Capillary Gas Chromatographic Determination of Methylglyoxal from Serum of Diabetic Patients by Precolumn Derivatization Using Meso-Stilbenediamine as Derivatizing Reagent

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Abstract

Stilbenediamine is used as derivatizing reagent for methylglyoxal (MGo) and dimethylglyoxal for the gas chromatographic (GC) determination of MGo from the serum of diabetic patients and healthy volunteers. The derivatization is obtained at pH 3. GC elution and separation are carried out on an HP5 column (30 m × 0.32 mm i.d.) at column temperature 150°C with a programmed heating rate of 50°C/min up to 250°C, and a total run time of 7 min. The nitrogen flow rate is 5 mL/min and detection is carried out by flame ionization detection. The linear calibration curves are obtained with a range of 0.076–0.760 µg/mL and the detection limit is 25 ng/mL MGo. The amounts of MGo found in the serum of healthy volunteers and diabetic patients are 0.025–0.065 µg/mL and 0.115–0.228 µg/mL, with coefficient of variation 1.3–3.1% and 1.4–3.3%.

Introduction

Various small molecular weight carbonyl compounds have been proposed as playing an important role as carbonyl stress in the pathogenesis of many chronic and age-related diseases (1). They accumulate in body fluids and tissue mainly by accelerated oxidative stress, and modify proteins and phospholipids to form biological active adducts such as glycation end products and lipoxidation end products (2). Methylglyoxal (MGo) has attracted attention because it may be a precursor for the adduct, owing to its high reactivity, and may exert various biological effects (3,4). MGo is formed by nonenzymatic and enzymatic fragmentation of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (5), the metabolism of acetone (6), and the catabolism of threonine (7). Dimethylglyoxal (DMGo) is another important dicarbonyl compound usually present in food products obtained by the fermentation process. It has a characteristic odor and taste that can affect the organoleptic quality of food (8).

A number of methods have been developed for the determination of MGo in biological samples. The high-performance liquid chromatography (HPLC) methods for the determination of MGo and DMGo mainly involve 1,2-diaminobenzene or 1,2-diamino-4,5-dimethoxybenzene as derivatizing reagents. The resulting quinoxaline compounds formed are monitored using a spectrophotometric or spectrofluorometric detector (9–13). Recently, a preliminary study was reported on the use of stilbenediamine (SD) for the HPLC determination of MGo, connected with UV detection with a detection limit of 76 ng/mL (14). Capillary gas chromatography (GC) has enormous ability for the separation of organic compounds with less running cost. The GC determination of MGo and DMGo involves mainly *o*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (15), cysteamine (16), and 4,5-dichloro-1,2-phenylenediamine (17) as

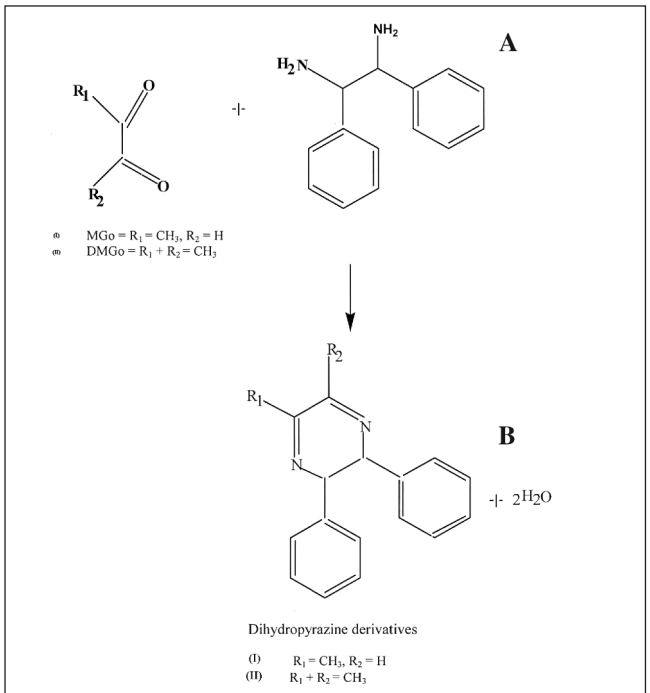

Figure 1. Structural diagrams of 2,3-diphenyl-5-methyl-2,3-dihydropyrazine (A) and 2,3-diphenyl-5,6-dimethyl-2,3-dihydropyrazine (B).

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derivatizing reagents. The determination is based on mass spectroscopic, thermionic, and electron capture detections using both packed and capillary columns. GC procedures based on *o*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine as derivatizing reagent are highly sensitive (linear calibration range 5.5–166 µg/L), but require long reaction time (1 h), long elution time (34 min), and analysis is based on the separation of two isomers (15,18).

The present work reports the use of SD as derivatizing reagent for the GC elution and separation of MGo and DMGo. The 5,6-diphenyl-5,6-dihydropyrazine compounds (Figure 1) formed are monitored by flame ionization detection (FID), frequently available with GC, and an increase in carbon number obtained in derivative generally enhances the response of the FID system.

Experimental

GC analytical procedure

To the solution (0.1–0.5 mL) containing MGo (0.076–0.760 µg/mL) and DMGo (0.071–0.71 µg/mL) was added SD (1 mL, 2%, w/v in methanol) and 1 mL acetic acid–sodium acetate (1M) buffer pH 3 in a well-stoppered test tube (Quick fit). The contents were warmed at 70°C for 20 min and chloroform (1 mL) was added. The layers were mixed well and allowed to separate. The organic layer solution (1 µL) was injected onto the HP-5 GC column (30 m × 0.32 mm i.d.) at column temperature 150°C with programmed heating rate 50°C up to 250°C with total run time 7 min. The nitrogen flow rate was 5 mL/min. The injection port and detector temperature were fixed at 250°C and 270°C, respectively. Hydrogen and nitrogen flow rates for the FID detection system were fixed at 40 mL/min and 45 mL/min, respectively. The split ratio was 10:1.

Determination of MGo from serum

A blood sample (5 mL) was collected from healthy volunteers and diabetic patients, and was allowed to rest at room temperature for 1 h. The contents were centrifuged at 3000 g for 30 min. The supernatant layer was separated and methanol was added twice in volume (5 mL). The contents were mixed well and again centrifuged at 3000 g for 30 min. The supernatant layer was transferred to a sample vial and the previously mentioned derivatizing GC procedure was followed. The quantitation was carried out using an external calibration curve.

Table I. Quantitative Analysis of MGo from the Blood Samples of Healthy Volunteers

Sample No.	Age (years)	Sex* (M = male) (F = female)	Blood glucose level at the time of collection of the sample (mg/dL)	Amount of MGo (µg/mL) (C.V.%) (*n* = 3)
1	26	M	125	0.065 (1.3)
2	23	M	140	0.055 (3.1)
3	21	F	130	0.050 (1.7)
4	23	M	127	0.035 (2.3)
5	32	M	131	0.040 (2.5)
6	19	F	115	0.025 (1.9)
7	24	F	122	0.030 (1.6)
8	30	M	149	0.055 (2.4)
9	24	F	122	0.045 (1.6)
10	30	M	138	0.060 (2.9)
11*	35	M	150	0.057 (1.8)

* Sample analyzed by internal standard technique.

Determination of MGo from serum using linear calibration with spiked sample

A blood sample (5 mL) collected from a diabetic patient (1) (Table I) was treated as described previously. After deproteinization with methanol, the solution was divided in two equal parts. One part was processed as described previously and other was added to 0.46 µg MGo and processed as described previously. The quantitation was carried out by an increase of the response with added MGo, and an external calibration curve.

Determination of MGo from serum using internal standard

Blood samples (5 mL) were collected from a healthy volunteer and a managed diabetic patient. The blood samples were treated as described previously and after deproteinization with methanol, the serum was added to DMGo (0.425 µg). The procedure was followed as described previously. The quantitation was carried out by the ratio of peaks of MGo and DMGo.

The chemicals MGo and DMGo (Fluka, Switzerland), methanol (RDH, Germany) chloroform (Merck, Darmstradt, Germany), hydrochloric acid (37%), potassium chloride, acetic acid, sodium acetate, ammonium acetate, sodium bicarbonate, sodium carbonate, and ammonium chloride were from E. Merck.

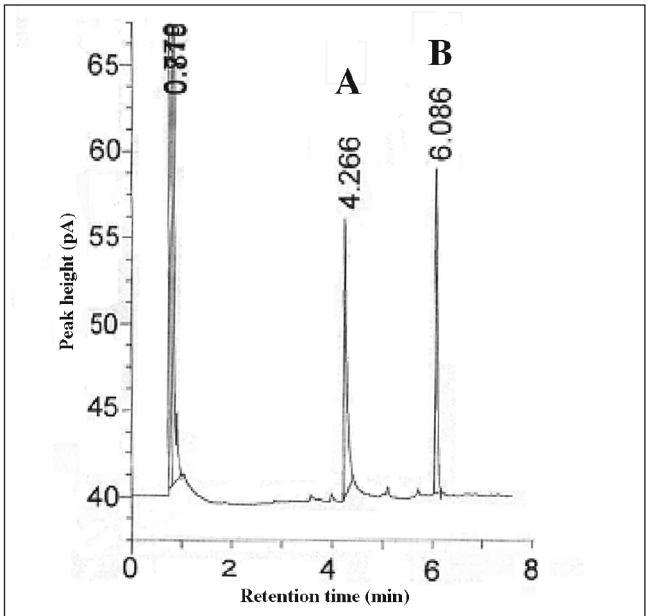

Figure 2. GC separation of MGo (A) and DMGo (B) derivatives of meso-stilbenediamine, from HP-5 column at a column temperature of 150°C with a programmed heating rate of 50°C/min up to 250°C, with total run time 7 min. Nitrogen flow rate was 5 mL/min. The injection port and detection temperatures were 230°C and 270°C, respectively. The detection was by FID and split ratio 10:1.

Meso-stilbenediamine was prepared as reported (19).

Buffer solutions within pH 1–10 at unit interval were prepared from the following: hydrochloric acid (0.1M), potassium chloride (1M), acetic acid (1M), sodium acetate (1M), ammonium acetate (1M), sodium bicarbonate (1M), sodium carbonate (saturated), ammonium chloride (1M), and ammonia (1M). The pH measurement was made with an Orion 420A pH meter with combined glass electrode and reference internal electrode.

GC studies were carried out on an Agilent model 6890 network GC system (Agilent Technology Inc., Palo Alto, CA), split/splitless injector operated with split mode, coupled with FID, hydrogen generator (Parker Baston Analytical Gas system H2-90, Parker Hannifin, Havor Hill, MA), and pure nitrogen (British Oxygen Company, Karachi). A computer with Chemstation software controlled the GC, and an HP Laser Jet 1300 printer was used throughout the study. An HP-5 capillary column with a film thickness of 0.25 µm (J&W Scientific Corporation) was used throughout the study.

Blood samples were collected in sterilized screw cap sample vials from the medical wards at Liaquat University of Medical and Health Sciences Hospital, Jamshoro and Hyderabad, by venipuncture from the patients suffering from diabetes and ketosis. The blood samples from healthy volunteers were collected from the Research Laboratory Dr. M.A. Kazi Institute of Chemistry, who had not taken any medicine for at least one week. The age, sex, and blood glucose levels of the patients were obtained from the records of the Liaquat University of Medical and Health Sciences Hospitals, Jamshoro and Hyderabad, with the permission of the duty doctor. The blood glucose level of healthy volunteers was determined on a Micro Lab 300 (Merck).

Results and Discussion

The reagent meso-stilbenediamine reacts with 1,2-diketones (MGo and DMGo) to form dihydropyrazine compounds (Figure 1).

When the solution of derivative MGo was injected on the HP 5 GC column, it eluted as a single peak and separated from the derivatizing reagent meso-SD. Therefore, the reaction conditions were checked for quantitative GC determination of MGo. The effects of pH, reagent meso-SD concentration added, and warming-up time were examined.

The pH was examined within 1–10 at unit interval, the amount of the reagent (2% w/v in methanol) was varied within 0.5–2.5 mL at an interval 0.5 mL, and warming time at 70ºC was varied for 5–30 min at an interval of 5 min. Each time, a constant volume (1 µL) was injected and average peak height (n = 3) was measured. The condition, which gave the maximum response, was selected. The maximum response was obtained in acidic solution within pH 2–4 and acetic acid-sodium acetate buffer pH 3 was selected. Similarly, the addition of 1 mL of meso-SD solution and warming time of 20 min were used, as reported previously (14).

DMGo also formed derivative with meso-SD and eluted from the GC column. GC conditions were then examined for the separation of MGo and DMGo derivatives. Complete separation was obtained when eluted from the HP-5 column at a column temperature of 150°C with a programmed heating rate of 50°C/min up to 250°C, with total run time 7 min. The nitrogen flow rate was 5 mL/min. The injection port and detection temperature was maintained at 230°C and 270°C, respectively. The detection was by FID and the split ratio was 10:1 (Figure 2). The resolution factor (Rs) between MGo and DMGo derivatives was calculated to 6. The retention factor (k') for MGo and DMGo derivatives was obtained at 4.54 and 6.89, respectively.

The linear calibration curves for MGo and DMGo derivatives were obtained by plotting the average peak height (n = 3) against concentration with five standard solutions (n = 5), and were observed with 0.076–0.760 µg/mL and 0.071–0.710 µg/mL, respectively. The coefficient of determination (r^2) for MGo and DMGo were 0.9977 and 0.9984 with regression equations y =

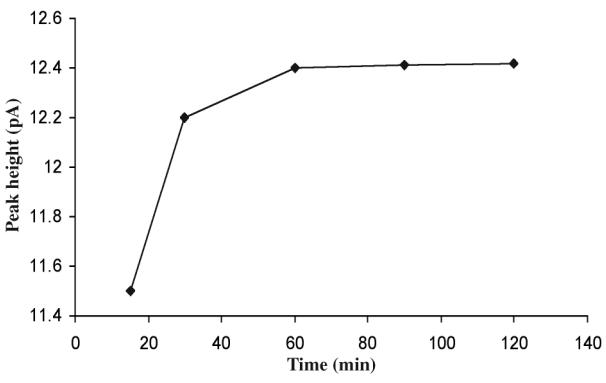

Figure 3. Effect of time (min) on the response of MGo from the blood of healthy volunteers after addition of MGo (0.456 µg). Dervatization was performed as described in the "Determination of MGo in serum" section. GC conditions as in Figure 2.

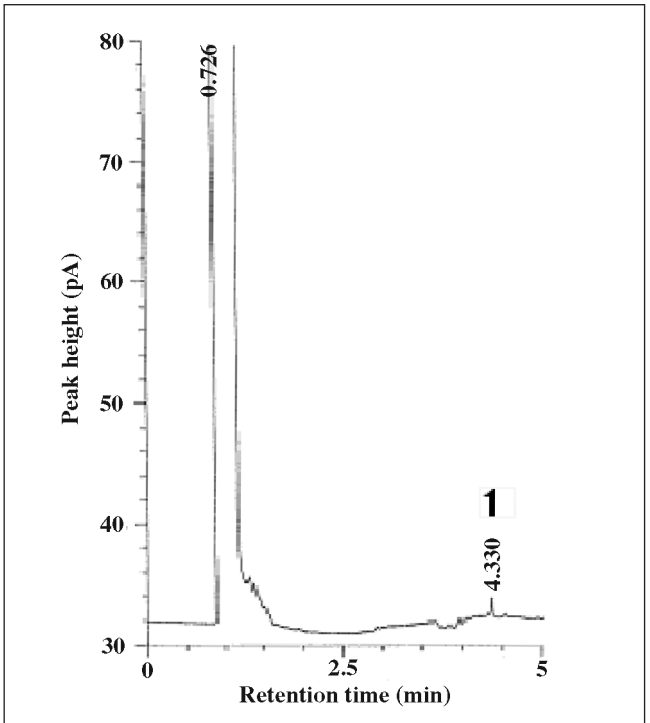

Figure 4. GC response of MGo from healthy volunteer 1 (Table I). GC conditions as in Figure 2.

25.126×-0.0329 and $y = 26.886 \times -0.5141$, respectively. The test solutions of MGo were analyzed ($n = 4$) to cover the entire calibration range, and relative errors were observed within ± 3.5%. The analysis of the replicate sample with MGo 0.456 µg/mL and DMGo 0.425 µg/mL ($n = 5$) was carried out in terms of peak height and retention time and coefficient of variations (C.V.) were observed at 1.1–1.5% and 1.3–2.5%, respectively. The detection limits, measured as signal-to-noise ratio 3:1, were observed at 25 ng/mL and 23 ng/mL, corresponding to 25 pg and 23 pg/injection (1 µL), and 2.5 pg and 2.3 pg reaching up to FID for MGo and DMGo, respectively.

The blood samples collected from diabetic patients and healthy volunteers were analyzed for the contents of MGo. Chaplen et al. (20) reported that the use of perchloric acid or trichloroacetic acid as deproteinizing agent results in the formation of MGo by the oxidation of nucleic acids. Therefore, deproteinization was carried out with methanol. The methanol was added twice, the volume of serum and contents were centrifuged for 30 min (10), and reproducible results were obtained with C.V. within 1.3–3.2%. The effect of allowing the blood sample to remain at room temperature was examined. A blood sample of a healthy volunteer (10 mL) was divided into 5 equal parts of 2 mL each. To each of the portions was added 0.456 µg MGo, and the sample was allowed to rest at room temperature for 15 min, 30 min, 1 h, 1.5 h, and 2 h. The remaining procedure was followed as previously described. A stable response was observed within 1 to 2 h (Figure 3), and 1 h of remaining at room temperature (30ºC) was selected before centrifugation. The results of the analysis are summarized in Tables I and II. The amount of MGo found in the serum of healthy volunteers was observed at 0.025–0.065 µg/mL with C.V. 1.3–3.1% (Table I) (Figure 4), as compared to diabetic patients within the range 0.115–0.228 µg/mL with C.V 1.3–2.8% (Table II, Figure 5A). A blood sample was spiked with 0.456 µg of MGo and quantitation was carried out from a linear calibration and an increase in the response. The amount found was 0.218 µg/mL, as compared to 0.228 µg/mL by direct calibration (Figure 5B). The results support the earlier observation that the serum of diabetic patients contains higher concentrations of MGo than that of healthy volunteers (21). The amounts of DMGo in blood samples were below quantitation limits and were not detected by GC. Therefore, DMGo was examined as the internal standard. The blood samples of a healthy volunteer and a managed diabetic patient were added to DMGo, and analysis was carried out following the analytical procedure. The amounts of MGo found were 0.057 µg/mL and 0.096 µg/mL with C.V. 1.8% and 3.1%, respectively. The amount of MGo found by internal standard (sample 11, Table I) was within the range observed for healthy volunteers.

Table II. Quantitative Analysis of MGo from the Blood Samples of Diabetic and Ketosis Patients

Sample No.	Age (years)	Sex* (M = male) (F = female)	Blood glucose level at the time of collection of the sample (mg/dL)	Amount of MGo (µg/mL) (C.V.%) ($n = 3$)
1*	63	M	515 spiked	0.218 (1.5)
	63	M	515 direct	0.228 (1.7)
2	59	M	508	0.214 (1.9)
3	62	M	488	0.205 (2.8)
4	50	M	481	0.201 (2.6)
5	57	M	337	0.130 (3.2)
6	45	F	319	0.115 (2.6)
7	49	F	469	0.190 (1.3)
8	48	F	392	0.160 (1.7)
9	53	F	418	0.165 (2.4)
10	54	M	503	0.210 (2.3)
11†	58	M	300	0.096 (3.1)

* Sample analyzed by standard addition.
† Sample analyzed by internal standard technique.

Conclusion

A GC method has been developed for the determination of MGo with meso-stilbenediamine as precolumn derivatizing reagent. The method indicated the sensitivity for its application in human serum with a detection limit of 25 ng/mL. The analysis of MGo from serum indicated a C.V. within 1.0–3.5%.

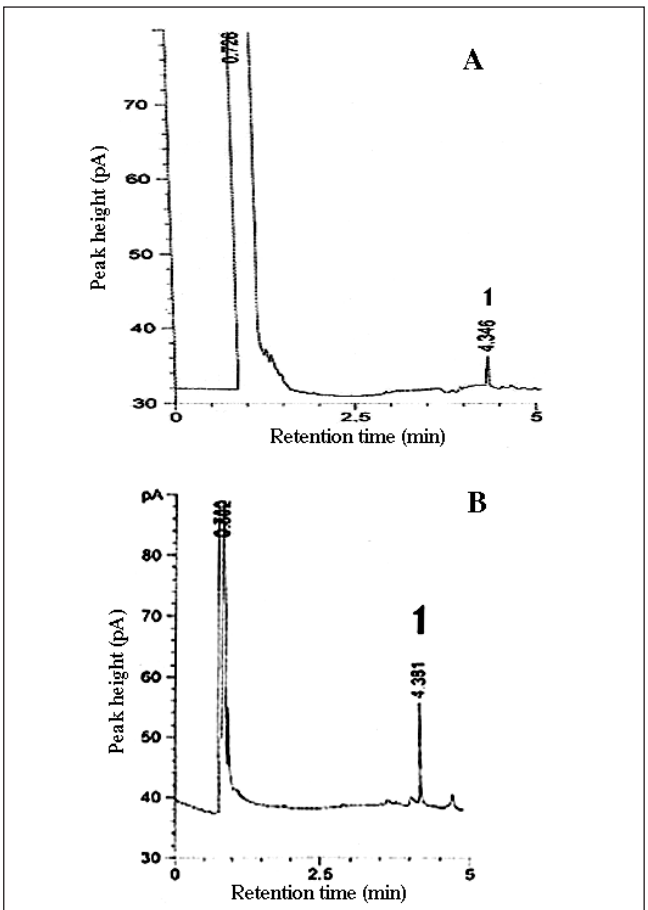

Figure 5. GC response for MGo as derivatives of meso-SD from the diabetic patient 1 (Table II). GC conditions as in Figure 2 (A). GC response for MGo as derivatives of meso-SD from the diabetic patient 1 (Table II) after spiking with 0.456 µg MGo. GC conditions as in Figure 2 (B).

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