A Novel Dual Labeling Approach Enables Converting Fluorescence Labeling Reagents Into Fluorogenic Ones *Via* Introduction of Purification Tags. Application to Determination of Glyoxylic Acid in Serum.

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Abstract

Pre-column derivatization with fluorescence labeling reagents involves many problems including crowded chromatograms, possibility of the introduction of analytical errors, and poor selectivity. Here in we report a novel purification tag/fluorophore dual labeling approach based on a multi-component reaction to solve this major problem. Glyoxylic acid was recently identified as an early biomarker for diabetes, thus it was selected as a model analyte for our new dual labeling approach. Using the multi-component Petasis reaction, we could introduce a fluorophore (1-pyreneboronic acid, 1-PyBA) and a purification tag (taurine) to our target analyte (glyoxylic acid) in one step reaction. Using taurine as the amine reactant in Petasis reaction leads to the formation of a reaction product with a terminal sulfonic acid group which can be selectively retained on an anion exchange sorbent allowing excess fluorescent 1-PyBA reagent and its fluorescent decomposition products to be washed away. Then, quantification of the formed analyte-fluorophore-purification tag adduct was carried out by a simple isocratic HPLC-fluorescence detection method. The newly developed technique allowed highly selective, very rapid and efficient determination of glyoxylic acid in human serum eliminating endogenous components and excess reagent interference. Glyoxylic acid was determined in serum at a final concentration down to 30 nM (600 fmol/injection) with good recovery (87.0%), accuracy (-2.2 – 9.2) and precision (%RSD ≤8.7).

Keywords: Glyoxylic acid; purification tag; taurine; dual labeling; Petasis reaction; serum

Introduction

The analysis of biological fluids encountered many complications and difficulties that are common in HPLC technique. These include; the presence of various components in biological matrices which make it difficult to clearly resolve the analyte of interest, the presence of endogenous components such as proteins which may deteriorate and shorten the lifetime of the column, the existence of the analyte in the biological matrices in a very low concentration which represents a detection difficulty. Hence, considering all of the above problems, it is not a surprise that most of the reported bioanalytical methods depend on multi-step sample pretreatment and preconcentration procedures which lead to relatively pure material that can be introduced to the final separation step by HPLC or other techniques [1,2].

The main traditional sample preparation methods are extraction (liquid-liquid extraction and solid-phase extraction (SPE)), deproteinization (precipitation with an organic solvent, insoluble salt formation, ultrafiltration, and dialysis), and chemical modification (derivatization and decomposition of analyte-protein conjugates). Yet, the classical approach of sample pretreatment exhibits some limitations such as the need for a long time, the high opportunity of sample loss, possibility of the introduction of analytical errors, and need for refined sample handling. However, despite performing sample pretreatment steps, there may be interference from endogenous components with the analyte [2,3]. Also, Pre-column derivatization with fluorescence labeling reagents involves many problems including crowded chromatograms, and poor selectivity [4]. Hence there is a need for a novel sample treatment method that overcomes the previously mentioned limitations. This raised the issue of developing a novel purification tag/fluorophore dual labeling approach to combine rapid and highly efficient sample cleaning with minimum interferences from endogenous biological components and the high sensitivity

of fluorescence detection. In this case, labeling based on a multi-component reaction allows simultaneous introduction of the fluorophore and purification tag in one step.

Petasis reaction is a multi-component reaction of a carbonyl compound with arylboronic acid and amine to form β-aryl-α-amino acid. Based on the Petasis reaction, our research group has developed a fluorescence labeling derivatization technique for determination of glyoxylic acid [5]. In such method, glyoxylic acid has been derivatized into a fluorescent derivative using 1-pyreneboronic acid (1-PyBA) in the presence of *N*-methylbutylamine. However, as the former method was fluorescent labeling one, it suffered from having crowded chromatogram by the peaks from either excess fluorescent reagent or endogenous compounds. This motivated us to make a full use of the Petasis multi-component reaction and develop a novel dual labeling technique *via* introducing a fluorophore and a purification tag to the analyte in one reaction step for the first time.

In this approach, glyoxylic acid reacted with 1-PyBA as a fluorophore in the presence of taurine as a purification tag forming a fluorescent derivative with a terminal sulfonic acid group (Fig 1). Then, by proper selection of a strong anion exchange sorbent that can efficiently adsorb the fluorescent derivative through interaction with its sulfonic acid group, it becomes possible to completely wash out excess fluorescent reagent (Fig 2). The use of this novel dual labeling technique resulted in a clear chromatogram free from any interfering peaks. Therefore, the developed method combined the advantages of Petasis reaction including mild reaction conditions, stability and relative safety of arylboronic acids, and proceeding of the reaction in the presence of water, and the advantages of simple, rapid and highly efficient extraction gained by using a purification tagging reagent. Thus, 1-PyBA will be changed from a fluorescence labeling reagent into a fluorogenic one *via* the removal of the interfering blank peaks.

The target analyte in this method is glyoxylic acid which is a metabolite produced endogenously through different metabolic pathways[6–9]. As well, it is well known as a mitochondrial toxin[10] and an inhibitor of the citric acid cycle[11]. The enzymatic metabolism of glyoxylic acid results in the formation of oxalic acid which is the main reason of kidney stones[7–9]. Additionally, glyoxylic acid is the main source of most of the urinary oxalate[12]. Moreover and most importantly, glyoxylic acid was recently discovered as an early biomarker for type 2 diabetes. A recent study revealed that the level of glyoxylic acid is significantly increased in prospected patients up to eighteen months even before their diagnosis with diabetes[13]. Hence, it is obvious that the determination of glyoxylic acid in biological fluids is of great importance.

The reported analytical methods for determination of glyoxylic acid in biological and environmental samples include HPLC-UV detection[14–16], HPLC-fluorescence detection (HPLC–FL)[5,17,18] and GC-flame ionization and mass spectrometric detection[19–21]. Though, these HPLC methods have some weaknesses including poor sensitivity and selectivity (Table S1, supplementary file). Despite the GC–MS methods[20] have high sensitivity, the GC–MS instrument is very complex with limited availability in laboratories. The newly proposed HPLC-FL method could increase both detection sensitivity and selectivity using the novel dual labeling technique allowing a convenient and rapid mean for determination of serum glyoxylic acid level eliminating endogenous components interference.

Experimental

Chemicals and reagents

The reagents used were of analytical grade and the solvents were of HPLC grade. Water purified by Yamato Autostill WG 203 (Yamato Science Co., Ltd., Tokyo, Japan) was used in

all experiments. Methanol, Acetonitrile (for HPLC) and dimethylsulfoxide (DMSO) were obtained from Kanto Chemical Co. (Tokyo). Glyoxylic acid monohydrate, tetrahydrofuran (THF), 1,4-dioxane, and 2-aminoethylphosphonic acid were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Ethanol, 2-propanol (for HPLC), *N,N*-dimethylformamide (DMF), L-alanine, and L-glycine were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Taurine was obtained from Sigma-Aldrich (St. Louis, MO, USA). 1-PyBA and 6-aminocaproic acid were from Tokyo Chemical Industry Co., Ltd. (Tokyo). Serum was purchased from Kohjin Bio Co. Ltd (Saitama, Japan).

Acetate buffer (50 mM, pH 4.0) and phosphate buffer (50 mM, pH 2.0) were prepared by mixing proper volumes of sodium acetate (50 mM) and acetic acid (50 mM) and proper volumes of sodium dihydrogen phosphate (50 mM) and phosphoric acid (50 mM), yielding pH of 4.0 and 2.0, respectively. A solution of 1-PyBA was prepared in ethanol (50 mM). Glyoxylic acid was prepared in ethanol (10 mM) and further diluted with the same solvent to obtain the working solutions. Taurine was prepared in 70 % aqueous ethanol (10 mM). Glyoxylic acid and reagent solutions were found to be stable for at least one month when kept in the refrigerator.

Instrumentation and HPLC conditions

Separation was done using a Shimadzu HPLC system (Kyoto) consisted of a Shimadzu LC-10AS pump, a Shimadzu RF-10AXL fluorescence detector, Chromato-PRO recorder, and a Rheodyne injector valve with 20 μL sample loop. Separation was performed on a Cosmosil 5C$_{18}$-MS-II column (4.6 mm i.d. × 250 mm, 5 μm particle size) from Nacalai Tesque Inc. (Kyoto), by isocratic elution with mobile phase composed of acetate buffer (50 mM, pH 4.0): acetonitrile (65:35, v/v%) at a flow rate of 1.0 mL/min. Gradient elution was carried out in the case of analysis of SPE non-treated samples. The gradient system included using the above mentioned mobile phase as A and 100 % acetonitrile as mobile phase B. The % of A was kept

as 100 % for 5 min, then decreased gradually to 0 % A and 100 % B in 0.5 min, then kept at 100 % B till 15 min. After that, re-equilibration of the column was carried out with A for 5 min. The fluorescence detection was carried out at 335 and 381 nm as excitation and emission wavelengths, respectively.

The pH measurement and sonication were carried out using a 9615-10D Horiba pH meter (Kyoto) and an SND US-102 Sonicator (Nagano, Japan), respectively. SPE was accomplished using Bond Elut SAX anion exchange sorbent (500 mg, 3 mL) from Agilent Technologies (CA, USA).

Derivatization procedure of standard glyoxylic acid

Aliquots of 100 µL glyoxylic acid were mixed with 50 µL of 1-PyBA (50 mM) and 50 µL of taurine (10 mM) in a screw-capped vial with polypropylene sticking packed cap, then it was heated at 100 °C for 60 min. After cooling, 300 µL of phosphate buffer (50 mM, pH 2.0) was added and the solution was 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. A reagent blank was prepared simultaneously in the same way without using glyoxylic acid.

Procedure for solid-phase extraction

A Bond Elut SAX sorbent was conditioned with 5 mL of acetonitrile followed by 5 mL of phosphate buffer (50 mM, pH 2.0). Then, 400 µL of derivatized glyoxylic acid sample was loaded into the SPE cartridge. Washing was performed with 10 mL chloroform followed by 5 mL of phosphate buffer (50 mM, pH 2.0) then the reaction product was eluted with 400 µL of NaOH (1.0 M). Twenty µL of the eluent was injected into the HPLC-FL system.

Serum sample analysis

A volume of 50 µL of human serum was deproteinized using 950 µL of ethanol. One hundred µL of the supernatant was mixed with 50 µL of 1-PyBA (50 mM) and 50 µL of taurine (10 mM). The mixture was heated at 100 °C for 60 min, then 300 µL of phosphate buffer (50 mM, pH 2.0) was added and the solution was filtered with cellulose acetate membrane filter (0.45 µm). Then 400 µL of the derivatized serum sample was loaded into the cartridge and the SPE procedure was continued as mentioned above. Then, 20 µL of the eluent was injected into the HPLC-FL system.

Method Validation

For method validation study, we followed the FDA guidelines for Bioanalytical Method Validation [22]. Calibration curves, limit of quantification (LOQ), accuracy and precision were carefully studied.

Construction of calibration curves was done using serum samples spiked with glyoxylic acid giving a final injected concentrations covering the range of (0.1-5.0 µM), following the procedure described under "*Serum sample analysis*", then plotting the peak area *versus* glyoxylic acid concentration (µM). The lower limit of detection (LOD) was calculated at a signal to noise ratio (S/N) = 3.

The accuracy and precision of the method were investigated by analysis of serum samples spiked with glyoxylic acid to yield final concentrations of 0.1, 0.5 and 2.0 µM. Three repetitive measurements were done at each concentration in the same day and on three successive days. The average concentration was calculated and the deviation of the average from the actual concentration denotes the accuracy. The %RSD of the concentrations calculated from three repetitive measurements in the same day expresses the intra-day precision, and

the %RSD of the concentrations calculated for three replicate measurements on three successive days represents the inter-day precision.[22]

In addition, recovery study was conducted to evaluate the efficiency of the SPE extraction method by comparing the peak areas of glyoxylic acid extracted from spiked serum samples and those of glyoxylic acid standard.

Results and Discussion

Due to their high selectivity and sensitivity, fluorescence detection methods have been widely developed and used for the analysis of different kinds of targeted analytes either environmental or biological ones. The majority of targeted analytes are not fluorescence in nature, hence derivatization with fluorogenic and fluorescent labeling reagents is mandatory in this case. Fluorescence labeling reagents are more common in use as they are more easy and straightforward in their design than fluorogenic ones as they can be easily created by combining fluorescent and tagging moieties together. However, they have undesirable properties such as interferences emerged from the fluorescence of these reagents [23]. Separation techniques, such as HPLC, partially solved this problem, however, these reagents still results in crowded chromatograms with many possible interfering peaks that could cause analytical errors and poor selectivity [4,24]. Hence, we designed a novel dual labeling technique that is capable of introducing a purification tag together with the fluorescence labeling reagent to the analyte. Thus converting it into a fluorogenic one *via* the removal of the interfering blank peaks after simple sample treatment selective for the tagging reagent. Herein we discuss the application of this new approach for determination of glyoxylic acid in serum as a model analyte using 1-PyBA as a model for fluorescence labeling reagent via the use of Petasis multi-component reaction.

Optimization of derivatization conditions in Petasis reaction

The Petasis reaction is a multi-component one that depends on the reaction of a carbonyl compound with amine and aryl boronic acid to form a substituted amine. Hence, for method optimization, different parameters contributing to the reaction efficiency were carefully studied.

First, different amines were studied in order to select the best one to perform a dual role; a purification tag and a reactant in Petasis reaction at the same time. For this reason, this study focused on amines having an acidic functional group that can interact with anion exchange sorbent including; taurine (having a sulfonic acid group), 6-aminocaproic acid, alanine, glycine (having a carboxyl group), 2-aminoethyl phosphonic acid (having a phosphoric acid group). Among these amines, only taurine and 6-aminocaproic acid reacted to give good fluorescent derivatives of approximately the same peak areas (Fig 3a). However, taurine was selected as the optimum amine for further experiments since the interaction of its sulfonic acid group with anion exchange sorbent is stronger than that of the carboxyl group of 6-aminocaproic acid. Studying the influence of taurine concentration on the peak area of the formed fluorescent product (1.0-100.0 mM) showed that the highest and constant peak area was obtained using 10.0 mM taurine and a further increase of its concentration did not produce additional enhancement (Fig 3b). So, 10.0 mM of taurine was used in this study. Taurine is practically insoluble in absolute ethanol, hence we conducted a study to choose the best % of aqueous ethanol (60-90%) that could be used as a solvent yielding highest fluorescence. The best one was 70 % aqueous ethanol.

The effect of the fluorescent derivatizing reagent 1-PyBA concentration was also studied over the range of 1.0-200.0 mM. A maximum peak area was obtained using 50.0 mM or more (Fig 3c). Hence, 50.0 mM of 1-PyBA was chosen as the optimum concentration in this study.

The influence of the solvent in which the reaction is carried out was investigated using ethanol, methanol, 2-propanol, 1,4-dioxane, DMF, DMSO, and THF. The maximum peak area was achieved using ethanol (Fig 3d).

The reaction temperature and time are also important factors that affect the reaction yield. Studying the reaction temperature (25-110 °C) revealed that the highest peak area was obtained at 100-110 °C (Fig 3e). Then, the reaction time was studied and the highest peak area was achieved after 60 min and no further increase was obtained after longer heating time (Fig 3f). So, the reaction was performed at 100 °C for 60 min.

Selectivity of Petasis reaction under the optimized towards glyoxylic aid

To assess the selectivity of Petasis reaction towards glyoxylic aid, we tried different types of α-hydroxy aldehydes (including reducing sugars such as glucose, fructose, and galactose) and α-keto acid (including pyruvic acid, phenyl pyruvic acid, and glyoxylic acid) as analytes. Only glyoxylic acid gave a new fluorescent peak beside the blank reagent ones. This clearly proves the selectivity of the proposed method for glyoxylic acid.

Chromatogram illustrated in Fig 4a was obtained under the optimum derivatization conditions for standard glyoxylic acid without the SPE cleanup. Although the peak of the glyoxylic acid derivative can be clearly detected at a retention time of 3.5 min, blank peaks derived from the fluorescent reagent 1-PyBA and its fluorescent decomposition products are detected in the chromatogram and it took about 15 min using a gradient elution mode to elute all of the blank peaks. Therefore, we conducted an SPE procedure to remove unnecessary peaks prior injection to the HPLC-FL system.

Solid-phase extraction method

For the purpose of sample clean up to remove excess fluorescent reagent and its decomposition products, SPE method was developed depending on the purification tag introduced to the fluorescent reaction product. A strong anion exchange sorbent Bond Elut SAX was chosen in this study to interact efficiently with the sulfonic acid group in the fluorescent reaction product. Excess 1-PyBA and its decomposition products do not possess sulfonic acid groups, so they did not interact with the anion exchange sorbent and can be washed out easily (Fig 2).

Under the used SPE conditions described in the experimental section, a clear chromatogram of glyoxylic acid derivative free from peaks of reagent and its decomposition products can be obtained using a simple isocratic elution within 4 min only (Fig 4b). Moreover, when this protocol was applied to human serum samples a clear chromatogram for glyoxylic acid was obtained without any interference from the endogenous component (Fig. 4c).

Thus, it is obvious that the dual labeling technique combined with a proper SPE can significantly diminish the interfering peaks from excess fluorescence labeling reagent and its decomposition products as well as interfering peaks from endogenous components, and hence improves the selectivity of the method and shortens the analysis time. Using this technique we can say that the fluorescence labeling reagent, 1-PyBA, was changed into a fluorogenic one *via* eliminating the interfering blank peaks.

Results of validation in serum

A good linear correlation was achieved between glyoxylic acid concentration in human serum and peak area over a final concentration range of 0.1-5.0 μM with a correlation

coefficient of 0.998 (Fig. S1, supplementary file) according to the following regression equation (mean ± SD, *n = 3)*:

$Y = (1.35\times10^{6}\pm0.11\times10^{6})\ X + (1.6x10^{4}\pm0.6x10^{4})$

Y = the average peak area and X = glyoxylic acid concentration (µM).

The LOD was also calculated at S/N = 3 and found to be a final concentration of 30 nM (600 fmol/injection) and LOQ, at S/N = 10, was 100 nM.

Table 1 lists the accuracy and precision results of the proposed method. The accuracy ranged from -2.2 to +9.2%. Meanwhile, the precision represents as %RSD was ≤ 8.7%. These results indicate that the accuracy and precision of the proposed method are in accordance with the FDA guidelines for Bioanalytical method validation[22].

As well, the results of the recovery study indicated the efficiency of the developed SPE method as indicated by the good average %recovery (87.0%).

The excellent selectivity owing to the high efficiency of purification tag/fluorophore dual labeling-SPE technique and rapidness gained by the short runtime (< 4 min) made the proposed HPLC-FL method well-suited for determination of glyoxylic acid in serum. Thus, the proposed dual labeling method was applied for glyoxylic acid level determination in human serum (n=3) and its concentration (mean ± SE) was found to be 16.9 ± 1.4 µM which is in accordance with previous methods that reported a plasma level ranged from 1-25 µM [13,15,17].

Comparison of the developed method and the reported one for glyoxylic acid determination

Comparing the novel dual labeling technique with the previously reported methods for determination of glyoxylic acid (Table S1, supplementary file), our method is 3-650 times more sensitive than most of the previously reported ones[14–18,21] and it shows a comparable sensitivity to the GC-FID method [19]. The only method which offered higher sensitivity than

ours was the two-dimensional GC-TOF-MS[20], which is a very complicated and expensive technique and moreover, it was not applied to biological fluids but only to a bacterial extract.

Most remarkably, the dual labeling technique could detect glyoxylic acid in human serum without any interference from endogenous serum component or from the fluorescence labeling reagent itself. Moreover, the developed method used a simple one-pot reaction of benign and stable reagents; 1-PyBA [25] and taurine [26], giving a stable water-soluble fluorescent derivative with a terminal sulfonic acid group that could be easily purified using ion exchange SPE with high extraction efficiency reached 87 % and the eluate could be injected directly into the HPLC-FL system without any need for evaporation/reconstitution.

Conclusions

A novel dual labeling technique capable of converting fluorescence labeling reagents into fluorogenic ones *via* the introduction of a purification tag together with a fluorophore to the analyte was established. Using the multi-component Petasis reaction, this technique has been developed for the first time and applied for the determination of glyoxylic acid in human serum. Taurine (purification tag) and 1-PyBA (fluorophore) were simultaneously reacted with glyoxylic acid yielding a fluorescent derivative with a terminal sulfonic acid group that could be easily purified using ion exchange SPE. This new technique resulted in the exclusion of any interferences encountered from endogenous components and the fluorescent labeling reagent and its decomposition products. Thus, this newly developed approach allowed the use of a very selective and fast isocratic HPLC-FL method for determination of glyoxylic acid in human serum with high sensitivity (LOD = 30 nM, 600 fmol/injection) in a total run time not exceeding 4 min. The use of the newly developed dual labeling technique will open a window for an easier sample cleaning way and getting rid of the crowded chromatogram problem arising from the use of fluorescence labeling reagent.

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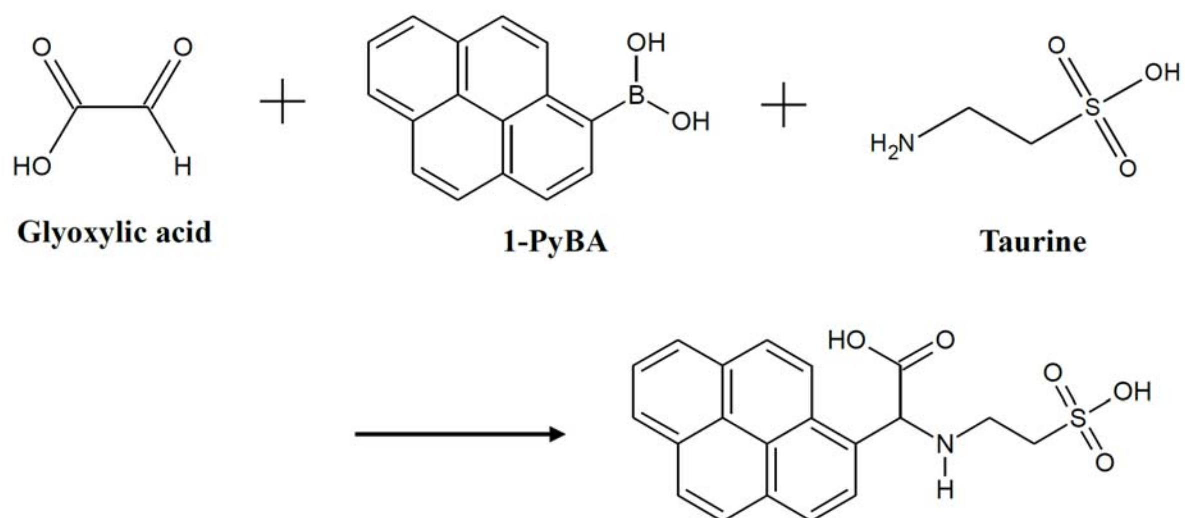


Fig. 1: Fluorescence derivatization of glyoxylic acid with 1-PyBA and taurine based on the Petasis reaction.

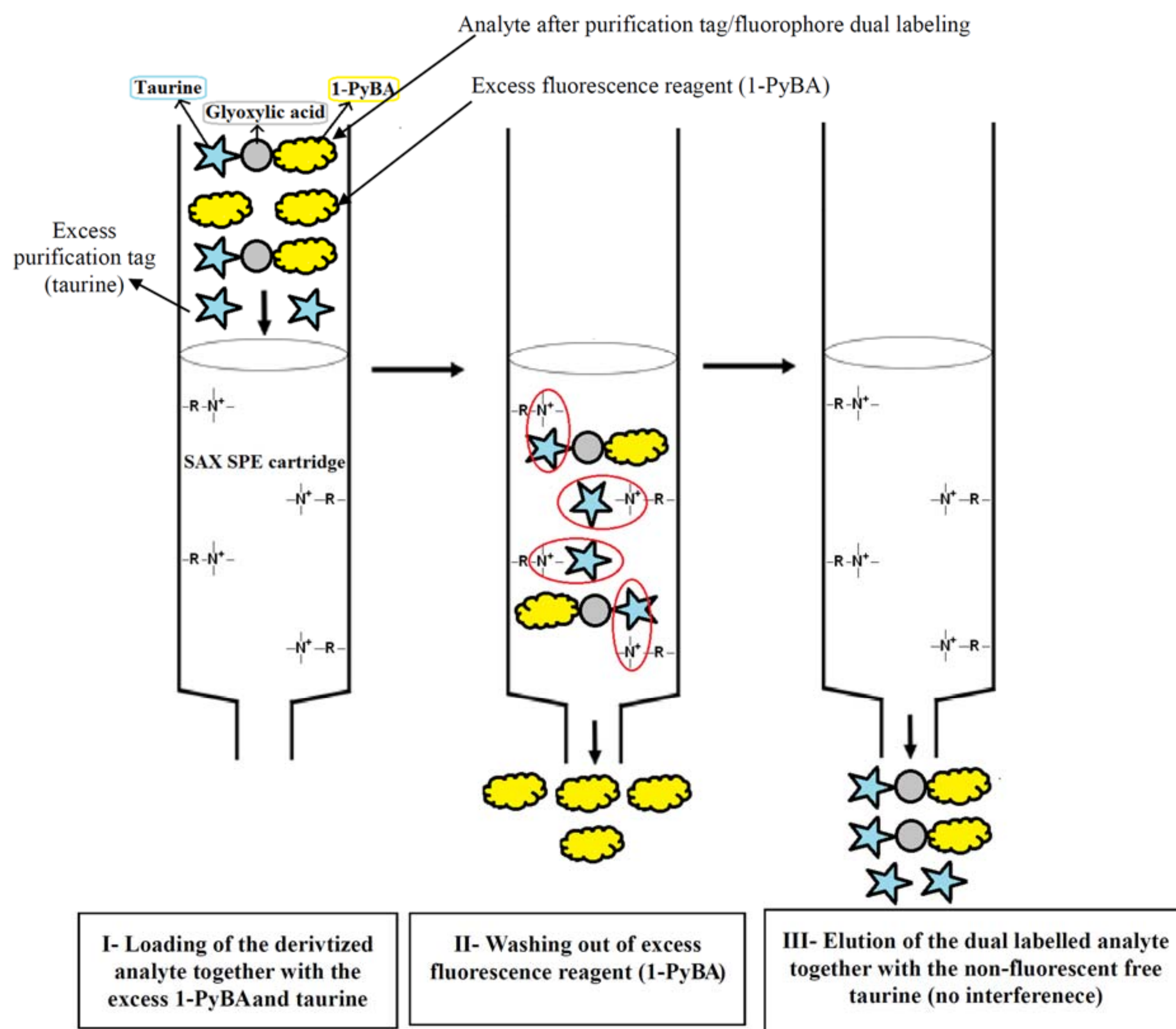


Fig.2 Separation of the derivatized analyte from excess fluorescence reagent by solid-phase extraction after purification tag/fluorophore dual labeling.

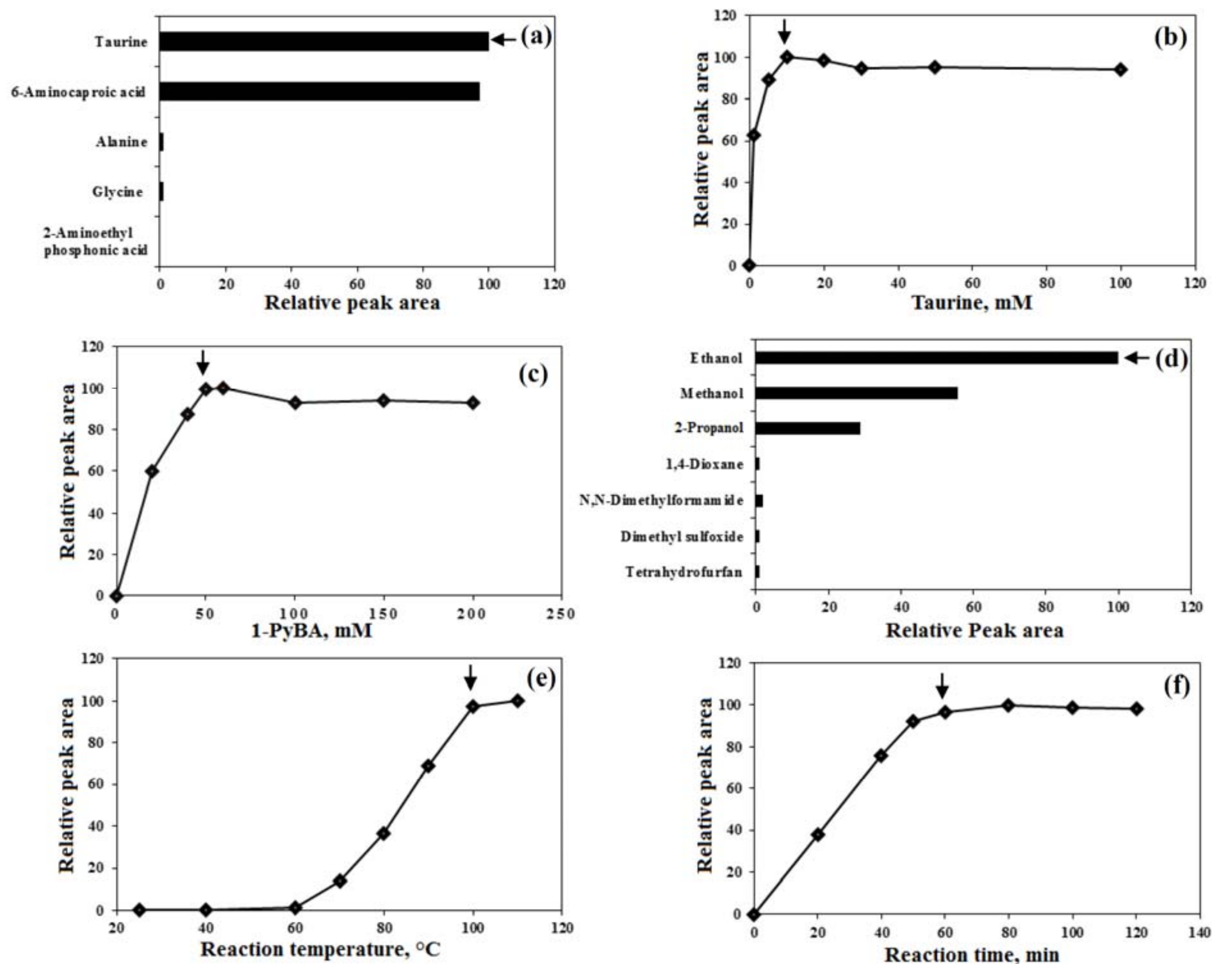


Fig. 3: Effect of different factors on the fluorescence intensity of the reaction product of glyoxylic acid (5 μM) with 1-PyBA and amine compound based on Petasis reaction. (a) effect of the type of amine compound, (b) effect of selected amine (taurine) concentration, (c) effect of concentration of 1-PyBA, (d) effect of reaction solvent, (e) effect of heating temperature, and (f) effect of reaction time.

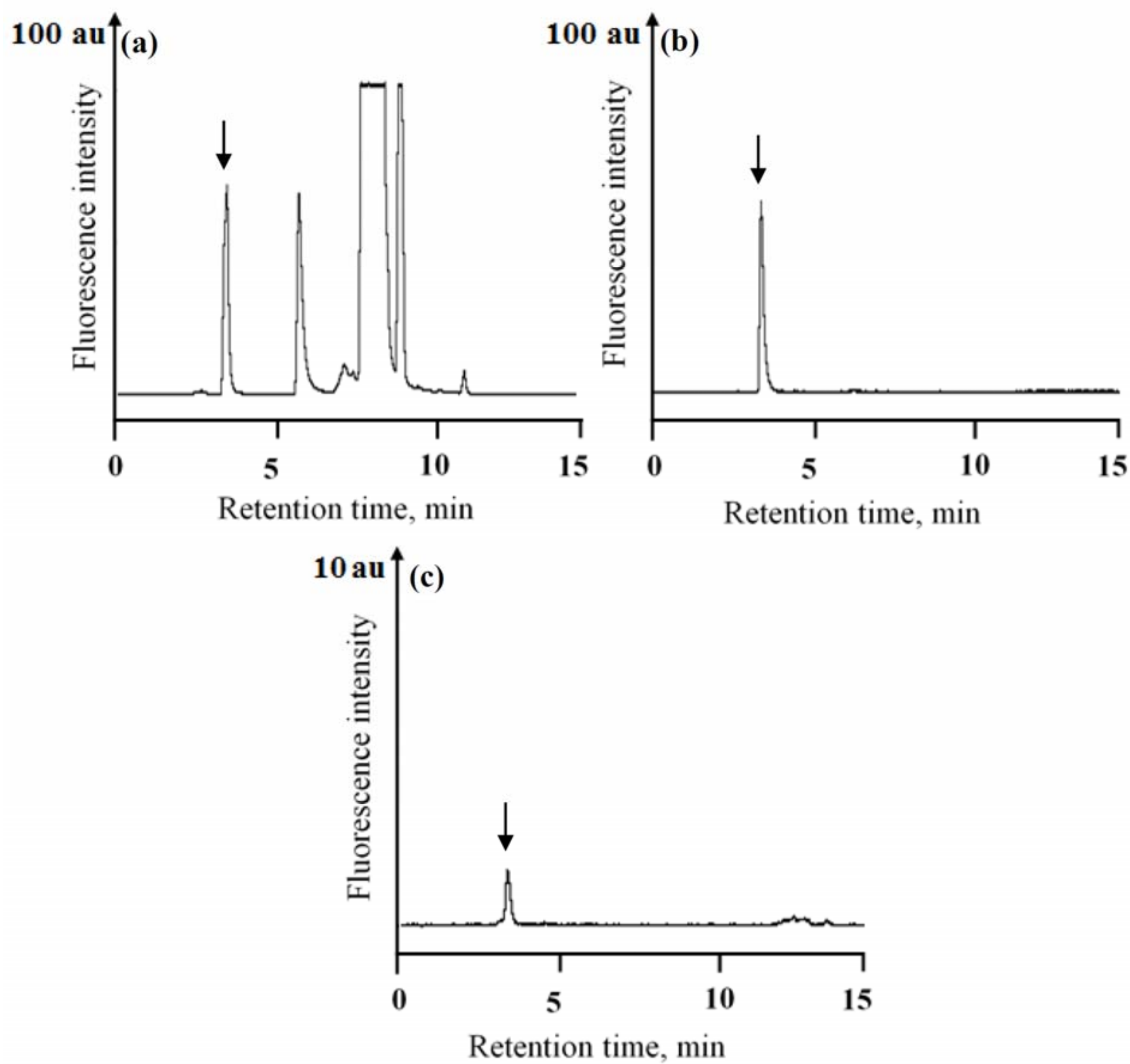
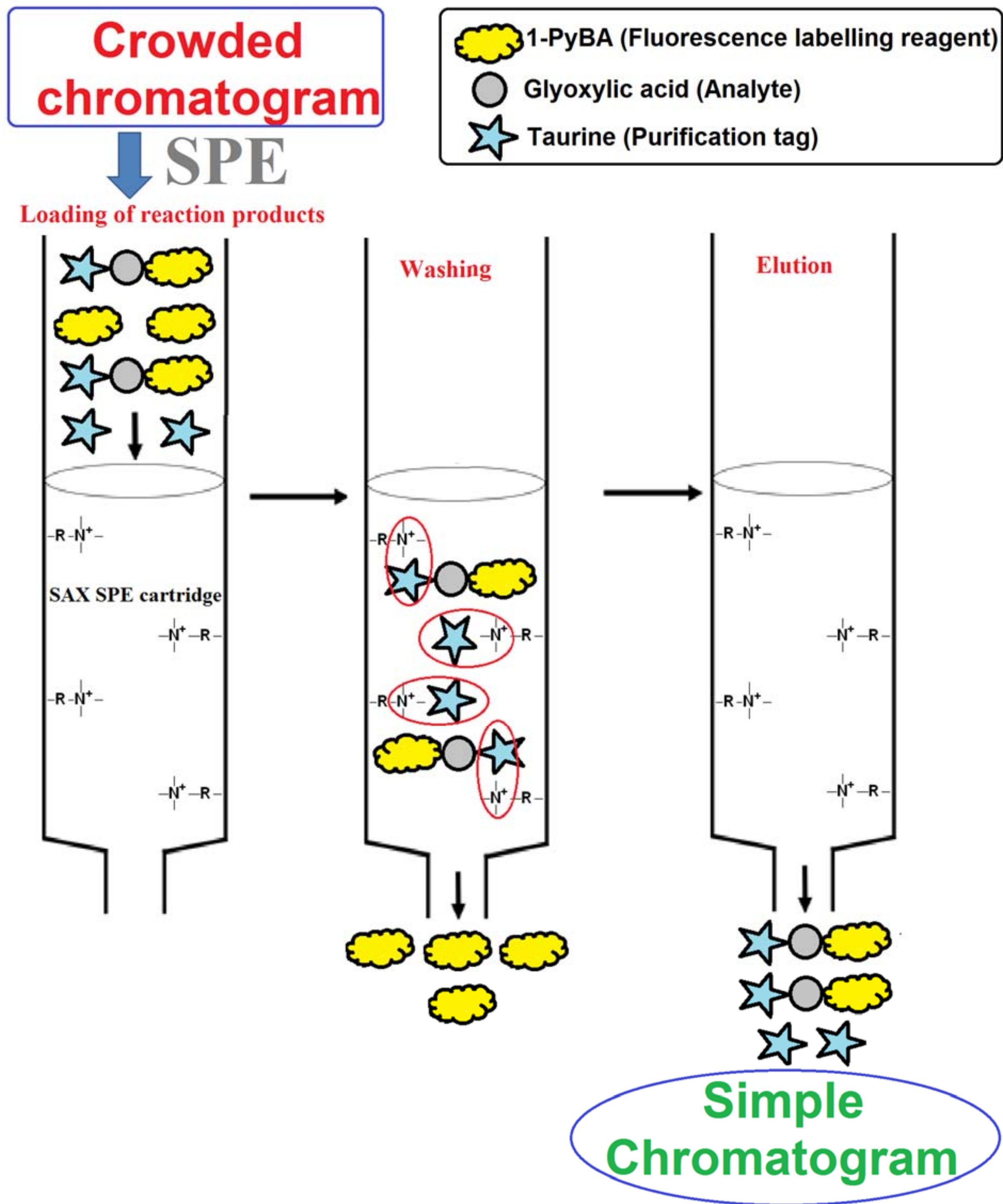


Fig. 4: Chromatograms of standard glyoxylic acid (5 μM) (a) before SPE procedure and (b) after SPE procedure. While (c) is the chromatogram of human serum sample adopting the optimized procedure. The glyoxylic acid peak is indicated by an arrow.

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

Glyoxylic acid (μM)	**Intra-day (n=3)**		**Inter-day (n=3)**	
	Accuracy (%)	**Precision (RSD, %)**	**Accuracy (%)**	**Precision (RSD, %)**
0.1	-2.2	7.8	5.3	7.2
0.5	4.3	8.7	2.5	6.5
2.0	9.2	7.3	-1.8	8.2

Graphical Abstract



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Supplementary Material

A Novel Dual Labeling Approach Enables Converting Fluorescence Labeling Reagents Into Fluorogenic Ones *Via* Introduction of Purification Tags. Application to Determination of Glyoxylic Acid in Serum.

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Table S1: Comparison of the developed purification tagging method with the previously reported ones for determination of glyoxylic acid.

Method	Reagent[a]	Matrix	Extraction method[b]/ *(Recovery)*	Run time (min)	LOD (µM)	Limitations	Ref
HPLC-FL	1-PyBA/ N-methyl-butylamine	Urine	PPT / (103%)	30	0.015	Crowded chromatogram and comparatively long run time	6
HPLC-UV	PHZ	Urine	None/ (78%)	>15	0.5	Crowded chromatogram, non-selective and low sensitivity	15
HPLC-UV	PHZ	Urine and plasma	PPT/ (104%)	20	0.1	Low sensitivity, non-selective	16
HPLC-UV	DNPH	Urine	None/ (92%)	20	6	Poor sensitivity, non-selective	17
HPLC-FL	*o*-PDA	Urine and plasma	PPT and LLE/ (95%)	>20	1	Poor sensitivity, non-selective, tedious extraction	18
HPLC-FL	DNPH & *o*-PDA	Rat liver	SPE and LLE/ (86%)	30	0.2	Double derivatization and tedious extractopn procesure	19
GC-FID	BF_3/1-butanol	Sea Water	SPE/ (31%)	>15	0.027	Low recovery	20
GC×GC–TOF-MS	(BSTFA : TMCS, 99:1) and methoxy-amine HCl	Bacterial extract	PPT/ (N/M[c])	N/M[c]	0.005	Tedious and very expensive technique	21
GC-MS	BF3/ methanol complex	Plasma	LLE/ (N/M[c])	15	19.6	Very poor sensitivity, tedious and very expensive technique	22
HPLC-FL	1-PyBA/ Taurine	Serum	PPT and SPE/ (87%)	<5	0.03	Very selective using dual labeling technique for glyoxylic acid for the first time	Proposed method

[a] PHZ: Phenylhydrazine, DNPH: 2,4-dinitrophenylhydrazine, *o*-PDA: *o*-phenylenediamine, BSTFA: N,O-bis(trimethylsilyl)trifluoroacetamide, and TMCS: trimethylchlorosilane

[b] PPT: protein precipitation and LLE: liquid-liquid extraction

[c] N/M: Not mentioned

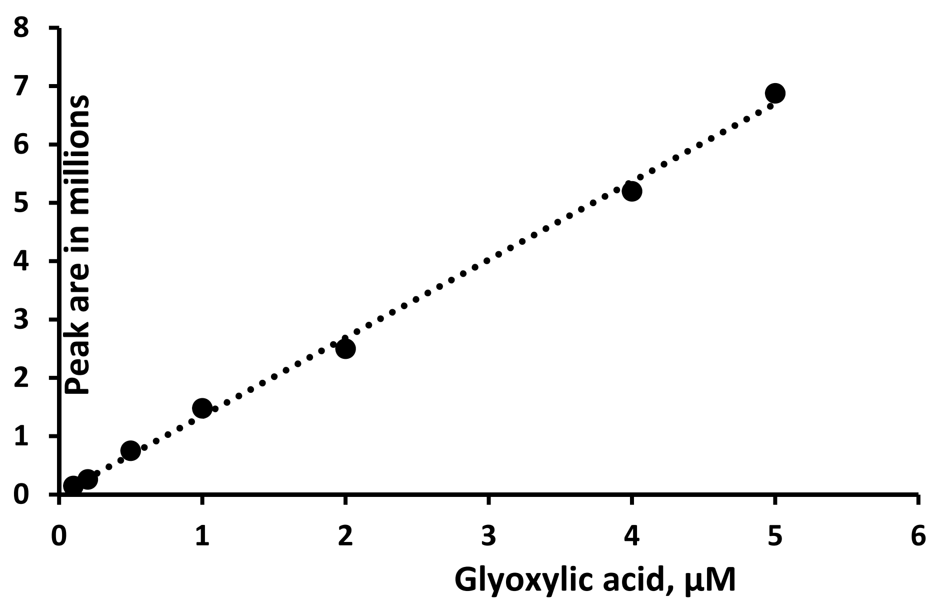

Fig. S1. Calibration curve for glyoxylic acid in spiked human serum samples