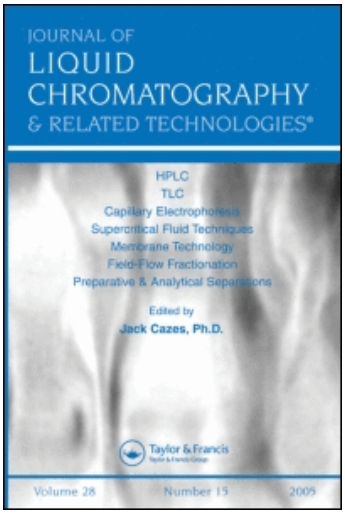

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N-Hydroxysuccinimidyl-3-indolylacetate As Pre-column Derivatizing Reagent to Separate and Determine Peptides and Their Hydrolysates by Reversed-Phase High Performance Liquid Chromatography

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N-HYDROXYSUCCINIMIDYL-3-INDOLYLACETATE AS PRE-COLUMN DERIVATIZING REAGENT TO SEPARATE AND DETERMINE PEPTIDES AND THEIR HYDROLYSATES BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

N-Hydroxysuccinimidyl-3-indolylacetate (SIIA) was firstly synthesized in a very simple route and used as a pre-column derivatizing reagent, which can react with amino compounds selectively under mild conditions. Its derivatives of peptides and their hydrolysates were separated and determined by reversed-phase high performance liquid chromatography (HPLC). In a mobile phase of methanol-water (17:83, v:v) containing 10 mmol/L citric acid-sodium phosphate buffer (pH 3.8), the derivatives of glutathione (GSSG), glycyl glycine (Gly-Gly), glutamic acid (Glu), cystine ($(Cys)_2$), and glycine (Gly) were eluted within 10 min on an ODS column. The detection wavelength was 260 nm. The detection limits were in the picomole range when the ratio of signal to noise (S/N) was 3.

INTRODUCTION

Small peptides and amino acids are the products of proteometabolism, which play an important role in life activities. And there is some association between the content of small peptides or amino acids in the body fluid and diseases. Therefore, the separation and determination of small peptides and their hydrolysates are of great importance in the study of life science. HPLC combined with sensitive detection is an effective technique in the separation and determination of these substances.[1] But for those peptides and amino acids with no or lower detective signals, chemical derivatizations of them with suitable derivatizing reagents are necessary in order to gain high sensitivity and selectivity.

So far, the derivatizing reagents used in the separation and determination of amino acids or small peptides by HPLC include *ortho*-phthalaldehyde (OPA),[2,3] naphthalene 2,3-dicarboxaldehyde (NDA),[4-6] 5-dimethylaminonaphthalene-1-sulfonyl chloride (DNS-Cl),[7,8] 4-dimethyl-aminlazobenesulfonyl chloride (DABS-Cl),[9] 9-fluorenylmethyl chloroformate (9-FMOC),[10,11] 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F),[12] phenyl-isothiocyanate (PITC),[13] and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (6-AQC),[14,15] etc.

The research on novel derivitizing reagents is still an attractive field in the separation and determination of amino acids or small peptides by HPLC. A new reagent, N-hydroxysuccinimidyl-3-indolylacetate (SIIA) with the reactive group of N-hydroxysuccinimidyl was synthesized in our lab. The advantage of this reagent is that it can react with biological activated substances containing amino groups selectively under mild conditions and can be synthesized in a very simple route with cheap materials.

In this paper, SIIA was used as the pre-column derivativing reagent in the separation and determination of small peptides and their hydrolysates by HPLC. The derivatization conditions and their chromatographic behaviours were invested. In the mobile phase consisting of methanol-water (17:83, v:v) with 10 mmol/L pH 3.8 citric acid- sodium phosphate buffer, glutathione (GSSG), glycyl glycine (Gly-Gly), glutamic acid (Glu), cystine ($(Cys)_2$), and glycine (Gly) were separated within 10 min on C_{18} column at 260 nm. The detection limits were in picomole grade at S/N=3. This method is simple, rapid and sensitive, which offer an example for the further application of these carboxy activated ester reagents in the determination of biological activated substances such as DNA, proteins etc. by HPLC.

EXPERIMENTAL

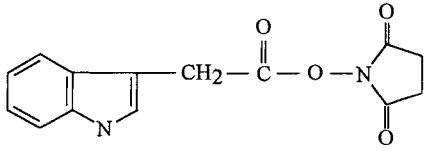
Apparatus and Reagents

An LC-10 HPLC apparatus (Japan Analytical Ind. Co.) with an S-3702 UV-VIS detector (AIC, Japan) and an ODS column (C_{18}, 10±1 μm, 150×4.6 mm i.d., Beijing Analytical Instrument Factory) were used.

SIIA was synthesized in our laboratory and its 1.845 mmol/L solution was prepared in dried acetonitrile. The standard solutions of GSSG, Gly-Gly, Glu, $(Cys)_2$, and Gly were prepared by dissolving each of them in water with the concentrations of 0.898, 1.002, 0.698, 0.416, and 1.146 mmol/L, respectively. De-ionized water was used for all solution preparation. Other reagents were of analytical reagent grade.

Synthesis of N-Hydroxysuccinimidyl-3-Indolylacetate (SIIA)

Indolylacetic acid (5 g) and N-hydroxyl succinimide (3.22 g) were dissolved in a flask containing 150 mL of tetrahydrofuran (THF), then a solution of 5.8 g dicyclo-hexylcarbodimide (DCC) in 20 mL THF was dropped slowly into it. After being stirred for 12 hr at 0°C, the mixture was filtered. SIIA residue was recovered from the reddish filtrate by the reduced-pressure distillation and recrystallized in absolute ethanol. A white crystal was obtained with an m.p. of 140°C. The results of elemental analysis of SIIA were as follows: $C_{14}H_{12}N_2O_4$ cal. C, 61.72; H, 4.44; N, 10.29%. Found C, 62.03; H, 4.39; N, 9.7%. The infrared spectrum (KBr pellet) were $\nu_{O\text{-}H}$ 3400-2400 cm^{-1}, $\nu_{N\text{-}H}$ 3495 cm^{-1}, $\nu_{C=O}$ 1760 cm^{-1}, $\nu_{C\text{-}N}$ 1000 cm^{-1}, $\nu_{C\text{-}O}$ 1100 cm^{-1}. The structure of SIIA is:



Analysis Procedure

A 5.0 mL volume of 40 mmol/L pH9.0 lithium carbonate buffer (modified with HCl) and 2.5 mL of 1.845 mmol/L SIIA solution were mixed with a known volume of the standard solution containing amino acids and small

peptides. The solution was heated in a water bath at 85°C for 5 min, and diluted to 25.00 mL with de-ionized water after being cooled to room temperature.

Before the analysis, the ODS column was pre-equilibrated with the mobile phase until the baseline was smooth. The derivatives were eluted at a flow rate of 1.0 mL/min and detected at 260 nm. The detector sensitivity was set at 0.02 AUFS. The peak areas were measured for quantitative calculations. The ODS column was employed at room temperature.

RESULTS AND DISCUSSION

Absorption Spectrum

In a basic medium, GSSG, Gly-Gly, Glu, $(Cys)_2$, and Gly can react with SIIA to form stable derivatives by heating. The maximum absorbances of them were reached at 268, 264, 255, 257 and 258 nm, respectively. A high sensitivity for each derivative was acquired at 260 nm, which was chosen as the detection wavelength.

Separation of SIIA Derivatives

The SIIA derivatives were injected into a ODS reversed-phase column and the mobile phase was methanol aqueous. The effect of methanol concentration on capacity factor (k') was shown in Figure 1.

As methanol concentration lower than 13% (v/v), it was difficult to separate the $(Cys)_2$ peak from the Gly-Gly peak. Whereas methanol concentration was higher than 19% (v/v), the peak of GSSG was overlapped with the injection peak. The separation was obtained with aqueous 17% (v/v) methanol as the mobile phase. Thus, 17% was chosen as an optimum concentration of methanol.

The effect of sodium acetate buffer and citric acid-Na_2HPO_4 buffer in the mobile phase on k' was tested. The citric acid-Na_2HPO_4 buffer is more suitable. The effect of pH on k' is shown in Figure 2. When pH of the mobile phase was between 3.6-3.9, the difference among the retention of each derivative was large enough to maintain a good separation. And pH3.8 was used as optimum pH value of the mobile phase.

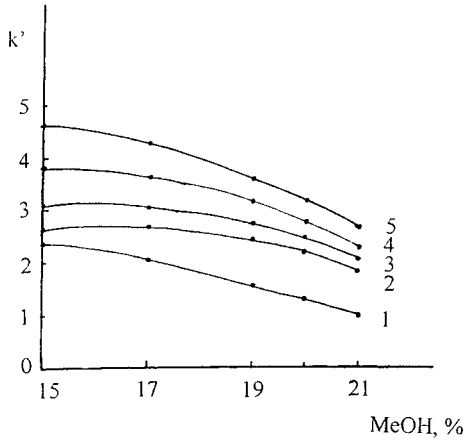


Figure 1. Effect of methamol comcemtration in mobile phase on k'. Mobile phase: 10 mmol/L, pH3.8 citric acid-sodium phosphate buffer in varible proportion methanol-water solution; Flow rate: 1.0 mL/min; Detection wavelength: 260 nm; Column: C_{18}, 10±1 μm, 150×4.6 mm i.d.; Detection sensitivity: 0.02 AUFS; Injection volume: 20 μL; Room temperature. 1. GSSG 2. $(Cys)_2$ 3. Gly-Gly 4. Gly 5. Glu.

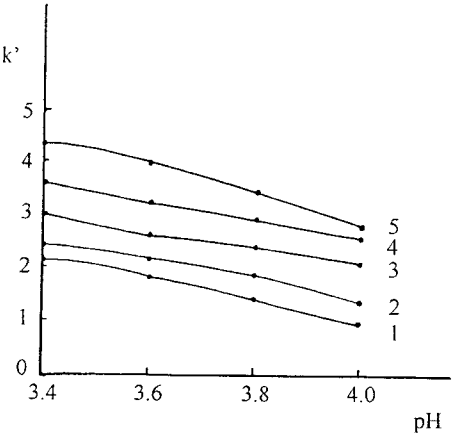


Figure 2. Effect of pH of the mobile phase on k'. Mobile phase: 10 mmol/L, citric acid-sodium phosphate buffer with different pH in methanol-water (17/83, v/v) solution; The other conditions were the same as in Figure 1. 1. GSSG 2. $(Cys)_2$ 3. Gly-Gly 4. Gly 5. Glu.

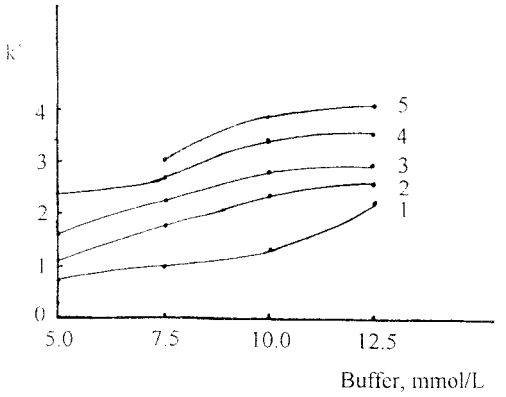


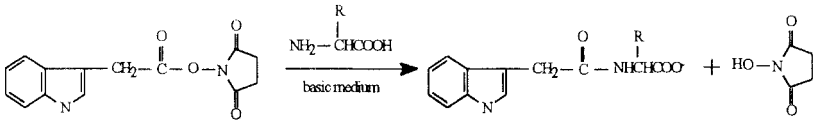
Figure 3. Effect of buffer concentration on k'. Mobile phase: pH 3.8 citric acid-sodium phosphate buffer in different concentration in methanol-water (17/83, v/v) solution; The other conditions were the same as in Figure 1. 1. GSSG 2. $(Cys)_2$ 3. Gly-Gly 4. Gly 5. Glu.

The retention time of each derivative slightly increased with an increase of buffer concentration in mobile phase. Experimental results were shown in Figure 3. When buffer concentration was higher than 12.5 mmol/L, the analysis time was longer and the peak became broader. In the range of 8-12 mmol/L, the better separation was obtained. Therefore, 10 mmol/L of the buffer was available for the separation.

A typical chromatogram was given in Figure 4 under the selected separation conditions. The five SIIA derivatives were separated in 10 min. The reproducibilities of their retention times and peak areas were very good.

Derivatization Conditions

Activated esters reagent N-hydroxysuccinimidyl-3-indolylacetate (SIIA) reacts with amino compound as below:



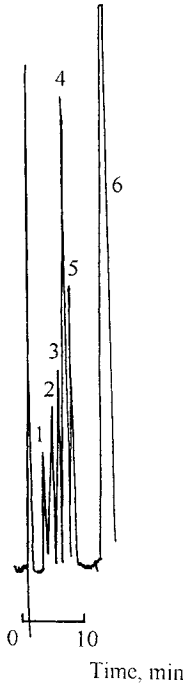


Figure 4. Typical chromatogram of five SIIA derivatives. Mobile phase: methanol-water (17/83, v/v) solution containing 10 mmol/L citric acid-sodium phosphate buffer (pH 3.8); The other conditions were the same as in Figure 1. 1. GSSG 2. $(Cys)_2$ 3. Gly-Gly 4. Gly 5. Glu 6. Reagent.

The derivatization reaction was affected by the amount of SIIS derivatization reagents, pH buffer concentration and temperature, etc. Our first experiment to test the validity of the reaction conditions described in the experimental section involved a study of peak area value as a function of derivativing reagent volume. In Figure 5, representative plots of peak value vs. derivativing reagent volume were shown. Maximum and constant peak area for the five SIIA derivatives were obtained when SIIA solution volume was greater than 2.0 mL. So a 2.5 mL SIIA acetonitrile solution was chosen.

Lithium carbonate was found to be more effective than potassium carbonate and borate buffer. Lithium carbonate buffer (modified with HCl) was used to facilitate the derivatization of GSSG, Gly-Gly, $(Cys)_2$, and Gly with SIIA. A high yield of the SIIA derivatives was achieved with the pH value increase of the derivative reaction. Whereas pH value was higher than pH10,

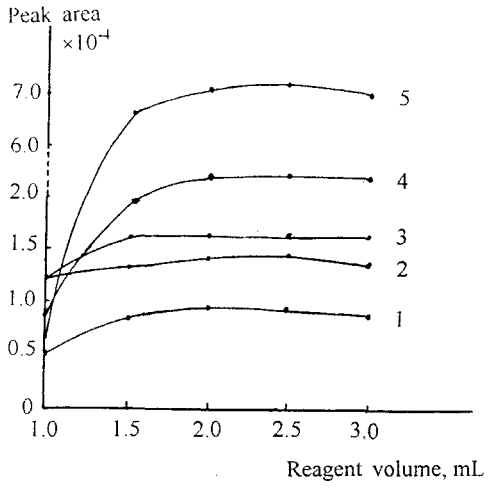


Figure 5. Effect of SIIA volume on the peak area. Mobile phase: methanol-water (17/83, v/v) solution containing 10 mmol/L citric acid-sodium phosphate buffer (pH 3.8); The other conditions were the same as in Figure 1. 1. $(Cys)_2$ 2. Glu 3. GSSG 4. Gly-Gly 5. Gly.

except that of GSSG, the yields of the other SIIA derivatives decreased. Therefore, pH9.0 was used as optimum acidity of reaction. The most intense peaks of the peptides and amino acids examined were achieved by the addition of lithium carbonate solution within 4.0-6.0 mL. 5.0 mL of the lithium carbonate solution was added to the reaction mixture.

Effect of temperature on the derivatization reaction under the conditions employed was also studied in this paper. In the range of 25-95°C, an increase of reaction temperature accelerated the reaction; 85°C was used. Maximum and constant peak areas of the corresponding peptides and amino acids were obtained at 85°C for 3-7 min. Therefore, 5 min was selected as optimum time.

Calibration Ranges and Detection Limits

The relationships between the peak areas and the amounts of the GSSG, Gly-Gly, $(Cys)_2$, Gly, and Glu were linear in the range of 3.593-25.14, 0.333-3.578, 0.802-8.016, 0.917-9.168, and 1.675-5.584 μmol/L, respectively. The absolute detection limits (S/N=3) were 1.36, 0.94, 0.68, 1.74, and 2.72 pmol, respectively.

CONCLUSIONS

SIIA has been firstly used as pre-column derivatizing reagents in the HPLC analysis of peptides and their hydrolysates. The derivatization reaction conditions are gentle and the derivatives stable. Excess derivatization reagent does not disturb the separation and determination because it is easy to separate from the derivatives. The detection limits are low and the analysis time short. The proposed reagent can be applied to the determination of amines in body fluids and biological activated substance such as DNA, proteins, etc., too. Further studies are being undertaken.

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