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Solid-support fluorescent derivatization of picomoles of protein at low concentration with FITC

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ABSTRACT

A new method based on solid-support reaction is described to realize fluorescent derivatization of proteins at concentrations as low as 10^{-8} M. A simple, low-cost homemade capillary C18 cartridge was fabricated as the solid-support reactor. Using bovine serum albumin (BSA) as a test protein, we demonstrated that the protein can be captured by this reactor and then labeled by fluorescein isothiocyanate (FITC, isomer I) on solid-support. Unwanted fluorescent intruder (excrescent FITC and products of secondary reactions) were removed from target easily. The analysis by nano-HPLC with laser-induced fluorescence (LIF) detection was described. The effect of reaction conditions on the derivatization has been evaluated and discussed. The use of the solid-support reactor allows easy handling of as little as 8.5 pmol of BSA. A fraction from weak anion-exchange chromatography (WAX) of human liver extract was used as an illustrative example of application to real samples.

1. Introduction

Proteins are basic of life and always play important roles in many physiological functions. Their analysis would lead to a better understanding of life. In many cases, the amount of protein sample is not enough for normal analysis, because some proteins of physiological importance are usually present at extremely low concentrations and large sample volumes are required, which makes their detection difficult. Nano-high performance liquid chromatography (nano-HPLC) is well suited for protein analysis at the micro-scale [1–4], which can provide higher detection sensitivity than traditional HPLC. UV absorbance is a common detector of HPLC, but the sensitivity of this technique is usually limited to the 10^{-6} M range for proteins due to the short optical pathlength across the capillary [5]. Laser-induced fluorescence (LIF) detection provides signal-to-noise ratios 100 times greater than those obtained in UV absorbance detection [6], and has led to limits of detection (LODs) of about 10^{-10} M for proteins [7]. But this low concentration sensitivity is obtained only for part of proteins containing tryptophan, phenylalanine or tyrosine that exhibits intrinsic fluorescence. Very expensive lasers with emission in the range of 270–280 nm are also needed [8,9].

To overcome this drawback, derivatization has been accepted as an alternative method for enlarging detection scale to overall proteins. With the help of fluorescent derivatization, other less expensive lasers, such as He–Cd, non-frequency-doubled Ar^+ or semiconductor, can also be used [10]. Sample derivatization involves the use of a labeling reagent that reacts with an amino group of the protein to form a fluorescent derivative. Labeling reagents such as fluorescein isothiocyanate, 5-carboxytetramethylrhodamine succinimidyl ester and 9-fluorenylmethyl chloroformate have been widely used as fluorescent tags [5,11,12]. Reactions are usually performed in aqueous environment with proteins and labeling reagents both at millimolar concentrations followed by dilution by several orders of magnitude before analysis. Proteins at low concentration below 10^{-6} M are much more difficult to be fluorescently labeled, which is a major limitation in the case of real samples [5]. Although fluorescent reagent at higher concentration is propitious to derivatization, the high background signal from excess fluorescent reagent, hydrolysis products and impurities of the chemicals involved in the reaction create dozens of background peaks that inevitably interfere with the protein signal, or even cover it. Dialysis and size exclusion chromatography (SEC) are useful methods to eliminate small molecule interferences, but they always required milliliters of sample and were hard for smaller volume such as micro-liters of sample handling.

One solution is the use of fluorogenic reagents. Different from other fluorescent reagents, these fluorogenic reagents are non-fluorescent, but form fluorescent products when they react with primary amines. 3-(2-Furoyl)-quinoline-2-carboxaldehyde (FQ) was demonstrated as a good reagent in this type [13,14]. But sample dilution cannot be avoided and cyanide was usually needed to react with the peptides to form a fluorescent FQ-peptide derivative.

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The potential toxic risk of the later reagent constitutes a problem for its application. Reagents also react slowly to form secondary fluorescent products [15].

Another strategy is the solid-support derivatization [16], which combines solid phase extraction (SPE) and derivatization. Complex sample handling aimed at removing unreacted reagents, sample/analyte purification and enrichment can be integrated into single step. Sample loss during transportation can also be saved. This methodology has been used for different amines and obviously improved their detection limits [17–22]. Pinto et al. [23] developed a derivatization method based on an immobilon CD membrane for insulin chain B, which allowed handling of the fluorescent labeling of peptides at concentrations as low as 10^{-8} M. However, fluorescent derivatization of proteins in capillary solid-support has not been reported.

In this paper, we described a simple method of solid-support derivatization for picomoles of proteins in diluted solution. A simple and low-cost homemade capillary C18 cartridges was fabricated for proteins capture and subsequent derivatization with a fluorescent reagent, fluorescein isothiocyanate (FITC, isomer I). The use of the solid-support reactor allows easy handling for protein enrichment and purification. Proteins of interest were separated from interferences before injection and analyzed by nano-HPLC with laser-induced fluorescence (LIF) detection. Using bovine serum albumin (BSA) as a test protein, we have labeled and detected 8.5 pmol BSA. This method was applied to analyze the fraction from weak anion-exchange chromatography (WAX) of human liver extract.

2. Experimental

2.1. Materials and chemicals

Fused silica capillary (100/250 μm i.d. × 375 μm o.d.) were purchased from Yongnian Optical Fiber Factory (Yongnian, Heibei, China). Packing materials of C18 particles (Kromasil, 5 μm, 300 Å) and 5 μm spherical silica gel were obtained from Eka Nobel (Switzerland). Acetonitrile were HPLC grade reagent purchased from Fisher Scientific (Fair Lawn, NJ). BSA, FITC, phenylmethanesulphonylfluoride (PMSF), dithiothreitol (DTT), trifluroacetic acid (TFA) and methyltriethoxysilane (MTES) were purchased from Sigma–Aldrich (St. Louis, MO). Water was purified by a Milli-Q system (Millipore, Molsheim, France). Menzel buffer (Na_2CO_3/$NaHCO_3$, pH 9.4) was prepared with a total carbonate concentration of 20 mM carbonate. Other chemicals were analytical-reagent grade, and all solvents were HPLC grade.

2.2. Preparation of capillary solid-support derivatization reactor

The capillary solid-support derivatization reactor was homemade by employing a slurry-packing procedure as previously described [24]. Briefly, on-column frits were fabricated by sol–gel technology using 5 μm spherical silica gel particles into capillaries with inner diameters of 250 μm. Then 5 μm C18 particles slurry was driven by pressure and packed into the capillary to form 10 mm column bed.

2.3. Solution derivatization

Fresh dilutions (10^{-4} to 10^{-7} M) of BSA in water were prepared before each experiment. FITC was prepared in Menzel buffer. A 20 μL aliquot of the BSA dilution was mixed with 10 μL of 2.5 mM FITC and 10 μL Menzel buffer (pH 9.4). The mixture was blending for 30 s, and allowed to react at ambient temperature for 10 h in the dark. A dilution with Menzel buffer (pH 9.4) of the fluorescent FITC-BSA derivative (10^{-4} to 10^{-7} M) was injected as sample.

2.4. Solid-support derivatization

Solid-support derivatization reactor was initialized by drawing with 50 μL of 100% ACN, followed by 50 μL of 40% ACN solution and then 50 μL of water. An aliquot of BSA was then applied, and passed slowly through the cartridges. Afterward, 2 μL FITC was loaded onto the cartridge and made sure the cartridge was immersed in the reagent. The reaction was allowed to proceed at defined temperature from 0 °C to 70 °C. After a given reaction time, unreacted FITC and impurities were washed away by 10 μL 10% ACN solution. The labeled protein was desorbed from the cartridges with 10 μL 40% ACN solution and injected into the chromatographic system.

2.5. Nano-HPLC-LIF

The nano-HPLC-LIF included a pump system, a 6 port injection valve with a 200 nL loop, a reversed-phase column of 20 cm × 100 μm i.d. packed with 5 μm Kromasil C18 as made in our previous work [25], and a home-made DPSS laser-induced fluorescence detector as reported before [26]. Fluorescence was monitored at an emission wavelength of 520 nm by an excitation with a solid blue laser at 473 nm (15 mW).

The Agilent 1100 series capillary pumping system was used for mobile phase delivery. Binary solvents of A (5% ACN) and B (95% ACN) were used in the elution. Gradient elution for the ramification sample was as follows: 0–50% B in 25 min, maintained for 10 min and further increased to 90% B in 15 min. The flow rate was adjusted to 500 nL/min by splitting.

2.6. Analysis of real sample

The normal human liver specimens were provided by Liver Cancer Institute of Zhongshan Hospital, Fudan University. All manipulations in liver specimen collection were approved by the Institutional Bioethics Committee and informed consent was given by all participants. Extraction of liver tissue was performed as previously described [27]. Generally, the tissue specimens were suspended in the protein soluble extraction buffer (1 mM PMSF, 50 mM DTT, 0.1% TFA and protease inhibitor cocktail dissolved in the lysis buffer according to the usage specification) and homogenized in an ice bath. The resulting homogenate was swirled for 30 min and centrifuged for 15 min at 18,000 × *g*. The supernatant was collected. Protein concentration of the sample was measured by the Bradford assay, using BSA as standard [28].

Weak anion-exchange chromatography separation was performed with a LC-2010A system and fractions were collected using FRC-10A fraction collector combined with a SCL-10A controller (Shimadzu, Kyoto, Japan). Shimadzu Class-VP station was used to acquire and process data. The separation procedure was fulfilled in 25 °C. Protein sample of 2 mg was loaded onto a WAX column (75 mm × 7.5 mm, TSK-gel, DEAE-5-PW, Tosoh, Tokyo, Japan). The buffer solutions used were 10 mM Tris–HCl (pH 7.5, buffer A) and 10 mM Tris–HCl/500 mM sodium chloride (pH 7.5, buffer B). A linear gradient elution had the following profile: 15 min of 100% buffer A, 80 min linear gradient from 0% to 30% buffer B, then 20 min linear gradient from 30% to 100% buffer B, 5 min of 100% buffer B and 5 min back to 0% buffer B. The flow rate is 0.5 mL/min. The chromatograms were monitored using the absorption wavelength λ_A = 215 nm. Fractions were collected every 2 min automatically from 3 to 125 min, and 61 fractions were obtained. Fraction 25 was selected randomly and used for solid-support derivatization.

The no. 25 fraction was diluted by 20-fold and 100 μL aliquot was passed slowly through our solid-support reactor. Afterward, 2 μL FITC (2.5 mM) was loaded onto the cartridge. The reaction was allowed to proceed at 60 °C for 30 min. Unreacted FITC and impurities were washed away by 10 μL 10% ACN solution. The labeled

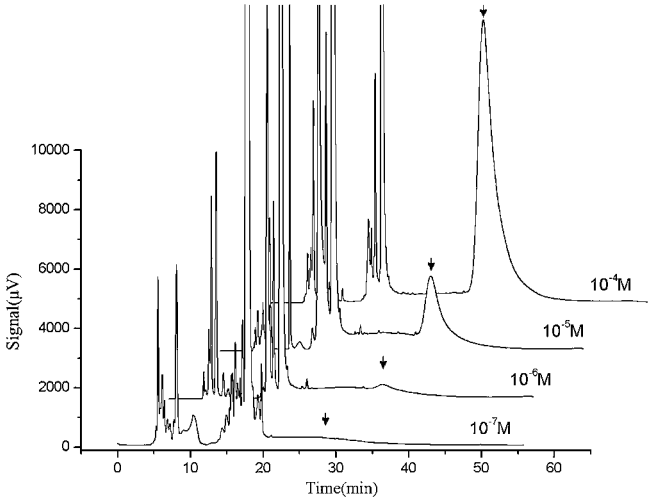

Fig. 1. Chromatograms obtained for BSA of different concentrations in solution derivatization. Arrow indicated FITC-labeled BSA. Reaction conditions: 20 μL of BSA (1.7×10^{-7} M to 1.7×10^{-4} M), 10 μL of 2.5 mM FITC and 10 μL Na_2CO_3/$NaHCO_3$ buffer (pH 9.4); reaction time, 10 h at room temperature in the dark; sample was diluted 1:100 in Na_2CO_3/$NaHCO_3$ buffer (pH 9.4) prior to injection. Separation conditions: nano-HPLC 20 cm × 100 μm; flow rate: 500 nL/min; mobile phases: (A) 5% ACN in water, (B) 95% ACN in water, gradient: 0–50% B in 25 min, maintained for 10 min and further increased to 90% B in 15 min; injection of volume: 200 nL; 20 °C; detection with fluorescence detector: excitation wavelength of 473 nm and emission wavelength of 520 nm.

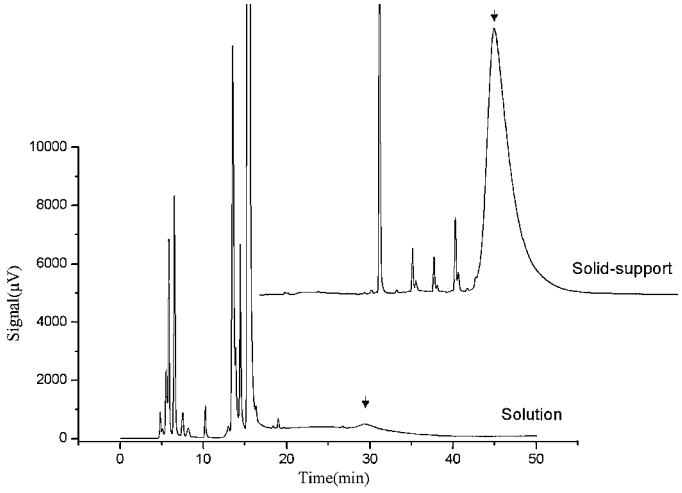

Fig. 2. Chromatograms of solid-support derivatization and solution derivatization of 1.7×10^{-6} M BSA. Arrow indicated FITC-labeled BSA. Solution derivatization conditions: same as Fig. 1. Solid-support derivatization conditions: 100 μL of BSA, 2 μL of 2.5 mM FITC; reaction time, 1 h at room temperature in the dark; washed: 10 μL 10% ACN solution; extraction: 10 μL 40% ACN solution. Separation conditions are given in Fig. 1.

protein was desorbed from the cartridges with 10 μL 80% ACN solution. Before analysis by nano-HPLC-LIF, this fraction was further purified by a homemade capillary size exclusion chromatography (SEC) column (20 cm × 250 μm i.d.) packed with HW-40s (TSK, Tokyo).

3. Results and discussion

3.1. Limitation of solution derivatization

As a common amine-reactive probe, FITC has been widely applied for the derivatization of amino acids, bioamines, peptides and proteins [29–31]. High fluorescence quantum yield of FITC in aqueous solutions makes it compatible with HPLC separation and very high sensitivity up to zeptomole mass detection limits can be obtained [32]. However, the labeling has to be done at high concentration, because the chemistry of FITC derivatization of amines is slow and strongly concentration dependent. Thus, the limit of detection (LOD) of real samples can be given in two ways, i.e., the lowest protein concentration with satisfactory derivatization, or the lowest concentration of the conjugated substance detected by a fluorescent detector [5].

The LOD of a highly derivatized sample is satisfactory. Reaction was performed in aqueous environment with BSA at 1.7×10^{-4} M followed by dilution by several orders of magnitude before analysis. The plot shows linearity between log of the fluorescent signal and log of BSA concentration (linear dynamic range is from 10^{-9} M to 10^{-5} M; slope = 1.01876 ± 0.01402; intercept = 9.85033 ± 0.09306; r^2 = 0.99886). The LOD (S/N = 3) is about 2×10^{-9} M (0.1 ng/μL) and only femtomolar amounts of sample were needed for analysis.

But the lowest protein concentration used in the conjugation was unsatisfactory. Fig. 1 shows a series of chromatograms for the labeling of BSA from 1.7×10^{-7} M to 1.7×10^{-4} M. BSA at concentration lower than 10^{-6} M can hardly be detected, which greatly reduced the advantage of laser-induced fluorescence detection. In addition, dilution of sample by derivatization reagent is inevitable, which leads to worse results.

3.2. Fluorescent background interferences

The fluorescent background interference is a common problem in fluorescent detection, which generally comes from excess fluorescent reagent, hydrolysis products and impurities of the chemicals involved in the reaction. Dozens of background peaks and high baseline usually interfere with the target signals, or even cover it yield overlapping. On the other hand, excess fluorescent reagent would decrease separation efficiency and resolution. Dilution of sample before injection was necessary to prevent overloading of analytical column and saturation of the detector.

An advantage of solid-support derivatization is that it can reduce the fluorescent background interferences easily, thus cleaner chromatograms can be obtained and dilution of sample is not required. We employed a simple step elution to eliminate unwanted fluorescent interferences. The majority of interferences was eliminated by using a 10% acetonitrile solution. Here in this case, the volume of washing solution should be as low as possible to avoid breakthrough of the analytes, so 10 μL was selected. After that, targets were desorbed from solid-support by 40% acetonitrile solution. Fig. 2 shows chromatograms resulting from solution derivatization (trace A) and solid-support derivatization (trace B) of same BSA samples (1.7×10^{-6} M). In order to prevent overloading of analytical column and saturation of the detector, sample of solution derivatization was diluted by 100-fold before injection. Thus its signal intensity of target is much smaller. It can also be observed that the background of products from solution derivatization is complex and target peak is inconspicuous compared with the baseline, but the potential overlap of target and fluorescent interferences is minimized obviously in the case of the solid-support derivatization.

3.3. Optimization of solid-support derivatization conditions

Solid-support derivatization combined solid phase extraction and fluorescent labeling reaction. Its efficiency is affected by many parameters, such as packing materials, concentration of fluorescent reagent or reaction temperature and time.

A satisfactory solid-support should have the following qualities: (1) high affinity of protein; (2) good capability to differentiate labeled protein and unreacted derivatization reagent or other fluorescent interferences; and (3) inertness toward the sample and

the derivatization reagent. Commercial reversed-phase particle is a good choice, because its properties closely match the requirements above and is easy to obtain. C18 particle had been used widely for SPE and was selected in our experiment.

A complete fluorescent derivatization of FITC with protein in aqueous environment was usually performed for hours or even over night [33]. Following the time course of solid-support derivatization, a quicker saturation curve was obtained (Fig. 3a). It can be explained by the molecular collision theory, since both protein and FITC were enriched at the head-column of solid-support reactor after loading, and higher concentration increased the reaction probabilities. As shown in Fig. 3a, the conjugation proceeded fast at beginning and obviously slowed down after about 30 min. Therefore, a short time of 30 min was used for further work.

Reaction temperature was another critical parameter and its effect was examined. As shown in Fig. 3b, a higher reaction temperature was also favorable to increase molecular collision probabilities. Thus, 60 °C was used for further work.

We also evaluated the effect of the fluorescent reagent concentration. As shown in Fig. 3c, the increase of FITC concentration results in a fluorescence signal increase of the labeled protein, which improved the method sensitivity. This improvement is not evident for concentrations beyond about 2.5 mM, which was induced by SPE capacity limitation. In this case (FITC > 2.5 mM), only part of FITC took part in the reaction and excess FITC permeated the solid-support reactor. Thus efficient amount of fluorescent reagent was not increased along with concentration. In order to decrease the fluorescent background interferences, a FITC concentration of 2.5 mM was used for further work.

3.4. Solid-support derivatization for protein sample in micro-liter scale

As mentioned above, a regular method for fluorescent derivatization of proteins usually required milliliters of sample, which is a limitation for sample of micro-volume. We tested the application of solid-support derivatization for low concentration protein sample in micro-liter scale by employing capillary solid-support reactor and step elution. This method was proved to be efficacious. 5–100 μL BSA sample with the same concentration (1.7×10^{-6} M) were loaded on the reactor and eluted after fluorescent derivatization for analysis. It can be observed a linear relationship of fluorescent signal versus the concentration of BSA (linear dynamic range is from 8.5×10^{-7} M to 1.7×10^{-5} M; slope = 1421.6 ± 71.6; intercept = 1619.3 ± 620.9; r^2 = 0.98994).

3.5. Solid-support derivatization for low-concentration protein samples

The fluorescent derivatization and detection of protein in lower concentration can be realized by increasing loaded sample volume in solid-support derivatization. In the chromatograms of BSA solutions (not shown), the fluorescent signal intensities of two samples with the same BSA amount (8.5 pmol), but very distinct concentrations (1.7×10^{-6} M and 8.5×10^{-8} M) were almost equal (about 2304 and 2519 μV), which means the LOD of this method is related to the sample amount rather than concentration. Thus it can be calculated that the LOD is about 50 fmol (S/N = 3).

3.6. Application to real sample

We have developed a method for the analysis of diluted fraction from weak anion-exchange chromatography (WAX) of human liver extract. According to WAX chromatogram (not shown), there are about 30–50 μg/mL proteins in selected fraction. After dilution by 20-fold, the protein concentration is about 2–3 μg/mL. Some small molecular in human liver extract may hardly be separated from proteins by 10% acetonitrile elution. In order to further eliminate these interferences, a capillary SEC was used after derivatization. As shown in Fig. 4, the chromatogram can be obtained with nanogram level injection, and only 0.1 mL (including several hundred ng protein) of sample was needed for protein fluorescent derivatization. A

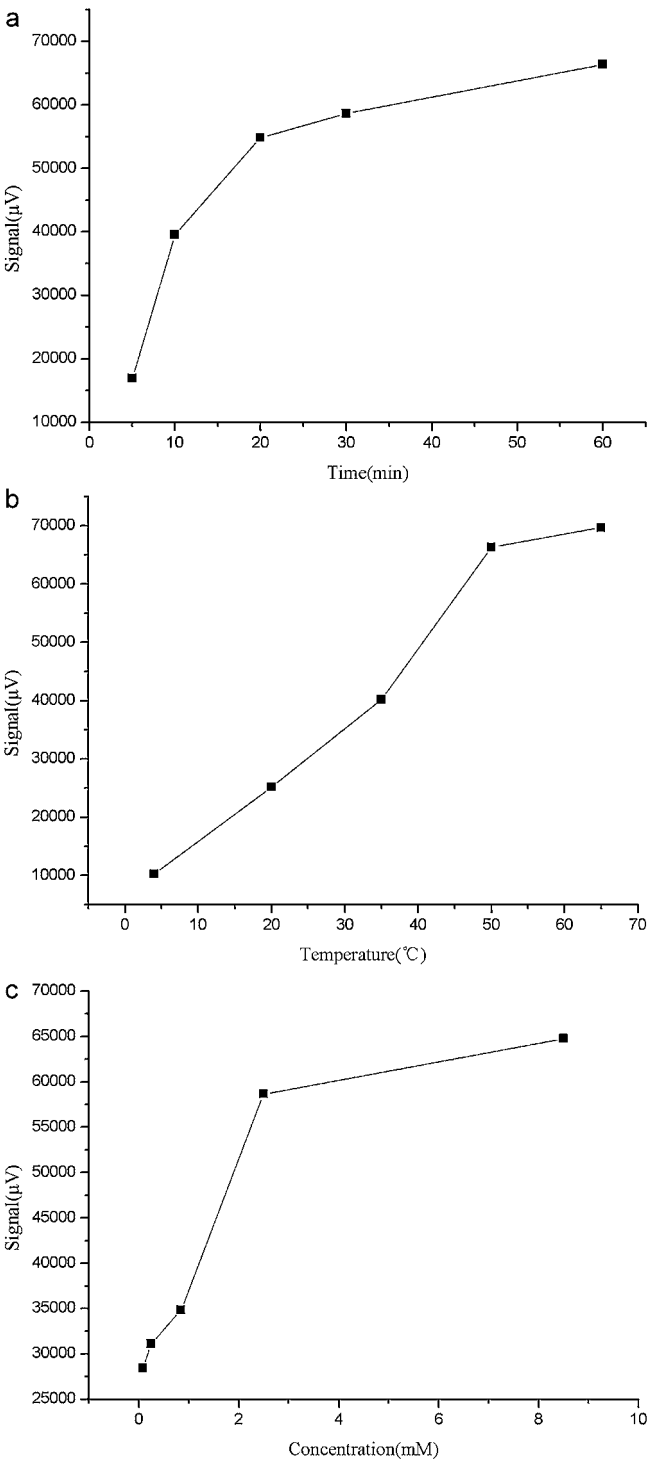

Fig. 3. Effect of (a) reaction time, (b) reaction temperature, (c) fluorescent reagent concentration on BSA-FITC responses. Solid-support derivatization conditions: 250 μL of 1.7×10^{-6} M BSA; for (a): 2 μL of 2.5 mM FITC, reaction temperature at 60 °C; for (b): 2 μL of 2.5 mM FITC, reaction time 60 min; for (c): reaction temperature at 60 °C, reaction time 30 min; washed: 10 μL 10% ACN solution; extraction: 10 μL 40% ACN solution. Separation conditions: same as Fig. 1.

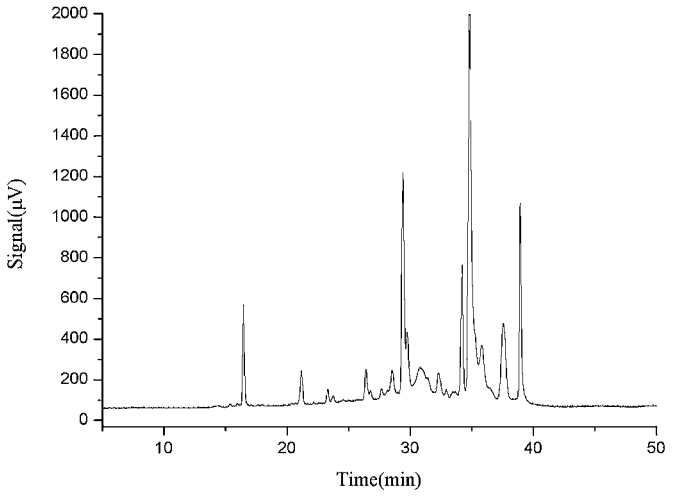

Fig. 4. The nano-HPLC elution profile of Fraction 25 from WAX of human liver extract. Solid-support derivatization conditions: 100 μL of diluted fraction, 2 μL of 2.5 mM FITC; reaction time, 30 min at 60 °C; washed: 10 μL 10% ACN solution; extraction: 10 μL 80% ACN solution. Separation conditions: same as Fig. 1.

harsh requirement of amount for real sample analysis is decreased. Additional process/time for concentration, such as freeze drying, can be saved. Sample with lower concentration can also be analyzed by increasing loaded sample volume. This demonstrated this method's promising potential in application to real sample.

4. Conclusions

This paper describes a different way to utilize solid-support fluorescent derivatization for the analysis of protein in low concentration and picomoles amount by nano-HPLC-LIF. The other advantages of this method included simple derivative purification and ease in building. A capillary reactor employing commercial C18 particles is constructed and operation procedure is established. The derivatization conditions have been optimized (reaction time 30 min, reaction temperature 60 °C, FITC concentration 2.5 mM). The use of the solid-support reactor allows easy handling of as little as 8.5 pmol of BSA and 10^{-8} M BSA can be labeled by FITC and analyzed, which surpasses the traditional solution labeling technique. This method also shows an encouraging potential in application to real sample. A very important feature to use this method in the future is the small amount of samples needed to carry out an analysis, and it can be integrated with lab-chip easily, which is favorable for biomarker or single cell analysis.

Acknowledgments

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