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Capillary gas chromatographic determination of methylglyoxal from serum of diabetic patients by precolumn derivatization with 1, 2-diamonopropane

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ABSTRACT

Capillary gas chromatographic (GC) determination of methylglyoxal (MGo) was developed on the basis of precolumn derivatization with 1,2-diaminopropane (DAP) at pH 3. The elution was carried out on an HP-5 (30 m × 0.32 mm i.d.) connected with FID. The linear calibration curve was obtained for MGo within 0.09–1.04 μg/ml with detection limit of 40 ng/ml. Dimethylglyoxal (DMGo) also formed derivative with DAP and eluted and separated from MGo at column temperature 100 °C for 1 min with heating rate 30 °C/min up to 200 °C with run time 4.6 min. The nitrogen flow rate was 1.5 ml/min with split ratio of 10:1, v/v. MGo was determined from serum and urine of diabetics and healthy volunteers. The amounts of MGo from serum and urine of diabetic patients were 0.180–0.260 μg/ml and 0.170–0.250 μg/ml with relative standard deviation (R.S.D.) within 1–4% and 1–3%, respectively. The amounts of MGo from serum of healthy volunteers were 0.032–0.054 μg/ml with an R.S.D. of 1.5–3%. DMGo was not detected from the biological fluids and was used as an internal standard.

1. Introduction

Various reactive carbonyl compounds with low molecular weight have been proposed to play an important role in pathogenesis of many chronic and age related diseases as carbonyl stress [1,2]. Chronic hyperglycemia leading to tissue damage is the primary etiological factor in the pathogenesis of diabetic macrovascular disease and may contribute to macrovascular complications [3–5]. Hyperglycemia-induced tissue damage in diabetes may occur through different mechanisms. One putative mechanism is the formation of advanced glycation end products (AGEs). Different AGE compounds have been characterized including reactive carbonyls such as methylglyoxal (MGo) [6–8]. It is present in all biological systems, but the levels are increased in diabetes, and it has been linked to diabetic late complications [9]. MGo is formed by the nonenzymatic and enzymatic fragmentation of dihydroxyacetone phosphate and glyceraldehydes-3-phosphate [10], the metabolism of acetone [11] and the catabolism of threonine. MGo is detoxified by the glyoxalse system, which catalyze the conversion to D-lactose [12] using reduced glutathione as an essential co-factor. Dimethylglyoxal (DMGo) is an important dicarbonyl compounds usually present in food products obtained by the fermentation process. It has characteristic odor and taste which can affect the organoleptic quality of food [13].

The measurement of circulating levels of MGo generates medical interest because it may help to evaluate the oxidative phenomenon in biological system [14]. It requires selective and sensitive determination at trace levels. Various analytical methods have been proposed for the determination of the MGo from biological samples. Shibamoto has reviewed analytical methods for trace level of reactive carbonyl compounds formed in lipid peroxidation systems [15]. Deng and Yu [16] have reported high performance liquid chromatography (HPLC) for the determination of MGo from urine using 2,4-dinitrophenylhydrazine as a derivatizing reagent. The HPLC methods for the determination of MGo primarily involve 1,2-diaminobenzene, 1,2-diamino-4,5-dimethoxybenzene or 2,3-diaminonaphthalene as derivatizing reagent. The resulting quinoxaline compounds formed are monitored using a spectrophotometeric or spectrofluorimetric detector [17–21]. The methods proposed are based on the quantification of dicarbonyl adducts with 2,3-diaminonaphthalene or 2,4-dinitrophenylhydrazine using electrospray ionization liquid chromatography/mass spectrometry (ESI/LC/MS) or (ESI/LC/MS-MS) [19,22]. These methods are highly sensitive, but require time-consuming solid phase extractions. Alvaro Do Rosario et al. analyzed MGo in water and biological

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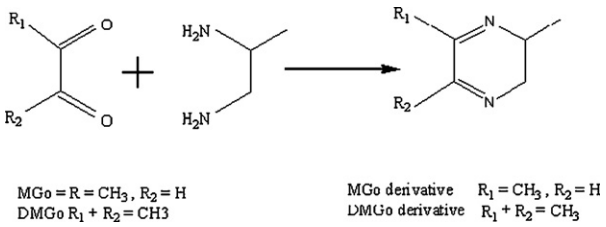

Fig. 1. Structural diagrams of reaction of MGo and DMGo with DAP.

matrix by capillary zone electrophoresis with diode array detection and using *o*-phenylenediamine as derivatizing reagent with linear dynamic range from 0.1–3.6 μg/ml with analytical time of 10 min [23]. Recently, stilbenediamine (1,2-diamino-1,2-diphenylethane) has been used as a derivatizing reagent for HPLC determination of MGo using UV detection with detection limit of 76 ng/ml [24]. The gas chromatographic determination of MGo and (DMGo) involve mainly *o*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride [14,25,26], cystamine [27], *o*-phenylenediamine [28], or 4,5-dichloro-1,2-phenylenediamine as a derivatizing reagents [29]. The determination is based on mass spectroscopic, thermoionic or electron capture detections. The GC analytical procedure involving *o*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride is sensitive (calibration range 5.5–166 μg/l MGo), but requires long reaction time (1 h), multiple solvent extractions, long elution time (32–33 min for MGo) and observation of two peaks for each component [24]. A simple method involving ordinary laboratory chemical with short reaction and analysis time with acceptable sensitivity for its possible use for the analysis of biological fluids could be of value. The present work examines inexpensive laboratory chemical 1,2-diaminopropane (DAP) as derivatizing reagent for GC determination of MGo from biological samples. The dihydropyrazine compounds formed (Fig. 1) are monitored by flame ionization detection (FID), frequently available with gas chromatograph and derivatization increased the carbon number, which could enhance the FID response.

2. Experimental

2.1. Materials

The chemicals MGo, DMGo and DAP (Fluka, Switzerland), methanol (RDH, Germany), chloroform (E-Merck, Germany), hydrochloric acid (37%), potassium chloride, acetic acid, sodium acetate, sodium bicarbonate, sodium carbonate, ammonium chloride and ammonia (28%) were supplied by E-Merck (Germany). The buffer solutions at unit interval within pH 1–10 were prepared from hydrochloric acid (0.1 M)–potassium chloride (1 M) pH (1 and 2), acetic acid (1 M)–sodium acetate (1 M) (pH 3–6), ammonium acetate (1 M) (pH 7), sodium bicarbonate (1 M)–sodium carbonate (saturated) (pH 8 and 9), and ammonium chloride (1 M)–ammonia (1 M) (pH 10). The pH was measured made with Orion 420A pH meter with a combined glass electrode and reference internal electrode. Gas chromatographic studies were carried out on Agilent model 6890 Network GC system (Agilent Technologies Inc., USA) coupled with FID, split/splitless injector operated in a split-mode, hydrogen generator (Parker Balston, Analytical Gas system H2-90, Parker Hannifin, Havorhill, MA, USA) and pure nitrogen (British Oxygen Company, Karachi). The computer with Chemstation software controlled the gas chromatograph, and was used throughout the study. A capillary column HP-5 (30 m × 0.32 mm i.d.) with a layer thickness of 0.25 μm (J & W scientific GC columns, USA) 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 (LUMHS) Hospital, Jamshoro and Hyderabad by vein puncture from the patients suffering from diabetes. The blood samples of healthy volunteers were collected from the Research Laboratory of Dr. M.A. Kazi Institute of Chemistry, who had not taken any medicine at least during one week. The age, sex and blood glucose levels of patients were obtained from the records of (LUMHS) Hospital, Jamshoro and Hyderabad with permission of duty doctor. The blood glucose level of healthy volunteers was determined on Micro lab 300 (E-Merck, Germany).

2.2. Gas chromatographic procedure

The solution (1 ml) containing MGo (0.09–1.04 μg) and DMG (0.1–1.17 μg) was added to DAP (1.5 ml, 2%, v/v in methanol) and 1.5 ml acetic acid–sodium acetate buffer pH 3 in a well stoppered test tube. The contents were warmed at 60 °C for 10 min and 1 ml chloroform was added to it. The contents were mixed well and layers were allowed to separate. An aliquot of the organic layer was transferred to a screw-cap sample vial and the solution (1 μl) was injected on to the GC column HP-5 (30 m × 0.32 mm i.d.) at column temperature of 100 °C for one min, followed by programmed heating rate 30 °C up to 200 °C with a total run time 4.5 min. Nitrogen flow rate was 1.5 ml/min. The injection port and detector temperatures were fixed at 200 and 250 °C, respectively. Hydrogen and nitrogen flow rate for FID system were fixed to 40 and 45 ml/min, respectively. The split ratio was 10:1, v/v.

2.3. Determination of MGo from serum

The blood sample (5 ml) collected from healthy volunteers and diabetic patients were allowed at room temperature for 1 h and were centrifuged at 3000 × *g* for 30 min. The supernatant layer was separated and methanol was added twice in volume (5 ml) volume. The contents were mixed well and again centrifuged at 3000 × *g* for 20 min. The supernatant layer was transferred to sample vial and DMGo 0.47 μg was added to it and derivatizing GC procedure of Section 2.2 was followed. The quantification was carried out using an external calibrations curve and from the ratio of the peak with an internal standard.

2.4. Determination of MGo from serum using linear calibration with spiked sample

A blood sample (5 ml) collected from a diabetic patient was treated as Section 2.3. The serum after deprotinozation with methanol was divided in two equal parts. A part was processed as Section 2.2 and the other was added 0.28 μg MGo and again processed as Section 2.2. The quantitation was carried from the increase in the response with added MGo and comparing the response with internal standard.

2.5. Determination of MGo from urine samples

The urine (2 ml) collected from the diabetic patients in stoppered test tube (Quick fit) was diluted with 1 ml methanol and was centrifuged at 3000 × *g* for 10 min. To the clear solution, 0.47 μg DMGo was added to it and derivatizing GC procedure of Section 2.2 was followed. The quantification was carried out by calibrations curve and with internal standard.

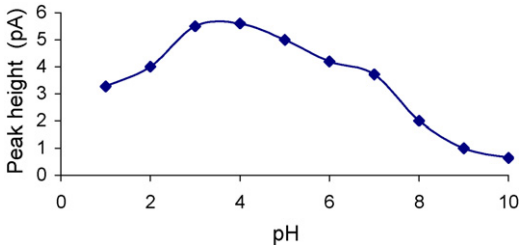

Fig. 2. Effect of pH on the GC elution of DAP–MGo derivative. GC conditions: The column HP-5 (30 m × 0.32 mm i.d.) with film thickness 0.25 μm at an initial column temperature 100 °C for 1 min with heating rate 30 °C up to 200 °C. The run time was 4.5 min. Nitrogen flow rate was 1.5 ml/min and split ratio 10:1. Detection was by FID. Injection port and detector temperatures were 200 and 250 °C. Nitrogen make-up flow rate was 45 ml/min. FID air and hydrogen flow rates were 450 and 40 ml, respectively.

3. Results and discussion

3.1. Optimization of derivatization and separation

The reagent DAP reacts with 1,2-diketones MGo and DMGo to form 2,6-dimethyl-5,6-dihydropyrazine and 2,3,6-trimethyl-5,6-dihydropyrazine compounds (Fig. 1). The compounds formed are highly stable and the solution of derivatives when injected on the gas chromatographic column HP-5 eluted as a single peak and separated from derivatizing reagent and solvent. It was therefore analytical conditions were optimized for the quantitative determination of MGo. The effects of pH, the amount of the reagent DAP added and warming time was examined. Each time constant volume (1 μl) was injected and average peak height ($n = 4$) was measured and the condition that gave maximum response was selected. The pH was examined between 1 and 10 at unit interval. It was observed that the reaction was favored in acidic medium and maximum response was obtained with pH 3–4 and pH 3 was selected (Fig. 2). The acetic acid–sodium acetate buffer pH 3 was used since it covered the pH range satisfactorily. The concentration of reagent DAP added (1.5 ml) was changed from 1.0–4.0% at an interval 0.5%. It was observed that maximum response was obtained with 1.5–2.0% and addition of 2% was selected. The variation in volume of 2% of reagent DAP added was examined within 0.5–2.5 ml at an interval of 0.5 ml. A similar response was obtained with 1.0–1.5 ml and 1.5 ml was selected. The reaction of DAP with MGo and DMGo was slow at room temperature (30 °C) and required 1–2 h to reach at maximum response. It was therefore reaction mixture was warmed at 60 °C for 5–20 min at an interval of 5 min. A similar response was observed after heating for 10 min and this time was selected. The derivatives once formed were highly stable and did not indicate any change in average peak height up to 24 h. MGo and DMGo as derivatives of DAP eluted from the GC column; therefore operating GC conditions were optimized for complete separation. Base line separation was obtained when eluted from the column HP-5 at a column temperature 100 °C for 1 min followed by heating rate 30 °C/min up to 200 °C with total run time 4.6 min. The nitrogen flow rate was 1.5 ml/min (Fig. 3).

3.2. Quantitation and validation

The linear calibration curves for MGo and DMGo as derivatives of DAP were obtained by plotting average peak height ($n = 4$) against concentration with seven standard solutions. The calibrations were linear within 0.09–1.04 μg/ml MGo and 0.16–1.17 μg/ml DMGo with co-efficient of determination (r^2) 0.9919 and 0.9954, and linear regression equations $Y = 12.286x - 0.0914$ and $Y = 12.161x + 0.6415$, respectively. The detection limits observed as signal to noise ratio (3:1) were 40 ng/ml and 50 ng/ml corresponding to 40 and 50 pg/injection (1 μl) and 4 and 5 pg reaching to the FID for MGo and DMGo, respectively. The test solutions of MGo ($n = 4$) were analyzed to examine, the validity of the entire calibration range and the relative % errors were obtained within ±1–3%. The precision in terms of repeatability of the separation with 0.47 μg/ml MGo and 0.50 μg/ml DMGo was examined in terms of peak height and retention time ($n = 5$) and relative standard deviation (R.S.D.) were obtained 2.0–2.2% and 1.0–1.2%, respectively. The resolution factors (Rs) calculated between MGo and DMGo

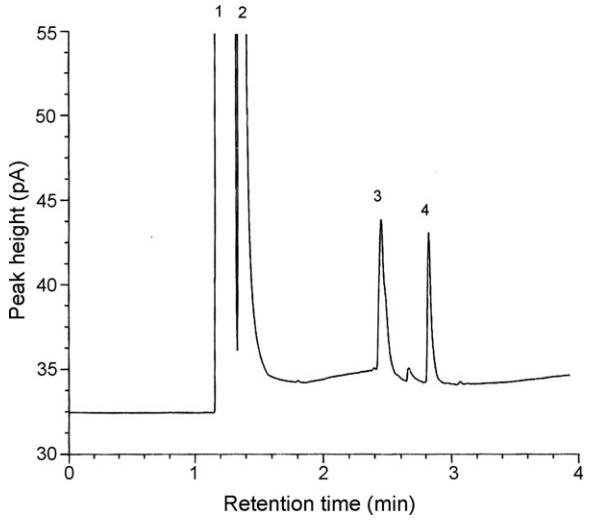

Fig. 3. GC separation of (1) solvent (2) DAP (3) MGo (4) DMGo as derivative of DAP. GC conditions as Fig. 2.

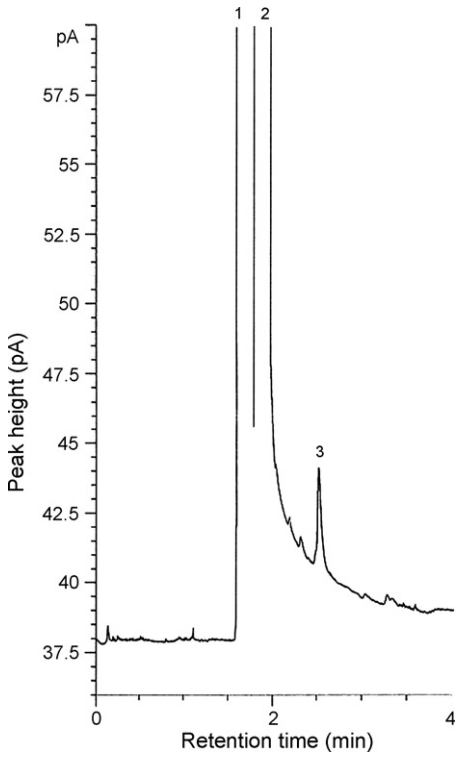

Fig. 4. GC response for MGo from blood sample of diabetic patient. GC conditions as Fig. 2.

Table 1
GC analysis of MGo from serum of diabetes patients (1–7) and healthy volunteers (8–15)

S.No.	Age (sex)	Blood glucose level (mg/dl)	Amount of MGo (μg/ml) found (R.S.D.%), $n = 4$
1	40 (male)	350	0.170 (3)
2	30 (male)	400	0.190 (1)
3	40 (male)	380	0.219 (2)
4	36 (female)	380	0.225 (2)
5	45 (female)	370	0.190 (3)
6	50 (male)	480	0.205 (1)
7*	30 (male)*	490, 490*	0.130 (1), 0.126 (2)*
8	30 (male)	150	0.038 (2)
9	27 (male)	145	0.035 (1.5)
10	28 (female)	140	0.032 (3)
11	29 (male)	160	0.054 (2)
12	28 (male)	158	0.062 (3)
13	30 (female)	145	0.053 (1.5)
14	30 (male)	130	0.048 (1.5)
15	31 (male)	155	0.054 (3)

* Spiked sample.

Table 2
GC determination of MGo from the urine of diabetes patients

S.No.	Age (sex)	Blood glucose levels (mg/dl)	Amount of MGo (μg/ml) found (R.S.D.%), $n = 4$
1	38 (male)	350	0.170 (2)
2	41 (female)	380	0.188 (1)
3	32 (male)	480	0.212 (1)
4	30 (male)	370	0.180 (3)
5	40 (female)	410	0.250 (1)

derivatives was 3.1 and capacity factors (k') was 1.19 and 1.54 for MGo and DMGo, respectively. Intra-day variation for the analysis of 0.47 μg MGo ($n = 6$) was examined and an R.S.D. was observed 3.5%.

3.3. Sample analysis

The blood and urine samples were collected from patients suffering from diabetes and were analyzed for the contents of MGo. It was observed that DMGo in the serum and urine samples was below the detection limit; therefore DMGo was used as an internal standard. The amount of the MGo from the serum and urine samples of diabetic patients was 0.210–0.278 μg/ml and 0.170–0.250 μg/ml with an R.S.D. of 1.5–2.0% and 1–3%, respectively (Tables 1 and 2) (Figs. 4 and 5). The amount of MGo observed in the serum of healthy volunteers was found within 0.046–0.080 μg/ml with an R.S.D. of 1.2–2.0% (Table 1). A serum sample of diabetic patient was spiked with 0.28 μg/ml of MGo and analysis was carried out using an analytical procedure. The amount of MGo found in serum by standard

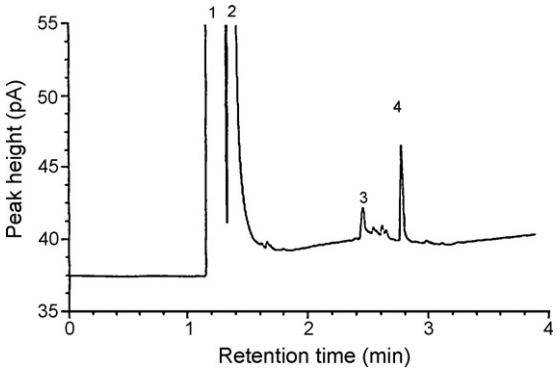

Fig. 5. GC response for MGo from urine sample of diabetic patient. DMGo was used as internal standard. GC condition as Fig. 2.

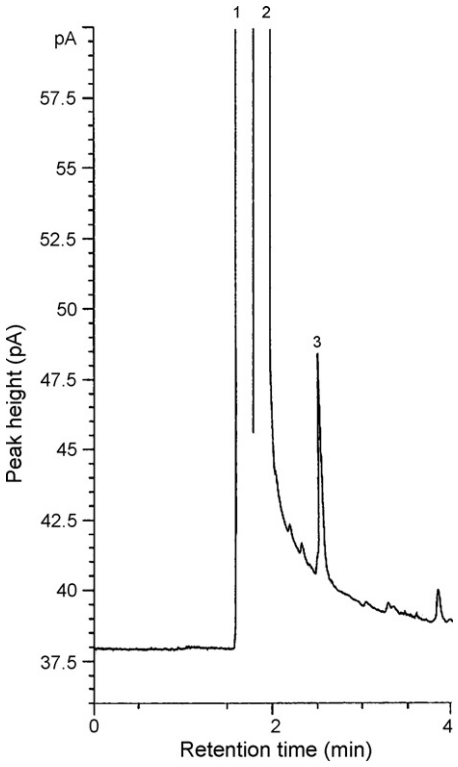

Fig. 6. GC response for spiked MGo from blood sample of diabetic patient. GC condition as Fig. 2.

Table 3
Comparative data for analytical procedures for determination of MGo

S.No.	Analytical method	Derivatizing reagent	Calibration range	Limit of detection	Optimal reaction time derivatization	Retention time	Detection	Ref.
1	HPLC	1,2-diamino-4,5-dimethyoxy-benzene	14.4–72.0 ng/ml	4.9 ng/ml	2 h	6.3 min	UV	18
2	GC/MS	*o*-(2,3,4,5,6 pentafluorobenzyl) hydroxylamine	5.5–166 ng/ml	1.6 ng/ml	1 h	31.79 min, 32.20 min	MS	14
3	Capillary zone electrophoresis	*o*-phenylenediamine	0.1–3.6 μg/ml	7.2 ng/ml	1 h	10 min	Photo diode array	23
4	GC	1,2-diaminopropane	0.09–1.04 μg/ml	40 ng/ml	10 min	2.6 min	FID	Present method

addition was 0.126 μg/ml with an R.S.D. of 2% (Fig. 6) as compared to 0.130 μg/ml calculated directly from linear calibration curve. The percentage recovery of MGo from serum was calculated to 96.9% with an R.S.D. of 2%. The results obtained were observed within the board range of reported values of 2.7–13.2 ng/ml MGo in plasma samples from healthy subjects [25], 26.9 ± 6.4 ng/ml (n = 3) in the blood samples of control diabetic group [30], 8.5 ± 0.5 μg/ml in the plasma of control and 29.3 ± 5.5 μg/ml of diabetes [14]. Table 3 indicates comparative data for analytical procedures for the determination of MGo, and use of inexpensive laboratory chemical with short reaction time and elution time are the advantages of the presented method.

4. Conclusion

The analytical capillary GC method with flame ionization detection has been developed for the determination of MGo from the serum and urine samples using DAP as a derivatizing reagent at pH 3. The total GC run time for each analysis was 4.5 min. Detection limit was observed 40 ng/ml corresponding to 4 pg reaching up to FID with split ratio 10:1. The amount of MGo in serum was observed 0.21–0.278 μg/ml in diabetic patients higher than healthy volunteers within 0.046–0.080 μg/ml with an R.S.D. of 3%. The percentage recovery of MGo was calculated to 96.9% with single extraction. DMGo was used as an internal standard. The observed value of MGo in diabetes and healthy volunteers were within the range for the reported values for MGo.

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