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Applying hollow fibres for separating free and bound label in continuous-flow immunochemical detection

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Abstract

On-line liquid chromatography–immunochemical detection (LC–ICD) provides the possibility to individually monitor cross-reactive compounds overcoming the need of tedious fraction collection. ICD is performed as a post-column reaction detection system and is based on a two-step immunoreaction. In the first step unlabelled antibodies are added to the LC effluent and allowed to react with antigens (analytes) eluting from the LC column. The amount of analytes bound to the antibodies is measured by adding, in a second step, labelled antigen to the reaction mixture. For quantitation, free and bound label need to be separated prior to detection. The present paper describes a hollow fibre module (HFM), which can be used for this purpose. Separation of free and bound label occurs on discrimination by size. Using biotin as a model compound, a detection limit of 30 nmol/l can be reached employing anti-biotin antibodies and a low-molecular-mass fluorescence label in the LC–ICD system. Additional to low-molecular-mass labels, the HFM allows the use of small enzyme labels. In this context, horseradish peroxidase-labelled biotin was used as a label in combination with antibodies in the immunochemical detection of biotin. This allows future implementation of commercially available enzyme immunoassay kits in continuous-flow immunochemical detection.

Keywords: Immunochemical detection; Detection, LC; Hollow fibres; Membranes; Biotin

1. Introduction

Combining the separation power of liquid chromatography (LC) with the selectivity and sensitivity of immunochemical detection (ICD) overcomes problems of sensitive detection in LC on the one hand and cross-reactivity, frequently found in immunochemical techniques, on the other hand [1]. The detection principle of ICD is based on recognition of the analyte by a suitable antibody and the use of labels to monitor this affinity reaction.

LC–ICD systems can be developed for analytes lacking good detection characteristics, e.g., peptides, leukotrienes [2,3]. Cross-reactivity has proven to allow simultaneous detection of the drug digoxin and its active metabolites [4]. This implies that metabolites of new drugs may be found through LC–ICD. By using receptor molecules as affinity proteins, it is further possible to screen for unknown receptor ligands, both for the purpose of drug discovery as well as for recognition of hormone abuse in doping.

Continuous-flow LC–ICD systems have been developed using antibodies [5], receptors [6] as well as avidin [7] as affinity proteins. In order to detect the

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affinity reaction in the on-line LC–ICD system, fluorescence-labelled antibodies [5] as well as fluorescence-labelled antigens [7] have been employed. When using fluorescent antigens, manipulation of the antibody is avoided. Additionally, labelled antigens are commercially available for a wide range of applications.

Both, homogeneous and heterogeneous systems can be developed for continuous-flow ICD. In a homogeneous set-up, no separation of free and bound label is performed, whereas in a heterogeneous set-up both species are separated prior to detection. Hence, the homogeneous set-up is easier. However, such systems require significant differences in detection characteristics between free and bound label, e.g., a change of quantum yield or of Stokes shift for a fluorescence label. Additionally, high background signals are often obtained.

In comparison, the heterogeneous set-up is generally applicable, independent of the label characteristics. A crucial part in continuous-flow heterogeneous systems is the separation of free and bound label. A previously described on-line LC–ICD system based on antibodies and labelled antigens uses a C_{18} restricted-access column material for this purpose. Free label molecules are retained at the hydrophobic inner surface of the restricted-access support [4]. Antibody-bound label passes the restricted-access column unretained and is detected by means of fluorescence detection. Separation of free and bound label is based on the difference in molecular mass and the hydrophobicity of the free label. This system requires the use of soluble affinity proteins, since insoluble or suspended affinity proteins would clog the restricted-access column. It further requires labels, which are small enough to reach the inner surface ($M_r < 10\,000$) and are hydrophobic enough to be efficiently retained on the restricted-access column [8].

Our aim is to broaden both the range of affinity proteins as well as the range of labels which can be used for LC–ICD. Insoluble or suspended proteins, like e.g., membrane-bound receptors, may function as affinity protein additionally to soluble proteins used so far. The range of labels which can be used is increased, when their hydrophobicity does not influence the separation of free and bound label. So far, the necessity for small labels in ICD using a restricted-access column for separating free and bound label excludes the use of enzyme labels.

The present paper describes the use of membranes for separating free and bound label in continuous-flow LC–ICD for the model compound biotin. Membranes are typically used for separating high- and low-molecular-mass compounds. Due to their common use in protein purification, membrane characteristics with respect to non-specific binding are favourable. Additionally, they are compatible with insoluble components. These characteristics have been employed in sample preparation techniques in combination with LC [9,10] as well as in post-column dialysis for the purpose of continuous buffer exchange [11]. For post-column separation of free and bound label in an LC–ICD system capillary cross-flow membranes (hollow fibres) are considered. Expected benefits when using membranes for separating free and bound label in ICD are the independence of label polarity and label size, continuous operation without the need of regeneration and the use of preparations containing insoluble components, e.g., cell membrane fragments.

2. Materials and methods

2.1. Chemicals

D-(+)-Biotin was obtained from Merck (Darmstadt, Germany), 5-(biotinamidocaproylamido)-pentylthioureidylfluorescein (fluorescein-biotin) from Sigma (St. Louis, MO, USA), and immunopure goat anti-biotin from Pierce (Rockford, IL, USA). Tween 20, sodium chloride, sodium phosphate, potassium chloride and potassium phosphate were purchased from Merck. HPLC-grade water was produced in a Milli-Q system (Millipore, Bedford, MA, USA) and used throughout this work.

2.2. Instrumentation

The flow injection (FI) system consisted of a Gilson (Villiers-le-Bel, France) XL autosampler equipped with a Rheodyne (Cotati, CA, USA) six-port injection valve (injection loop 20 μl), one Gilson 305 and two Gilson 306 LC-pumps which delivered the carrier (mobile phase), the antibody

solution and the fluorescein-biotin solution at a flow-rate of 0.2 ml/min each. A Jasco (Tokyo, Japan) fluorescence detector FP 920 (λ_{exc}=486 nm, λ_{em}= 516 nm) was used for detection. Data acquisition occurred either with a Kipp and Zonen (Delft, Netherlands) BD 40 recorder or with Gilson 715 software. The analytical system was controlled by Gilson 719 Turbo Pascal software.

Phosphate-buffered saline containing 0.5% Tween 20 (PBST) was used throughout as FI carrier. The antibody (6.7 nmol/l) and fluorescein-biotin (13.3 nmol/l) solutions were prepared in PBST and added to the FI carrier via inverted Y-type mixing unions (Upchurch, Oak Harbor, WA, USA). Knitted 0.5 mm I.D. PTFE reaction coils with a volume of 900 and 600 μl for reaction 1 and 2, respectively, were used. The system was operated at room temperature. LC separations were carried out on a 100×3.0 mm I.D. stainless-steel column packed with Nucleosil C_{18} (5 μm particles, Macherey–Nagel, Düren, Germany) using methanol–aqueous triethylammonium acetate (10 mmol/l, pH 6.6) (10:90, v/v) as mobile phase as described previously [7]. The mobile phase was delivered at a flow-rate of 0.4 ml/min.

2.3. Hollow fibre module

Separation of free and antibody-bound antigen was performed with a custom-made hollow-fibre module (HFM) consisting of one capillary cross-flow membrane (X-Flow BV, Almelo, Netherlands, type UFM, M5) of 12 cm length, 1.5 mm I.D. and M_r 100 000 cut-off, or 0.5 mm I.D. and M_r 50 000 cut-off.

The HFM containing the 0.5 mm I.D. hollow fibre is depicted in Fig. 1. The module was manufactured from Plexiglass with a cylindrical channel and screw inlets at both ends as well as at the side of both ends. Fused-silica of 320 μm I.D. was inserted into the PTFE tubing and the hollow fibre. Two mm of silicone tubing was put around both the hollow fibre and the tubing, a fingertight nut with a flat end was put on the tubing and screwed into the Plexiglas. The silicone tubing serves as ferrule, tightening both the hollow fibre and the PTFE tubing.

The HFM containing the 1.5 mm I.D. hollow fibre was manufactured in a similar way. The connection between the PTFE tubing and the hollow fibre was accomplished by inserting the PTFE tubing directly into the hollow fibre. Silicone tubing again served as ferrule.

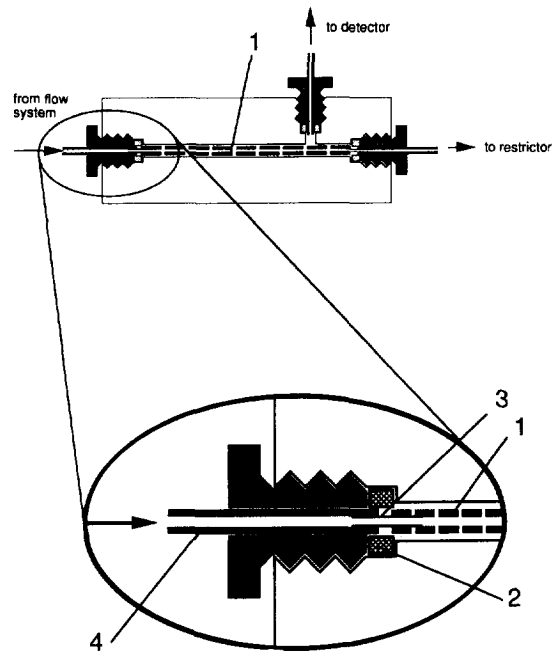

Fig. 1. Hollow fibre module: (1) hydrophilic cross-flow hollow fibre, (2) silicone ferrule, (3) fused silica (320 μm I.D.), (4) PTFE tubing (0.5 mm I.D.).

The outlet was connected to a Nupro (Willoughby, OH, USA) fine metering valve restrictor forcing the fluid through the membrane to the side connection at the outlet leading to the fluorescence detector.

3. Results and discussion

3.1. ICD with membrane separation

The ICD presented here consists of a two-step immunoreaction (see Fig. 2). First, antibodies are added post-column and allowed to react with analyte eluting from the LC column for 2 min. Hereafter, fluorescence-labelled antigen is added to the reaction mixture. In the short reaction time available (1 min) the label binds primarily to free antibody-binding sites. The analyte concentration can then be measured as the concentration of either free or antibody-bound label. For homogeneous assays this is performed by the different detection characteristics of

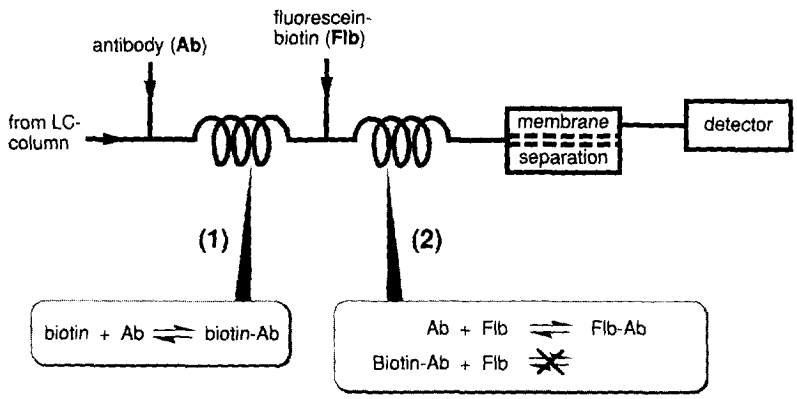

Fig. 2. Scheme of the ICD system.

the free and bound label [7]. However, when indistinguishable, free and bound label have to be separated prior to detection. By introducing a membrane into the system with a cut-off between the size of the large antibody and the label, free label (with a molecular mass typically below 1000) passes the membrane freely, whereas the antibody-bound label ($M_r \approx 150\,000$) will remain in the retentate stream and ultimately be washed out of the capillary. The separation occurring as a consequence of this phenomenon is solely based on size.

The flux through the membrane is induced by applying a pressure drop (<5 bar) over the membrane. Thus, a split is created between the retentate and permeate outlet, whereby the compositions of the retentate and permeate stream vary (see Fig. 3). The permeate contains free label only, whereas the retentate contains free label as well as antigen–antibody complex. Simultaneously, the retentate volume is reduced thereby concentrating antibodies and antibody–antigen complexes. A selective mass flow split is thus created in the HFM.

As mentioned above, both free or antibody-bound label can be used for quantifying the original analyte concentration. Since the permeate stream only contains free label, whereas the retentate stream consists of a mixture of free and bound label, we chose to use the permeate stream for detection. When measuring the amount of free label to quantify the immunoreaction, positive signals are obtained in the presence of analyte. This is in contrast to the decrease of signals obtained in systems measuring bound label, e.g., when a restricted-access column is used for separating free and bound label [7]. The set-up chosen here limits the concentration of label, which can be added to the reaction mixture (see reaction 2

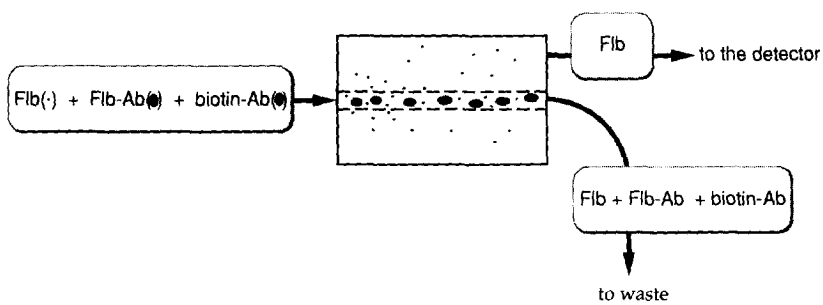

Fig. 3. Scheme of the separation of free and antibody-bound label.

Table 1
Estimated volumes of the HFMs

	0.5 mm I.D. HFM	1.5 mm I.D. HFM
Donor compartment	24 μl	213 μl
Acceptor compartment	65 μl	261 μl

in Fig. 2). Even though a large excess of label would be advantageous to obtain fast reaction kinetics, it would also lead to an increased background signal.

3.2. Continuous-flow ICD conditions

Using biotin as a model compound, a flow injection (FI) system was set up to investigate factors influencing the HFMs performance. These comprise fibre dimensions, residence times, and concentration of reactants.

3.2.1. Influence of the HFMs dimensions on bandbroadening

Extra-column bandbroadening introduced by the HFM into the ICD system depends on the dimensions of the retentate and permeate compartments, since they determine the dead volume as well as the residence time in the HFM. The retentate and permeate compartments of the HFMs employing a 1.5 and 0.5 mm I.D. hollow fibres are specified in Table 1. The estimated volumes differ nine-fold for the retentate compartments and four-fold for the permeate compartments. These differences are apparent when evaluating the bandbroadening in the system, see Table 2. Adding a flowing stream and passing a reaction coil leads to a bandbroadening of $\sigma_v = 95$ μl and $\sigma_v = 165$ μl for reactions 1 and 2, respectively. Introducing the HFM leads to a further increase of peak width. For the 1.5 mm I.D. HFM this is an extra $\sigma_v = 358$ μl, whereas the 0.5 mm I.D. HFM leads to an extra $\sigma_v = 207$ μl. The extra-column bandbroadening of the presented ICD system amounts to a total peak width of $\sigma_{v(tot)} = 282$ μl. This is in a similar range as for a previously described system [7]. Such bandbroadening is acceptable if the increase in selectivity is considered.

Table 2
Bandbroadening in the ICD system

	σ (μl)[a]	$\Delta\sigma$ (μl)
After injection port	15	-
After first reaction coil	96	95
After second reaction coil	191	165
After HFM with 1.5 mm I.D.	406	358
After HFM with 0.5 mm I.D.	282	207

[a] σ determined as half peak width at 0.6 of peak height.

Small dimensions of the HFM are advantageous in ICD, since less bandbroadening occurs as a consequence of dead volume and residence time. Satisfactory results were obtained with a module containing a hollow fibre of 12 cm length and 0.5 mm I.D.. Further developments reducing the retentate and permeate volumes of the HFM, e.g., by shortening the HFM, will be advantageous for limiting extra-column bandbroadening.

3.2.2. Influence of residence time on the immunochemical reactions

Additionally to influencing bandbroadening, residence times in the HFM may also influence the immunochemical reaction. The residence time in the retentate compartment virtually lengthens the time for reaction 2 (see Fig. 2). As described above, during a short reaction period for reaction 2, label binds primarily to free antibody-binding sites (association). However, when prolonging this reaction time, dissociation of analyte– and label–antibody complex may also occur.

The influence of this effect on the signal is shown in Fig. 4. The residence time in the retentate compartment was varied by using different flow-rates at constant reaction times using the 0.5 mm I.D. HFM. At each flow-rate the back pressure was adjusted to keep the flow-rate of the permeate constant. At residence times shorter than 4.6 s, no influence on the signal can be observed for different analyte concentrations. However, at a residence time of 9 s, the signal is altered at high analyte concentration. When antigen–antibody-complex dissociation occurs as a consequence of a long residence time in the retentate compartment, free antibody binding sites become available. At low analyte concentrations, mainly free label will be present, associating again with the antibody-binding sites. At high concentrations, however, both free analyte and free label will be present and compete for the

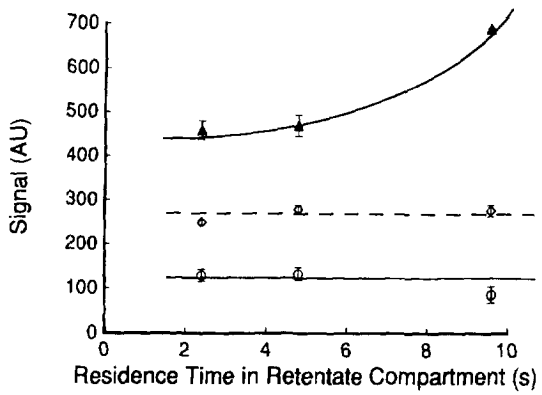

Fig. 4. Influence of the residence time in the retentate compartment on the signal for (○) 200, (◇) 400 and (▲) 800 nmol/l biotin.

antibody-binding sites. In this way, label molecules originally bound to antibodies may be displaced by analyte molecules, leading to an increased concentration of free label and eventually to a higher signal. Since no influence on the signal could be observed at a retentate residence time shorter than 4.6 s, work was continued under these circumstances.

The influence of the residence times in the permeate phase was investigated by changing the backpressure at constant total flow-rate using the 0.5 mm I.D. HFM. Assuming limited influence of the concentration effect at the limited retentate residence time of 4.6 s, the permeate residence time should not influence the immunochemical reaction, since only free label is present. This is supported by data for different analyte concentrations represented in Fig. 5.

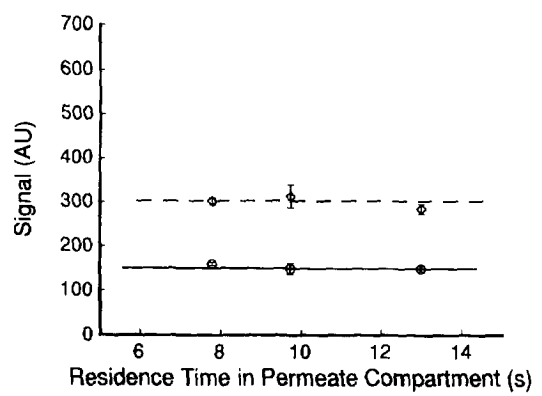

Fig. 5. Influence of the residence time in the permeate compartment on the signal for (○) 200 and (◇) 400 nmol/l biotin.

Short retentate residence times are favourable for the separation of free and bound label without affecting the immunochemical reactions. Therefore, further developments reducing the retentate volume of the HFM will limit its influence on the immunochemical reactions.

3.2.3. *Influence of immunoreagents*

Special attention needs to be paid to the immunoreagents used in ICD. The antibodies used should exert high affinities ($K_d \geq 10^7$ mol^{-1}) to the analytes as well as to the label. For the model system described here, pure antibodies are commercially available and proved to be applicable for ICD. Additionally, we expect that the HFM will allow the use of crude sera or other matrices containing affinity proteins in continuous-flow ICD. On the other hand, the labelled antigen needs to be of high purity in order to limit the background signal and allow accurate quantification of the immunoreaction.

The concentrations of the immunoreagents influence reaction kinetics, background signal and cost. In order to achieve fast reaction kinetics, high concentrations are desirable allowing an excess of binding sites to react with the analyte in reaction 1 and an excess of label to react with the left-over free binding sites in reaction 2 (see Fig. 2). The high cost limits the amount of affinity proteins used in ICD. A previously described system using bound label as a measure for the analyte concentration therefore used a limited amount of binding sites, but an excess of label [7]. In the set-up described here, also a limited amount of binding sites was used in the same concentration range as in previously described systems [6,7]. In contrast to the previously described method, here free label is used as a measure for the analyte concentration. Under these circumstances, an excess of label creates a high background signal. It is therefore also necessary to limit the concentration of label added.

In Fig. 6 the advantageous effect of a higher label concentration is clearly seen. At higher concentrations of label, higher signals are obtained. The label concentration was however not further increased in order to limit the background.

Under such circumstances, an increase in binding site concentration leading to faster reaction kinetics will be counteracted by a decreased excess of label.

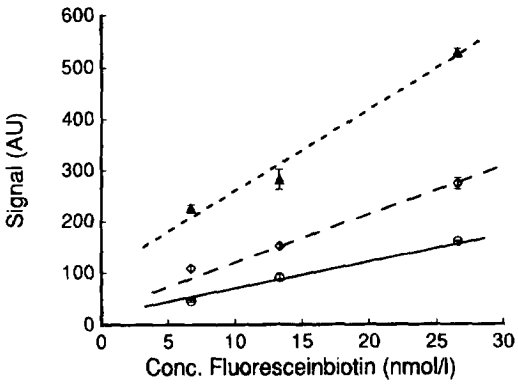

Fig. 6. Influence of label concentration on the signal of (○) 200, (◇) 400 and (▲) 800 nmol/l biotin.

This is reflected in results (not shown) giving similar signals for biotin when increasing the antibody concentration at constant label concentration.

As mentioned above, many affinity proteins are still rather expensive and will therefore determine the overall cost of analysis. The set-up described here consumes 6.6 pmol antibodies per minute. With an assay time of 5 min, which is sufficient in the flow injection mode, a total of 6.6 pmol (=1 μg) antibodies are consumed. This is in the same range as antibody consumption in a microtiter-assay per well [12]. At a purchase price of, e.g., US$ 100 per mg, each measurement would amount to the cost of US$ 0.1. However, when coupling the ICD to LC, immunoreagent consumption will be increased as a consequence of longer assay times.

3.3. On-line coupling to liquid chromatography

The ICD system was coupled on-line to reversed-phase LC using C_{18}-bonded silica as stationary phase. The eluent necessary for the chromatographic separation consisted of a 10 mmol/l tetraethylammonium acetate buffer pH 7.0 containing 10% of methanol. It thus differs from the previously used FI carrier with respect to ions and the presence of organic modifier. The effect of the eluent on the ICD system was investigated in the FI system. Responses obtained when using the eluent are identical to those obtained with the fully aqueous system (results not shown). This corresponds with previous investigations, which have shown that a modifier content up to 30% does not influence the antibody–antigen interaction [5]. Since the presented ICD system comprising a HFM is not sensitive to breakthrough of label, the modifier content may be increased up to a level which is harmful either to the antibody–antigen interaction or to the hollow fibre. Chemical resistance of membranes to organic solvents is limited. For the membranes applied in this work resistance to ethanol contents up to 12% is assured, they are however not resistant to ethanol contents above 40%. Such chemical resistance will suffice for most applications, especially when considering the fact that the modifier content of the LC effluent is diluted before reaching the HFM.

Compared to the FI set-up, the flow-rate of the LC mobile phase was increased to 0.4 ml/min. The increased flow-rate reduces reaction times as well as antibody and label concentrations, which lead to less reaction efficiency and consequently to lower signals.

The advantages of ICD in comparison with UV are clearly shown in Fig. 7, which contains chromatograms of (a) LC–UV detection of a 400 μmol/l biotin standard solution omitting the HFM, (b) LC–ICD of a 400 nmol/l biotin standard solution, and (c) LC–ICD of a blank solution. The front in chromatogram (a) as well as the dips at t=6 min in chromatograms (b) and (c) are caused by the DMSO in the solutions. Additional to strongly increased selectivity, the signal-to-noise ratio is drastically improved.

3.4. Analytical data

For the LC–ICD system a linear calibration curve was obtained in the range of 80–4000 nmol/l (20 μl injections, r=0.998). The detection limit estimated as a signal-to-noise ratio of three is 30 nmol/l for a 20-μl injection volume. Repetitive injections indicate a satisfactory stability of the system. When injecting a 400 nmol/l solution of biotin for twenty times an R.S.D. of 5.5% is calculated.

The stability of the ICD system in combination with a hollow fibre module largely depends on the robustness of the membrane. Both leakage and fouling of the membrane will seriously affect the performance of the system. Leakage might occur due to pressure changes, whereas fouling might be caused by non-specific binding of proteins present in

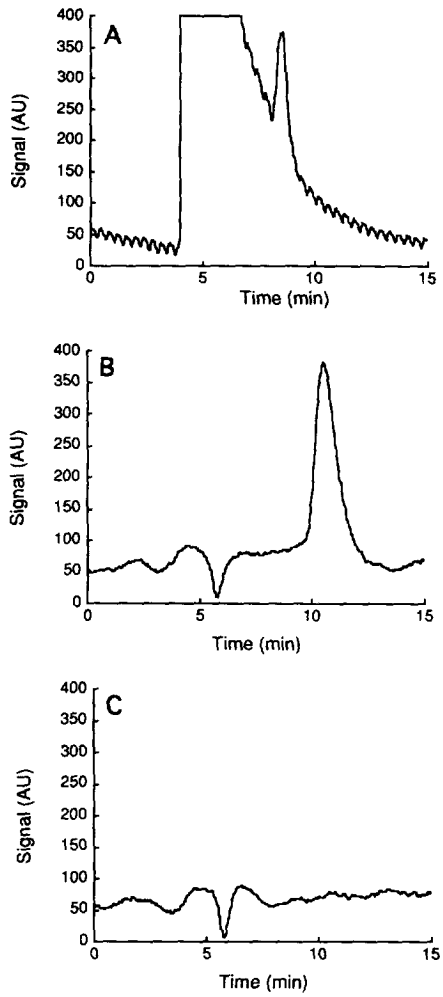

Fig. 7. (A) LC–UV detection at 210 nm of 400 μmol/l biotin; (B) LC–ICD of 400 nmol/l biotin and (C) of a blank solution using fluorescence labelled biotin as reporter molecule.

the FI carrier. In order to prevent non-specific binding of proteins, the carrier contains Tween 20 as well as a high salt concentration. Should the membrane foul nevertheless, a decrease of flux is expected in time. However, no changes in flux were observed. Therefore, we assume a constant flux and no, or very limited, fouling of the membrane. Accordingly, no reconditioning procedures were carried out. Future investigations will evaluate these findings with respect to long term stability, injection of real life samples and the use of crude affinity protein solutions.

3.5. Enzyme-labelled antigen as reporter molecule

The hollow fibre with a cut-off of M_r 100 000 used here can in principle separate antibodies with a molecular mass of around 150 000 from compounds up to M_r 80 000. This indicates that by using membranes for separating free and bound label, larger labels can be used additionally to the low-molecular-mass labels employed so far in continuous flow ICD. Horseradish peroxidase (HRP) fulfils the requirements for such a label, being a small ($M_r \approx$ 44 000) and active enzyme. Additionally, many HRP-labelled antigens are commercially available, since HRP is a frequently used enzyme label in batch enzyme immunoassays.

In order to test the applicability of an enzyme label in continuous-flow ICD, 6.7 nmol/l HRP-labelled biotin was added as reporter molecule to the main stream. The amount of free enzyme label is determined by adding fluorogenic substrate to the flowing stream after the HFM. The substrate chosen here consisted of 83.3 μmol/l hydroxyphenylpropionic acid and 20 mmol/l hydrogen peroxide yielding a fluorescent product which is detected at 320 nm excitation and 404 nm emission wavelength. Fig. 8 represents signals obtained for 20 μl injections of 40 μmol/l biotin. So far, signals have been obtained for analyte concentrations down to 200 nmol/ l. Current work is focused on optimising the enzymatic reaction step with the aim to amplify the signal and thus lower detection limits.

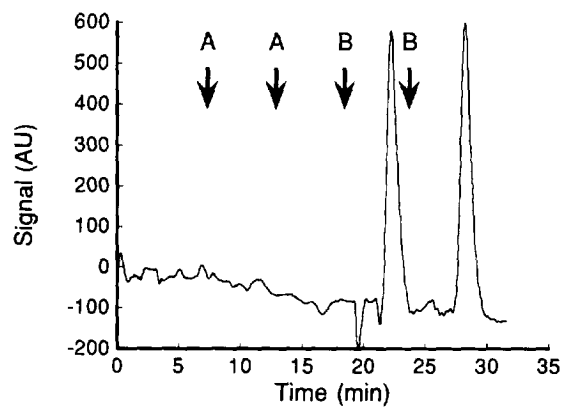

Fig. 8. Signals obtained using horseradish peroxidase-labelled biotin as reporter molecule: (A) blank injection, (B) 40 μmol/l biotin injection.

4. Conclusions

The presented system establishes an efficient separation method for free and bound label in ICD by using a hollow fibre module. The separation is solely based on size and does not require regeneration. Additionally, enzyme-labelled ligands can be employed as long as the enzyme label has a molecular mass well below that of the affinity protein. As shown, for example, a membrane with a cut-off of M_r 100 000 separated the antibody ($M_r \approx 150\,000$) from horseradish peroxidase ($M_r \approx 44\,000$), which is commonly used as a label in enzyme immunoassays. This introduces the opportunity to implement commercially available enzyme-immunoassay kits into an LC–ICD system.

Since the risk of fouling is low, this system should also be compatible with non-soluble proteins, such as membrane-bound receptors or even cell systems, for the biorecognition of the analyte.

Increased flexibility in comparison to previously described systems is therefore obtained with this set-up for the application of on-line LC–ICD.

Acknowledgments

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