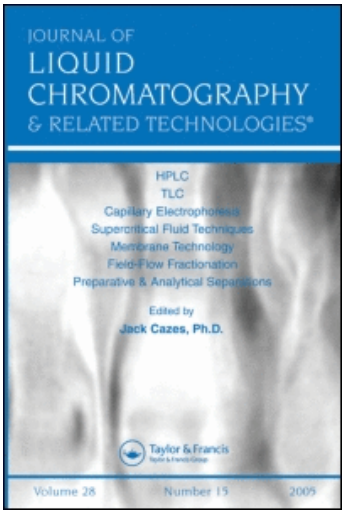

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SENSITIVE DETERMINATION OF GABAPENTIN IN HUMAN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH UV-VIS AND FLUORESCENT DETECTION

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SENSITIVE DETERMINATION OF GABAPENTIN IN HUMAN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH UV–VIS AND FLUORESCENT DETECTION

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□ *Two rapid, sensitive, and reproducible methods for the determination of gabapentin (GBP) in urine based on high-performance liquid chromatography (HPLC) with UV-Vis and fluorescent detection, respectively, were developed for the first time using a new synthesized fluorescent label, 6-oxy-(N-succinimidylacetate)-9-(2′-methoxycarbonyl) fluorescein (SAMF). The highest derivatization efficiency was obtained in borate buffer (pH 7.25) at room temperature for 10 min. The chromatographic separation was carried out using a mixture of methanol and water containing 5 mmol* l^{-1} *sodium citrate buffer (pH 5) as a mobile phase with UV-Vis detection at* $\lambda = 455$ *nm nm and fluorescent detection (FLD) at* $\lambda_{ex}/\lambda_{em} = 488/520$ *nm, respectively. A calibration curve ranging from* 1×10^{-8} *mol* l^{-1} *to* 1×10^{-6} *mol* l^{-1} *was shown to be linear. The concentration limit of detection (LOD) was* 1×10^{-8} *mol* l^{-1} *and* 2.5×10^{-10} *mol* l^{-1} *(signal to noise ratio = 3) for HPLC-UV-Vis and HPLC-FLD, respectively. The proposed methods were applied to the determination of GBP in urine samples with satisfying results.*

Keywords fluorescent detection, gabapentin, high-performance liquid chromatography, 6-oxy-(*N*-succinimidyl acetate)-9-(2′-methoxycarbonyl) fluorescein, UV-Vis detection

INTRODUCTION

Gabapentin, 1-(aminomethyl) cyclohexaneacetic acid (GBP), is a novel antiepileptic drug recently being employed for the treatment of partial seizures.[1] As shown in Figure 1A, GBP is a structural analogue of γ-aminobutyric acid (GABA), an inhibitory neurotransmitter. GBP is also found to be effective for patients who are resistant to conventional antiepileptic drugs, whereas its mechanism of action is not fully understood. There are only a few instances in literature which have reported the determination of GBP in human biological fluids.[2–4] Therefore, developing new

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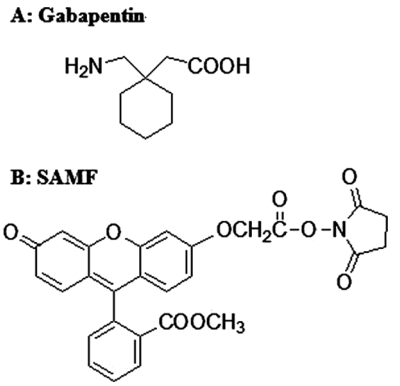

FIGURE 1 Chemical structure of (A) GBP, (B) SAMF.

analytical methods for the measurement of GBP is critically important in understanding its efficacy and action mechanism.

Several methods have been reported to monitor GBP using spectrophotometry,[5] gas chromatography (GC),[6] high-performance liquid chromatography (HPLC),[7,8] and capillary electrophoresis (CE).[9,10] Among them, HPLC with UV-Vis absorption (HPLC-UV-Vis) or fluorescent detection (HPLC-FLD) is a fast, reliable, sensitive, and most widely used method. Besides, the separation process comprised in the chromatography can effectively exclude the interference from coexisting compounds to exhibit high selectivity and sensitivity. As GBP exhibits no significant native fluorescence and has only weak absorption at $\lambda = 276$ nm,[11] it is difficult to detect it with UV-Vis or FLD with satisfied sensitivity. Consequently, chemical derivatization using chromogenic or fluorescent labels is an efficient method.

Up to now, there are several reagents reported for labeling GBP including phenylisothiocyanate (PITC),[12] 1-fluoro-2,4-dinitrobenzene (FDNB),[13] *o*-phthalaldehyde (OPA),[8,14] 4-chloro-7-nitrobenzofurazan (NBD-Cl),[15] (2-naphthoxy) acetyl chloride (NAC),[16] 9-fluorenylmethyl chloroformate (FMOC-Cl),[17] fluorescein isothiocyanate (FITC),[18] 6-carboxyfluorescein succinimidyl ester (CFSE),[2] etc. However, as PITC and FDNB exhibit no significant fluorescence, they can only been used for chromogenic label. Additionally, the derivatizing reaction of FDNB is not achieved at room temperature and the temperature should reach 65°C. As for OPA, the derivatives exhibit poor stability, which strongly affects its application. As a fluorescent label, NBD-Cl provides good stability, sensitivity, and selectivity with amino compounds; nevertheless the derivatization is usually taken within strong alkaline aqueous-organic phase system and the temperature is always about 60°C. NAC, an acetyl chloride reagent, reacts with GBP under mild conditions with rapid reaction, but the fluorescent detection is performed at short wavelength ($\lambda_{ex}/\lambda_{em} = 225/360$ nm). In this UV

district, the method is badly compromised because of the strong background interference especially when biological samples are analyzed. FMOC-Cl has been introduced to label GBP with certain merits, but in the separation the column temperature of 60°C is needed for the resolution of adducts.

FITC and CFSE are the most frequently used fluorescein-based derivatives, which have relatively high absorptivity and excellent fluorescence quantum yield. As it is well known, FITC needs tedious reaction time. Furthermore, a clean chromatogram can hardly be obtained because of the strong peaks of excessive FITC, fluorescein amines and other by-products. Using CFSE, a *N*-hydroxysuccinimidyl fluorescein active ester as a label can overcome these drawbacks, and Chang[2] introduced this application with satisfied results. However, it is worthy to note that these fluorescein-based reagents have poor photostability and pH-dependent fluorescence. In acidic medium, the fluorescence intensity was badly weakened. What is more, the HPLC mobile phase is acidic in many cases. It means that the sensitivity might be seriously weakened when these probes are used in HPLC. Herein, to develop good chromogenic and fluorescent derivatizing labels for GBP with satisfactory derivatizing and spectral characters is of great importance.

6-Oxy-(*N*-succinimidyl acetate)-9-(2′-methoxycarbonyl) fluorescein (SAMF) is a new tagging reagent which has been synthesized in our laboratory (the structural formula of SAMF is shown in Figure 1B). Unlike other fluorescein-based labels, it has good photostability and relatively pH-independent fluorescence (pH 4–9) with satisfying reactive selectivity. SAMF has been successfully used for the assay of amino acid neurotransmitters,[19] aliphatic amines,[20] etc., with HPLC-FLD or CE-LIF in our previous work. However, it has not been exploited as a reagent for GBP. On the other hand, although the sensitivity of UV-Vis absorption detection is lower than fluorescent detection, UV-Vis absorption detector is still the most common detector equipped with HPLC. So, it is also of great significance to research excellent chromogenic reagents. It is found experimentally that SAMF hydrolyzate and SAMF-GBP derivatives have strong absorption at 455 nm. Although SAMF has not been reported as UV-Vis absorption label, these experimental data demonstrates its enormous prospect in this aspect. Hence, the aim of this study was to investigate the possibilities of SAMF as a labeling reagent for GBP using HPLC-UV-Vis and HPLC-FLD, respectively. In this paper, optimization of the separation and derivatization conditions, such as reagent concentration, pH value, derivatizing temperature, and reaction time, was studied in detail. Compared with the rigid reaction conditions and interference from impurities when the reagents discussed previously were used as labeling reagents, labeling with SAMF has attractive advantages in the derivatizing reaction,

HPLC separation and detection sensitivity as well. The new methods were successfully applied to assay of GBP in urine samples.

EXPERIMENTAL

Apparatus

An LC-10AD HPLC system (Shimadzu, Japan) with an SPD-10A UV-Vis detector (Shimadzu, Japan) was used. The separation was performed on a Hypersil C_{18} column (10 μm, 250 mm × 4.6 mm i.d., Elite, Dalian, China). An Agilent 1100 HPLC system (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) with a G1321A fluorescence detector was used. The separation was performed on a Lichrosorb C_{18} column (5 μm, 250 mm × 4.6 mm i.d., Agilent, USA). The spectrum was determined using a 970CRT fluorescence spectrophotometer (Shanghai Precision & Scientific Instrument Co., Ltd., Shanghai, China) and a TU-1900 UV-Vis spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., Beijing, China).

Chemicals and Reagents

Gabapentin was obtained from Acros Organics (Gell, Belgium) and a 1×10^{-4} mol l^{-1} stock solution of GBP was prepared in water. SAMF was synthesized in our laboratory and a 1×10^{-4} mol l^{-1} solution was prepared in anhydrous acetonitrile. Methanol was purchased from Tedia Company Inc., (Fairfield, USA). All other reagents were of analytical reagent grade. Borate buffer (0.1 mol l^{-1}) was prepared by mixing certain concentrations of $Na_2B_4O_7$ or H_3BO_3 with 1 mol l^{-1} HCl or 1 mol l^{-1} NaOH to the required pH. Sodium citrate buffer (0.1 mol l^{-1}) was prepared by mixing certain concentrations of Na_3Cit_3 solution with 1 mol l^{-1} HCl or 1 mol l^{-1} NaOH solution to the required pH value. All aqueous solutions were prepared from ultrapure water purified with a Milli-Q system (Millipore, Bedford, MA, USA).

Derivatization Procedure

To a 10 ml vial containing appropriate amount of GBP and borate buffer (pH 7.25, 1 ml), SAMF (1×10^{-4} mol l^{-1}, 0.4 ml) was added. The whole solution was diluted to the mark with water and was kept at 25°C for 10 min.

Sample Preparation

Fresh urine samples were collected from volunteers. A certain amount of urine sample was deproteinized by adding methanol and was centrifuged

at 9000× g for 10 min. Before analysis, the samples were filtered through a 0.45 μm membrane filter. Pipe the processed urine of specified volume to a 10 ml vial to make a sample which can be directly used for the following derivatization as described previously.

Determination of Molar Absorption Coefficient

A series of different concentrations of SAMF hydrolyzate and SAMF-GBP derivatives were prepared according to the procedures described previously and then the spectra was determined with the UV-Vis spectrophotometer at $\lambda = 455$ nm. Molar absorption coefficient was calculated according to the formula ($A = \varepsilon bc$) and the obtained data.

Photostability Study

According to the procedure described previously, a concentration of 1×10^{-6} mol l^{-1} SAMF-GBP derivatives was prepared and kept in the dark at room temperature, 4°C, respectively, and chromatograms were recorded on aliquots taken at different storage time.

RESULTS AND DISCUSSION

Spectral Properties of SAMF and SAMF-GBP Derivatives

It has been proved that SAMF have excellent fluorescence properties, such as pH-independent fluorescence (pH 4–9), and excellent photostability.[20] For example, the fluorescence quantum yield of FITC derivatives is only 0.17 at pH 5, while the fluorescence quantum yield of SAMF derivatives reaches 0.30, which is almost double that of FITC derivatives. The fluorescence of SAMF and its derivatives also exhibit much more excellent photostablity than FITC.

In this work, we studied the UV-Vis absorption performance of SAMF in detail. It was found that SAMF hydrolyzates and SAMF-GBP derivatives had same spectra whose maximum absorption was at 455 nm (as shown in Figure 2). The molar absorptivity of SAMF hydrolyzate and SAMF-GBP derivatives were $2.67 \times 10^4 l \cdot mol^{-1} \cdot cm^{-1}$ at 455 nm, thus both compounds had relatively strong absorption in the visible district. In view of its good UV-Vis absorption spectra and derivative properties, SAMF is not only an excellent fluorescence reagent for amino compounds, but also has great potential in chromogenic label.

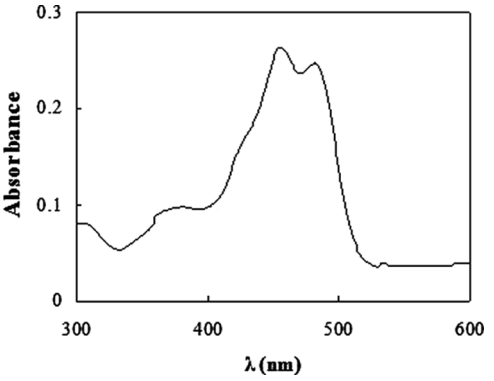

FIGURE 2 UV-Vis spectra of SAMF hydrolyzate and SAMF-GBP. Maximum absorption wavelength: 455 nm. Concentrations of SAMF: 1.2×10^{-5} mol l^{-1}.

Optimization of HPLC Separation Conditions

High-Performance Liquid Chromatography with UV-Vis Detection

The parameters affecting separation were optimized. Methanol and water were selected as mobile phase. It was found that there were several small unknown peaks except for the peak of hydrolyzate. The retention time of one unknown peak was similar to the derivative peak, which might affect the separation. Therefore, the effect of methanol content was studied in detail. As shown in Fig. 3, when methanol content was above 71%, the peaks could not be separated on the baseline. If the methanol content was lower than 70%, longer analysis time was needed. Thus, 70:30 (v/v) was employed as the optimum methanol content.

In this experiment, it was demonstrated that adding buffer solution to the mobile phase can reduce the retention time of derivatives and improve

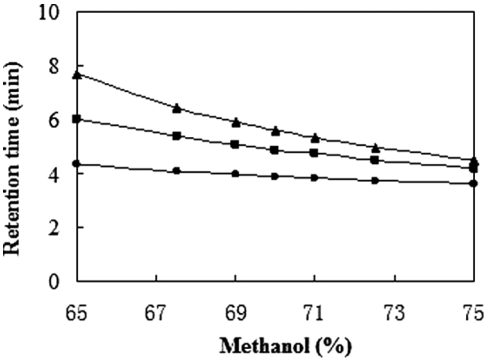

FIGURE 3 Effect of methanol content on the retention time of the derivatives with HPLC-UV-Vis. (◆) SAMF-GBP; (●) the hydrolyzate of SAMF; (■) unknown peak.

the separation. Consequently, a certain amount of citrate buffer solution was added in the mobile phase. Different pH values (4.25, 4.5, 5, 5.5, 6) of mobile phase were tested in detail. It was found that the retention time decreased with the increasing of pH. When pH was above 4.5, the retention time was almost stable and the best resolution was obtained at pH 5.0. As a result, pH 5.0 was employed as optimum.

The effect of citrate buffer concentration on the separation was also examined. It was demonstrated that buffer concentration had slight influence on the resolution. $5\,\text{mmol}\,\text{l}^{-1}$ sodium citrate buffer was selected for further investigation of separation efficiency.

The flow rate was also studied in detail. Increasing the flow rate led to shorter retention time, but the column pressure was too large to have good separation when the flow rate was above $1.1\,\text{ml}\,\text{min}^{-1}$. $1.1\,\text{ml}\,\text{min}^{-1}$ was employed as the optimum flow rate.

In conclusion, the best separations of HPLC-UV-Vis were achieved with methanol-water (70:30, v/v) containing $5\,\text{mmol}\,\text{l}^{-1}$ sodium citrate buffer (pH 5). The flow rate was $1.1\,\text{ml}\,\text{min}^{-1}$. The UV-Vis detection was performed at $\lambda = 455\,\text{nm}$.

High-Performance Liquid Chromatography with Fluorescent Detection

As the UV-Vis detector and fluorescence detector were connected to different HPLC equipments, which had great effect on separation, the separation conditions of HPLC-FLD were also optimized. The final option of the mobile phase was methanol-water (78:22, v/v) containing $5\,\text{mmol}\,\text{l}^{-1}$ sodium citrate buffer (pH 5), and the flow rate was $1.0\,\text{ml}\,\text{min}^{-1}$. The fluorescent detection was performed at $\lambda_{ex}/\lambda_{em} = 488/520\,\text{nm}$.

Optimization of Derivatization Conditions

Derivatization conditions such as reagent concentration, reaction pH, reaction temperature, and time, play important roles in detection sensitivity and quantitative research. So, these conditions were optimized carefully.

For succinimidyl ester labeling, the derivatizing reaction is often completed in aqueous phase. Under the circumstances, there is a competition between the derivatizing and the hydrolysis. To ensure the reproducibility and efficiency of derivatization, an excess of labeling reagent should be used for quantitative analysis of derivatives. Simultaneously, too much reagent leads to a strong peak of the hydrolyzates and interferes with the determination. The influence of the SAMF amount on the derivatization was tested. When the concentration of reagent was in the range of $7–9\,\mu\text{mol}\,\text{l}^{-1}$, the peak area of the SAMF-GBP derivative was highest and

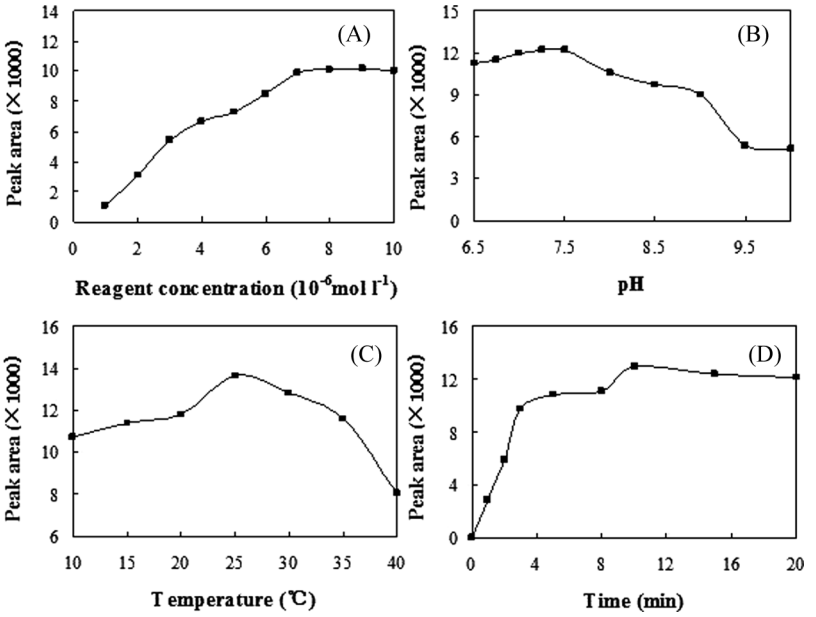

FIGURE 4 Effect of (A) SAMF concentration, (B) buffer pH, (C) reaction temperature, and (D) reaction time on the peak areas of the GBP derivatives. (■) SAMF-GBP.

stable (Figure 4A). As a result, 8 μmol l^{-1} was selected as the optimal concentration.

The influence of diverse pH values on the peak areas was also researched using borate buffer. For succinimidyl ester labeling, the reaction is usually performed in basic medium. It might be due to that in acidic conditions the amino protonation of GBP reduced the derivative efficiency. The pH value (6.5–10) was studied in detail and the results are illustrated in Figure 4B. It was found that the peak area of the derivative reached maximum at pH 7.25. When pH value was above 7.5, the derivatization yield decreased, obviously. This was in part ascribed to the instability of GBP in strong alkaline conditions. Thus, pH 7.25 was used in this work.

The effect of temperature on peak area was investigated in the range of 10 to 40°C (Figure 4C). It was found that temperature made great influence on derivatizing reaction. Generally, high temperature could accelerate the derivatization reaction as well as the hydrolysis of the reagent. It was demonstrated that the peak area of the derivative reached a maximum at 25°C. Hence, 25°C was selected for derivatization.

At 25°C, the effect of the reaction time, varying from 1 to 20 min was also exploited. From the kinetics curves shown in Figure 4D, it was clear that the reaction was completed and peak area reached the highest in 10 min. So, 10 min was chosen as derivatization time. It is well-known that

FITC is one of the most frequently used fluorescein derivatives, which usually requires more than 12 h at room temperature to complete the label process.[21] As a contrast, the derivatization reaction of SAMF with GBP is very rapid and possesses desirable labeling chemistry.

To sum up, 8 $\mu mol\,l^{-1}$ was chosen as the optima reagent concentration. The derivatizing reaction was performed at room temperature (25°C) for 10 min in borate buffer (pH 7.25) in the experiment.

Interference

As other amino compounds such as amino acids, aliphatic amines, also react with SAMF, their existence may interfere with the analysis of GBP. In order to verify that this method has good selectivity toward our target compound, we researched the interference under the optimized separation conditions. Although GBP is similar to GABA in structure, it was found that the peaks of GABA derivatives and the hydrolyzates overlapped in the chromatogram. Therefore, the existence of GABA has no interference with the determination of GBP. A mixture of common amino acids and aliphatic amines was also analyzed under the optimized separation conditions. It was discovered that the retention time of these compounds were markedly different from that of GBP. Therefore, these substances mentioned previously do not make any influence on the analysis. On account that active ester exhibits low reaction activity toward aromatic amines and alcohols,[22] this two kinds of compounds have no interference in the determination of GBP.

Stability Studies

For the chromogenic and fluorescent labeling of trace substances, the good stability of reagents is essential for high-sensitivity detection. To ensure the highest derivative yield, an excess of labeling reagent should be used during the reaction process. However, if the reagent stability is poor, the strong baseline noise can not be ignored in comparison with the signal of target molecules. What is more important is that the existence of decomposition products might seriously interfere with the separation and resulting detection sensitivity. Accordingly, the stability of SAMF-GBP derivatives was studied particularly. It was found that the GBP derivatives were stable for at least 3 days when stored at room temperature in the dark, as decrease in the peak area of SAMF-GBP was only 3.1%. If lower storage temperature was chosen such as 4°C, the stability time was at least 25 days, and the decrease in the peak area was 4.5%. All these data suggest that

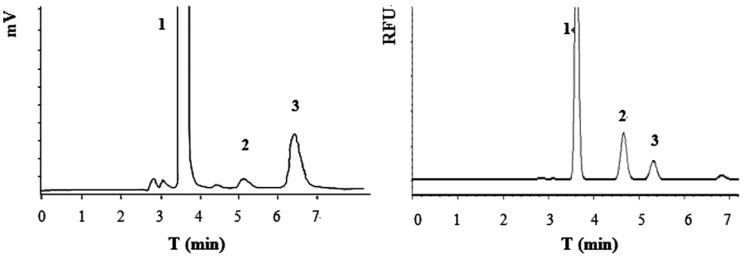

FIGURE 5 Typical chromatograms of SAMF and GBP derivatives. (A) HPLC-UV–Vis (B) HPLC-FLD. (A) Peaks: 1, the hydrolyzate of SAMF; 2, unknown peak; 3, SAMF-GAP. Mobile phase: methanol-water (70:30, v/v) containing 5 mmol l^{-1} sodium citrate buffer (pH 5); UV–Vis detection at 455 nm. Concentrations of GBP: 1×10^{-6} mol l^{-1}. (B) Mobile phase: methanol–water (78:22, v/v) containing 5 mmol l^{-1} sodium citrate buffer (pH 5); Fluorescence detection at $\lambda_{ex}/\lambda_{em} = 488/520$ nm. Peaks and other conditions are same as (A).

SAMF and its GBP derivatives have excellent stability characteristic, which is much advantageous to detect GBP with satisfied sensitivity.

Linear Range, Reproducibility, and LODs

The standard chromatograms (Figure 5) were obtained under the optimized derivatization and separation conditions. As illustrated in Figure 5, the SAMF derivatives and the hydrolysis were baseline separated both within 7 min. A comprehensive study on reproducibility, linearity, and detection limit for determination of GBP has been performed. Reproducibility was evaluated by repeating five consecutive runs of the derived standard GBP solution for the peak area and retention time. As showed in Table 1, the RSD (relative standard deviation) for the SAMF-GBP derivatives indicated excellent reproducibility.

Under the optimized conditions, quantitative analysis of GBP was preformed by derivatizing various amounts of standard GBP solutions.

TABLE 1 Calibration Range, Regression Equations, Correlation Coefficients, RSDs, and Detection Limits

Detection Method	Calibration Range (n mol l^{-1})	Regression Equation[a]	r	RSD (%, n = 5)[b]		LOD (nmol l^{-1})
				PA	RT	
UV-Vis	10–1000	$y = 168.63 \times 10^8 x + 351.51$	0.9992	2.40	3.30	10.00
FLD	10–1000	$y = 1.4823 \times 10^8 x + 2.1222$	0.9994	0.80	1.60	0.25

[a]x is the concentration of GBP (mol l^{-1}) and y is the peak area of GBP derivatives in the chromatogram.

[b]PA is the peak area and RT the retention time.

The linearity, correlation coefficients, and lowest detect limits of the method were shown in Table 1. The lowest detect limits were obtained on the basis of the minimum analyte concentration, which provided a peak three times higher than the baseline noise. The detection limits for the labeled GBP was 1×10^{-8} mol l^{-1} with HPLC-UV-Vis and was 2.5×10^{-10} mol l^{-1} with HPLC-FLD.

Sample Analysis

The developed method was also applied to the determination of GBP in urine samples. To exclude the native interference of these samples, the fluorescence of urine samples was examined. It was demonstrated that they show no native fluorescence at $\lambda_{ex}/\lambda_{em} = 488/520$ nm and no UV-Vis absorption was detected at $\lambda = 455$ nm. Sample chromatograms were shown in Figures 6 and 7. The analytical results were summarized in Table 2. The recoveries range from 95.7 to 102.3% and the RSDs range from 0.2 to 4.5%. The experimental results show that two methods could be applied to the analysis of GBP in urine.

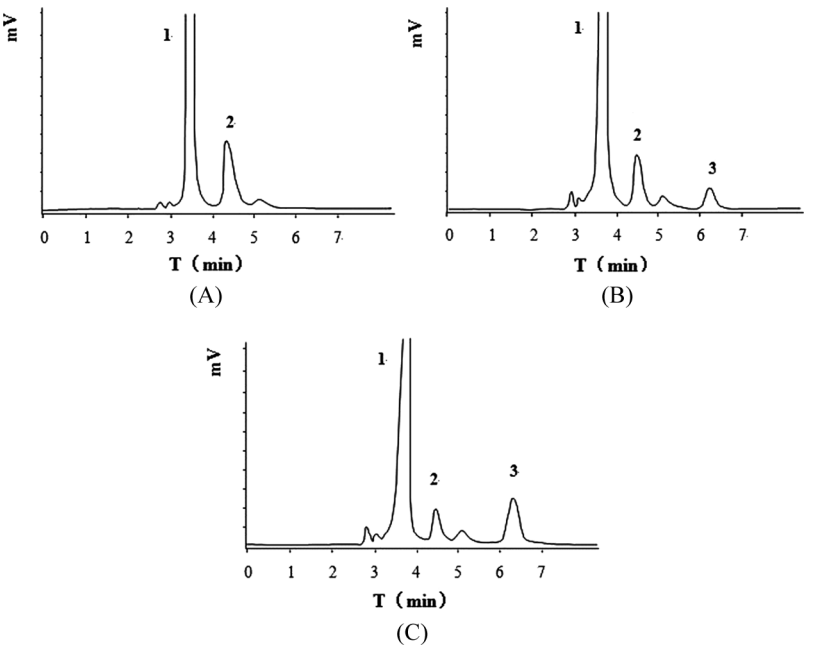

FIGURE 6 Chromatograms obtained from urine samples with HPLC-UV-Vis. (A) urine sample, and (B) and (C) urine sample spiked with GBP standard. Other conditions are same as in Figure 5A.

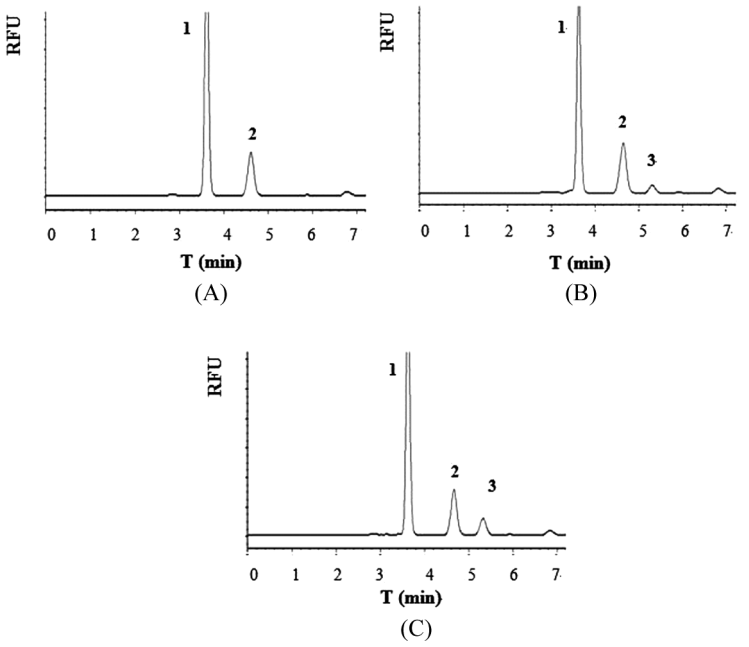

FIGURE 7 Chromatograms obtained from urine samples with HPLC-FLD. (A) urine sample, and (B) and (C) urine sample spiked with GBP standard. Other conditions are same as in Figure 5B.

Comparison with Other Methods

The utility of the method can be assessed by several factors: (1) derivatization conditions such as reaction temperature and time, (2) separation and detection conditions in terms of interference, detection wavelength, and so on, and (3) detection limits. Table 3 shows a summary of the comparison of the methods presented here with other reported approaches.

TABLE 2 Recoveries and RSDs of the SAMF Derivatives in Urine Samples

				RSD (%, n = 5)[a]	
Method	Added ($mol l^{-1}$)	Found ($mol l^{-1}$)	Recovery (%)	PA	RT
HPLC-UV-Vis	0	0		0	0
	8.0×10^{-7}	7.7×10^{-7}	96.0	2.5	3.2
	4.0×10^{-7}	3.8×10^{-7}	95.7	4.1	4.5
HPLC-FLD	0	0		0	0
	8.0×10^{-7}	8.2×10^{-7}	102.3	0.2	0.4
	4.0×10^{-7}	3.9×10^{-7}	98.5	2	0.3

[a]PA is the peak area and RT is the retention time.

TABLE 3 Comparison of These Methods with Other Techniques

Derivative Reagents	Methods	Detection Wavelength (nm)	Reaction Time (min)	Reaction Temperature (°C)	LOD ($nmol l^{-1}$)	References
PITC	HPLC-UV	254	20	25	175	[12]
OAC	CZE-UV	300	10	30	5000	[9]
FDNB	HPLC-UV	360	10	65	58.48	[13]
NQS	HPLC-UV-Vis	458	20	60	300	[23]
OPA	HPLC-FLD	$\lambda_{ex}/\lambda_{em} = 230/420$	<1	–	300	[14]
OPA-MPA[a]	HPLC-FLD	$\lambda_{ex}/\lambda_{em} = 330/440$	5	25	3000	[24]
FMOC-Cl	HPLC-FLD	$\lambda_{ex}/\lambda_{em} = 260/315$	10	60	58.48	[15]
NAC	HPLC-FLD	$\lambda_{ex}/\lambda_{em} = 225/360$	15	30	0.23	[16]
NBD-Cl	HPLC-FLD	$\lambda_{ex}/\lambda_{em} = 470/537$	10	60	1.75	[17]
FITC	CE-LIF	$\lambda_{ex}/\lambda_{em} = 488/520$	14 h	25	1	[18]
CFSE	CE-LIF	$\lambda_{ex}/\lambda_{em} = 488/520$	30	–	60	[2]
SAMF	HPLC-UV	455	10	25	10	This work
SAMF	HPLC-FLD	$\lambda_{ex}/\lambda_{em} = 488/520$	10	25	0.25	This work

[a]OPA-MPA is *o*-phthalaldehyde-3-mercaptopropionic acid.

Among these label reagents, PITC, ofloxacin acyl chloride (OAC), FNDB, and 1,2-naphthoquinone-4-sulphonic acid sodium (NQS)[23] are restricted in UV absorption detection and show no significant fluorescence signals. In addition, PITC degrades in contact with water and the extraction residue from plasma should be completely free from water before the addition of reagent. The derivatizing reaction of FDNB with GBP is not achieved at room temperature and the temperature should reach 65°C. As a fluorigenic reagent, the reaction time of OPA with GBP was less than 1 min, however, the derivatives were instable. Resultantly, automated procedure is needed and the derivative should be injected immediately after preparation. The reaction time for FMOC-Cl and GBP is about 10 min but the column temperature of 60°C is needed for separation of the adduct. In terms of OPA, FMOC-Cl, and NAC, the fluorescent detection is performed at UV district, which might introduce strong interference from biologic samples. Bahrami et al. presented a successful precolumn derivatization technique using NBD-Cl, nevertheless the derivatization is carried out at rigid conditions such as 60°C and strong alkaline conditions. FITC as a labeling reagent takes 16 h to complete the derivatization reaction and forms some by-products.

In our methods, SAMF reacts with GBP at room temperature for 10 min to produce stable derivatives. The procedure is easy to perform, and the obtained sensitivity is superior to or competitive with those reported. Compared to other labels, the proposed methods with SAMF exhibit advantages such as mild reaction conditions, rapid labeling reaction, excellent stability, pH-independent fluorescence (pH 4–9), little peak interference, and satisfactory sensitivity.

CONCLUSIONS

6-Oxy-(*N*-succinimidyl acetate)-9-(2′-methoxycarbonyl) fluorescein, a new sensitive fluorescent labeling reagent synthesized in our laboratory, was successfully used to label and determine GBP with HPLC-UV-Vis and HPLC-FLD for the first time. The derivatization was performed at room temperature for 10 min in borate buffer (pH 7.25). The derivatives of GBP were baseline separated within 7 minutes with methanol–water, containing 5 mmol l^{-1} sodium citrate buffer (pH 5) as mobile phase. The methods were applied to the determination of GBP in urine samples. Two methods are simple, rapid, sensitive, and efficient.

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