Development of HPLC method for estimation of glyoxylic acid after pre-column fluorescence derivatization approach based on thiazine derivative formation: A new application in healthy and cardiovascular patients' sera

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Abstract

Glyoxylic acid (GA) is the intermediate metabolite in various mammalian metabolic pathways. GA showed high reactivity towards formation of advanced glycation end-products (AGEs); the main cause of pathogenesis and complications of many diseases. The presented study aimed to detect GA in healthy and cardiovascular patients' (CV) sera; however analysis of GA in biological fluid is a challenge and requires chemical derivatization. Hence, a new, highly sensitive, time saving and reproducible pre-column fluorescence derivatization procedure coupled with high performance liquid chromatography (HPLC) method was developed. The derivatization method was based on reaction of 2-aminobenzenthiol (2-ABT), a fluorogenic reagent, with GA in acidic medium to form highly fluorescent thiazine derivative (290 and 390 nm for excitation and emission wavelengths respectively). The fluorescent derivative was separated within 6 min on a reversed-phase ODS column using an isocratic elution with a mixture of methanol–water (70:30, v/v %). The proposed method parameters were optimized and the method was validated. A good linearity in the concentration range (0.05-5.0 μM) was obtained with detection limit (LOD) of 10 nM (200 fmol/injection), which is more sensitive than several previous methods. Moreover, the recovery results were within the range of 85.0-95.5 % and the intra- and inter-day precision results were ≤3.5%. It should be emphasized that this method is the first one for monitoring of GA in CV patients; to investigate its role for diagnosis and monitoring the severity and complications of this disease in clinical laboratory.

Keywords: Glyoxylic acid; 2-Aminobenzenthiol; High Performance Liquid Chromatography (HPLC); Pre-column fluorescence derivatization; Serum analysis

1. Introduction

Glyoxylic acid (GA) is α-dicarbonyl compound and the intermediate metabolite in different mammalian metabolic pathways [1]. GA can be produced *in vivo* from deamination of glycine in peroxisomes, by oxidation of glycolate and through catabolism of hydroxyproline in liver mitochondria [1,2]. GA is oxidized into oxalate in mammals as a final metabolite by lactate dehydrogenase resulting in high concentration of oxalate which is susceptible to urinary calculus formation [1]. GA has an aldehydic group which is highly reactive towards formation of advanced glycation end-products (AGEs) [1,2]. Meanwhile, AGEs have been involved in the pathogenesis of different diseases such as diabetes mellitus (DM) [1,2], cardiovascular (CV) [3-7], neurodegenerative and renal diseases [5]. Studies revealed that GA was found to be more reactive toward formation of AGEs than D-glucose, D-fructose and DL-glyceraldehyde [1,2].

Several analytical methods were reported for determination of GA in different matrices including high performance liquid chromatography (HPLC) [8-17], gas chromatography (GC) [12, 18, 19], thin layer chromatography (TLC) [20], electrophoresis [21], voltammetric [22], polarography [20] and electrochemiluminescence [23]. However, most of these methods have several drawbacks either low sensitivity [9-11, 13, 14, 16, 20, 23], utility of expensive and sophisticated equipment that are not available in most laboratories [12, 18, 19] or long analysis time [11, 15-17].

Till now, few HPLC with fluorescence detection were reported [8, 15, 17], two of them were performed in our laboratory based on Petasis reaction [15, 17], where GA reacted with a fluorescence labeling reagent: 1-pyreneboronic acid. Although high sensitivity of this method, it suffered from having long analysis time (1hr), besides a crowded chromatogram had been obtained due to excess reagent

[15]. In order to clear up the chromatogram of GA to be free from reagent peaks, a clean up procedure using solid phase extraction (SPE) method was conducted [17].

Owing to the high importance in detection and quantitation of GA in biological fluids, a derivatizing reagent; 2-aminobenzenthiol (2-ABT), a fluorogenic reagent, was utilized for derivatization of GA. The presented reagent 2-ABT has no fluorescence itself, however by reaction with GA in acidic medium it forms a highly fluorescent thiazine derivative after 25 min.

Different parameters affecting the derivatization reaction and chromatographic separation were optimized. In addition, the proposed method was validated according to ICH guidelines where linearity, sensitivity parameters and reproducibility were studied. The proposed method showed higher sensitivity over previously reported one following a simple and time saving derivatization procedure and utilizing instruments available in many laboratories. Further, the proposed method was applied for the first time to determine GA in healthy and cardiovascular (CV) patients' sera samples in order to ensure its suitability to diagnose and monitor severity of CV disease in clinical laboratories.

2. Experimental

2.1. Chemicals and reagents

Glyoxylic acid monohydrate (GA) was purchased from Sigma Aldrich (Osaka, Japan). 1,2-Di(2-furyl)-1,2-ethanedione used as an internal standard (I.S.) was purchased from TCI Chemical company (Tokyo, Japan). 2- Aminobenzenthiol (2-ABT), hydrochloric, acetic and perchloric acids were obtained from Nacalai Tesque Chemical Company (Tokyo, Japan). Nitric, phosphoric acids, methanol and acetonitrile (HPLC grade solvent) were obtained from Kanto Chemical Company (Tokyo, Japan). All reagents used for measurements were of analytical grade. Water

used in all chromatographic measurements was purified by Yamato Autostill WG 203 (Yamato Science Co., Ltd., Tokyo, Japan).

Stock solutions of GA (1 mM) and I.S. (5 mM) were prepared in methanol and further dilutions with the same solvent to obtain the working solutions were performed. The solution of 2-ABT reagent (50 mg/mL) was prepared in methanol and further dilutions with methanol were performed to obtain the optimum concentration. The standard and reagent solutions were stable for one month when kept in the refrigerator at 4° C. Pooled human serum samples were obtained from Kohjin Bio Co. Ltd (Saitama, Japan).

2.2. Instrumentation and chromatographic conditions

Chromatographic measurements were performed using a Shimadzu HPLC system (Kyoto) which consisted of a Shimadzu LC-9A pump, a Shimadzu RF-10AXL fluorescence detector, a Chromato-PRO recorder, and a Rheodyne injector valve with 20-µL sample loop. Chromatographic separation was performed on a Daisopak (SP-120-5-ODS-AP column (4.6 mm i.d.× 250 mm, 5-µm particle size) from Daiso CO., Ltd., Osaka, Japan) by an isocratic elution with a mobile phase composed of methanol: water (70:30, v/v%) with a flow rate of 1.0 mL/min. The fluorescence detection was performed at 290 and 390 nm as excitation and emission wavelengths, respectively. The HPLC-FL system was connected to an EZChrom Elite chromatography data acquisition system (Scientific software, Pleasanton, CA, USA). Fluorescence spectra were measured using Shimadzu RF-1500 spectrofluorometer. Quattro micro TM triple-quadruple mass spectrometer (Waters Co., Milford, MA, USA) was used for recording the mass spectrum (MS) of the fluorescent derivative after positive electro spray ionization (ESI^+).

2.3. Fluorescence derivatization procedure

One hundred microliters of standard solution of GA were transferred into a screw-capped vial, where 100 µL of 2-ABT (2 mg/mL) and 100 µL of hydrochloric acid (0.05 M) were added. After vortex-mixing, the vial was heated at 90 °C for 30 min. After cooling, the vial contents were filtered with 0.45-µm cellulose acetate membrane filter (Advantec Toyo Co., Tokyo) and 20-µL of the filtrate were injected into the HPLC-FL system.

2.4. Spiked human serum sample pretreatment

A volume of 50 µL of pooled human serum was transferred into 2-mL eppendorf tube, where 50 µL of GA standard solutions using different concentrations and 50 µL of I.S. (50 µM) were added. Further, 500 µL of methanol was added for protein precipitation, the contents were vortex mixed for 20 sec, centrifuged at 2200 ×*g* for 20 min and filtered through 0.45-µm cellulose acetate membrane filter (Advantec Toyo Co., Tokyo). After that, A 100 µL of supernatant was taken and then introduced into the derivatization reaction as described in section 2.3.

2.5. Real sample collection and pretreatment

Real sample analysis was conducted in accordance with established guidelines and approved by the Ethics Committee of the School of Biomedical Sciences, Nagasaki University (approval number 22). Serum samples were obtained from 5 healthy volunteers (2 females, 3 males; age range, 40–62; mean age 52.2 ± 5.9) and from 5 CV patients (3 females, 2 males; age range,69–85; mean age 77 ± 6.5) attending Sasebo Chuo Hospital. The serum samples were frozen at −80 °C before analysis.

A 50 µL of healthy or CV patient serum sample was transferred to 2-mL eppendorf tube; further 50 µL of I.S. was added. After that, 550 µL of methanol was added for protein precipitation, the contents were vortex mixed for 20 sec, centrifuged at 2200 ×*g* for 20 min and filtered through 0.45-µm cellulose acetate

membrane filter. A 100 µL of supernatant was taken and then introduced into the derivatization reaction as described in section 2.3.

2.6. Method validation

For method validation, ICH guidelines [24] was followed where calibration curves, linearity range, limit of detection (LOD), limit of quantification (LOQ), accuracy, and intra- and inter-day precision were carefully studied.

3. Results and discussion

Owing to the absence of chromophore and fluorophore in GA structure, derivatization reaction is mandatory for its determination either in biological or environmental samples. In the presented method, simple and highly sensitive fluorescence derivatization method was developed based on reaction of GA with 2-ABT; a fluorogenic reagent in acidic medium after heating at 90 °C for 30 min. Fig. S1 (Supplementary Material) shows the fluorescence spectra of the derivatized product obtained from the derivatization of GA with 2-ABT in acidic medium. The fluorescence thiazine derivative was detected at 290 and 390 nm as excitation and emission maxima, respectively.

Mass spectrometer was used for recording the mass spectrum (MS) of the fluorescent derivative after positive electro spray ionization (ESI^+). The most abundant ion peak was found at *m/z* 166, this peak attributed to the formed fluorescent thiazine derivative, the derivatization reaction mechanism is represented in Fig. 1. The derivatization mechanism is in agreement with the results obtained from our previous article [25].

3.1. Optimization of fluorescence derivatization parameters

In order to obtain higher sensitivity for the proposed method, derivatization parameters including type of acid, concentration of both acid and 2-ABT reagent, reaction temperature and time were optimized using a standard solution of GA (1.5 μM). The derivatization reaction requires acidic medium, so different acids such as hydrochloric, sulfuric, nitric, phosphoric, acetic and perchloric acids were examined. As shown from Fig.S2 (Supplementary Material), hydrochloric acid is the best acidic medium giving the highest results; therefore hydrochloric acid was used for subsequent measurements. Further, different concentrations of hydrochloric acid in the range from 0.025 to 0.15 M were studied. From the results presented in Fig.2A, the relative peak height of GA was increased by increasing the concentration of hydrochloric acid and 0.075 M showed the maximum results after that a decrease was observed and hence 0.075 M was selected as optimum concentration. Moreover, the effect of different concentrations of 2-ABT in the range of 0.5-3.0 mg/mL were tested. It was found that the maximum peak height was obtained in the presence of 2 mg/mL of 2-ABT and it was selected as optimum concentration as represented in Fig.2B.

Reaction temperature has a great effect on the fluorescence derivatization procedure and different temperatures in the range from 70-100 ^{0}C were studied. It was observed that by increasing the temperature, the peak height of GA was increased until reaching stable results at 90 ^{0}C as shown in Fig.2C. Further, different reaction time from 10 to 35 min were examined and maximum results were obtained after 25 min, hence 30 min was selected as the optimized reaction time as shown in Fig.2D. A chromatogram of reagent blank and standard solution of GA after fluorescence derivatization is shown in Fig.3.

3.2. Method Validation

3.2.1. Using standard solution of GA

Under the optimized reaction conditions described above, a standard solution of GA with different concentrations was analyzed using the proposed method. The constructed calibration curve showed good linearity between GA concentration and peak area ratio over the concentration range 0.05-5.0 μM with correlation coefficient of 0.998, according to the following regression equation:

$$Y = -0.0117 + 1.148\ X$$

Y = the peak area ratio and X = GA concentration (μM), n= 3

LOD at a signal-to-noise ratio (S/N) of 3 was 10 nM (200 fmol/injection) and LOQ at (S/N) of 10 was 33 nM.

The sensitivity of the proposed method was higher than previously reported one as shown in Table 1. Although the proposed method was less sensitive than some reported methods. However, some of these methods suffer from several drawbacks as described in Table 1.

Further, intra- and inter-day precision of the proposed method were studied using six replicates of standard solution of GA at three different concentration levels (0.08, 2.0 and 4.0 μM). It was observed that RSD values for intra-day (n= 6) and inter-day (n= 6) precision were in the range 1.5–2.6% and 2.2–3.1%, respectively.

3.2.2. Estimation of GA in human serum samples

Pooled human serum sample was used in this validation study, where pre-analysis step was performed to determine GA content before spiking with standard GA solution.

Calibration curve of pooled serum samples spiked with GA was constructed in the concentration range from 0.08 to 3.8 μM to assess the linearity of the proposed method in biological fluids. A good linearity was achieved between GA

concentration in human serum and peak area ratio with a correlation coefficient equal 0.997, LOD was 12 nM at S/N of 3, while LOQ was 40 nM at S/N= 10.
The regression equation:

$$Y = 0.013 + 1.114\ X$$

Y = the peak area ratio and X = GA concentration (μM), n=3

Intra and inter-day precision were examined using six replicates of serum samples spiked with three different concentration levels of GA within the calibrated concentration range (0.1, 2.5 and 3.8 μM). From data represented in Table 2, it was observed that RSD values for intra-day (n = 6) and inter-day (n = 6) precision were 1.9–3.1% and 2.9–3.5%, respectively. The recovery of the proposed method was determined and it was expressed as [(found amount/spiked amount) × 100]; the results were ≥ 85%, which indicate good accuracy of the proposed method.

Further, the proposed method was applied for determination of GA in sera of healthy and CV patient human. Fig. 4 represents typical chromatograms of blank serum without derivatization, serum spiked with standard solution of GA and serum samples from healthy and CV patients determined by the proposed method. The peak of GA was detected clearly without any interference from biological components, which indicates the high sensitivity of the presented method.

An increase in the relative peak area of GA was observed in CV patient serum sample when compared with healthy sample. Hence, a statistical analysis was conducted, where the level of GA was compared among healthy control subjects and CV patients. Five healthy control sample and five CV patient serum samples were used for comparison study, the level of GA in healthy subjects was found to be in the range 1.5-2.3 μM which show good agreement with previous article [1]. On the other hand, GA concentration in CV patient samples was found to be in the range of 2.3-3.6 μM.

The statistical analysis using *t*-test at the 95% confidence level was conducted to compare the level of GA in healthy and CV subjects as shown in Table S1 (Supplementary Material). The data presented revealed the presence of significantly higher level of GA in CV patients when compared with healthy controls as shown in Fig.5. The increased level is attributed to ability of GA to form AGEs [1,2] which considered as important mediators for development and complications of CV diseases [3-6]. AGEs accumulate in blood vessels altering their structure and function through cross-linking with collagen [5]. In addition, activation of AGE receptors can cause complex signaling pathways that increase inflammation, oxidative stress, enhance the vascular smooth muscle apoptosis, enhance calcium deposition, which lead to development and complication of atherosclerosis [3]. Meanwhile, AGEs have a damage effect on human aortic endothelial cells which lead to apoptosis [6]. Hence, monitoring GA concentration in biological fluids can be used for detection of presence and severity of CV diseases. The proposed method proved its suitability to be utilized in clinical laboratories to monitor severity and complication of cardiovascular diseases.

4. Conclusion

A simple pre-column fluorescence derivatization process was developed for determination of GA. The method based on reaction of GA with 2-ABT, a fluorogenic reagent, to form fluorescent derivative separated on HPLC-FL system within 6 min. The proposed method was more sensitive than several previous methods, cost–effective and time saving method. All experimental parameters were optimized; the method was validated and showed good linearity in the concentration range 0.05-5.0 μM with correlation coefficient equal 0.998 and low detection limit of 10 nM (200 fmol/injection).

The presented work is the first study that examined concentration level of GA in cardiovascular patients' sera, where a significant higher concentration of GA was observed when compared with healthy control samples. The method proved its suitability for monitoring GA in CV patient with simple pre-treatment procedure and without interference from complex matrices. The method is highly recommended for routine analysis of large numbers of clinical samples for monitoring severity and complication of cardiovascular diseases.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Acknowledgment

The authors would like to thank Dr. Takahiro Imazato (Sasebo Chuo Hospital, Sasebo, Japan) for his valuable help in this article.

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Figure Captions

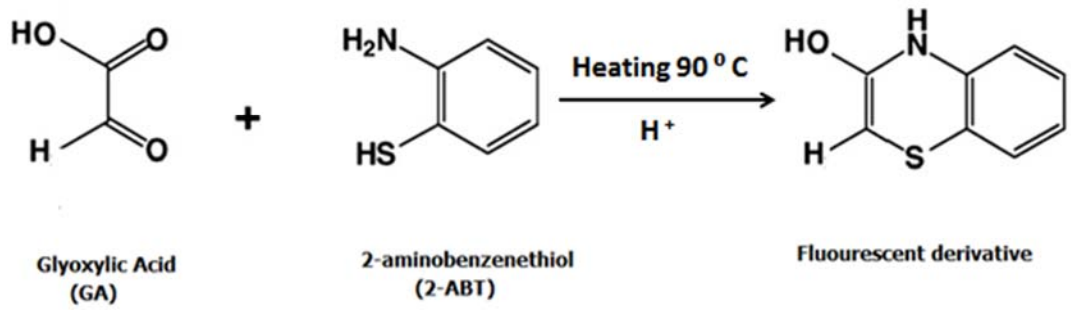
Fig.1. Fluorescence derivatization reaction mechanism of GA with 2-ABT

Fig.2. Optimization parameters of derivatization reaction of GA (1.5 μM): effects of (A) concentration of hydrochloric acid, (B) concentration of 2-ABT reagent, (C) reaction temperature, and (D) reaction time on relative peak height of GA

Fig.3. Chromatograms obtained from (A) reagent blank and (B) standard solution of GA (1.5 μM) after derivatization with 2-ABT reagent.

Fig.4. Chromatograms from (A) blank serum sample without derivatization, (B) spiked serum sample with GA (1.0 μM), (C) serum from healthy human, and (D) serum from cardiovascular patient.

Fig.5. A boxplot showing the concentration level of GA in healthy and cardiovascular subjects


HO
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Heating 90 0 C
H+
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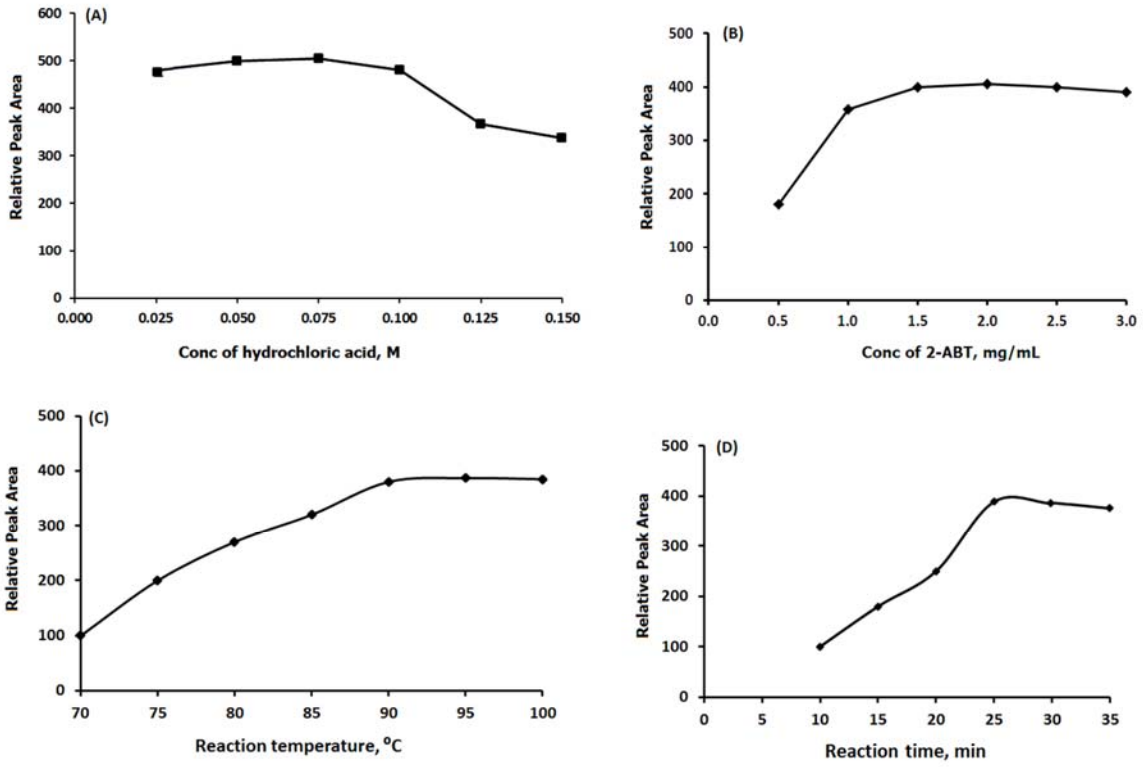
Glyoxylic Acid
(GA)
2-aminobenzenethiol
(2-ABT)
Fluourescent derivative

Fig. 1


(A)
Relative Peak Area
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500
400
300
200
100
0
0.000
0.025
0.050
0.075
0.100
0.125
0.150
Conc of hydrochloric acid, M
(B)
Relative Peak Area
500
400
300
200
100
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Conc of 2-ABT, mg/mL
(C)
Relative Peak Area
500
400
300
200
100
0
70
75
80
85
90
95
100
Reaction temperature, °C
(D)
Relative Peak Area
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400
300
200
100
0
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5
10
15
20
25
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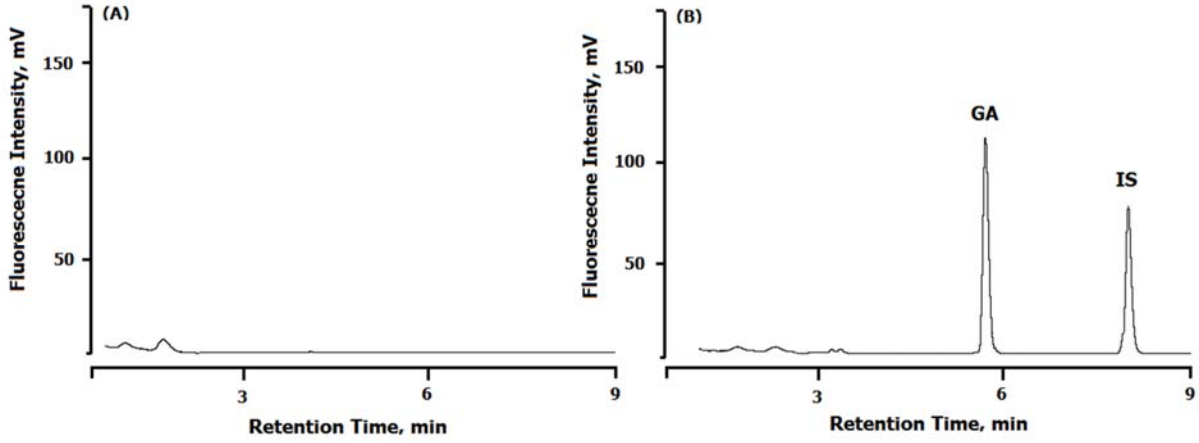
35
Reaction time, min

Fig. 2


(A)
Fluorescecne Intensity, mV
150
100
50
3
6
9
Retention Time, min
(B)
Fluorescecne Intensity, mV
150
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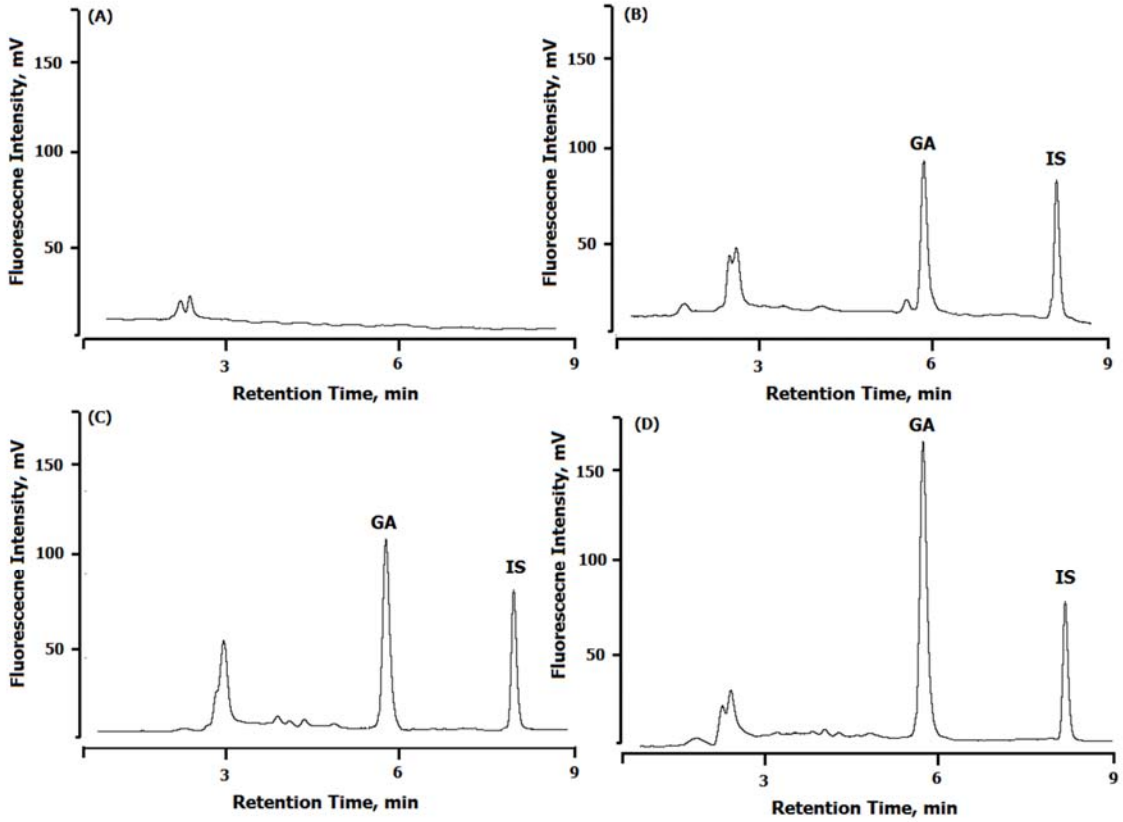
GA
IS
3
6
9
Retention Time, min

Fig. 3


(A)
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Fluorescecne Intensity, mV
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Retention Time, min
GA
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Fig. 4

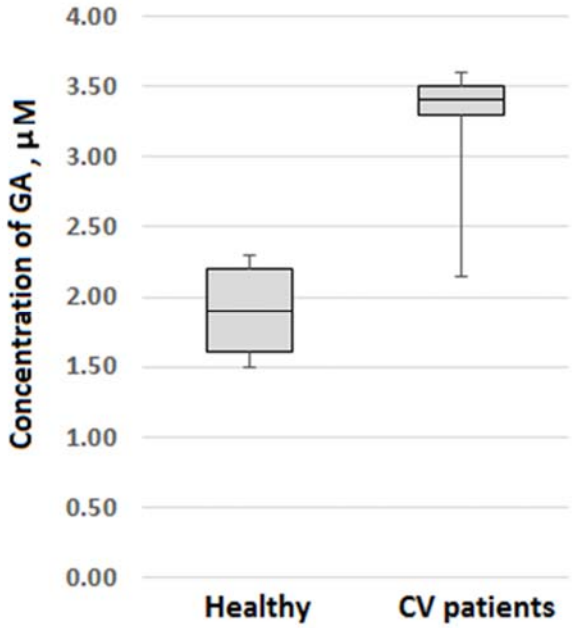
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Concentration of GA , µM
Healthy
CV patients

Fig. 5

Table 1

Comparison between the proposed method and previously reported one

Analytical Method	Matrix	LOD (µM)	Drawbacks	Ref
HPLC -UV, using phenyl hydrazine reagent	Biological fluids	0.10	Low sensitivity, hydrazine reagents are flammable, irritant to skin, nerves, and respiratory tract and unstable [26]	10
GC-TOF-MS	Methylobacterium extorquens	0.002	Complex and expensive equipment.	12
HPLC-refractive index	----------	1.60	Low sensitivity	13
HPLC-UV, using meso stilbenediamine reagent	Human serum	0.94	Low sensitivity	14
HPLC-FL, Petasis reaction	Human urine	0.005	Long reaction time and the chromatogram suffer from interfering peaks from excess reagent.	15
HPLC-ESI-MS/MS, using 2,4-dinitrophenylhydrazine reagent	Drinking water	0.025	Sophisticated instruments not available in most laboratories. Besides derivatization using hydrazine reagents suffers from analytical errors due to formation of *syn* and *anti* isomers [26]	16
HPLC-FL, Petasis reaction	Human serum	0.030	Low sensitivity, long analysis time (1hr)	17
GC-Flame ionization detector	Sea water	0.025	Low sensitivity, expensive and sophisticated instruments	18
GC-MS	Human plasma	0.005	Complex instruments need special expertise and the procedure described is tedious and time consuming	19
Differential pulse polarography	----------	6.70	Low sensitivity	20
Electrochemiluminescecne	Human serum and urine	0.87	Low sensitivity	23
Presented method	Human serum	0.01	----------	----

Table 2

Spiked amount (μM)	Intra-day precision (n=6)			Inter-day precision (n=6)		
	Found	RSD	%Recovery	Found	RSD	%Recovery
0.1	0.088	3.0	88.0	0.085	3.5	85.0
2.5	2.37	2.5	94.8	2.32	3.1	92.8
3.8	3.63	1.9	95.5	3.57	2.9	93.9

Recovery and precision results of GA in pooled human serum sample

Supplementary Material

Development of HPLC method for estimation of glyoxylic acid after pre-column fluorescence derivatization approach based on thiazine derivative formation: A new application in healthy and cardiovascular patients' sera

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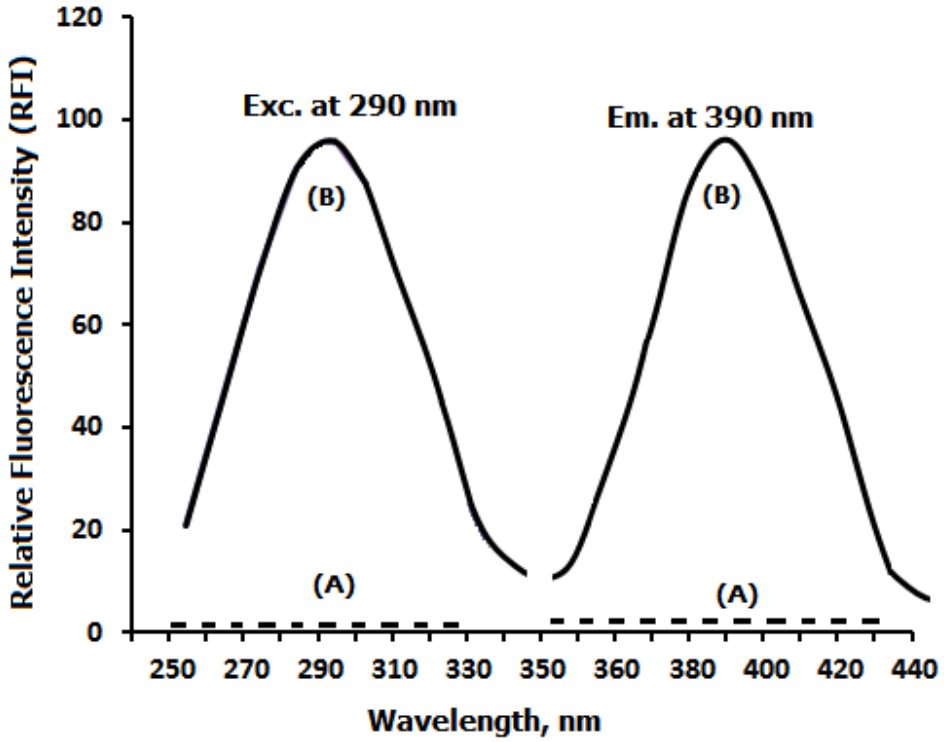

Fig.S1.Excitation and emission spectra of: (A) reagent blank, and (B) the fluorescent derivative from reaction of GA (5 µM) with 2-ABT reagent

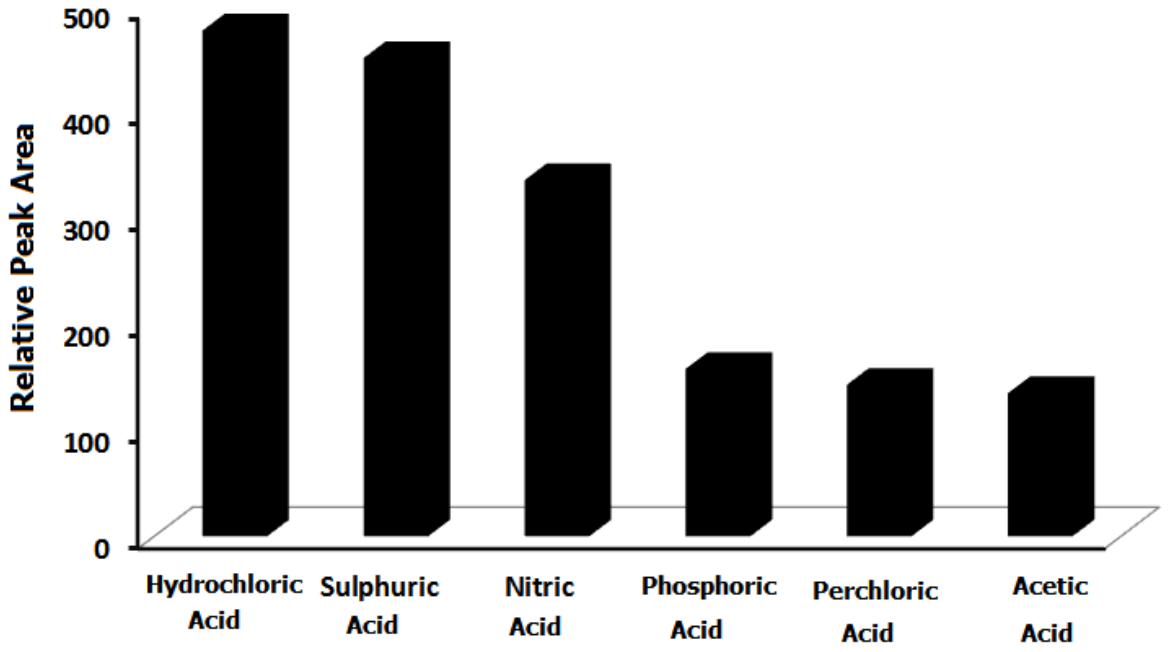

Fig.S2. Effect of different acids on relative peak height of GA

Table S1

Glyoxylic acid concentration in healthy and cardiovascular patients' sera

Groups	Conc. range (μM)	Mean ± SE [a]
Healthy (n=5)	1.5-2.3	1.9 ± 0.36
CV patients (n=5)	2.3-3.6	3.3 ± 0.45
Statistical Analysis [b]	Control Vs CV patient, *p* value= 0.00477	

[a] Standard error

[b] *t-test was used for comparison at a significant level α= 0.05, there is significant difference at P<0.05 value was less than 0.05*