

**Determination of glyoxylic acid in urine by liquid chromatography and fluorescence detection using a novel derivatization procedure based on the Petasis reaction**

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## Abstract

The Petasis reaction is the multi-component reaction of carbonyl compound, amine and arylboronic acid to form  $\alpha$ -amino acid or  $\beta$ -aminoalcohol. In the present work, as a first analytical application of the Petasis reaction, a high-performance liquid chromatography (HPLC) with fluorescence detection method was developed for the determination of glyoxylic acid. The glyoxylic acid was derivatized with 1-pyrene boronic acid as a fluorescent arylboronic acid in the presence of *N*-methylbutylamine as an amine to give the fluorescent  $\alpha$ -amino acid. The HPLC separation of fluorescent derivative was performed within 30 min on an octyl column with a gradient elution of acetonitrile and 50 mM acetate buffer (pH 4.0). The detection limit (S/N=3) for glyoxylic acid was 5.0 nM (20 fmol/injection). Finally, the proposed method could be applied to determine the glyoxylic acid concentration in human urine without interferences from biological components.

keyword: Petasis reaction, Fluorescence derivatization, Glyoxylic acid, Fluorescent arylboronic acid

## 1. Introduction

Compounds containing carbonyl groups are widely distributed in the environment, in both plants and living organisms. Since it is thought that carbonyl compounds play significant roles in the many pathological processes, a sensitive method for determination of carbonyl compounds in biological samples is of great interest. High-performance liquid chromatography (HPLC) with fluorescence detection is a powerful analytical tool for the sensitive determination of trace amount of compounds in biological samples [1-4]. However, most carbonyl compounds are non-fluorescent or weakly fluorescent. In order to determine the carbonyl compounds fluorimetrically, several fluorescence derivatization reagents having a hydrazine group have been developed, e.g., HCPI [5], DBD-H [6] and dansyl hydrazine [7]. However, hydrazine reagents are unstable and should be prepared just before analysis. In addition, hydrazine reagents are flammable and irritate nerves, skin and respiratory tract [8]. Also, *p*-aminobenzoic acid [9] and dansylacetamidooxyamine [10] were used for fluorescence derivatization of carbonyl compounds, but the reagents and the derivatives are relatively unstable. Therefore, in this study, a stable and safe derivatization reagent for the carbonyl group based on the Petasis reaction was developed.

The Petasis reaction is the multi-component reaction of a carbonyl compound, an amine and an arylboronic acid to form an  $\alpha$ -amino acid or  $\beta$ -aminoalcohol [11-16]. The Petasis reaction proceeds under mild conditions without additional coupling reagents. Also, the Petasis reaction can proceed even in the presence of water [15, 16]. In addition, arylboronic acids are stable and relatively benign compounds [17]. From these aspects, a fluorescent arylboronic acid can be used as a stable and safe

fluorescence derivatization reagent for carbonyl compounds. Until now, there have been no reports of a fluorescence derivatization technique based on the Petasis reaction.

In this study, a fluorescent arylboronic acid, 1-pyreneboronic acid (1-PyBA, Fig. 1) was used to develop a pre-column derivatization HPLC method for the determination of glyoxylic acid as a model carbonyl compound.

Glyoxylic acid is a metabolite synthesized by various metabolic pathways [18-21]. It has been reported that glyoxylic acid is mitochondrial toxin [22] and citric acid cycle inhibitor [23]. Moreover, glyoxylic acid is enzymatically metabolized to oxalic acid, which causes kidney stone disease [19-21]. Calcium oxalate is the major constituent of kidney stones [24], and almost half of urinary oxalate is derived from glyoxylic acid [25]. Several analytical methods to determine glyoxylic acid concentration in biological and environmental samples have been reported, such as HPLC with UV absorbance detection (HPLC-UV) [26-28] and fluorescence detection (HPLC-FL) after derivatization [29, 30] and GC with mass spectrometric detection (GC-MS) [31-33]. However, these HPLC methods have limitations due to their low sensitivity and selectivity. Although GC-MS method is highly sensitive, the GC-MS instruments such as GC with time-of-flight MS (GC-TOF-MS) [32] and GC with quadrupole MS [33] are very complicated and less common.

Glyoxylic acid can be converted to a fluorescent derivative after reaction with 1-PyBA in the presence of *N*-methylbutylamine (MBA), and the derivative can be determined sensitively by fluorescence detection. Furthermore, the developed method was successfully applied to the determination of glyoxylic acid in human urine samples without any interference from biological components.

## **2. Experimental**

### **2.1 Materials and reagents**

1-PyBA was obtained from Tokyo Chemical Industries (Tokyo, Japan) and used without further purification. MBA was purchased from Acros Organics (Geel, Belgium). Ethanol (extra pure grade), sodium acetate and acetic acid were from Nacalai Tesque (Osaka, Japan). Glyoxylic acid monohydrate and acetonitrile (HPLC grade) were from Wako (Tokyo, Japan). Creatinine Companion Kit was from Exocell (Philadelphia, PA, USA). Water was distilled and passed through a Pure Line WL21P system (Yamato, Tokyo, Japan). All other chemicals were of the highest purity and quality available. Stock solution of glyoxylic acid (10 mM), 1-PyBA (500 mM) and MBA (200 mM) were prepared in ethanol and stored at 4 °C.

### **2.2 Equipment**

The HPLC system consisted of two Shimadzu LC-10AT pumps (Kyoto, Japan), a Shimadzu RF-10AXL fluorescence detector, a Rheodyne 7125 injector with a 20- $\mu$ L loop (Cotati, CA, USA), and an EZChrom Elite chromatography data acquisition system (Scientific Software Inc., USA).

Melting point was measured by Yanagimoto MP-53 melting point apparatus (Kyoto, Japan). Elemental analysis was carried out using Perkin Elmer 2400II (Norwalk, CT, USA). Mass spectrum was obtained by using JEOL JMS-700N mass spectrometer (Tokyo, Japan).

### **2.3 HPLC conditions**

Chromatographic separation was performed on a COSMOSIL 5C8-MS (250×4.6 mm i.d.) with a gradient elution program using solvent A (acetonitrile-50 mM acetate buffer (pH 4.0) (35:65, v/v) and solvent B (acetonitrile). The gradient elution was programmed as follows: 0% B (0–15.0 min), 0% B to 100% B linearly (15.0–15.5 min), and 100% B (15.5–26.0 min). The flow-rate was set at 1.0 mL/min at ambient temperature. The excitation and emission wavelengths were set at 335 nm and 381 nm, respectively.

### **2.4 Fluorescence derivatization procedure for standard glyoxylic acid**

To 100  $\mu$ L of 0.01-50  $\mu$ M glyoxylic acid in ethanol, 50  $\mu$ L of 100 mM 1-PyBA in ethanol and 50  $\mu$ L of 10 mM MBA in ethanol were successively added. After vortex-mixing, the reaction mixture was heated at 80 °C for 1 h. To the reaction mixture, 300  $\mu$ L of water was added in order to remove excess 1-PyBA by formation of precipitate of 1-PyBA. A 20  $\mu$ L aliquot of the supernatant was injected into HPLC system after filtration through an Advantec 0.45  $\mu$ m cellulose acetate membrane filter (Tokyo, Japan).

### **2.5 Human urine analysis**

Urine samples were obtained from healthy male volunteers (21–35 years, n = 7) in

our laboratory. After the collection, the urine samples were kept at -80 °C until analysis. A 50 µL aliquot of urine sample was diluted with 950 µL of ethanol. To 100 µL of the diluted urine, 50 µL of 100 mM 1-PyBA in ethanol and 50 µL of 200 mM MBA in ethanol were successively added and mixed well. After heating at 80 °C for 1 h, 300 µL of water was added to the reaction mixture. A 20 µL aliquot of the supernatant was injected into HPLC system after filtration through an Advantec 0.45 µm cellulose acetate membrane filter. For normalization, the concentration of urinary creatinine was determined by Jaffe reaction using Creatinine Companion Kit.

### **3. Result and discussion**

#### **3.1 Optimization of fluorescence derivatization conditions**

Fig. 2(A) and (B) shows the typical chromatograms obtained from reagent blank and the reaction mixture of glyoxylic acid with 1-PyBA and MBA, respectively. The peak observed at 10.3 min on the chromatogram was identified as the derivative of glyoxylic acid because the retention time was identical with that of 2-(*N,N*-methylbutylamino)-2-(1-pyrenyl)acetic acid. The peak was only detected in the presence of both 1-PyBA and MBA, while the peak was not detected without either 1-PyBA or MBA. These result indicated that glyoxylic acid was successfully converted to the fluorescent derivative by the Petasis reaction.

In order to increase the reactivity, the conditions of derivatization reaction were optimized using a standard solution of glyoxylic acid. The effect of reaction solvent on the reaction yield was examined with ethanol, 1,4-dioxane, acetonitrile,

dichloromethane, dimethylformamide and 2-propanol. Among these solvents, tetrahydrofuran, ethanol and dichloromethane gave higher reaction yield than other solvents (Fig. S1). Considering the toxicity of dichloromethane, ethanol was selected as the reaction solvent. The concentration of 1-PyBA was examined over a range of 2-500 mM, and the maximum peak area of glyoxylic acid was obtained at a concentration over 50 mM (Fig. 3); 100 mM 1-PyBA was selected. However, the use of excess amount of 1-PyBA gave a considerable reagent blank peak on the chromatogram. To decrease the interference from reagent peak, water was added to the solution after derivatization reaction in order to precipitate unreacted 1-PyBA. Even though the addition of water to the reaction mixture, large blank peak of 1-PyBA was still observed in the chromatogram. However, without the addition of water, the peak of glyoxylic acid could not be detected clearly by the interferences with the excess 1-PyBA. As a co-reactant, the effects of amine species on retention times of the derivative were investigated using MBA, morpholine, *N*-ethylbenzylamine, *N*-ethanolbenzylamine, *N*-phenylpiperazine and *N*-cyclohexylpiperazine. The retention time increased with increasing hydrophobicity of the amine (Fig. S2). The result indicated that the retention time can be controlled by changing the hydrophobicity of the amine. In this study, MBA was selected as amine because the use of MBA resulted in baseline separation of the derivative peak and the reagent peak. Then, the concentration of MBA was studied over a range of 4-200 mM, and the maximum peak height of derivative was obtained at 10 mM; 10 mM of MBA was selected (Fig. S3). The effects of reaction temperature and time on the peak area were examined. Although the peak area increased with increasing the temperature until 90 °C (Fig. S4), 80 °C was selected considering the boiling point of ethanol (78 °C). As shown in Fig.



4, the constant and maximum peak area was achieved by heating for more than 50 min, 60 min was selected as reaction time.

### **3.2 Synthesis of the fluorescent derivative derived from glyoxylic acid, 1-PyBA and MBA**

The fluorescent derivative, 2-(*N,N*-methylbutylamino)-2-(1-pyrenyl)acetic acid, was prepared as follows: glyoxylic acid monohydrate (13.8 mg, 0.15 mmol), 1-PyBA (36.9 mg, 0.15 mmol) and MBA (13.1 mg, 0.15 mmol) were dissolved in 1.8 mL of ethanol and the mixture was heated at 80 °C for 5 h. After the reaction, the precipitate was filtered and washed with 15 mL of ethanol and 9 mL of water successively, to give white crystal; yield: 19.6 mg, 37.9%. The melting point of the crystal was determined as 215 °C. The results of elemental analysis (C, 79.73%; H, 6.61%; N, 4.02%) were in good agreement with the theoretical value of C<sub>23</sub>H<sub>23</sub>NO<sub>2</sub> (C, 79.96%; H, 6.67%; N, 4.06%). In addition, the molecular ion peak was observed at m/z 346.30 (FAB-MS) and the value was consistent with the molecular weight of the expected product (346.17, [M+H]<sup>+</sup>). Therefore, the formation of 2-(*N,N*-methylbutylamino)-2-(1-pyrenyl)acetic acid was confirmed.

### **3.3 Calibration curve, detection limit and repeatability**

A calibration curve was prepared by diluting a standard solution of glyoxylic acid. A linear relationship ( $r=0.998$ ) between the peak area and the concentration of

glyoxylic acid was obtained by measuring 6 levels in the range from 0.01 to 50  $\mu\text{M}$ . The regression equation of calibration curve (mean $\pm$ standard error, n=5) was  $y=(1.20\times 10^5\pm 2.67\times 10^3)x + 2.46\times 10^4\pm 2.39\times 10^4$ , where y was the peak area (a.u.) and x was the concentration of glyoxylic acid ( $\mu\text{M}$ ). The intra-day (n=3) and inter-day (n=3) precisions were evaluated using different concentrations of standard solution (0.5, 2.5 and 10  $\mu\text{M}$ ). The relative standard deviations of intra-day measurements were 5.1, 5.4 and 4.4%, respectively, and those of inter-day measurements were 6.7, 7.0 and 3.8%, respectively. The detection limit of glyoxylic acid was 5.0 nM (20 fmol/injection) at signal to noise ratio (S/N) of 3. The sensitivity of the proposed method was 40, 500 and 15 times higher than those of HPLC-UV (0.1  $\mu\text{M}$ , 0.83 pmol/injection) [27], HPLC-FL (10 pmol/injection) [30] and GC with quadrupole MS (1.5  $\mu\text{M}$ , 0.3 pmol/injection) [33], respectively and almost equal to that of GC-TOF-MS (50 nM, 50 fmol/injection) [32]. The proposed method proved highly sensitive determination of glyoxylic acid without requiring expensive and elaborate instrumentation. The reaction yield, which was estimated by comparing the peak areas of the derivative in the reaction mixture and authentic 2-(*N,N*-methylbutylamino)-2-(1-pyrenyl)acetic acid, was 77%.

### **3.4 Determination of glyoxylic acid in human urine samples**

In order to evaluate the applicability of the proposed derivatization method to biological analysis, the method was applied to the determination of glyoxylic acid in human urine. In contrast with the reaction of standard solution, the derivatization reaction of glyoxylic acid could not proceed well in urine. This phenomenon may be

attributed to competing reaction of MBA and biological amines in urine because the reactivity was improved by the increasing in MBA concentration. The concentration of MBA was studied for the derivatization of glyoxylic acid in urine over a range of 10-400 mM, and the maximum peak area of derivative was observed at 200 mM; 200 mM of MBA was selected (Fig. S5). Typical chromatograms of human urine and human urine spiked with 200  $\mu$ M glyoxylic acid are shown in Fig. 5(A) and (B), respectively. The peak of glyoxylic acid was clearly observed on the chromatogram without any interference from biological components, employing only a dilution for pretreatment.

The spiked calibration curve of glyoxylic acid obtained by measuring 6 levels was linear ( $r= 0.998$ ) over a concentration range of 10-500  $\mu$ M of glyoxylic acid in urine. The regression equation of calibration curve (mean $\pm$ standard error,  $n=5$ ) was  $y=(5.51\times 10^3\pm 1.52\times 10^2)x$  and  $1.58\times 10^4\pm 1.81\times 10^3$ , where  $y$  was the peak area (a.u.) and  $x$  was the concentration of spiked glyoxylic acid ( $\mu$ M). Accuracy and precision were evaluated by analyzing urine samples spiked with three different concentrations of standard glyoxylic acid (10, 50 and 200  $\mu$ M). As shown in Table 1, the intra- and inter-day accuracy of glyoxylic acid ranged from 96.2% to 107.0% with precision values less than 9.0%. These results indicate that the proposed method is both precise and accurate in human urine analysis.

This method was applied to the determination of glyoxylic acid in human urine samples obtained from healthy subjects ( $n=7$ ). The urinary concentration of glyoxylic acid (mean  $\pm$  SD) was  $219.4 \pm 110.9 \mu$ M ( $148.5 \pm 41.1$  nmol/ mg creatinine). The results obtained by the proposed method were quite similar to those obtained by the reported methods [28, 29]. Therefore, the proposed derivatization reaction was proved

to have sufficient practicality.

In addition, the proposed derivatization method has a potential to be more selective because the Petasis reaction is a multi-component reaction. If fluorescent amine is applied to the proposed derivatization reaction together with fluorescent arylboronic acid, the derivative containing two fluorophores can be obtained by one-step reaction. The derivative can be detected more selectively based on interaction between two fluorophores such as fluorescence resonance energy transfer (FRET) [34] or excimer fluorescence [35].

## **Conclusion**

In this study, a novel fluorescence derivatization reaction for glyoxylic acid based on the Petasis reaction was developed. The glyoxylic acid was converted to a fluorescent derivative after reaction with a fluorescent arylboronic acid, 1-PyBA in the presence of amine, MBA, and the derivative could be determined sensitively by fluorescence detection. The proposed derivatization method was successfully applied to the determination of glyoxylic acid in human urine without any interference from the biological matrices. The proposed reaction can be applicable for the development of determination methods for other kinds of carbonyl compounds.

Further investigation will attempt to develop a derivatization method, which can introduce two fluorophores simultaneously based on the Petasis reaction.

## **Reference**

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## Figure captions

Fig. 1. Fluorescence derivatization reaction of glyoxylic acid with 1-PyBA and MBA based on the Petasis reaction.

Fig. 2. Chromatograms of (A) reagent blank and (B) standard solution of 50  $\mu\text{M}$  glyoxylic acid after derivatization.

Fig. 3. Effect of 1-PyBA concentration on the peak area of glyoxylic acid. Derivatization conditions: concentrations of glyoxylic acid and MBA were 50  $\mu\text{M}$  and 200 mM, respectively. Reaction temperature and reaction time were 80  $^{\circ}\text{C}$  and 60 min, respectively.

Fig. 4. Effect of reaction time on the peak area of glyoxylic acid. Derivatization conditions: concentrations of glyoxylic acid, 1-PyBA and MBA were 50  $\mu\text{M}$ , 100 mM and 200 mM, respectively. Reaction temperature was 80  $^{\circ}\text{C}$ .

Fig. 5. Chromatograms of (A) human urine sample and (B) human urine sample spiked with 200  $\mu\text{M}$  glyoxylic acid.



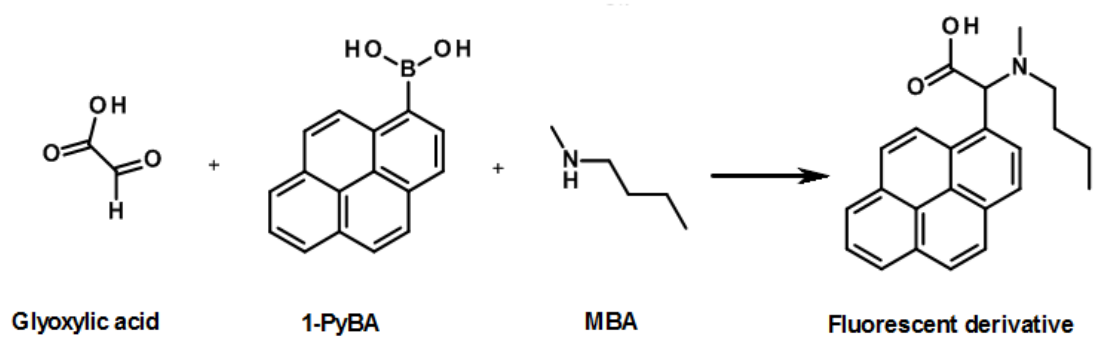


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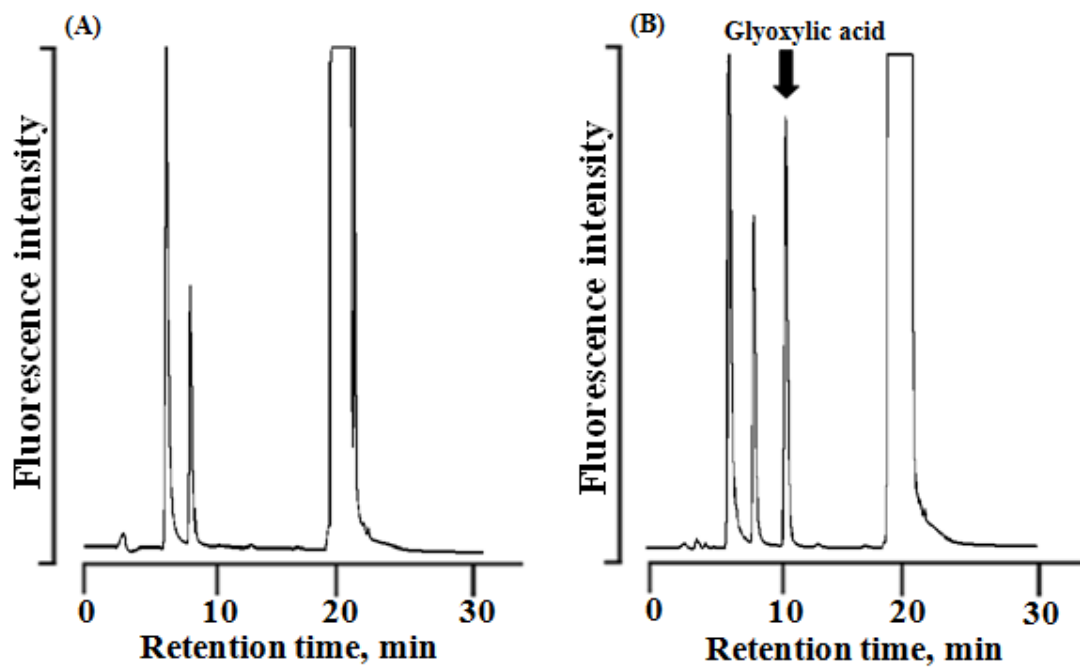


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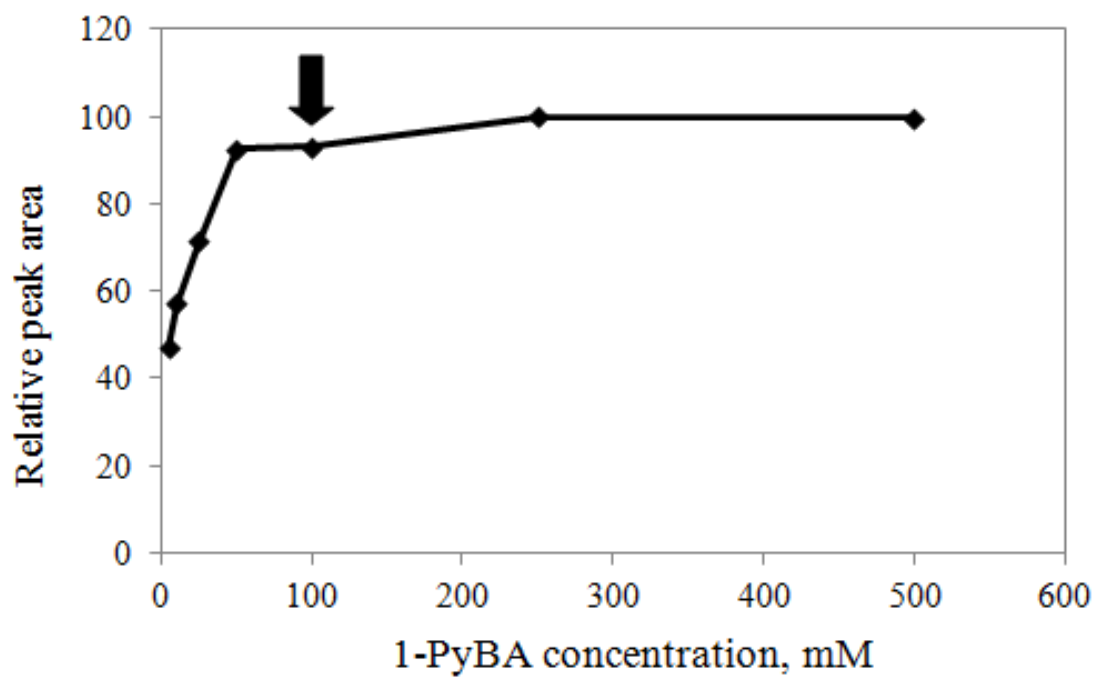


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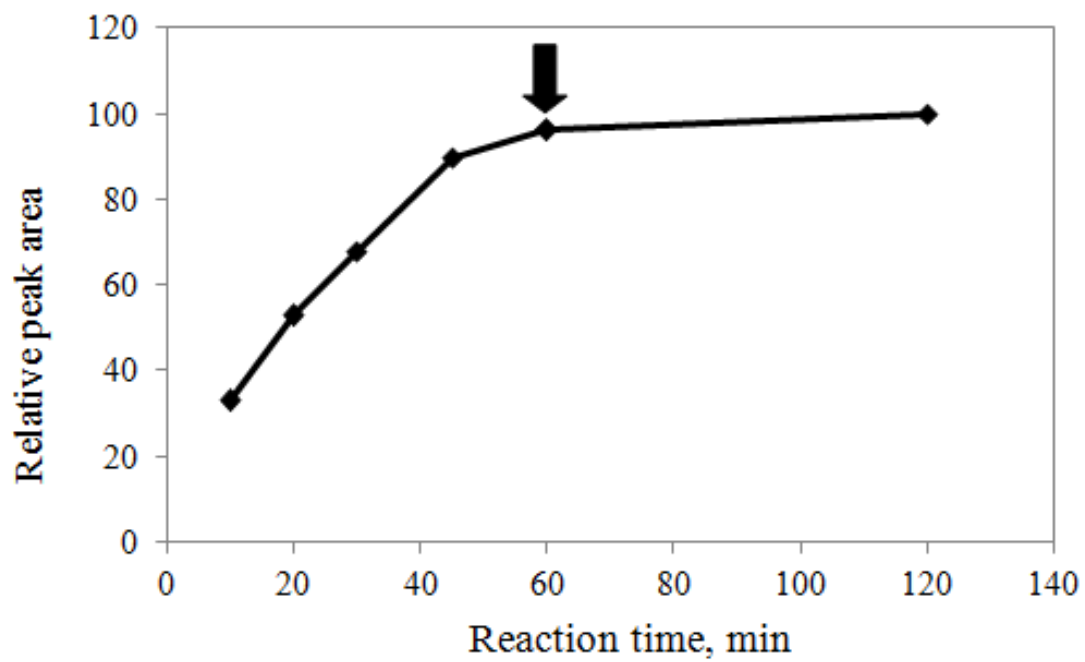


Fig. 4. Effect of reaction time on the peak area of glyoxylic acid. Derivatization conditions: concentrations of glyoxylic acid, 1-PyBA and MBA were 50  $\mu$ M, 100 mM and 200 mM, respectively. Reaction temperature was 80  $^{\circ}$ C.

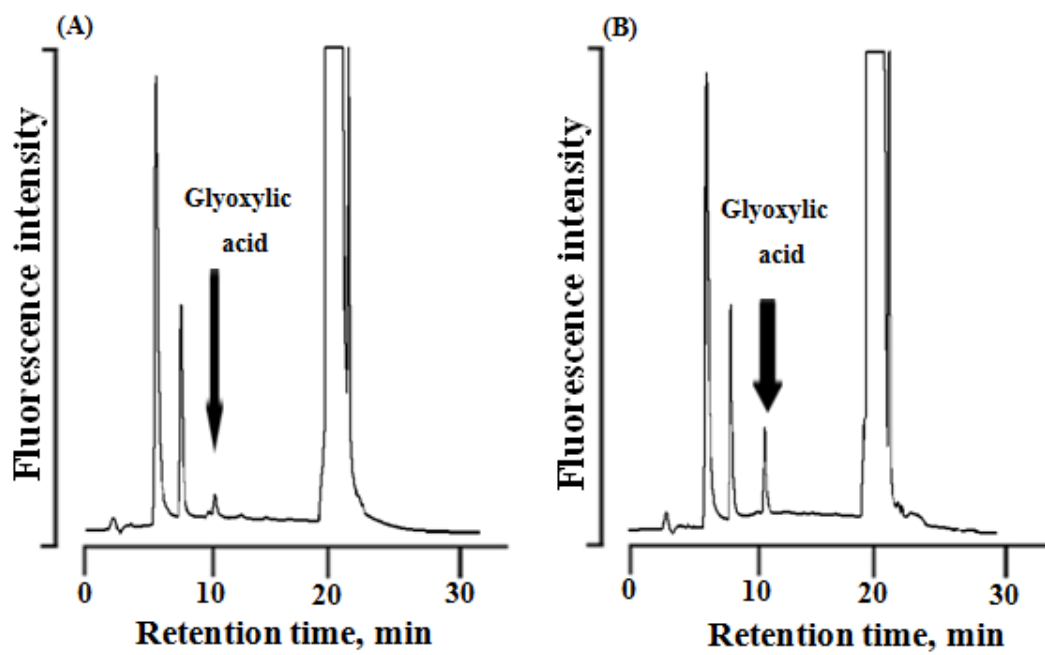


Fig. 5. Chromatograms of (A) human urine sample and (B) human urine sample spiked with 200  $\mu\text{M}$  glyoxylic acid.

**Table 1.** Intra- and inter-day accuracy and precision of the proposed method for determination of glyoxylic acid in human urine.

| Glyoxylic acid<br>( $\mu\text{M}$ ) | Intra-day (n=5) |                        | Inter-day (n=3) |                        |
|-------------------------------------|-----------------|------------------------|-----------------|------------------------|
|                                     | Accuracy        | Precision              | Accuracy        | Precision              |
|                                     | (%)             | (RSD <sup>a</sup> , %) | (%)             | (RSD <sup>a</sup> , %) |
| 10                                  | 102.7           | 8.2                    | 102.5           | 6.6                    |
| 50                                  | 96.2            | 7.2                    | 100.9           | 7.9                    |
| 200                                 | 107.0           | 9.0                    | 106.2           | 7.6                    |

<sup>a</sup> Relative standard deviation

## **Figure captions for supporting information**

Fig. S1. Effect of various reaction solvents on the peak area of glyoxylic acid. Derivatization conditions: The concentrations of glyoxylic acid, 1-PyBA and MBA were 50  $\mu$ M, 1.0 mM and 2.0 mM, respectively. Reaction temperature and reaction time were 80  $^{\circ}$ C and 60 min, respectively.

Fig. S2. Effect of hydrophobicity of amine on retention factor of the derivative. Derivatization conditions: The concentrations of glyoxylic acid, 1-PyBA and amine were 50  $\mu$ M, 1.0 mM and 2.0 mM, respectively. Reaction temperature and reaction time were 80  $^{\circ}$ C and 60 min, respectively.

Fig. S3. Effect of MBA concentration on the peak area of glyoxylic acid. Derivatization conditions: concentrations of glyoxylic acid and 1-PyBA were 50  $\mu$ M and 100 mM, respectively. Reaction temperature and reaction time were 80  $^{\circ}$ C and 60 min, respectively.

Fig. S4. Effect of reaction temperature on the peak area of glyoxylic acid. Derivatization conditions: concentrations of glyoxylic acid, 1-PyBA and MBA were 50  $\mu$ M, 100 mM and 200 mM, respectively. Reaction time was 60 min.

Fig. S5. Effect of MBA concentration on the peak area of glyoxylic acid in urine sample. Derivatization conditions: concentrations of 1-PyBA was 100 mM. Reaction temperature and reaction time were 80  $^{\circ}$ C and 60 min, respectively.

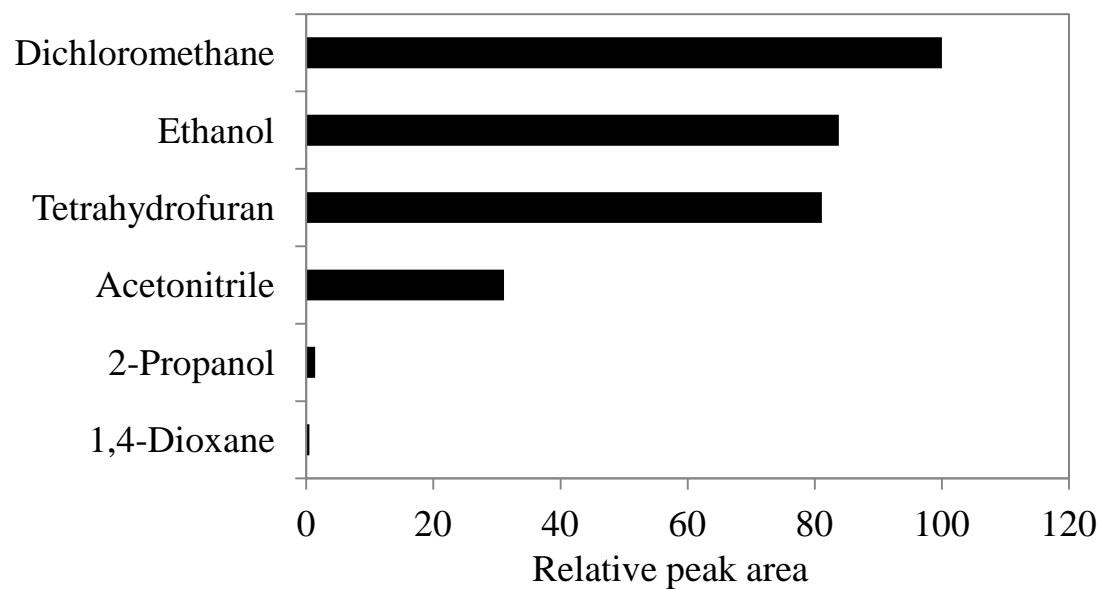


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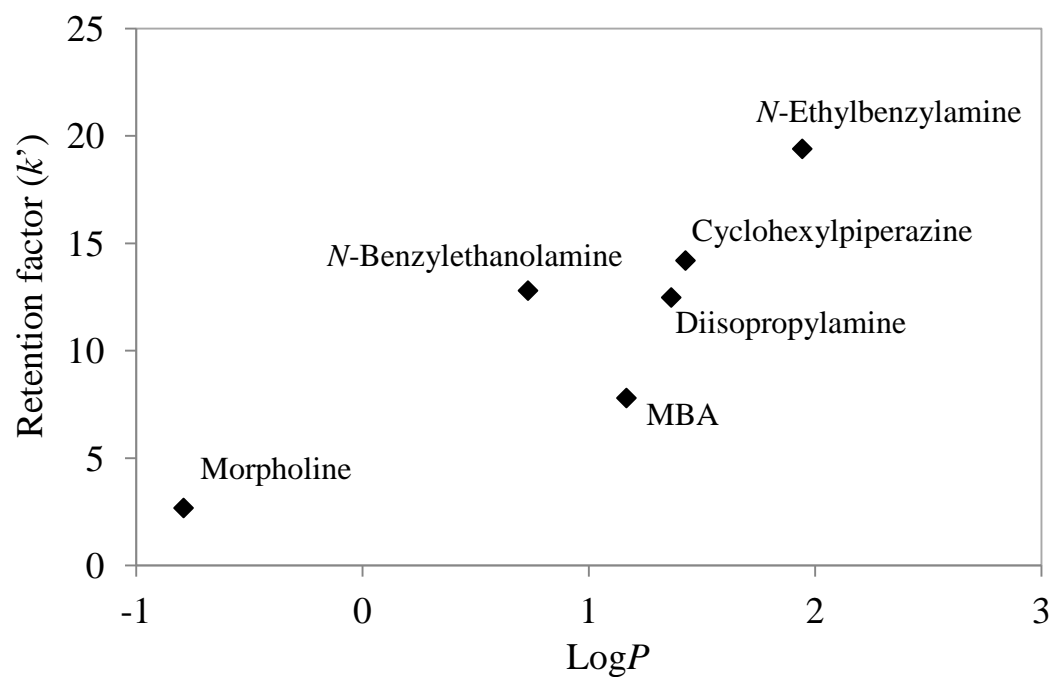


Fig. S2 Effect of hydrophobicity of amine on retention factor of the derivative.

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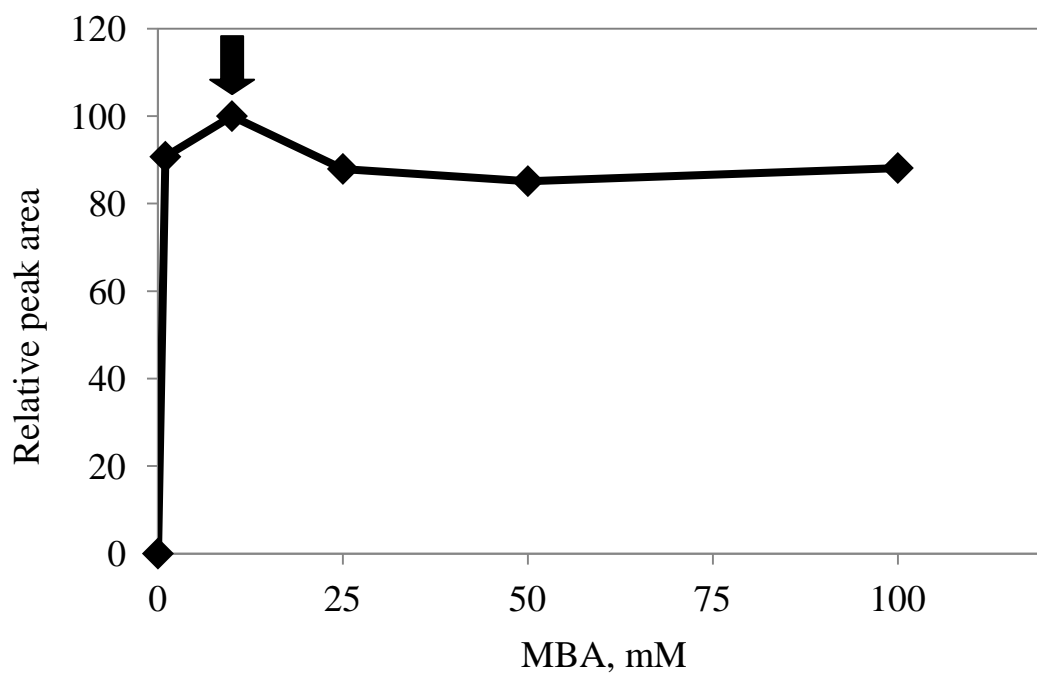


Fig. S3 Effect of MBA concentration on the peak area of glyoxylic acid.

Derivatization conditions: concentrations of glyoxylic acid and 1-PyBA were 50  $\mu$ M and 100 mM, respectively. Reaction temperature and reaction time were 80  $^{\circ}$ C and 60 min, respectively.

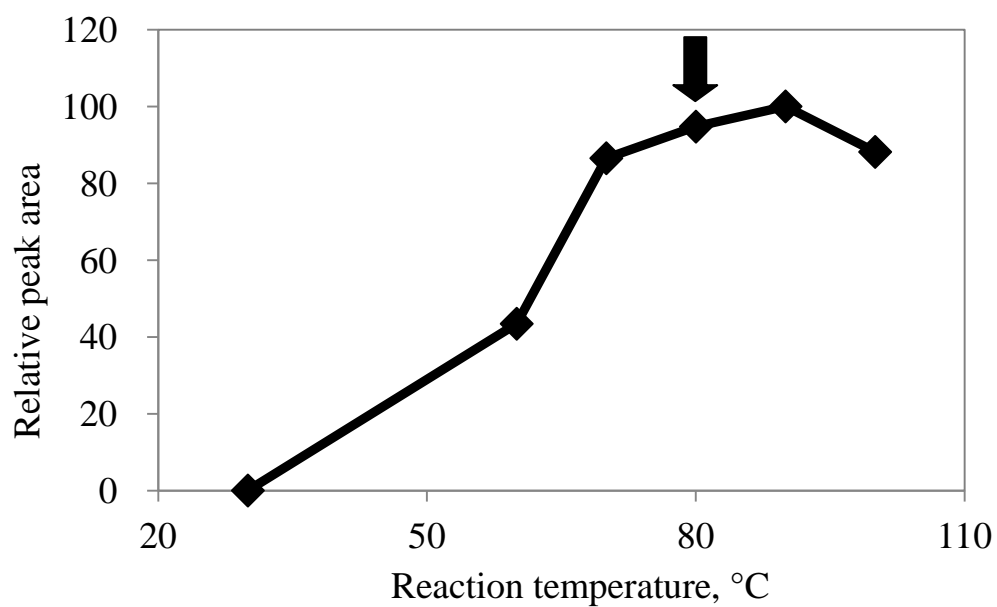


Fig. S4 Effect of reaction temperature on the peak area of glyoxylic acid.

Derivatization conditions: concentrations of glyoxylic acid, 1-PyBA and MBA were 50  $\mu\text{M}$ , 100 mM and 200 mM, respectively. Reaction time was 60 min.

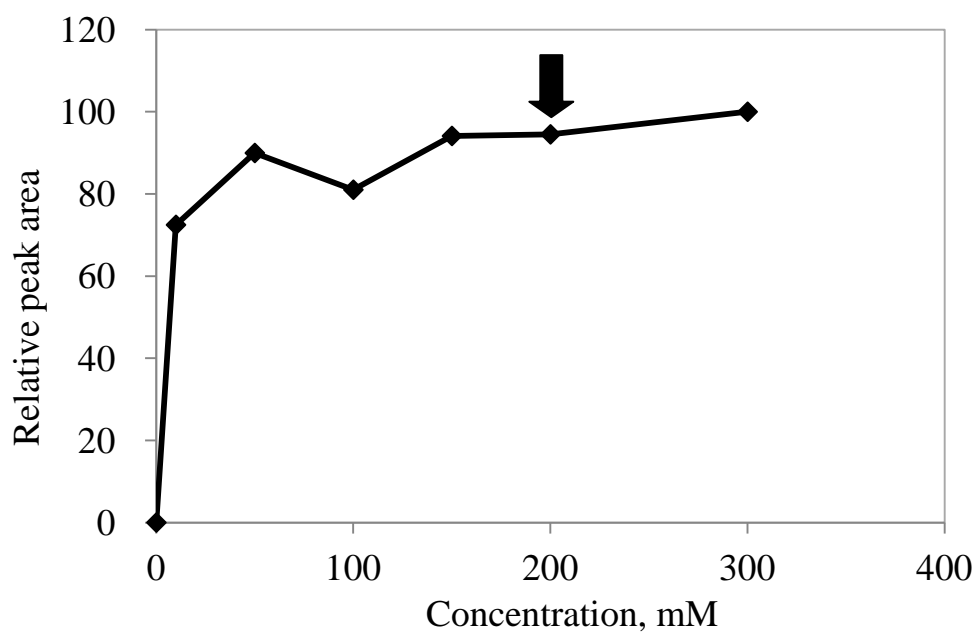


Fig. S5 Effect of MBA concentration on the peak area of glyoxylic acid in urine sample.

Derivatization conditions: concentrations of 1-PyBA was 100 mM. Reaction temperature and reaction time were 80 °C and 60 min, respectively.