ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
26 (2001) 975–985

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Potential of on-line micro-LC immunochemical detection in the bioanalysis of cytokines

T. Schenk [a], H. Irth [a,*], G. Marko-Varga [b], L-E. Edholm [b], U.R. Tjaden [a], J. van der Greef [a]

[a] *Leiden/Amsterdam Center for Drug Research, Division of Analytical Chemistry, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands*
[b] *Department of Bioanalytical Chemistry, Astra DracoAB, PreClinical Research & Development, P.O. Box 34, S-221 00 Lund, Sweden*

Received 10 April 2001; received in revised form 12 May 2001; accepted 13 May 2001

Abstract

An on-line liquid chromatography-immunochemical detection (LC-ICD) system for the quantification of cytokines in cell extracts has been developed using a post-column continuous-flow reaction detection system using fluorescence labelled antibodies. Cytokines eluting from the micro-HPLC column react with antibodies to form fluorescent complexes. In a second step the excess of free antibody is trapped on a cytokine bound support prior to fluorescence detection. The concentration detection limit of the flow injection-ICD system was 50 pM (20 μl injection volume) for interleukine 4 (IL-4). An absolute detection limit of 1 fmol was obtained for IL-4. Similar to ICD systems for small non-protein analytes developed earlier, reaction times were in the order of 1 minute. The immobilised cytokine affinity columns can easily be regenerated and used for months. The present ICD system for interleukine 4, 6, 8 and 10 was coupled to ion exchange-, size exclusion- and reversed phase chromatography. Important parameters (reaction times, reaction conditions) were investigated to get a better understanding of post-column ICD systems for macromolecules.

Keywords: Immunochemical detection; Cytokine; Micro LC; Immunoaffinity sample clean-up

1. Introduction

Recent developments in molecular biology have increased the availability of recombinant proteins. Hence, proteins are found to be of great importance in future drug development [1–6]. Proteins also are important biomarkers to monitor certain physiological or pathological processes in the human body [7–9]. Cytokines are a group of (glyco)proteins of 10–30 kDa which play an im-

* Corresponding author. Present address: Department of Analytical Chemistry and Applied Spectroscopy, Division of Chemistry, Vrije University Amsterdam, De Boelelaan 1083, 1081 Amsterdam, The Netherlands. Tel.: +31-71-527-4220; fax: +31-20-444-7543.
E-mail address: irth@chem.vu.nl (H. Irth).

0731-7085/01/$ - see front matter
PII: S0731-7085(01)00464-2

portant role in regulating immune responses, sleep, and have been associated with several inflammatory diseases [10–16]. Therefore, cytokines are important biomarkers to monitor the effect of drugs influencing the immune system or inflammation. Due to the high biological activity of most cytokines their concentration in body fluids is rather low [17–20]. In order to measure these low concentrations, extremely sensitive and selective analytical methods must be available to detect picomolar concentrations of the cytokine of interest in the presence of micromolar concentrations of other endogenous proteins. In the past decades several methods have been developed to determine protein concentrations [21–28]. Of these, only immunoassays are sufficiently sensitive and, to a certain extent, selective enough to meet the stated requirements. Hence, immunoassays are nowadays the most widely used method to quantify cytokines in biological matrices. However, immunoassays suffer from some disadvantages [29–38]. The antibodies used are often not 100% specific for the cytokine of interest, which causes cross-reactivity and consequently erroneous results. Also, other compounds, e.g. soluble cytokine receptors or cofactors of the cytokine, could interfere with the immunorecognition and reduce the accuracy of the quantification. Several cytokines are able to form dimers, trimers or oligomers which can differ considerably in biological activity and affinity towards the antibodies used in the immunoassay, resulting in inaccurate quantification data. Using immunoassays only one analyte can be quantified at a time and due to the long incubation times, even when automated, an immunoassay takes 8–24 h.

The earlier mentioned problems of cross-reactive compounds, soluble receptors and oligomer formation could be overcome by isolating the cytokines by HPLC combined with fractionation prior to the immunoassay. However, this would further dilute the samples and makes the procedure even more time consuming. A less laborious approach is to couple immunoassays post-column on-line to HPLC separations. Irth et al. [39] developed such a system for small analytes, e.g. digoxin. This immunochemical detection system was based on the addition of an excess of fluorescently labelled Fab fragments to the column effluent. Digoxin-antibody complex was then separated from remaining free antibodies by passing through a digoxin bound support. A review of biospecific detection in liquid chromatography (LC) has been given by Emneus and Marko-Varga [40]. Combining the high separation efficiencies and speed of HPLC with the extreme sensitivity of biochemical detection (LC-BCD) has proven to be a very powerful and fast analytical tool for small, non protein, molecules. Recently, Miller and Herman [41] presented a LC-BCD system for human methionyl granulocyte colony stimulating factor (GCSF) using both labelled antibodies and receptors. Oosterkamp et al. [42] developed a receptor affinity detection system coupled on-line to HPLC for urokinase plasminogen activator.

The physiological effects of cytokines often depend on the relative concentrations of several cytokines. Therefore, it is crucial to quantify all relevant cytokines present in one biological sample to obtain a full cytokine profile. Additionally, the information generated on activity-structure relations is of mandatory importance. Quantification of each cytokine using a different immunoassay for each cytokine can be highly prone to error.

The aim of the present work was to design techniques allowing the quantification of several cytokines at the same time circumventing the earlier mentioned problems of cross-reactivity, soluble receptors and oligomer formation in a relatively short time. In the present paper we describe a new analytical tool for the simultaneous, accurate and fast determination of cytokines using LC on-line coupled to immunochemical detection (LC-ICD). The developed ICD technique for proteins is analogous to the ICD system for digoxin developed by Irth et al. [39]. The influence of the reaction times in the coil and column on the performance of the system was investigated to get a better understanding of the reaction kinetics of macromolecules in post-column reaction detection techniques. This detection principle was coupled on-line to ion exchange, size-exclusion- and reversed phase separations. To be able to detect several cytokines in a

single chromatographic run the immunoreagent consisted of a mixture of labelled antibodies against the cytokines, while the excess of free antibodies was removed by placing the corresponding cytokine affinity columns in series. Furthermore, we investigated the impact of different LC parameters like ionic strength and organic modifier content on the performance of the ICD system.

2. Experimental

2.1. Materials

All recombinant human cytokines and monoclonal antibodies were purchased from R&D Systems Europe (Abingdon, UK). POROS-CM, POROS-EP, POROS-R1 10 μm were purchased from PerSeptive Biosystems (Cambridge, USA). TSK G2000 SWxl was purchased from Tosohaas (Stuttgart, Germany), acetic acid from J.T. Baker (Deventer, Netherlands). The antibodies were fluorescently labelled using a FLUOS labelling kit from Boehringer Mannheim (Mannheim, Germany). ELISA blocking reagent was also purchased from Boehringer Mannheim and all other chemicals from Merck (Darmstadt, Germany).

2.2. Instrumentation

All experiments were carried out using a Gilson (Villiers-le-Bel, France) ASPEC XL equipped with a Rheodyne (Cotati, CA) sixport injection valve, a Jasco (Tokyo, Japan) FP 920 fluorescence detector (excitation wavelength 488 nm, emission wavelength 520 nm) was used for detection, while data was collected using Gilson 715 Pascal Software and a Kipp & Zonen (Delft, Netherlands) BD40 recorder. A Pharmacia (Uppsala, Sweden) P3500 pump was used for adding the immunoreagent. All tubing consisted of 0.13 mm i.d. PEEK, except for the reaction coil, which was made of 0.2 mm i.d. × 0.4 mm o.d. PTFE. The analytical system was controlled by Gilson 719 Pascal Software. When the immunochemical detection system was coupled to a chromatographic system a LC-Packings (Amsterdam, The Netherlands) IC-400-var flowsplitter, two additional Gilson pumps, 305 and 306 and a Gilson 811C dynamic mixer were used to create the gradient.

2.3. Immobilisation of the cytokines and anti-cytokine antibodies

POROS-EP (40 mg) was mixed with 25 μg cytokine or 500 μg anti-cytokine antibody in 0.5 ml 0.1 M carbonate buffer pH 8.0 and rotated end-over-end for 72 h at room temperature. After 72 h the remaining active groups on the POROS-EP were blocked by transferring the material to 1 ml of 1 M ethanolamine adjusted to pH 8.0 with HCl and rotating the mixture for 2 h. Finally, the product was washed with and stored in phosphate-buffered saline (PBS): 10 mM phosphate buffer pH 7.4 with 150 mM NaCl and 20 mM KCl at 4 °C. Blocked-POROS-EP was obtained by omitting the IL-4. All affinity columns were packed according to the manufacturers prescription in a 1.6 × 60 mm PEEK column.

2.4. Flow injection immunochemical detection (FI-ICD)

A scheme of the ICD is displayed in Fig. 1. The reaction coil consisted of knitted 0.2 mm i.d. × 0.4 mm o.d. PTFE tubing with an internal volume of 200 μl. The carrier flow consisted of PBS containing 0.5% (w/w) Tween 20 (PBST) and 1% (w/w) ELISA blocking reagent (BR). The immunoreagent solution consisted of 1 nM labelled antibod-

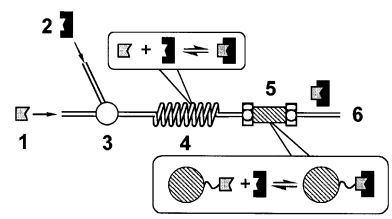

Fig. 1. Scheme of the continuous flow immunochemical detection system for cytokines. (1) Carrier flow (100 μl/min) consisting of BR and injected cytokine, (2) immunoreagent solution (100 μl/min) consisting of 1 nM fluorescently labelled anti-cytokine antibodies, (3) mixing-T, (4) reaction coil, (5) immobilised cytokine column, (6) flow to fluorescence detector. For other conditions, see Section 2.

ies in BR. Mixing of the two buffer streams, with a mixing ratio of 1:1, was performed using an inverted Y-type low-dead-volume mixing union. All experiments were performed at room temperature. All cytokine samples were dissolved in BR and stored at −20 °C.

2.5. Coupling of liquid chromatography to immunochemical detection (LC-ICD)

For gradient cation-exchange- and reversed phase separations a flowsplitter (splitratio: 1/25) was used to obtain a flow rate of 40 μl/min in the micro LC-system. All separations were performed at room temperature. The flow rate of the immunoreagent was 160 μl/min. For the LC-ICD analysis of two cytokines in a single run the two corresponding cytokine-columns were placed in series.

2.6. Cation-exchange chromatography

A 0.75 × 50 mm PEEK column was packed with CM-POROS. Elution was performed using a salt gradient, starting 10 min after injection, from PBST to PBST containing 0.4 M NaCl in 15 min. All samples (20 μl) were dissolved in PBST. Cation-exchange chromatography was also performed with pH step gradient elution, using the same HPLC column. Elution was performed using a step gradient, 5 min after injection, from 10 mM acetate buffer pH 4.5 containing 0.5% Tween 20 and 0.2 M NaCl to 10 mM carbonate buffer pH 10.0 containing 0.5% Tween 20 and 0.2 M KCl. The samples were dissolved in starting buffer.

2.7. Size exclusion chromatography

A 0.75 × 300 mm PEEK column was packed with TSK G2000 SWxl. Elution was performed using PBST as mobile phase at a flow rate of 40 μl/min. All samples (5 μl) were dissolved in PBST.

2.8. Reversed phase chromatography

A 0.5 × 50 mm PEEK column was packed with POROS-R1 10 μm stationary phase. Elution was performed using linear gradients of 60% A (0.1% TFA in 10% MeOH) to 100% B (0.1% TFA in 90% MeOH) in 15 min. All samples (20 μl) were dissolved in BR unless stated otherwise.

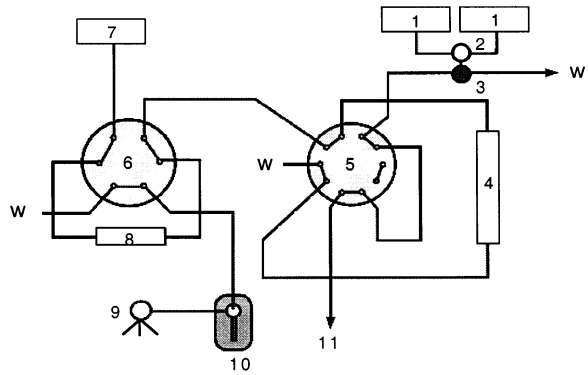

Fig. 2. Scheme of the immunoaffinity sample clean-up coupled to on-line micro HPLC-immunochemical detection of cytokines. (1) Gradient pumps, (2) mixing-T, (3) flow splitter, (4) micro LC-column, (5) 10-port valve, (6) 6-port valve, (7) elution-pump, (8) immunoaffinity pre-column, (9) solvent select valve, (10) dilutor, (11) flow to ICD system, (w) waste. For other conditions, see Section 2.

2.9. Sample clean-up

A scheme of the immunoaffinity sample clean-up coupled to on-line micro HPLC-immunochemical detection of cytokines is displayed in Fig. 2. Cell extract samples consisted of 95 μl human monocyte cell extract spiked with 5 μl cytokine in PBS or blank PBS and were loaded on a 1.6 × 60 mm anti-cytokine affinity column using PBS as carrier at 0.5 ml/min. The immunoaffinity precolumn was washed for 2 min with PBS at 0.5 ml/min. The cytokines were eluted using 0.1% TFA for 4 min at 100 μl/min. The precolumn was regenerated with 1 M NaCl at 0.5 ml/min for 5 min and equilibrated using 0.5 ml/min PBS for 3 min.

3. Results and discussion

The post-column ICD system (Fig. 1) presented here is based on the fast association of fluorescently-labelled antibodies with their target cy-

tokine. In the first step, the antibodies are added to the mobile phase. Cytokines react with the antibodies to form fluorescent immunocomplexes. In a second step free antibodies are removed prior to fluorescence detection via passage through a small column packed with cytokine-bound support.

3.1. Immobilisation of cytokines

POROS-EP was used as the solid support for immobilising the cytokines because of its better resistance against pressure and organic modifiers compared to Sepharose. POROS-EP is commonly used for covalent immobilisation of proteins by their amino groups. This immobilisation-step of the protein is a random immobilisation, only a certain percentage of the immobilised protein molecules will have the right orientation to bind the antibody. For the synthesis of the cytokine-supports only 25 μg cytokine (2 nmol) was used. In comparative studies it has been established that ca. 10% of the proteins used for the immobilisation are able to bind an antibody after immobilisation. Theoretically, this will yield a cytokine-column with a total capacity of 200 pmol. Since the immunoreagent solution of 1 nM antibodies is pumped at a flow rate of 100 μl/min during flow injection experiments, the affinity-column should theoretically be able to bind 200 ml immunoreagent solution, corresponding to 33 hours of analysis. This indicates that the capacity of the cytokine-columns is limited and therefore regeneration has to be possible.

3.2. Flow injection ICD

Interleukine 4 (IL-4) was used as a model cytokine to develop a multi-cytokine immunochemical detection system coupled on-line to an HPLC separation. First a flow injection immunochemical detection system was developed for IL-4. After immobilisation of IL-4, binding of the labelled anti-IL-4 antibodies (anti-IL-4*) to the IL-4-support was tested using a continuous flow of antibodies. Fig. 3 shows the increase in baseline when 1 nM fluorescently labelled antibodies are continuously added to a carrier flow and subsequently pass through a Blocked-POROS-EP column (from situation 1 to situation 2 in Fig. 3). Exchanging the Blocked-POROS-EP column for the IL-4 column results in a 90% reduction of the fluorescence (from situation 2 to situation 3 in Fig. 3), indicating that 90% of the fluorescently labelled antibodies are trapped by the affinity column. The 10% of the fluorescence signal which was not trapped by the IL-4-support is probably caused by protein impurities in the antibody preparation or antibodies that lost their activity caused by labelling at or near the binding site. Injection of an excess of antigen (100 nM IL-4) results in a peak of 95% of the maximum signal, while the blank shows no peak at all. This points out that the antibodies can be prevented from binding to the IL-4-support by blocking them with 100 nM IL-4 during a one minute reaction in the reaction coil. This proves that at least 95% of the antibodies binding to the IL-4-support bind

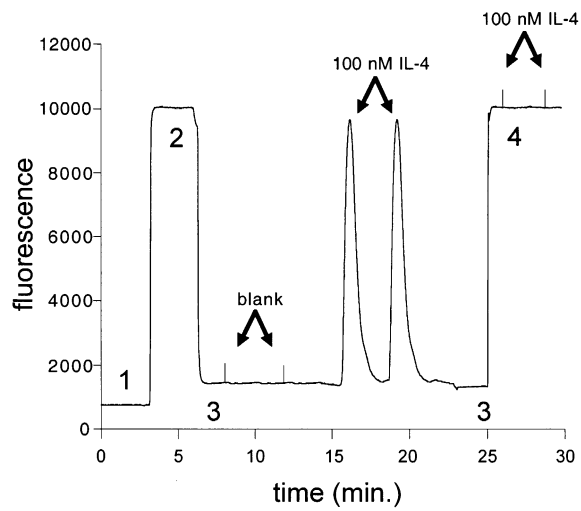

Fig. 3. Signal of continuous flow immunochemical detection systems. (1) carrier (100 μl/min) and immunoreagent (100 μl/min) both consist only of background buffer and pass through a Blocked-POROS-EP column resulting in a low fluorescence signal, (2) 1 nM fluorescently labelled antibody is added and still no cytokine column is present resulting in a strong increase in fluorescence background, (3) the Blocked-POROS-EP column is exchanged for a cytokine column, resulting in a 90% reduction of the background fluorescence by trapping of the labelled antibodies on the IL-4 column, (4) cytokine column is exchanged for the Blocked-POROS-EP column again, resulting in a high fluorescence signal. For other conditions, see Section 2.

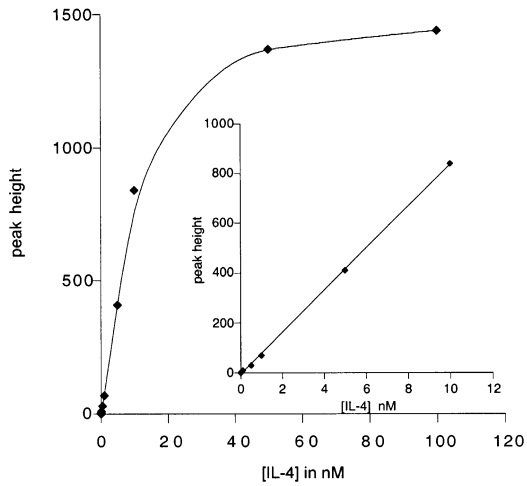

Fig. 4. Calibration curve of IL-4 in FI-ICD, injection volume, 20 μl. Both flows 100 μl/min. For other conditions, see Section 2.

specific. The IL-4 injected when the Blocked-POROS-EP column was on-line (situation 4 in Fig. 3) did not result in any signal. This proves that the IL-4 dependent signal was not caused by fluorescence enhancement by binding of the cytokine to the labelled antibody.

3.3. Analytical data

Fig. 4 shows a typical calibration curve of IL-4 ($n = 3$). As can be seen the detector response is non-linear to the IL-4 concentration over the full scale. This effect is produced by the saturation characteristics of the antibody/antigen interaction. However the first part of the calibration curve, between 50 pM and 10 nM of IL-4 is linear ($r = 0.999$, $n = 3$). The limit of detection (signal-to-noise ratio = 3) for IL-4 was 1 fmol (20 μl injection). It has been established that the labelling ratio of the antibodies was 3.5, indicating that the average amount of label molecules on each antibody was 3.5. Other experiments (data not shown) showed that the signal of IL-4 is proportional to the labelling ratio of the anti-IL-4* antibodies, while the baseline noise exhibits a smaller increase. This means that the limit of detection can probably be decreased using highly labelled antibodies.

3.4. Effect of reaction times and LC-conditions on the ICD system

The described ICD system for cytokines has to be coupled on-line to a liquid chromatographic protein separation system. In most normal-bore LC systems, however, the flow of the mobile phase is higher than the 100 μl/min used in the present ICD system for IL-4. Therefore the effect of higher flow rates (shorter reaction times in cytokine column) on the binding of the antibody to the IL-4-support was tested. In these experiments both flows (mobile phase and immunoreagent solution) were increased equally, so the mixing ratio remained 1:1 and the antibody concentration passing through the IL-4-support column was unchanged. This study proved that the binding of the labelled antibodies to the cytokine support was not affected by residence time in the cytokine column ranging from 6 s to 2 min. The excess of immobilised cytokine present in the column will trap the 1 nM antibodies almost instantaneously.

To get a better understanding of the reaction kinetics between macromolecules in postcolumn reaction detection systems the influence of the reaction time in the reaction coil was investigated. The reaction time in the coil was varied by using different coil lengths. Fig. 5 shows the influence of the reaction time in the coil on the peak height for the ICD system of IL-4. These results clearly show that the peak height increases with the reaction time until a maximum after approximately 2 min, after which the peak height decreases again. The initial increase of the signal is caused by the association reaction of the injected IL-4 with the labelled anti-IL-4 antibodies because the concentration of the antibody-antigen complex is proportional to the peak height. The kinetics of the reaction between the two macromolecules indicates that this reaction is not diffusion controlled but mass-transfer controlled. The decrease of the peak height is caused by the band broadening in the reaction coil, which counteracts the increase caused by the prolonged association reaction.

The described ICD system for proteins has to be coupled on-line to a liquid chromatographic

protein separation system. Ion-exchange chromatography is probably the most suited separation technique prior to ICD because of the mobile phase composition used (aqueous buffer, salts, moderate pH). As immunoreactions normally take place under physiological conditions at physiological ionic strength, the effect of different concentrations of sodium chloride on the immunoreaction was tested. Fig. 5 shows that an increasing ionic strength in the reaction coil causes a considerable decrease in peak height but does not influence the reaction kinetics because the shape of the three curves is identical with an optimum at a reaction time of 2 min. The decreased signal caused by the increased ionic strength is caused by a reduced affinity constant between the IL-4 and the antibodies.

To investigate the feasibility of coupling the ICD system for proteins to reversed phase chromatography the influence of methanol on the post-column immunochemical detection system was studied. In Fig. 6 the influence of methanol on the peak height is presented. This figure suggests an almost linear relationship between the IL-4 signal and the methanol concentration, indicating the negative effect of methanol on the interaction between IL-4 and the antibodies.

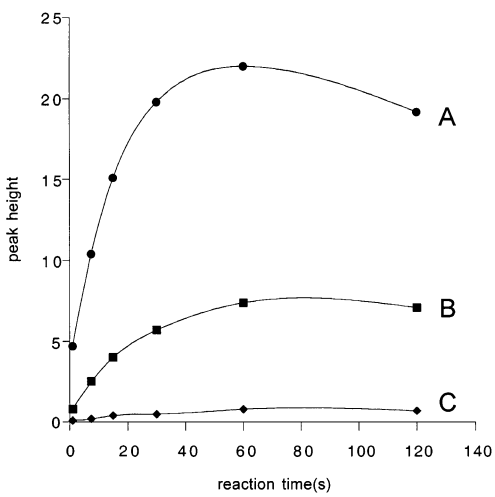

Fig. 5. Influence of reaction times and ionic strength on ICD of IL-4. (A) 165 mM NaCl, (B) 330 mM NaCl and (C) 660 mM NaCl. For conditions, see Section 2.

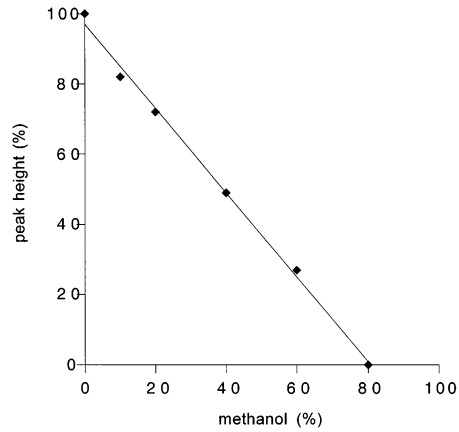

Fig. 6. Influence of methanol on ICD of IL-4. For conditions, see Section 2.

3.5. Regeneration of the cytokine-support

After using the cytokine-column for 1 day and re-using it a second day, the binding of the antibodies to the affinity column was reduced, resulting in a gradually increasing base-line. This was caused by saturation of the affinity support, corresponding to the theoretical capacity of 200 pmol. It was found that the labelled antibodies could be desorbed from the affinity column by flushing it overnight with PBS containing 1 M NaCl at a flow rate of 0.5 ml/min. Using this regeneration-step one cytokine-column could be used for 2 months without detectable loss of binding efficiency.

3.6. Coupling of LC-ICD

Micro-LC was chosen as the separation step prior to the ICD since it offers the possibility to maintain the low flow rates in the ICD system (minimising the reagent consumption) without post-column splitting. Micro-LC also offers the possibility to dilute the HPLC effluent (40 μl/min) with the immunoreagent solution (160 μl/min) and hereby decreasing the adverse effects of the salt or modifier gradients (Figs. 5 and 6) on the ICD system. A third advantage using micro-LC is the enhanced on-column concentration when gradient separations are employed in combination

with smaller elution volumes compared to normal bore LC.

3.7. Cation exchange chromatography-ICD

When using isocratic elution in cation-exchange chromatography it was extremely difficult to obtain a chromatogram for one cytokines. We were unable to perform a separation of different cytokines. The physio-chemical properties of the different cytokines are too different to obtain a separation using isocratic elution. After unsuccessful attempts to use isocratic elution in cation-exchange chromatography of IL-4, a salt gradient was used for elution. This resulted in an extremely increasing baseline caused by a decrease in affinity of the labelled anti-IL-4 for the IL-4-support (an increased dissociation rate constant). This decrease in affinity will not only decrease the binding efficiency of the column (the percentage of antibodies binding to the affinity support) but moreover will cause the dissociation of the already bound antibodies from the affinity support. This result is in accordance with the earlier discussed results shown in Fig. 5 and with the ability to regenerate the immobilised IL-4 support with 1 M NaCl. A less steep salt gradient for elution did not result in any better results. However, it was possible to retain IL-4 on the cation exchange column at pH 4.5, 0.2 M NaCl and elute the protein with a pH step gradient to pH 10.0, 0.2 M KCl, without any adverse effects on the ICD system. The disadvantage of this one step pH gradient is that at pH 4.5 most proteins will be retained by the cation-exchanger, and at pH 10.0 most proteins will elute from the column. This means that no actual separation will take place. A linear pH gradient would overcome this disadvantage. However, it is very difficult to obtain a linear pH gradient over a broad pH range. If this would be possible it would surely make ion exchange separations of proteins more compatible with immunochemical detection. Especially because the pH gradient can easily be buffered in the ICD system to physiological pH by addition of the immunoreagent. A second opportunity to make ion exchange chromatography compatible with ICD is on-line desalting of the HPLC effluent, using for example continuous-flow dialysis or cross flow membranes [43], prior to immunochemical detection.

3.8. Size exclusion chromatography-ICD

To overcome the problem of the adverse effects of the LC mobile phase on the ICD system, a separation technique which operates under stable physiological conditions should be used. Size exclusion chromatography is such a technique. However, chromatograms obtained after size-exclusion chromatography of 100 nM IL-4 and interleukine 10 (IL-10) showed that IL-4 and IL-10 co-elute after ICD. Considering the molecular mass difference between IL-4 (14 kDa) and IL-10 (18.5 kDa) and the specifications of the size exclusion material, a separation of the two cytokines was expected. However, the low resolution was diminished by the band broadening in the post column ICD system. Experiments to verify this using UV detection would consume large amounts of cytokine and were hence too expensive.

3.9. Reversed phase chromatography-ICD

Modifiers used in reversed phase HPLC such as methanol, ethanol or acetonitrile are capable of changing the tertiary structure of proteins, which might cause a loss of their biological activity. For this reason severe problems could be expected when coupling reversed phase HPLC to ICD. However, the denaturing effect of most common modifiers is reversible, implying that when the modifier concentration decreases, the proteins will return to their original conformation. This offer the possibility to couple reversed phase separations to ICD, namely by diluting the HPLC effluent with the immunoreagent to a modifier concentration, which is not harmful to the protein.

In the present reversed phase micro HPLC-ICD system the 40 μl/min. LC effluent was mixed with 160 μl/min. immunoreagent solution in the ICD system. As a consequence of this 4-fold dilution of the mobile phase the methanol concentration in the ICD system increases up to 22.5% during a gradient separation. During the actual reversed

phase separation the cytokines are exposed too much higher organic modifier concentrations. Fig. 7 shows chromatograms of interleukine 6 (IL-6) and interleukine 8 (IL-8) and a reversed phase separation between IL-4 and IL-10 coupled on-line to immunochemical detection using methanol gradients up to 95%. These results prove the compatibility of reversed phase protein separations with ICD. This is likely to hold true for (a broad range of) most proteins, especially because these four cytokines are structurally completely different. However, as can also be seen in Fig. 7, the organic modifier gradient does increase the base-line of the ICD system. This is in accordance with the earlier discussed reduction of the antibody affinity with increasing methanol concentrations (Fig. 6). The effect of the reduced affinity by methanol is comparable to the effect of reduced affinity caused by ionic strength. A decrease in binding efficiency of the cytokine column, but moreover, dissociation of the already bound antibodies from the cytokine support will result in a gradually increasing baseline.

For the LC-ICD analysis of two cytokines in a single run the corresponding cytokine-columns were placed in series. Attempts to co-immobilise two or more cytokines on one support were not successful. This limits the amount of different

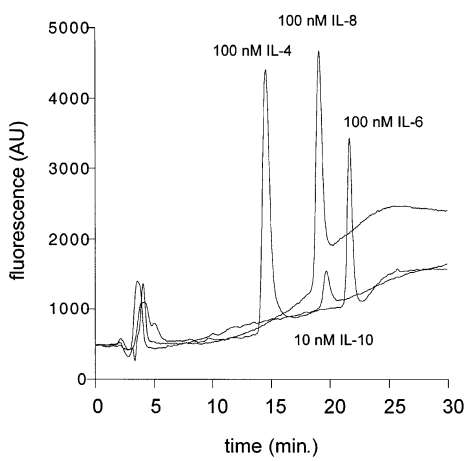

Fig. 7. Revered phase micro-LC separation between 100 nM IL-4 and 10 nM IL-10 and chromatograms of 100 nM IL-6 and 100 nM IL-8, 20 μl injections on 0.5×50 mm POROS-R1. For other conditions, see Section 2.

cytokines that can be detected in a single LC-ICD system because of the bandbroadening and increase backpressure when more than two cytokine affinity columns are placed in series. The cytokine samples, used to obtain the chromatograms shown in Fig. 7, contained 1 mg/ml blocking reagent, a 10 000 times excess of other proteins. This also confirms the extreme selectivity of the developed ICD system.

Identical FI-ICD system as presented for IL-4, IL-6, IL-8 and IL-10 were also developed for other cytokines like tumour necrosis factor-α, Interferon-γ, interleukine 2 and interleukine 12. These cytokines could successfully be immobilised on a solid support. The antibodies to the cytokines could also be labelled while still binding to immobilised cytokine column. However, when the cytokines were injected into the FI-ICD system only very small peaks appeared, resulting in very high detection limits of 100 nM. Different approaches to solve this problem have been unsuccessful so far.

3.10. Analysis of cytokines in cell extracts

Analysis of cytokines in cell extracts by direct injection of the samples on the micro LC-ICD system resulted in very broad peaks and bad separations. This was caused by overloading of the micro LC column due to the biological matrix of the cell extracts. A sample pre-treatment step was necessary to remove the excess of background and prevent overloading of the micro-LC column. Immunoaffinity sample clean-up was chosen because it is one of the most widely used sample pre-treatment steps prior to the LC analysis of proteins [44]. As can be seen from Fig. 8, analysis of 5 nM IL-4 in cell extract using immunoaffinity sample clean-up coupled to on-line LC-ICD results in almost the same signal as direct analysis of 5 nM IL-4 in aqueous buffer with LC-ICD. This indicates that the biological matrix hardly influences the selectivity of the analytical method and that only 15% of cytokine is lost during the sample clean-up procedure. The limit of detection ($s/n = 3$) was 500 pM for IL-4 in cell extracts. By mixing different cytokine antibody supports and packing them in one immunoaffinity precolumn

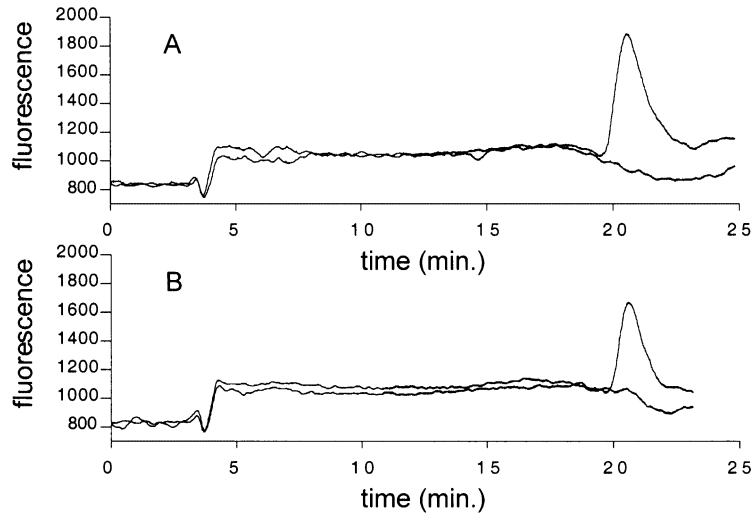

Fig. 8. (A) Revered phase chromatography ICD of 5 nM IL-4 in aqueous buffer with blank. (B) Immunoaffinity sample clean-up revered phase chromatography ICD of 5 nM IL-4 in cell extract with blank cell extract. For other conditions, see Section 2.

different cytokines can be analysed simultaneously in one cell extract.

4. Conclusions

Macromolecules behave similar to low molecular mass analytes in continuous flow biochemical detection system regarding their reaction kinetics. The small diffusion constant of large proteins does not negatively influents their reaction kinetics in a well-mixed continuous flow biochemical detection system.

The presented LC-ICD system also shows that reversed phase separations of proteins, using mobile phases with organic modifiers such as methanol or acetonitrile, are compatible with post-column immunochemical detection systems. The present system allows multi-cytokine analysis in complex biological matrices and the determination of cross-reactive compounds such as cytokine-like proteins and breakdown products. For a readout which is more comparable to the biological activity of the cytokines the labelled antibody can be replaced by a labelled soluble receptor, which exists for most cytokines. When receptors are used as biological targets this biochemical detection system can also be used for drug discovery, screening of complex samples, e.g. natural products or combinatorial chemistry products.

References

[1] M.J. Cho, R. Juliano, Trends Biotechnol. 14 (1996) 153–158.
[2] R.M. Bukowski, in: S. Wu-Pong, Y. Rojanasakul (Eds), Biopharmaceutical Drug Design and Development, N.J. Humana Press, Totowa, 1999, pp. 393–427.
[3] W.J. Britt, Trends Microbiol. 4 (1996) 34–38.
[4] H. Gershon, M.D. Growe, World J. Surg. 20 (1996) 1194–1199.
[5] M.E. Gosse, J.A. DiMasi, T.F. Nelson, Clin. Pharmacol. Ther. 60 (1996) 608–618.
[6] K. Koths, Curr. Opin. Biotechnol. 6 (1995) 681–687.
[7] M. Shaarawy, A.S. El-Shobokshy, A.I. El-Noury, Cytokine 7 (1995) 171–175.
[8] J. Bar, R. Maymon, M. Hod, Y. Manor, C. Moroz, Hypertens. Pregnancy 17 (3) (1998) 315–321.
[9] J. Xu, J. Menezes, U. Prasad, A. Ahmad, Int. J. Cancer 84 (1999) 396–399.
[10] R.C. Newton, J. Uhl, Pharmacological Methods in the Control of Inflammation, A.R. Liss, New York, 1989, pp. 83–99.
[11] J.T. Whicher, S.W. Evans, Clin. Chem. 36 (7) (1990) 1269–1281.
[12] M.C. Cohen, S. Cohen, Am. J. Clin. Pathol. 105 (1996) 589–598.
[13] M.E. Spurlock, J. Anim. Sci. 75 (1997) 1773–1783.

[14] M.J. Elliott, R.N. Maini, Bailliere's Clin. Rheumatol. 9 (4) (1995) 633–652.
[15] M.B. Widmer, Curr. Opin. Biotechnol. 2 (1991) 872–876.
[16] M.J. Krueger, L. Kapas, M. Kimura, M.R. Opp, Methods Neurosci. 17 (1993) 111–129.
[17] J. Rovensky, V. Lackovic, Z. Veselkova, M. Horvathova, J. Koska, S. Blazickova, M. Vigas, Int. J. Tissue React. Exp. Clin. Asp. 21 (1999) 43–49.
[18] J.P. de Bandt De, S. Chollet-Martin, A. Hernvann, N. Loiret, L.D. Du Roure, S.K. Lim, M. Vaubourdolle, J. Gueschot, R. Saizy, J. Giboudeau, L.J. Cynober, Trauma 36 (1994) 624–628.
[19] P. Damas, J.L. Canivet, D. de Groote, Y. Vrindts, A. Albert, P. Franchimont, M. Lamy, Crit. Care Med. 25 (1997) 405–412.
[20] J.S. Friedland, J.C. Porter, S. Daryanani, J.M. Bland, N.J. Screaton, M.J.J. Vesely, G.E. Griffin, E.D. Bennett, D.G. Remick, Crit. Care Med. 24 (1996) 1775–1781.
[21] S.P.J. Brooks, B.J. Lampi, G. Sarwar, H.G. Botting, Anal. Biochem. 226 (1995) 26–30.
[22] C.V. Sapan, R.L. Lundblad, N.C. Price, Biotechnol. Appl. Biochem. 29 (1999) 99–108.
[23] A.C. Lucarini, B.V. Kilikian, Biotechnol. Tech. 13 (2) (1999) 149–154.
[24] J.H. Waterborg, H.R. Matthews, Methods Mol. Biol. 32 (1994) 1–4.
[25] S. de Bernardo, M. Weigele, V. Toome, K. Manhart, W. Leimgruber, Arch. Biochem. Biophys. 163 (1974) 390–399.
[26] I.S. Krull, R. Strong, Z. Sosic, B.-Y. Cho, S.C. Beale, C.-C. Wang, S. Cohen, J. Chromatogr. B 699 (1997) 173–208.
[27] A. Qing Tang, K. Harrata, C.S. Lee, Anal. Chem. 68 (1996) 2482–2487.
[28] D. Figeys, R. Aebersold, Electrophoresis 19 (1998) 885–892.
[29] J.N. Murthy, R.W. Yatscoff, S.J. Soldin, Clin. Biochem. 31 (1998) 159–163.
[30] P.M. Rainey, K.E. Rogers, W.L. Roberts, Clin. Chem. 42 (1996) 1645–1653.
[31] F. Tejada, A. Cremades, F. Monserrat, R. Penafiel, Clin. Chim. Acta 275 (1998) 63–69.
[32] H. Brailly, F.A. Montero-Julian, C.E. Zuber, S. Flavetta, J. Grassi, F. Houssiau, J. van Snick, Clin. Chem. 40 (1994) 116–123.
[33] M.L.S. Tsang, J.A. Weatherbee, Aliment. Pharmacol. Ther. 10 (1996) 55–61.
[34] J.A.D. Bienvenu, G. Monneret, M.C. Gutowski, N. Fabien, Toxicology 129 (1998) 55–61.
[35] A. Sparreboom, H.A.H. Rongen, W.P. Bennekom, Anal. Chim. Acta 295 (1994) 1–26.
[36] A.R. Mire-Sluis, R. Gaines-Das, R. Thorpe, J. Immunol. Methods 186 (1995) 157–160.
[37] E. Moreau, J. Philippe, S. Couvent, G. Leroux-Roels, Clin. Chem. 42 (9) (1996) 1450–1453.
[38] A. Ledur, C. Fitting, B. David, C. Hamberger, J.M. Cavaillon, J. Immunol. Methods 186 (1995) 171–179.
[39] H. Irth, A.J. Oosterkamp, W. van der Welle, U.R. Tjaden, J. van der Greef, J. Chromatogr. 633 (1993) 65–72.
[40] J. Emneus, G. Marko-Varga, J. Chromatogr. A 703 (1995) 191–243.
[41] K.J. Miller, A.C. Herman, Anal. Chem. 68 (1996) 3077–3082.
[42] A.J. Oosterkamp, R. van der Hoeven, W. Glassgen, B. Konig, U.R. Tjaden, J. van der Greef, H. Irth, J. Chromatogr. B 715 (1998) 331–338.
[43] E.S.M. Lutz, H. Irth, U.R. Tjaden, J. van der Greef, J. Chromatogr. A 755 (1996) 179–187.
[44] D.S. Hage, Clin. Chem. 45 (1999) 593–615.