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High-performance liquid chromatography coupled to enzyme-amplified biochemical detection for the analysis of hemoglobin after pre-column biotinylation

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Abstract

The determination of proteins with enzyme-amplified biochemical detection (EA-BCD) coupled on-line with high-performance liquid chromatography (HPLC) is demonstrated. The EA-BCD system was developed to detect biotin-containing compounds. Hemoglobin, which was used as a model compound, was biotinylated prior to sample introduction. Several biotinylation parameters, such as pH and removal of excess biotinylation reagent, were investigated. After biotinylation samples were introduced to HPLC followed by EA-BCD. To the HPLC effluent, alkaline phosphatase label streptavidin (S-AP) was added, which possesses high affinity to biotin and biotin-containing compounds. Excess S-AP was removed by means of an immobilized biotin column followed by substrate addition. The non-fluorescent substrate is converted to a highly fluorescent product by the enzyme label. A detection limit of 2 femtomol biotinylated Hb was achieved with good reproducibility and linearity. However, biotinylation at low analyte concentration suffers from low yield due to slow reaction kinetics. Finally, Hb was successfully extracted from urine with a recovery of 94%. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

The increasing demands of analytical chemistry requires the continuous development of modern analytical systems which are able to detect multiple analytes with high sensitivity and selectivity. One of the most sensitive detection systems developed during the last decades takes advantage of enzyme-amplification. The application of enzymes for the detection of analytes at low concentration is possible due to the high turnover number of enzymes, such as alkaline phosphatase (AP) and horseradish peroxidase (HRP) and their possibility to convert substrates into detectable products [1–3]. Nowadays, most enzyme-amplified detection systems are based on the interaction of analytes with enzyme labeled affinity proteins and are performed in microtiter plates. Detection limits at the attomol level were achieved

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with the use of fluorescence or luminescence detection [4,5].

A disadvantage of the application of microtiter plates is the impossibility to distinguish between multiple, cross-reactive compounds in a sample. An additional separation technique, such as capillary electrophoresis or liquid chromatography, is needed to separate the different analytes of interest. However, the coupling of such separation techniques to microtiter plates analysis is difficult with regard to reproducibility and ease of automation. Furthermore, the reproducibility of microtiter plate analysis is rather poor (15–20% inaccuracy [1,6]) compared to flow detection systems such as flow injection analysis (FIA) or high-performance liquid chromatography (HPLC). To eliminate these problems, the on-line coupling of enzyme-amplified biochemical detection (EA-BCD) as a post-column detection technique to FIA or HPLC is necessary.

Several flow injection enzyme immunoassays were introduced during the last decade [7–9]. The presented systems were all based on the interaction of the analyte with enzyme-labeled antibodies. A limitation of the described systems, is the use of antibodies to the analytes of interest. Development, purification and enzyme labeling of antibodies are laborious and expensive procedures. A different approach involves the use of a universal probe, which can be used to label the analytes. Instead of enzyme-labeled antibodies to the analytes, enzyme-labeled affinity proteins to the universal probe can be applied. In principle, all analytes can be detected with the same enzyme-labeled affinity protein in such an EA-BCD system, if the analytes can be labeled with the universal probe. A suitable universal probe is biotin, which forms very stable complexes with avidin and streptavidin ($K_D = 1 \cdot 10^{-15}$ mol/l) [10,11]. Biotin consists of a bicyclic urea structure to which a valeric side chain is attached. However, this valeric side chain is not involved in the biotin–(strept)avidin interaction. Several reactive probes, such as hydrazide groups or succinimidyl esters, can substitute the carboxylic functionality of the valeric side chain without interfering in the biotin(strept)avidin interaction [10–13].

Recently, we have introduced an EA-BCD system coupled on-line with FIA for the detection of biotin-containing compounds at low concentration [14]. Alkaline phosphatase labeled streptavidin (S-AP) was added to the FIA effluent as affinity protein for the biotin-containing compounds. Excess S-AP was separated from biotin-bound S-AP by means of a immobilized biotin column. Next, the non-fluorescent substrate Attophos was added, which is converted by S-AP to a highly fluorescent product. Several parameters, such as concentration of affinity protein and substrate, buffer composition, incubation time, enzyme–substrate reaction time and temperature were investigated using FIA of biotin amino caproic hydrazide (BACH) coupled on-line to EA-BCD. With the described system, a limit of detection (LOD) of 2.5 fmol BACH was achieved. Several other biotinylation reagents were examined as well, with approximately the same LOD values, which confirmed the assumption that the substitution of the valeric side-chain hardly affects the affinity interaction.

In a subsequent paper, the on-line coupling of HPLC to EA-BCD for the detection of biotinylated compounds was presented [15]. The overall strategy can be divided into four sub-procedures i.e. biotinylation, sample clean-up, separation of the biotinylated compounds followed by EA-BCD. Naphthalene aldehyde and anthracene aldehyde were used as model compounds. In the described system, biotinylation was performed with BACH, which reacts with the aldehyde functionality of the analytes. Succeeding biotinylation and preconcentration by means of solid-phase extraction (SPE), HPLC was performed to separate the biotinylated compounds followed by EA-BCD. A LOD of 250 fmol on-column biotinylated anthracene aldehyde was achieved. A limitation of the overall procedure is the low yield of biotinylation at low analyte concentration. However, the described EA-BCD system could be used for both biotinylated analytes without adaptation of the system.

One of the most widespread applications in respect to biotin–streptavidin interaction is the biotinylation of proteins and peptides [13,16–19]. Proteins can be used as biomarkers for monitoring of different biological processes in the body. Furthermore, there is an increasing interest of the use of proteins in drug development. Therefore, detection of proteins in biological matrices at low concentration becomes more and more important.

In this paper we present a biotinylation procedure for proteins, followed by HPLC–EA-BCD. Hemoglobin (Hb) was used as a model compound. Several parameters for the solution-phase biotinylation, such as pH, reaction time and concentration of the biotinylation reagent had to be examined to achieve the maximal yield. An important aspect of the procedure is the biotinylation yield at low concentrations. Following biotinylation, preconcentration of the analytes was performed on a reversed-phase pre-column. Attention was paid to the removal of excess biotinylation reagent to avoid interference with EA-BCD. The reaction products were monitored with native fluorescence as well as with EA-BCD coupled on-line with HPLC. The influence of the composition of the HPLC effluent on the EA-BCD system was examined.

To demonstrate the feasibility of the system, the extraction of Hb from spiked urine, followed by biotinylation and HPLC–EA-BCD was investigated. The existence of Hb in urine is a symptom for Hemoglobinuria, caused by intravascular degradation of erotrocytes due to e.g. traumatic stress, malaria or toxicological effects [20,21]. Moreover, Hb in urine may be indicative of bladder or kidney cancer [22]. Determination of Hb is normally performed with dipsticks based on the peroxidase activity of Hb, which caused oxidation of a color indicator. However, the use of dipstick is a qualitative method and the sensitivity is rather low. Furthermore, interference of ascorbic acid (vitamin C) with the peroxidase indicator was observed, leading to false-negative results [23,24]. For a more sensitive and quantitative determination of Hb, several spectroscopic methods are developed, but these methods suffers from cross-reactivity of other proteins [25,26].

2. Experimental

2.1. Chemicals

Sulfosuccinimidyl 6-(biotinamido) hexanoate (BNHS, catalogue no. 21335) and alkaline phosphatase-labeled streptavidin (S-AP, immunopure, catalogue no. 21324) were obtained from Pierce (Rockford, IL, USA). Sodium chloride, sodium nitrate, Tween 20, disodium hydrogenphosphate, sodium acetate and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Tris–(hydroxymethyl)aminomethane (Tris) was acquired from GibcoBRL (Grand Island, NY, USA). Acetonitrile, HPLC grade, was from Rathburn (Walkerburn, UK). Methanol and di ethanolamine were purchased from Mallinckrodt Baker (Deventer, Netherlands). Aldehyde activated Poros 20, Poros 10 R1, Poros 10 R2 and Attophos substrate set were all acquired from Boeringer Mannheim (Mannheim, Germany). Biotin aminocaprioc hydrazide (BACH, 95%, catalogue no. B-3770) and human hemoglobin (Hb) were obtained from Sigma (St. Louis, MO, USA). All reagents were of analytical grade unless otherwise stated. All aqueous solutions were prepared with water purified with a Milli-Q system from Millipore (Bedford, MA, USA). All solutions were filtered through a 0.2 μm cellulose acetate filter (Sartorius, Groningen, Netherlands).

2.2. Biotinylation procedure

To the (purified) Hb, dissolved in 150 μl acetonitrile, 825 μl 10 m*M* disodium hydrogenphosphate (pH 9.5) was added. Next, BNHS was dissolved in phosphate buffer at a concentration of 5 mg/ml. A fixed volume of 25 μl of the BNHS solution was added immediately to the Hb solution and allowed to react at ambient temperature for one h. Biotinylated Hb was stored at 4 °C and protected from light prior to sample introduction. Under these conditions, biotinylated Hb was stable for at least 4 h.

2.3. Pre-column setup and preconcentration procedure

Preconcentration of the analytes was performed with a single pre-column setup. The time schedule of the procedure is given in Table 1. Sample handling was performed with an Aspec XL autosampler from Gilson (Villiers-Le-Bel, France) equipped with two Rheodyne six-port injection valves (Cotati, CO, USA) and a 402 dilutor (flow-rate 1 ml/min). A 5×3 mm I.D. pre-column packed with Poros R2 was connected to an injection valve and rinsed with 1 ml acetonitrile and 1 ml 30% acetonitrile in 0.1% TFA. Just prior to sample introduction, the reaction solution was diluted with 3 ml 30% acetonitrile in 0.1%

Table 1
Time schedule of the preconcentration procedure

Step (min)	Poros R2 pre-column position	Action
1 (0)	Load	Rinse pre-column with 1 ml acetonitrile and 1 ml 30% acetonitrile in 0.1% TFA
2 (2)		Dilute 1 ml sample with 3 ml 30% acetonitrile in 0.1% TFA
3 (3)		Load 1 ml of diluted sample
4 (4)		Rinse pre-column with 10 ml 30% acetonitrile in 0.1% TFA
5 (14)	Inject	Desorb retained analytes with HPLC gradient
6 (25)	Load	Start new procedure

TFA. A volume of 1 ml of the diluted sample was introduced to the pre-column. Following sample introduction, the pre-column was rinsed with 10 ml 30% acetonitrile in 0.1% TFA to remove excess BNHS. Next, the injection valve was switched and coupled on-line with a 10x1 mm I.D. analytical column, packed with Poros R2. The analysis was performed with gradient elution delivered by a Merck 655A-12 gradient pump equipped with an L-5000 LC controller. To the gradient pump, an accurate (LC Packings, Amsterdam, Netherlands) was connected to split the HPLC flow, resulting in a flow-rate of 40 μl/min to the analytical column. The flow-rate was monitored with a Liqui-flow from Bronkhorst Hi-Tec (Ruurlo, The Netherlands). Two different gradient profiles were used, which are given in Table 2. Detection was performed by a Jasco FP 920 fluorescence detector (Tokyo, Japan). Monitoring of the analytes was done either by native fluorescence of Hb or by EA-BCD, which is described in the next section.

2.4. Preparation of immobilized biotin column

Biotin was immobilized according to the following procedure. BACH (20 mg) was dissolved in 3 ml 0.1 mol/l sodium acetate (pH 5.6) and 1 ml acetonitrile. Next, 40 mg aldehyde activated Poros was added and allowed to react overnight in the dark under continuous stirring. The suspension was centrifuged at 1000 *g* for 5 min. The supernatant was removed and 3 ml 0.1 mol/l sodium acetate (pH 5.6) was added to the precipitate. The washing procedure was repeated four times. Subsequently, 2 ml 0.2 mol/l Tris was added to quench the residual aldehyde functionality. The suspension was stirred for two h, followed by centrifugation at 1000 *g* for 5 min. The supernatant was removed and a 5×2 mm I.D. column was slurry-packed with the immobilized biotin. The immobilized biotin column was rinsed with 2 ml water and 2 ml methanol prior to use. The packed column and the immobilized biotin were stored in the refrigerator when not in use.

Table 2
Gradient profiles[a]

Time (min)	Gradient profile 1		Gradient profile 2	
	A (%)	B (%)	A (%)	B (%)
0	70	30	70	30
1.0	70	30	70	30
7.0	20	80	0	100
12.0	20	80	0	100
15.0	70	30	70	30

[a] Buffer A consists of 10% acetonitrile in 0.1% (V/V) TFA, buffer B consists of acetonitrile.

2.5. Enzyme-amplified biochemical detection

The EA-BCD setup previously described [14,15] was used with some minor modifications, see Fig. 1. To the HPLC or FIA effluent (40 μl/min) 1 unit/liter S-AP in carrier buffer was added via a mixing union at a flow-rate of 170 μl/min supplied by a Pharmacia LKB 2150 HPLC pump (Uppsala, Sweden). The carrier buffer consist of 20 m*M* Tris, 150 m*M* sodium chloride, 150 m*M* sodium nitrate and 0.5% (w/v) Tween 20. The pH of the carrier buffer was adjusted at 8.2. The incubation of enzyme-labeled streptavidin with biotin-containing compounds takes place in a knitted PTFE reaction coil with a volume of 745 μl (250 μm I.D.) resulting in a incubation time of 212 s. Next, excess S-AP was separated from biotin bound S-AP by means of an immobilized biotin column (5×2 mm I.D.). Sub-

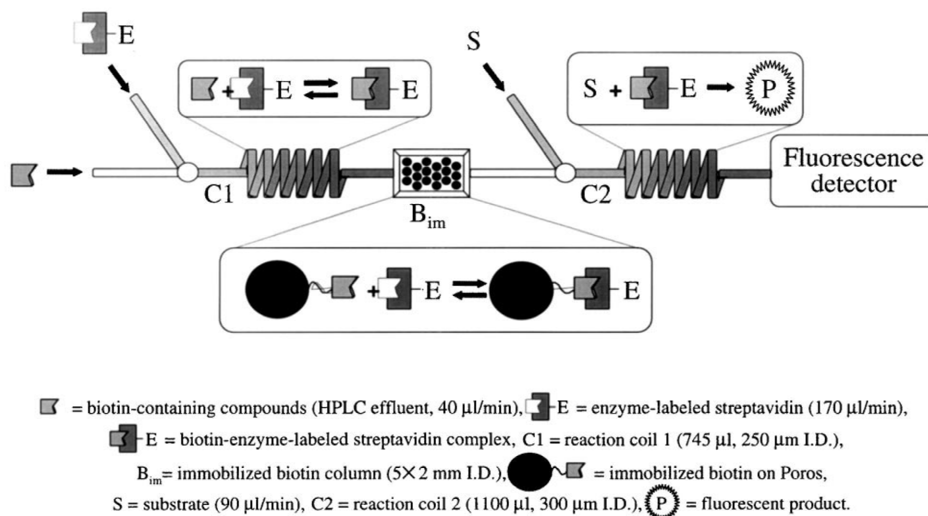

Fig. 1. Schematic representation of enzyme-amplified biochemical detection.

sequently, 20 µmol/l Attophos in 0.1 mol/l diethanolamine, pH 9.9 was added via a second mixing union with a flow-rate of 90 µl/min. Substrate was delivered by a Microstar K-100 pump (Knauer, Berlin, Germany). The Attophos was converted by the enzyme label in a knitted PTFE reaction coil with a volume of 1.1 ml (300 µm I.D.), resulting in a reaction time of 220 s. The enzyme–substrate reaction was performed at 50 °C. Detection of the enzyme product was done by fluorescence detection at 440 nm excitation (18 nm slit) and 550 nm emission (40 nm slit). The S-AP and Attophos solutions were prepared freshly daily.

2.6. Solid-phase extraction of hemoglobin from urine

Human urine (500 µl) was spiked with Hb and diluted with 500 µl 25% acetonitrile in 0.1% TFA. The diluted samples were filtered through a 0.2 µm nylon Acrodisk filter (Gelman, Ann Arbor, MI, USA) to eliminate particles. Preconcentration and purification was performed on a Poros R1 pre-column (5×2 mm (I.D.)), connected to an injection valve. Preconcentration solvent, 25% acetonitrile in 0.1% TFA was supplied by a Spectroflow 400 pump (Kratos, Rotterdam, Netherlands). Prior to sample introduction, the pre-column was flushed with preconcentration solvent for 5 min at a flow-rate of 0.25 ml/min. Next, 500 µl of the filtered sample was introduced via an injection loop to the pre-column, followed by 5 ml preconcentration solvent. Desorption of the retained compounds with acetonitrile was performed by a second Spectroflow 400 pump at a flow-rate of 50 µl/min for 3 min. The desorbed samples were collected in polypropylene vials and stored at 4 °C prior to the biotinylation procedure. The purified samples were stable for at least a week when stored at 4 °C. After desorption, the pre-column was rinsed with 2.5 ml 100% acetonitrile at a flow-rate of 0.25 ml/min.

3. Results and discussion

3.1. Assay design improvement

The EA-BCD system described previously [14,15] was used with some minor modification (see Fig. 1). To the FIA or HPLC effluent, S-AP was added in carrier buffer. The on-line incubation of biotin-containing compounds and affinity protein occurs in reaction coil 1. In principle, the incubation should be complete before reaching the immobilized biotin column. Ideally, excess S-AP should be completely trapped by the immobilized biotin column, whereas the biotin-bound fraction elutes unretained. Previous experiments showed that the immobilized biotin

column retains 96% of the unbound S-AP [14]. Next, substrate is added and converted by the enzyme label in the second reaction coil. The flow-rate through and the volume of reaction coil 1 determine the incubation time of the biotin-containing compounds with the enzyme-labeled affinity protein. Previous experiments show an optimum volume of reaction coil 1 of 745 μl (250 μm I.D.) resulting in a reaction time of 212 s. The biotin-containing compounds were completely associated with the enzyme-labeled affinity protein. However, the use of large reaction coils lead to peak broadening due to dispersion [27]. Therefore, a shorter reaction coil was chosen in spite of the fact that the association in this coil was not complete. In the new set-up, the 745 μl coil was used resulting in a complete association of the biotin-containing compounds. The increase in peak broadening was fully compensated by replacing the 500 μm I.D. enzyme–substrate reaction coil by a reaction coil with an I.D. of 300 μm and a reaction volume of 1100 μl (reaction time, 220 s). The longer incubation time and enzyme–substrate reaction time lead to a decrease in LOD by a factor 2.5.

Another adaptation was made in respect to the concentration of the enzyme-labeled affinity protein and substrate. The concentration S-AP was decreased from 3 U/l to 1 U/l, which resulted in a lower signal-to-noise ratio (S/N) but also in a lower background. This background is mainly caused by breakthrough of free enzyme or S-AP, which shows little or no affinity to the immobilized biotin column [14]. At the same time, the concentration of substrate was increased from 10 μmol/l to 20 μmol/l. The combination of a decreased S-AP concentration with a lower background and the increase of substrate concentration resulted in a lower noise. Moreover, the increase of substrate concentration lead to a faster enzyme–substrate reaction and therefore in a higher S/N. However, a further increase of affinity protein or substrate concentration, leads to a higher background and therefore a lower S/N. With the modified setup, an LOD of 250 attomol BACH was achieved, which is an improvement of a factor 10 compared to the previously published system [14]. An additional advantage of decreasing the S-AP concentration is that the operation time of the immobilized biotin column (with the same capacity) is increased by a factor 3. With the concentrations used, the immobilized biotin can be used for 125 h without decrease of S-AP trapping efficiency.

3.2. Amplification factor

An important aspect of the EA-BCD system is the amplification factor achieved. To determine this amplification factor, a calibration solution of Attofluor was used, which was included with the Attophos substrate set. Attofluor is the fluorescent product after enzyme conversion of the substrate Attophos. An aliquot of the calibration solution was introduced by FIA to the EA-BCD. Carrier buffer and substrate buffer were pumped, omitting S-AP, substrate and the immobilized biotin column. The response obtained after injection of a fixed amount of 680 fmol Attofluor was compared to the response of biotinylated Hb. From these results it can deduced that an amplification factor of 700 was achieved. In principle, this amplification factor can be increased, by increasing the substrate concentration, which results in a faster enzyme conversion [7–9]. However, a higher concentration of substrate will lead to a higher background and noise and therefore a higher LOD.

3.3. Influence of HPLC effluent to EA-BCD

Most affinity interactions are influenced by conditions such as pH and concentration organic modifier [28,29]. The biotin–streptavidin association occurs at neutral pH. However, the HPLC effluent contains 0.1% TFA (pH 1.5), needed for an efficient protein separation. Therefore, the pH of the carrier buffer was increased to 8.2. After mixing of HPLC effluent and carrier buffer at appropriate flow-rate, the pH was 7.5. An additional problem is the concentration of organic modifier in the HPLC effluent, which during the gradient run increases to 100% acetonitrile. A mixing ratio of HPLC effluent and carrier buffer was chosen of 1:5, resulting in a maximum concentration of 20% acetonitrile after mixing. To investigate the influence of the increase of organic modifier, FIA was performed by injection of a fixed amount of 25 fmol BACH at increasing concentration acetonitrile. Fig. 2 shows the influence of the amount organic modifier in the carrier solvent on the peak area. The acetonitrile content in the FIA

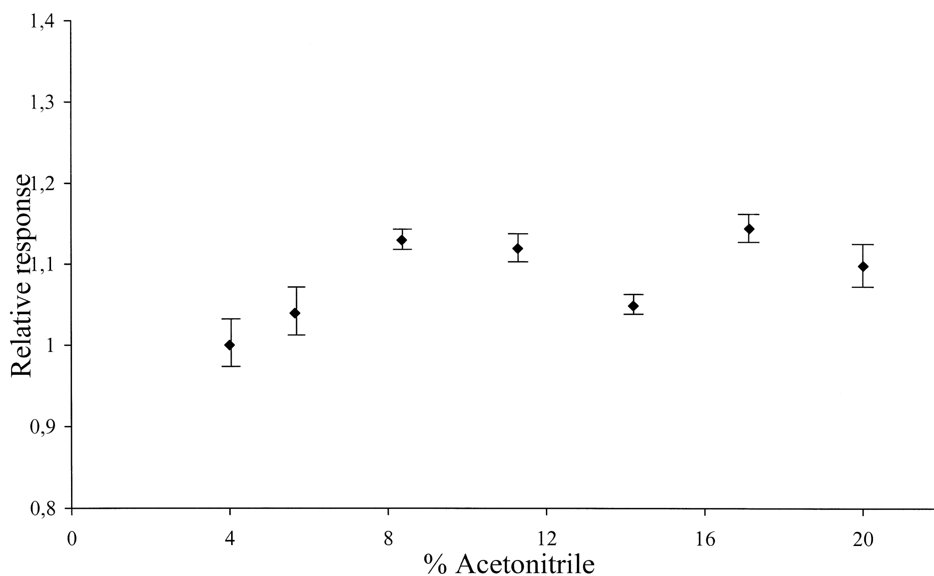

Fig. 2. Relative response of EA-BCD for BACH at different concentration acetonitrile after mixing. The values are related to the signal obtained with 4% acetonitrile. Error bars indicated the confidence interval (95%, $n=5$).

effluent was increased from 20% to 100%. Due to the mixing ratio chosen, the acetonitrile content increased from 4% to 20% after mixing. From Fig. 2 it can be deduced that the variation of peak area during the gradient was below 12.5%. Furthermore, the peak area at a fixed concentration organic modifier is relatively constant (relative standard deviation (RSD)$<$6%, $n=5$), which means that a biotinylated compound which elutes with a certain amount of organic modifier will give a constant signal.

3.4. Biotinylation of hemoglobin

Biotinylation of proteins with BNHS is usually performed at high pH due to the fact the NHS ester will react with the deprotonated form of primary amines. Unfortunately, the biotinylation reagent is not stable in aqueous solutions, because the NHS ester starts to hydrolyze immediately. Hydrolysis of the NHS ester group will be faster at a higher pH [30]. Therefore, the pH of the reaction solution should be a compromise between reaction velocity and the extent of hydrolysis of the NHS ester. To examine the optimum pH for the biotinylation of Hb, reactions were performed at pH values ranging from 7.5 to 10.5 (10 m*M* disodium hydrogenphosphate) at a concentration of 20 μmol/l Hb. After 1 h reaction, 2 μl of the reaction solution was introduced to the HPLC system via an injection loop. Reaction products were monitored by native fluorescence (excitation at 280 nm, emission at 340 nm) thereby omitting the EA-BCD system. Hb and biotinylated Hb were completely separated when gradient 1 (see Table 2) was used. The yield of the reactions at different pH values is given in Table 3. From these results it can be concluded that a maximum yield was obtained between pH 8.5 to 10.0. Therefore, pH 9.5 was used in further experiments. The reaction was terminated after 1 h due to the complete hydrolysis of BNHS.

In Fig. 3a and b the LC analysis of Hb and

Table 3
Biotinylation yield at different pH values

pH of reaction solution	Biotinylation yield (%)
7.5	66
8.0	84
8.5	98
9.0	97
9.5	99
10.0	98
10.5	73

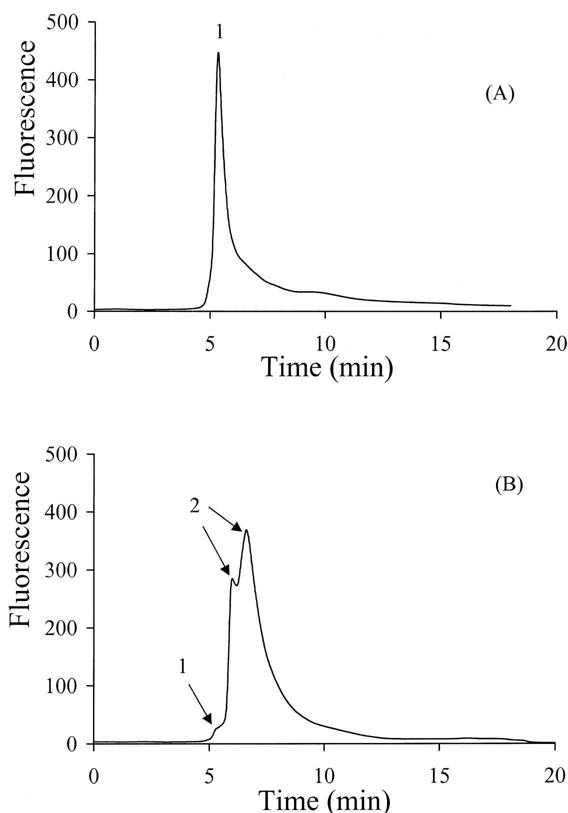

Fig. 3. Chromatograms obtained from Hb (A) and biotinylated Hb (B) with Gradient profile 1 (see Table 2). The analytes were monitored by native fluorescence. 1=Hb, 2=biotinylated Hb. For conditions, see text.

biotinylated Hb respectively is presented. With gradient profile 1 (see Table 2), different biotinylated Hb peaks were observed. When decreasing the slope of the gradient, even more peaks are observed, indicating that a mixture of biotinylated Hb arise during the labeling reaction. BNHS reacts with primary amines, which means that the N-terminal amine group as well as the free amine groups from lysine can be labeled. Depending on the amount of excess BNHS, biotin substitution of 8 – 14 moles biotin per mole protein are observed [18,30], resulting in a mixture of biotinylated protein. A complete separation of this mixture was not achieved, because of the relative small differences of chromatographic behavior of the differently labeled Hbs. An additional problem arises when the EA-BCD was connected to the HPLC system. Due to peak broadening in the EA-BCD system, resolution of the separation was lost, resulting in one broad peak. Furthermore, during this study we are interested of measuring the total amount of Hb, rather than the exact composition of the labeling mixture. Therefore, the slope of the gradient was increased (gradient profile 2, see Table 2) in such a way that one (relative narrow) peak only was observed.

Theoretically, the number of biotin substitution can be determinate [18,30]. However, as mentioned before, the substitution number depends on the amount of excess biotinylation reagent used. During the biotinylation of unknown concentrations Hb, the amount of excess BNHS is unknown. The biotin substitution number can vary with concentration of analyte, especially at low concentration. For this reason, no attempts were made to determine the substitution number. Several BNHS concentrations, ranging from 25 μg/ml to 200 μg/ml were used to investigate the yield of the biotinylation. An optimum BNHS concentration of 125 μg/ml was found. Although a higher BNHS concentration leads to a higher yield at low Hb concentration, excess BNHS could not be completely separated from the biotinylated Hb, thereby leading to erroneous results.

To examine the reproducibility of the derivatization procedure at low levels, Hb was biotinylated at a concentration of 20 nmol/l. Following biotinylation, samples were introduced to HPLC–EA-BCD via the Poros R2 pre-column as previously described. An RSD of 7.2% ($n=6$) was found. The biotinylated samples were stabile for at least 4 h, when stored in ice and protected from light, but should be introduced to the system as fast as possible to avoid erroneous results.

3.5. Preconcentration of biotinylated hemoglobin

Following the labeling procedure, biotinylated Hb was preconcentrated on a Poros R2 pre-column. During this preconcentration, excess BNHS was removed. To examine the recovery of the preconcentration procedure, Hb was biotinylated at a concentration of $1\cdot10^{-6}$ mol/l. After the reaction, the solution was diluted to a concentration of $2.5\cdot10^{-8}$ mol/l and 2 μl of the diluted sample was introduced directly to the HPLC–EA-BCD system. Next, the pre-column was connected and 2 μl of the diluted

sample was introduced according to the previously described procedure. The recovery of biotinylated Hb compared to direct injection was 96% (RSD= 3%, n=6). Approximately the same recovery was achieved when 35% acetonitrile in 0.1% TFA was used as preconcentration solvent. A higher amount of organic modifier resulted in improved removal of excess BNHS. However, due to the higher organic modifier content, peak broadening of the biotinylated Hb occurred, resulting in a decreased peak shape. For the following experiments, 30% acetonitrile in 0.1% TFA was used as the preconcentration solvent.

3.6. Calibration curves

To demonstrate the potential of the HPLC–EA-BCD system, a calibration curve was made of biotinylated Hb. Biotinylation was performed at a concentration of $1\cdot10^{-6}$ mol/l Hb. At this concentration, a yield of approximately 100% was obtained. The biotinylated samples were diluted and introduced directly to the HPLC system via a 2 μl injection loop. Fig. 4 presents the HPLC–EA-BCD chromatogram, which was obtained when 5 fmol biotinylated Hb was introduced. Linearity ranged from 2.5 nmol/l to 50 nmol/l with a correlation coefficient of 0.997. The RSD of all data points was below 3% (n=4). A detection limit of 2 fmol after dilution (S/N=3) was achieved. The biotinylation of undiluted samples yielded a detection limit of 250 fmol (S/N=3). This considerable increase in detection limits can be explained by the slower reaction rate of the biotinylation reaction at low Hb concentrations. When using on-line preconcentration after the biotinylation step (preconcentration volume, 200 μl), a linear calibration curve of Hb at a concentration from 2.5 nmol/l to 50 nmol/l was obtained with a correlation coefficient of 0.994 (n= 4). The RSD of the biotinylation at low concentration was higher, but remains below 9% for all data points.

3.7. Preconcentration of hemoglobin from urine

To examine the feasibility of the presented procedure, urine was spiked at a concentration of $2\cdot10^{-8}$ mol/l Hb. To avoid non-specific binding of Hb to the 0.2 μm acrodisk filter, the sample was diluted with 25% acetonitrile in 0.1% TFA prior to filtration. The sample was purified off-line on Poros R1, as described in the Experimental section. Next, biotinylation was performed and the sample was introduced, after on-line preconcentration on a Poros R2 pre-column, to the HPLC–EA-BCD system. Fig. 5A and B present the chromatograms obtained after purification of Hb spiked with urine and blank urine

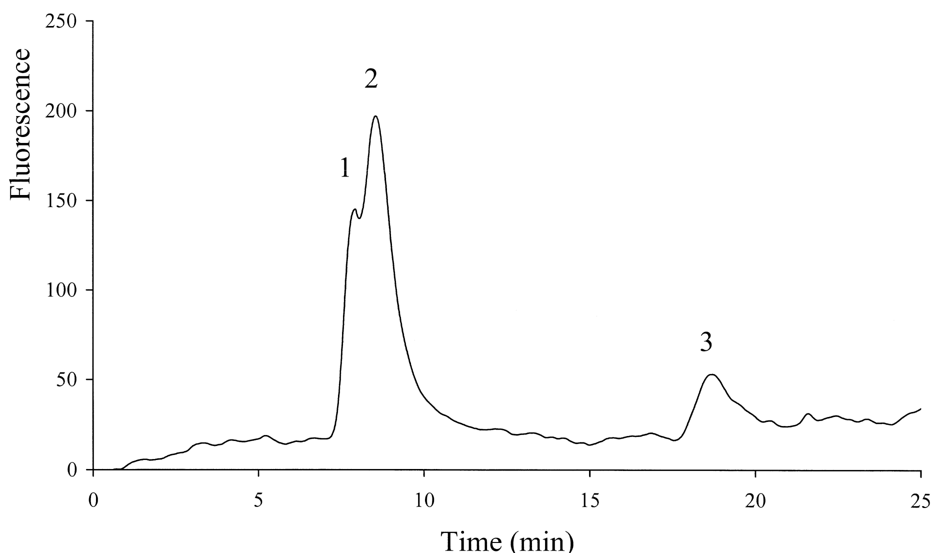

Fig. 4. HPLC–EA-BCD of 5 fmol biotinylated Hb. 1,2=Excess BNHS, 3=biotinylated Hb. For conditions, see text.

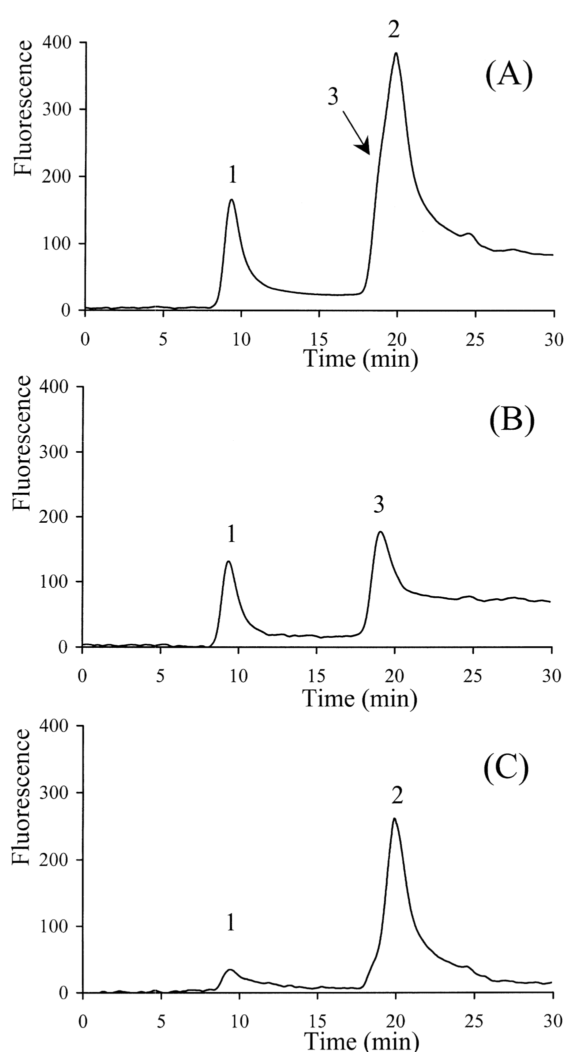

Fig. 5. HPLC–EA-BCD of Hb spiked urine (A) and blank urine (B) after extraction and biotinylation. Chromatogram C=Hb spiked urine, blank subtracted. 1=Excess BNHS, 2=biotinylated Hb, 3=matrix components. For conditions, see text.

respectively. Despite the extensive sample clean-up, the baseline of the blank urine was still disturbed by matrix effects, showing an additional peak eluting just before the biotinylated Hb peak. This peak is probably caused by micro-proteins, which normally occurs in urine [31]. The additional peak was not separated from the biotinylated Hb peak with the gradient profile used. By subtracting the blank urine baseline from the Hb spiked chromatogram, presented in Fig. 5c, a recovery for the total procedure of 94% was determined (RSD=4.9%, n=4). However, a blank urine sample is usually not available for pathological research. Therefore, the gradient profile has to be adjusted to separate the matrix components from the biotinylated Hb. A more convenient approach is the use of immobilized antibody pre-columns for the proteins of interest. The use of immobilized antibody pre-columns will considerably enlarge the applicability of the described procedure. In this case, not only relative hydrophobic proteins such as Hb can be analyzed, but also more hydrophilic proteins. Furthermore, isolation of proteins from plasma becomes possible.

4. Conclusions

A sensitive EA-BCD system was described, which can be used for the detection of Hb. An LOD of 2 fmol biotinylated Hb was achieved. The calibration curves for biotinylated Hb at high and low concentration show good linearity. However, biotinylation at low concentration suffers from low yield, due to slow reaction kinetics.

In principle, the yield of the labeling reaction can be increased by increasing the concentration of the biotinylation reagent. Unfortunately, the preconcentration procedure is not efficient enough to remove a higher amount of BNHS in the reaction mixture. A more selective purification method, e.g. preconcentration on an immobilized antibody column to Hb is necessary to eliminate a larger amount BNHS. However, even with a more selective preconcentration method, the yield at low concentration will be problematic. The low analyte concentration can not be fully compensated by just increasing the labeling reagent concentration [32,33]. A different approach is on-column biotinylation [15,32,33]. A higher yield can be achieved by preconcentration of the analyte prior to biotinylation on a pre-column, followed by rinsing the pre-column with biotinylation reagent. After removal of excess biotinylation reagent, the pre-column can be coupled on-line to the HPLC–EA-BCD. Future research will be devoted to on-column labeling to achieve higher biotinylation yields and therefore lower minimal detectable concentration. The large variety of commercially available biotinylation reagents makes the current meth-

odology suitable for the analysis of a wide range of analytes.

Hb was enriched from urine with good recovery and reproducibility. However, some matrix effect from urine was still observed. Therefore, in future research, solid-phase extraction will be performed with selective, immobilized antibody supports. Immobilized antibodies can not only be used for the isolation of Hb from urine, but also for a selective preconcentration of other analytes from different biological matrices.

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