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Gradient reversed-phase liquid chromatography coupled on-line to receptor-affinity detection based on the urokinase receptor

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Abstract

A postcolumn receptor-affinity detection (RAD) was developed for the detection of urokinase and cross-reactive compounds. The analytical method consisted of gradient reversed-phase HPLC coupled on-line to a RAD system based on fluorescein-labelled urokinase receptor (fluorescein–uPAR) as reagent. Fluorescein–uPAR was added continuously to the HPLC effluent to react with analytes eluting from the LC column. Unreacted fluorescein–uPAR was removed by a short affinity column packed with an immobilised urokinase support. The analyte-bound fluorescein–uPAR fraction passes the affinity column unretained and was detected downstream by means of a fluorescence detector. An absolute detection limit of 40 fmol urokinase was obtained in the flow injection mode. In the gradient HPLC–RAD system a detection limit of 40 n*M* (20-μl injection, absolute amount, 800 fmol) was obtained. The present method allowed the identification of active breakdown products of urokinase both in standard samples and biological matrices.

Keywords: Enzymes; Urokinase

1. Introduction

The development of novel protein therapeutic agents requires analytical methods which are able to determine both the parent drug and (active) metabolites in complex biological matrices. Immunoassays are currently the preferred analytical methods for the quantitative bioanalysis of proteins [1]. A variety of immunoassays, mostly based on radioactive or enzyme labels, are described in the literature. While providing high sensitivities, batch immunoassays provide solely information on the total amount of cross-reactive analytes in a particular sample. In situations where more than one cross-reactive analyte is present, immunoassays do not provide means to distinguish between the different cross-reactive compounds. Quantitation of individual analytes requires prefractionation of the sample using, for example, high-performance liquid chromatography (HPLC). Subsequently, after evaporation of the HPLC mobile phase each fraction is analysed by immunoassay.

In recent years, the on-line coupling of liquid chromatography to biological assays such as immuno- or receptor assays was reported, overcoming the tedious and error-prone fraction collection/evaporation steps [2–9]. A comprehensive overview

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on affinity-based techniques is given by Hage in the present issue [10]. Flow biochemical detection methods employ bioassays which are performed in a postcolumn reaction detection system. Using this method, the tedious and error-prone fraction collection/evaporation steps are avoided. On-line flow assays generally involve solution-phase biochemical reactions and, in heterogeneous assay formats, the separation of free and bound label prior to detection. The interaction of analytes with biomolecular targets such as antibodies or receptors is monitored by using suitable, most often fluorescent labels. Detection is performed using a conventional HPLC fluorescence detector.

By using suitable assay designs, principally all affinity-based interactions can be implemented in postcolumn detection systems. We have previously described assays based on antibody–antigen, receptor–ligand and (strept)avidin–biotin interactions; both labelled ligand and labelled affinity proteins were used to monitor these interaction [2–6]. Most assays were developed for low-molecular-mass ligands. By employing soluble receptors in postcolumn reaction detection systems, in the following denoted as receptor-affinity detection (RAD), a detector response is obtained reflecting the biological activity of the analyte. In the area of protein analysis, Miller and Herman [7] described on-line LC–RAD systems for the analysis of human methionyl granulocyte colony stimulating factor (GCSF) in rat serum. Both antibodies and GCSF-receptor were used as biomolecular target for analyte recognition. Absolute detection limits of the immunochemical method were 80 fmol. Cho et al. [6] used a sequential addition approach for the determination of bovine growth hormone releasing factor (GHRF). Alternatively, immobilised receptors can be used in affinity chromatography to separate and quantitate ligands [11–15].

In the present paper, we investigate the possibility to implement protein–receptor interactions in an on-line HPLC–RAD set-up. Urokinase plasminogen activator (uPA), which is involved in the proteolysis of biologically active peptides, was used as model protein [16]. The RAD system is based on the soluble receptor of uPA, in the following denoted as uPAR [17,18].

2. Experimental

2.1. Materials

uPA was obtained from Boehringer Mannheim (Mannheim, Germany). The soluble uPA-receptor (uPAR) was prepared at Boehringer Mannheim (Penzberg, Germany) and had an activity of >85%. Cyclic uPA peptide 19–32 (uPA_{19-32}) was synthesised at Boehringer Mannheim. PPACK (D-phenylalanyl-L-prolyl-L-arginine-chloromethylketone) was provided by Boehringer Mannheim. FLUOS (fluorescein N-hydrosucciminide ester), POROS-EP, POROS-AL and phosphate buffered saline (PBS) was from Boehringer Mannheim. Texas-Red-NHS and BODIPY-NHS were from Molecular Probes (Eugene, OR, USA). 8-Anilino-1-naphthalenesulfonate (ANS) was from Sigma Chemicals (Beerse, Belgium). Potassium phosphate was from Merck (Darmstadt, Germany). Acetonitrile and methanol were obtained from Rathburn Chemicals (Walkerburn, UK). All other organic solvents were purchased from Baker (Deventer, The Netherlands) and were of analytical grade.

2.2. Synthesis of uPA-column materials

Affinity column materials were prepared according to the manufactors' instructions [19]. Prior to coupling to the affinity column, the protease activity of uPA was deactivated with PPACK, an inhibitor which binds irreversibly to uPA. Briefly, 618 mg of uPA was incubated with a 2-fold excess of 20 m*M* PPACK (pH 8.0) for 30 min at 37°C. Afterwards, the solution was extensively dialysed against coupling buffer for the affinity column.

2.3. Synthesis of fluorescence-labelled uPAR

The uPAR was labeled with FLUOS according to the manufacturer's specifications for protein labelling with FLUOS. In a typical experiment, 1 ml of 618 mg/ml uPAR in 20 m*M* sodium borate, 500 m*M* sodium chloride (pH 8.0), was reacted with a 10-fold excess of FLUOS for 1 h. Subsequently, 100 ml 1 *M* lysine (pH 8.0) were added and the mixture was allowed to react for 30 min. The fluorescein–uPAR

conjugate was dialysed against phosphate buffered saline and stored at 4°C.

Affinity purification of fluorescein–uPAR was performed by loading the fluorescein–uPAR solution onto an affinity column. After washing the column with mobile phase, fluorescein–uPAR was eluted with 1% acetic acid in water. The recovered uPAR was dialysed against PBS.

2.4. Flow-injection and liquid chromatography combined with receptor-affinity detection

The present RAD system was similar to that described in [2] with a few modifications. The flow injection (FI) RAD system consisted of a Beckman Gold system and a Pharmacia (Uppsala, Sweden) P3500 pump used to deliver the a carrier solution and the uPAR solution, respectively. Sample handling and injection was performed using a Gilson (Villiers-le-Bel, France) ASPEC XL equipped with a Rheodyne six-port injection valve (20-μl injection loop). A Merck-Hitachi 1080 fluorescence detector (λ_{ex}=486 nm; λ_{em}=520 nm) was used for detection. The FI carrier solution consisted of binding buffer and was pumped at a flow-rate of 0.2 ml/min. The fluorescent uPAR solution (1 n*M* uPAR–flu) was prepared in PBS consisting of 0.4% Tween 20 and added the FI carrier solution via an inverted Y-type mixing union and was pumped at a flow-rate of 0.2 and 0.4 ml/min for the FI and LC step, respectively. Knitted 0.3-mm I.D. PTFE reaction coils were used for reaction detection. The reaction was performed at ambient temperature. The analytical system was controlled by Gilson 719 PASCAL software.

2.5. Liquid chromatography–receptor affinity detection

HPLC–RAD (scheme, see Fig. 1) was performed similarly to FI–RAD with a few modifications. HPLC separations were carried out on a 125×2.0 mm I.D. stainless-steel column packed with POROS-R2 (10-μm particles, Boehringer Mannheim). HPLC was operated in a gradient mode using 0.1% trifluoroacetic acid (TFA) in water as mobile phase A, and 0.09% TFA in 90% acetonitrile as mobile phase B, at a flow-rate of 0.4 ml/min. For analysis of uPA,

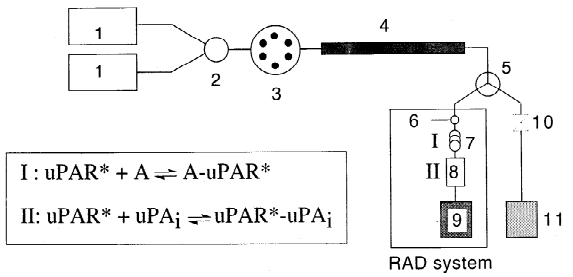

Fig. 1. Scheme of the gradient LC–RAD system. 1, HPLC pump; 2, dynamic mixer; 3, injection valve; 4, analytical column; 5, flow splitter; 6, reagent pump for fluorescein–uPAR solution; 7, reaction coil; 8, uPA-affinity column; 9, fluorescence detector; 10, flow restrictor; 11, UV detector. uPAR*, fluorescein-labelled uPAR; A, analyte, uPA$_i$, immobilised uPA.

a gradient system of 20–40% B was employed at a flow-rate of 0.4 ml/min using a Beckman Gold (Fullerton, CA, USA) programmable solvent module 126. The high flow-rate used for the separation could not be coupled directly to the RAD system due to the high content of organic modifier necessary for the separation. Therefore, a postcolumn split was performed using an Acurate (LC Packings, Amsterdam, Netherlands) resulting in a split ratio of 1:10. The high flow portion (360 μl/min) was connected to a UV absorbance detector (Beckman Gold programmable detection module 166) and the low flow portion was connected to the RAD system. In contrast to the FI system, the fluorescein–uPAR solution consisted of 2.5 n*M* fluorescein–uPAR and 20 m*M* K_2HPO_4 (pH 7.4) and was pumped at a flow-rate of 0.5 ml/min. After every run, the uPA affinity column was regenerated with 2 ml 0.1% TFA in water.

2.6. Mass spectrometry

All mass spectrometric experiments were performed on a Finnigan MAT 900 (San Jose, CA, USA) equipped with a Finnigan MAT API interface, operating in electrospray mode (ESI). Spectra were collected during constant infusion of the dialysed uPA with a Harvard 2400 syringe pump (Harvard Apparatus, South Natick, MA, USA), scanning the MS over an appropriate mass range. Deconvolution

was performed with the BIOMASS deconvolution program.

The ions which were detected were used for single ion monitoring experiments (SIM) during CE–MS experiments. CE was performed in a polyvinyl alcohol (PVA) coated fused-silica capillary (70 cm, 100 μm) whose outlet was coupled to the MS. Injection and electrophoresis was performed using a Prince programmable injector (Prince Technologies, Emmen, The Netherlands). Injection were performed hydrodynamically (30 s, 50 mbar). CE was performed with 30 kV combined with a pressure of 5 mbar. As a background electrolyte 1% of acetic acid in water was used. Before analysis, uPA was first dialysed overnight against pure water at room temperature.

3. Results and discussion

3.1. Design of the RAD system

Based on our previous work with fluorescein labelled antibodies, we developed a continuous-flow fluororeceptor assay in which the receptor is labelled with a fluorescence marker [2]. An identical approach was chosen by Miller and Herman for the determination of GCSF [7]. The principle of this assay is depicted in Fig. 1. A solution of fluorescent-labelled uPAR is added continuously to the LC effluent to react with uPAR-binding ligands, for instance uPA or uPA_{19-32}. After a reaction time of 60 s, the excess of unreacted labelled uPAR is separated from the bound uPAR–ligand complex by means of a short affinity column packed with an immobilised uPA support. The uPAR–uPA complex passes the affinity column unretained and is detected downstream by means of a conventional fluorescence detector.

The detection sensitivity of the RAD system depends strongly on the fluorescence quantum yield of the label, and, particularly, on the purity of the labelled receptor used as reagent. Direct derivatisation of the unpurified recombinant uPAR preparation with fluorescein as label yielded fluorescein-labelled uPAR which possessed 10% of active receptor and 3–4 fluorescein labels per receptor. We also evaluated Texas-Red and BODIPY as labels. For both labels, a significant lower fluorescence recovery was obtained, which is probably due to quenching of the fluorescence label by uPAR.

Although satisfactory detection limits can be obtained for high-affinity ligands such as uPA, it is desirable to work with a purer labelled receptor to improve the detection limits for low-affinity ligands. Two strategies were followed: (i) protection of the uPA binding site which involves reactive primary amino groups and (ii) purification of the labelled uPAR by affinity chromatography on an uPA column.

Fluorescein N-hydroxysucciminide ester (FLUOS, λ_{ex}=489 nm, λ_{em}=520 nm) was chosen. Protection of active amino-groups prior to fluorescein labelling was performed using dimethylmaleic anhydride [20]. In activity tests we found that the protection of active amino-groups had only a small effect on the binding affinity of the labelled uPAR (increase of activity from 10% to 15%). Attempts to purify the fluorescent uPAR preparation by means of affinity chromatography on an uPA-column were unsuccessful. For the subsequent measurements, fluorescein-labelled uPAR derived from unpurified, unprotected recombinant uPAR preparations were used as reagent.

3.2. Flow-injection RAD

The performance of the RAD system was tested using a flow-injection system. Fig. 2 shows a series of flow injections of uPA at a concentration range between 50 and 2000 ng/ml (1–40 n*M*). The concentration at which 50% of the maximum signal is obtained (EC_{50}) is approximately 8 n*M* (400 ng/ml). In the current system a concentration detection limit of 50 ng/ml was obtained (signal-to-noise: 3, injection volume: 20 μl). This corresponds to a mass detection limit of 1 ng uPA (20 fmol) which is approximately one order of magnitude better as previously observed with the antidigoxin system [1]. A reaction time of 1 min was required to obtain this detection limit.

In Fig. 3, a flow-injection RAD determination of a uPA_{19-32} is depicted in the concentration range 1–50 μg/ml (0.25–31 μ*M*). The measured EC_{50} of uPA_{19-32} was approximately 12.2 μ*M* (20 μg/ml). When the EC_{50} of uPA and uPA_{19-32} are compared with their respective affinities as measured in a microtitre plate assay, a similar shift in affinity is

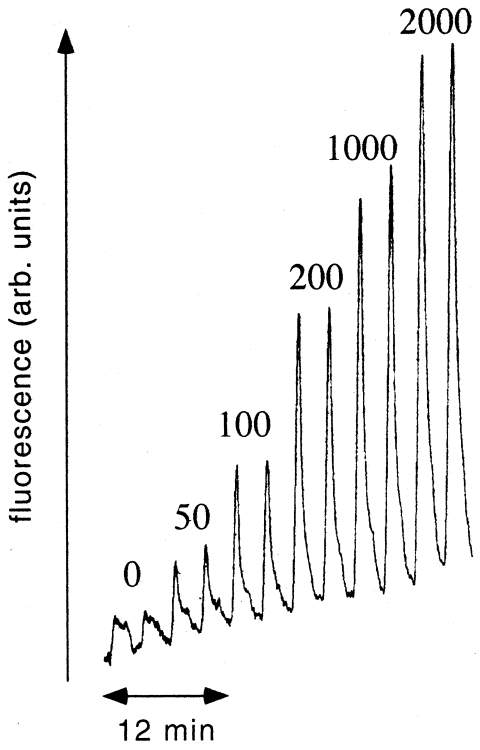

Fig. 2. Flow-injection RAD of uPA (all concentrations in ng/ml). Conditions: see Section 2.

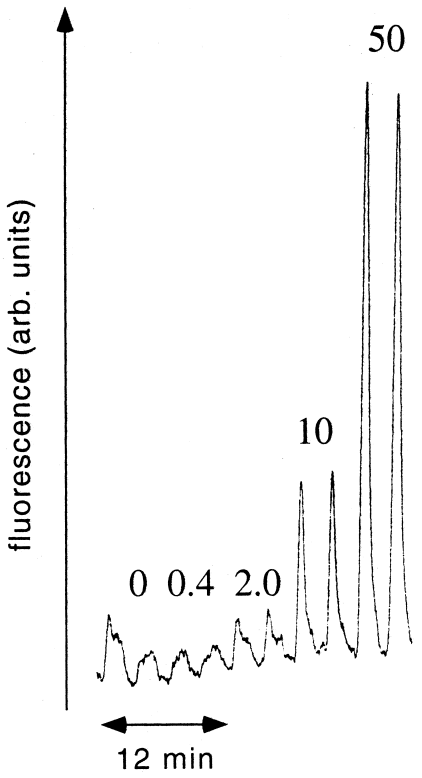

Fig. 3. Flow-injection RAD of cyclic uPA19-31 (all concentrations in μg/ml). Conditions: see Section 2.

observed (data not shown). This proves that with the present RAD method ligands with affinities in the micromolar range can be detected although at a significantly lower sensitivity than obtained for high-affinity ligands such as uPA. As a negative control bovine serum albumin (BSA) was injected into the FI–RAD system. No response was obtained for the injection of up to 2 μ*M* (120 μg/ml) BSA.

3.3. On-line coupling of the RAD system to LC

Most separations of proteins are based on gradient elution methods using C_8 or C_{18} analytical columns. In the case of uPA, it turned out that it was not possible to develop a suitable isocratic chromatographic system. We therefore chose to connect the RAD method directly to gradient HPLC. In an initial attempt, the RAD system was coupled directly to a reversed-phase analytical column, and a steep gradient from 10 to 90% acetonitrile in 15 min was applied. It appeared that a high flow-rate of the fluorescein–uPAR solution had to be chosen compensate for the organic modifier content of the HPLC mobile phase. Besides resulting in a substantial consumption of receptor solution, rather unsatisfactory detection limits for uPA were obtained.

We therefore decided to apply a postcolumn split and used only a small fraction of the total flow for the RAD system. The split was achieved by implementing an Acurate flow splitter operated at a split ratio of 1:10. This resulted in a flow-rate to the RAD system of 40 μl/min. The remaining flow of 360 μl/min was directed to a UV-absorbance detector allowing dual RAD and UV-absorbance detection. It should be noted that flow-splitter was designed for precolumn splitting and therefore not optimised for low dead volumes resulting in substantial band broadening. In the final system the organic modifier content in the reaction coil during the gradient run from changed 0.7 to 7%. These concentrations are in the same range as in previously developed LC–RAD

systems, in which up to 10% acetonitrile and methanol were shown to be compatible with receptor–ligand interactions [3].

Despite the small change in organic modifier content a considerable increase of the baseline was obtained during the gradient run (see Fig. 4). The same baseline drifts were reported by Miller and Herman [7] using both labelled antibodies and receptors as biochemical reagents. Since the slope of the baseline was very reproducible between different gradient runs we implemented baseline subtraction to facilitate the evaluation of the chromatograms.

In contrast to other RAD systems reported previously, the present system required the regeneration of the uPA affinity column (no. 8 in Fig. 1) between different runs. After prolonged operation, breakthrough of fluorescein–uPAR was observed resulting in a drastic baseline increase. The breakthrough is probably caused by the limited capacity of the uPA column. The regeneration was performed simultaneously to the re-equilibration of the reversed-phase LC column and did therefore not cause an increase of analysis time. Repetitive regeneration of the uPA affinity column did not result in any apparent decrease of efficiency, resulting in a column lifetime of at least 1 week.

A typical chromatogram of the determination of uPA using a POROS R-2 column is shown in Fig. 4 which depicts both the RAD and UV trace. UV analysis of standard uPA samples revealed a small peak eluting prior to the uPA peak and possibly represents a breakdown product. In the RAD chromatogram the breakdown product results in a peak of almost equal height to the uPA peak indicating that the breakdown products has a high affinity for the uPA receptor. To elucidate the structure of the breakdown product, additional mass spectrometry was carried out on fractions isolated from the gradient reversed-phase LC system. In addition, CE–MS was used. A molecular mass of 33 000 was found for the breakdown product. We assume that the breakdown product is the single chain fragment of uPA (sc-uPA) which has a molecular mass of 33 000. sc-uPA has a higher affinity for the uPA receptor compared to uPA [21,22]. Unfortunately, no standards of sc-uPA were available to confirm its presence by comparison of retention times. Further studies are required to unambiguously identify the breakdown product.

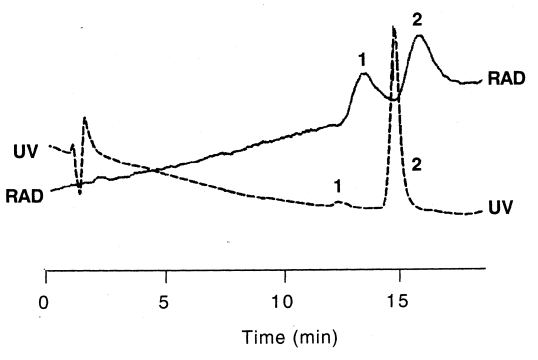

Fig. 4. Chromatogram of 100 n*M* uPA and its breakdown product (20-μl injection). Conditions: see Section 2.

The method was linear from 2 to 20 μg/ml uPA (r^2=0.99). Using nonlinear curve-fitting the dynamic range could be expanded to 150 μg/ml uPA. The rather narrow linear range is caused by the low concentration of fluorescein–uPA used in the present set-up. All peak areas were determined after applying baseline subtraction. The detection limit for uPA was 2 μg/ml uPA (signal-to-noise=3; 20-μl injections) corresponding to an absolute detection limit of approximately 40 ng (800 fmol). Miller and Herman [7] reported considerably lower detection limits for recombinant human methionyl GCSF, namely 80 fmol using labelled fab fragments of anti-GCSF antibodies as affinity protein. This difference can be explained by the fact that no postcolumn split was necessary due to the apparent stability of both Fab fragment and immobilised GCSF at the organic modifier concentrations used. They used an injection volume of 120 μl instead of the 20-μl volume used in the uPAR system. When using fluorescent-labelled GCSF-receptor as a reagent, considerably higher detection limits were obtained which is consistent with our measurements. Probably receptor proteins are less stable in the presence of high concentrations of organic modifier. This results in a considerable decrease of the active receptor concentration and, consequently, in a decreased concentration of receptor–analyte complex to be detected.

The selectivity of on-line HPLC–RAD in bioanalysis is demonstrated in Fig. 5. When analysing human plasma, no background signal can be seen

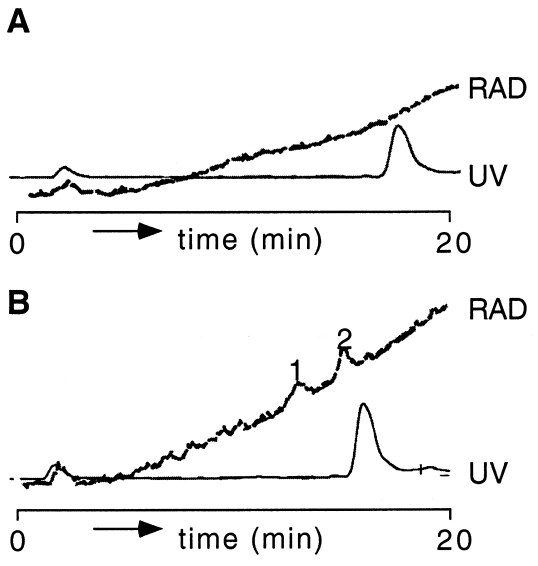

Fig. 5. Chromatogram of uPA in plasma (20-μl injection); (A) plasma blank, (B) plasma spiked with 100 n*M* uPA. Conditions: see Section 2.

in the fluorescence trace. The UV trace reveals one large peak at the end of the chromatogram. uPA and its conversion product can readily be detected in plasma at concentrations of 16 μg/ml while no signal at all is obtained in UV-absorbance detection.

4. Conclusions

The on-line coupling of HPLC with receptor assays allows the determination of analytes based on biological activity. Cross-reactive compounds can be distinguished. Thus, the present approach can be a useful tool, for example, in metabolite research or drug discovery. The on-line approach provides rather short analysis time of the order of 15–20 min and overcomes laborious fractionation and evaporation steps. Due to the high selectivity of the detection method, sample handling is limited to a minimum, often only centrifugation of particulate matter is required.

The detection limits which can be obtained with receptor-based systems are considerably lower as those obtained in immunochemical detection. Both receptor purity and stability are probably the main factors which cause a decrease in detection sensitivity. Labelling of receptor preparation which contain high amounts of inactive proteins results in a drastic increase of fluorescence background and unfavourable signal-to-noise ratios. Degradation of labelled receptors during biochemical detection results in both a decrease of the active receptor concentration and an increase of the fluorescence background. A particular problem is baseline drift caused by the changing organic modifier concentration in gradient HPLC. Baseline subtraction as applied in the present system, is possible for analytical systems where high sensitivity is not a major requirement, e.g., in stability analysis, in vitro metabolism studies and drug discovery. For high sensitivity analytical system, alternative approaches have to be chosen.

The postcolumn split provides simultaneous biochemical detection and, for example, UV, diode-array or mass spectrometric detection. In this way, a single analysis provides information on the distribution of biological activity in a particular sample, as well as providing structural data of active compounds. The most important application areas will clearly be drug discovery, particularly in the screening of complex samples such as natural product extracts.

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References

[1] B.L. Ferraiolo, M.A. Mohler, in: B.L. Ferraolo (Editor), Protein Pharmacokinetics and Metabolism, Plenum Press, New York, 1992, Ch. 1.
[2] H. Irth, A.J. Oosterkamp, W. van der Welle, U.R. Tjaden, J. van der Greef, J. Chromatogr. 633 (1993) 65.
[3] A.J. Oosterkamp, M.T. Villaverde Herraiz, H. Irth, U.R. Tjaden, J. van der Greef, Anal. Chem. 68 (1996) 1201–1206.
[4] A.J. Oosterkamp, L. Heintz, G. Marko-Varga, H. Irth, U.R. Tjaden, J. van der Greef, Anal. Chem. 68 (1996) 4101–4106.
[5] E.S.M. Lutz, H. Irth, U.R. Tjaden, J. van der Greef, J. Chromatogr. A 755 (1996) 179.

[6] B.-Y. Cho, H. Zou, R. Strong, D.H. Fisher, J. Nappier, I.S. Krull, J. Chromatogr. A 743 (1996) 181.
[7] K.J. Miller, A.C. Herman, Anal. Chem. 68 (1996) 3077.
[8] D. Wu, F. Regnier, Anal. Chem. 65 (1993) 2029–2035.
[9] R.A. Strong, B.Y. Cho, D.H. Fisher, J. Nappier, I.S. Krull, Biomed. Chromatogr. 10 (1996) 337–345.
[10] D.S. Hage, J. Chromatogr. A 795 (1998) 185.
[11] M. Nachman, A.R. Azad, P. Bailon, J. Chromatogr. 597 (1992) 155–166.
[12] P. Bailon, D.V. Weber, J.E. Smart, Bioprocess Technol. 12 (1991) 267–285.
[13] C. Spence, C.A. Schaffer, S. Kessler, S.P. Bailon, Biomed. Chromatogr. 8(5) (1994) 236–241.
[14] P.F. Ruhn, J.D. Taylor, D.S. Hage, Anal. Chem. 66 (1994) 4265.
[15] H. Zou, Y. Zhang, P. Lu, I.S. Krull, Biomed. Chromatogr. 10 (1996) 122–126.
[16] L. Novokhatny, A. Medved, P. Mazar, J. Marcotte, K. Henkin, K. Ingham, J. Biol. Chem. 267 (1992) 3878–3885.
[17] M. Ploug, J. Eriksen, T. Plesner, N.E. Hansen, K. Dano, Eur. J. Biochem. 208 (1992) 397–404.
[18] M. Behrendt, M. Ploug, E. Ronne, G. Hoyer-Hansen, K. Dano, Methods Enzymol. 223 (1993) 207–222.
[19] Data sheet for CNBr-activated Sepharose, Pharmacia/Biotech, Sweden.
[20] N. Endo, N. Uemoto, Y. Kato, Y. Taheda, T. Hara, J. Immunol. Methods 104 (1987) 253.
[21] J. Kuiper, D.C. Rijken, G.A. de Munk, T.J. van Berkel, J. Biol. Chem. 267 (1992) 1589–1595.
[22] A. Higazi, R.L. Cohen, J. Henkin, D. Kniss, B.S. Schwartz, D.B. Cines, J. Biol. Chem. 270 (1995) 17375–17380.