ from MG and o-PDA.

pared by hydrolysis of dimethylacetal from Aldrich (Milwaukee, WI, U.S.A.) according to the method of Kellum et al. [20].

Instruments

A Model 572 liquid chromatograph (Gaschro-Kogyo, Osaka, Japan) equipped with a Model 502 variable-wavelength detector (Gaschro-Kogyo) was used. Determination of MQ was performed on a 150×4.6 mm I.D. Unisil Q ODS T (particle size 5 μm) column (Gaschro-Kogyo) with isocratic elution using a mixture of 10 m*M* potassium dihydrogen phosphate in phosphoric acid (pH 2.1)–acetonitrile (80:20, v/v) and monitored at 315 nm. Spectrometry was performed with a Shimadzu double-beam UV-180 spectrometer (Kyoto, Japan).

Determination of MQ

MQ has three absorption peaks (200, 243, 335 nm) in 5 *M* perchloric acid, and its molar absorptivity is $9.72 \cdot 10^3$ l mol^{-1} cm^{-1} at 335 nm. MG was spectrophotometrically determined at 335 nm to establish the reaction conditions for the formation of MQ from MG and o-PDA, and its recovery.

MG in water

After 0.5 *M* perchloric acid was added to an aqueous solution of MG (10–50 μl of 100 μg/ml) to a total volume of 1 ml, 0.1 ml of a 1% aqueous solution of o-PDA was added. The mixture was allowed to stand at 25°C for 1 h. Then 0.2 ml of 5 *M* sodium hydroxide was added, and the reaction mixture was extracted with 3 ml of benzene using a Vortex mixer and centrifuged at 2200 *g* for 15 min. The benzene layer (2 ml) was extracted three times with 1 ml of 5 *M* perchloric acid, followed by determination at 335 nm.

MG in rat liver

Rat liver was homogenized with 0.5 *M* perchloric acid (2 ml per g of liver) in a Waring blender at 5000 rpm for 5 min and centrifuged at 6000 *g* for 30 min. Then 1 ml of the supernatant was analysed as described above, except that benzene was replaced by dichloromethane for the extraction. A 2-ml volume of the extract was placed in a vial, and 0.05 ml of 6 *M* hydrochloric acid was added. The vial was tightly capped and shaken well. The mixture was evaporated using a Savant Speed Vac concentrator (Model SVC-100H, New York, NY, U.S.A.) at room temperature. The residue was dissolved in 1 ml of the mobile phase and subjected to HPLC.

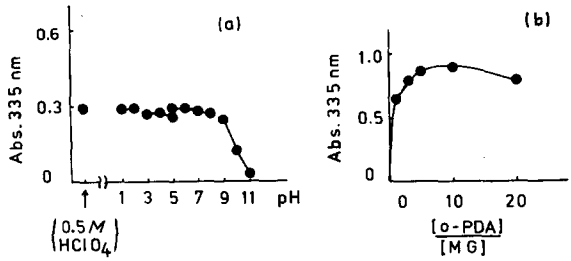


Fig. 2. Effect of pH and amount of o-PDA on the formation of MQ. In (a), MG (5 μg) in 1 ml of buffers of various pH was treated with 25 μl of a 0.1% solution of o-PDA in ethanol at 27°C for 1 h. Solid sodium hydrogen carbonate was added to the acidic reaction mixture. After extraction with 3 ml of benzene, the benzene layer (2 ml) was reextracted with 1.5 ml of 5 *M* perchloric acid, which was determined at 335 nm. In (b), MG (10 μg) in 1 ml of 0.5 *M* perchloric acid was treated with various amounts (13.5–270 μl) of a 0.1% aqueous solution of o-PDA at 27°C for 1 h; the reaction mixtures were determined at 335 nm.

RESULTS

Reaction conditions

Optimum reaction pH. MG (5 μg) in 1 ml of various pH solutions or 0.5 *M* perchloric acid was treated with 25 μl of 0.1% aqueous solution of o-PDA at room temperature for 1 h. Acidic reaction mixtures were made alkaline with solid sodium hydrogen carbonate, extracted with benzene and treated as described in *MG in water*. As shown in Fig. 2a, the average yield of MQ was 92.2±2.3% (n=8) between pH 1 and 7 and 94.4±1.2% (n=3) in 0.5 *M* perchloric acid. It gradually decreased from pH 7 to pH 9, declined rapidly from pH 9 and reached 0 at pH 11.

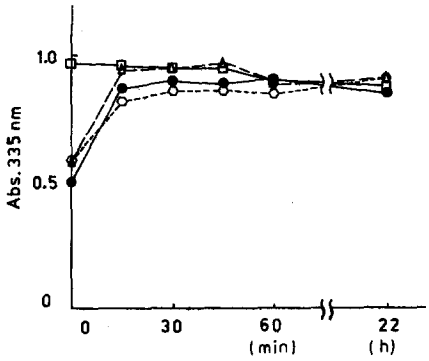
Optimum reaction temperature and time. MG (30 μg) was treated with o-PDA at 10, 27, 40 and 80°C and treated as described in *MG in water*. As shown in Fig. 3, the reaction proceeded readily in high yield (92.5% at 27°C, 1 h) and most readily at 80°C.

Optimum amount of o-PDA. MG (10 μg) in 1 ml of 0.5 *M* perchloric acid was treated with various amounts of o-PDA in aqueous solution at 25°C for 1 h and processed as above. The result is shown in Fig. 2b. The molar ratio of o-PDA to MG was 5–10 for the best yield of MQ.

Solvent for extraction. To MG (20 μg) in 1 ml of 0.5 *M* perchloric acid or the supernatant of rat liver homogenate prepared as above was added 0.1 ml of a 1% aqueous solution of o-PDA. The mixture was allowed to stand at 25°C for 1 h. After neutralization, the reaction mixture was extracted with 3 ml each of several organic solvents (benzene, toluene, n-heptane, n-hexane, chloroform and dichloromethane). As a result, benzene was chosen as the best solvent and MG was extracted 85.8±0.9% (n=3) from water and 92.5±1.7% (n=3) from the homogenate. Dichloromethane was also used.

High-performance liquid chromatography

Fig. 4 depicts the chromatograms of (a) authentic MQ and (b) MQ formed from MG in rat liver. The standard curve was linear up to at least 250 nmol. As



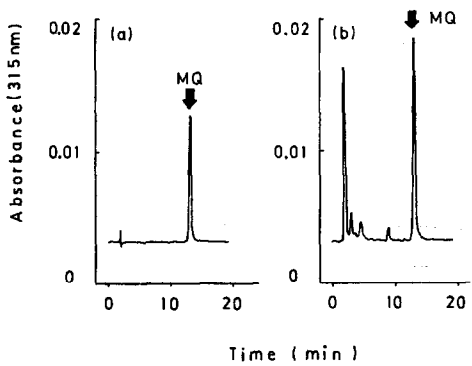


Fig. 3. Effect of reaction time and temperature on the formation of MQ. MG (30 μg) in 1 ml of 5% sulphuric acid (w/v) was preincubated at various temperatures (○ = 10°C; ● = 27°C; △ = 40°C; □ = 80°C) for 3 min, and then 0.1 ml of a 1% o-PDA solution in 50 m*M* sulphuric acid was added. After reaction at each temperature, 0.5 ml of 5 *M* sodium hydroxide was added and extracted with 3 ml of benzene. The benzene layer (2 ml) was reextracted three times with 1 ml of 5 *M* perchloric acid, and MG in the extract was determined at 335 nm.

Fig. 4. High-performance liquid chromatograms of (a) authentic MQ and (b) that obtained from MG in rat liver. (a) MQ (10 μg) was dissolved in 1 ml of the mobile phase, 20 μl of which were injected; (b) rat liver (6 g) was treated in the manner described in *MG in rat liver*, and 20 μl of dissolved sample were injected.

little as 10 nmol of MQ could be detected by the present procedure. As shown in Table I, recoveries of MG added to the supernatant of rat liver homogenate were 74.2 ± 1.8%.

Identification of the peak of MQ. Five brains of Wistar strain albino rats (male), in which MG was present in highest amounts, were treated in the manner described in *MG in rat liver*, and 100-μl aliquots were injected for HPLC. The peak corresponding to MQ was collected from the end of the column. This fractionation procedure was repeated ten times. The combined fraction (ca. 30 ml) was con-

TABLE I

RECOVERY TESTS OF MG ADDED TO SUPERNATANT OF RAT LIVER

After various amounts of MG were added to the supernatant of rat liver, the supernatant was treated with o-PDA, neutralized, extracted with dichloromethane and assayed by HPLC. The coefficients of variation are given as C.V. = (S.D./mean) · 100.

MG added (μg per 0.5 g wet weight)	MG determined (μg per 0.5 g wet weight)	Recovery (%)	C.V. (%)
0	0.79		
1	1.55	76.0	11.4
3	2.96	72.3	2.2
5	4.44	73.0	3.7
10	8.33	75.4	6.2
Mean ± S.D.		74.2 ± 1.8	

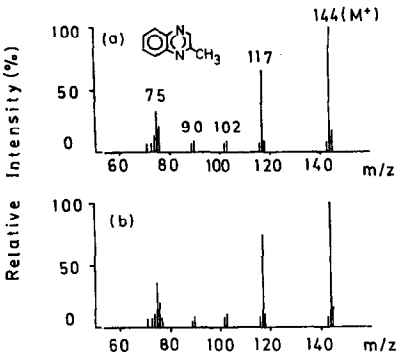


Fig. 5. Mass spectra of MQ. (a) Authentic MQ; (b) the reaction product of MG in rat brain. After the supernatant of the rat brain homogenate had been treated with o-PDA, the MQ formed was isolated by HPLC.

centrated to ca. 1 ml, and then 50 μl were injected for HPLC in order to check that it would produce a single peak on the chromatogram. The remaining concentrate was made alkaline with 0.1 ml of 5 *M* sodium hydroxide and extracted with 1 ml of dichloromethane. The extract was subjected to mass spectrometry (Fig. 5), indicating a parent peak (M^+) of 144 and the identification of the peak of MQ on the chromatogram.

MG in organs of diabetic and fasting rats.

Milligan and Baldwin [21] reported that myoglobin catalyses the oxidative decarboxylation of acetoacetate to MG in the presence of O_2 and Mn^{2+}. We thought that the MG level must rise in the organs of diabetic and fasting rats, owing to the rise in ketone body levels in the body. Male Wistar strain albino rats (five weeks old) were fasted for 72 h and allowed to drink water freely. Rats of a control group received normal solid food. After decapitation, organs were immediately removed and treated in the manner described in *MG in rat liver*. The results listed in Table II indicate that MG levels increased in the hearts of the fasting group. As a experimental diabetes, alloxan (200 mg/kg) was injected intraperitoneally into the rats and a control group was given only physiological saline. Each group was maintained on normal solid food and water ad libitum. After 72 h rats were decapitated and treated as above. All rats were examined for blood glucose. The MG contents in several organs are given in Table II. Under these conditions, MG levels rose in the heart and fell in skeletal muscle and liver.

DISCUSSION

Many studies of MG have been published. The biochemistry of MG is an enigma to this day. One reason for this may be the methods used to determine MG. The colorimetric method that uses 2,4-dinitrophenylhydrazine has generally been employed. However, it has been demonstrated clearly by us that the colour development that is the basis of this method is not specific for MG but positive for many substrates of MG. The evidence for this will be published elsewhere. The

TABLE II

MG CONTENTS IN VARIOUS RAT RISSUES IN UNUSUAL STATES

After decapitation of rats, organs were immediately removed and homogenized. The supernatants were treated with o-PDA, extracted with dichloromethane and assayed by HPLC. Values are mean ± S.D.

Tissue	MG control (μg/g wet weight)	*n*	MG experimental group (μg/g wet weight)	*n*	$P<0.05$★
Fasting rats					
Heart	1.10 ± 0.17	5	2.15 ± 0.49	5	↑
Liver	0.88 ± 0.13	5	0.60 ± 0.16	6	No difference
Muscle	0.90 ± 0.13	4	0.86 ± 0.20	4	No difference
Alloxan-treated rats					
Heart	0.46 ± 0.08	5	0.70 ± 0.13	4	↑
Liver	0.63 ± 0.13	4	0.35 ± 0.07	5	↓
Muscle	0.64 ± 0.05	4	0.39 ± 0.05	5	↓

★Data of both groups were checked with the *t*-test.

method presented here was specific for MG, because MQ was formed only by treating MG with o-PDA. Fodor et al. [22] have reported that MG can be isolated in the form of 2,4-dinitrophenylhydrazone from beef liver. By the same token, it is not certain whether the hydrazone was formed from MG or from triose, which is a precursor of MG. It could be said here that MG is present in liver. Since MG combines with proteins [23,24], we had to confirm that MG could be determined with good recovery from biological samples. For this purpose, various amounts of bovine serum albumin were added to MG in a buffer solution. After deproteinization with perchloric acid, MG was treated with o-PDA, and the reaction mixture was neutralized and extracted with dichloromethane. The MG was assayed by HPLC. In this case the recovery of MG was 92.9 ± 3.4%. On taking the results of Table I into consideration, the presence of protein evidently interferes little with the measurement of MG, which may be liberated from protein by adding strong acid.

ACKNOWLEDGEMENTS

This work was supported, in part, by a Grant-in-Aid for Scientific Research (No. 60571042) from the Ministry of Education, Science and Culture, Japan. Mr. A. Iwado is thanked for the mass spectrometry.

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