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Process monitoring by flow-injection immunoassay

Evaluation of a sequential competitive binding assay

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ABSTRACT

A new variation on the theme of flow-injection binding assays is presented, namely the sequential competitive binding assay, in which the sample containing the native antigen is first introduced into the flow system followed by a pulse of labelled antigen. The flow-injection binding assay was used in monitoring the effluent from column chromatographic separations of proteins. By applying a computer-based evaluation system, concentrations are automatically read and compensation is made for denaturation in the affinity sorbent.

INTRODUCTION

Downstream processing is regarded as a key area in biotechnology. In spite of this monitoring and controle are rather primitive in the sense that only indirect measurements are used to follow the process on-line; absorbance at 280 nm, pH and conductivity may be monitored, whereas specific monitoring of a target protein is normally carried out by off-line analysis completed several hours later. One limitation so far has been that immunological methods have been too slow and chromatographic techniques not sensitive enough and often slow. The developement of immunobased sensors offers alternatives to performing immunoassays in a rapid and reliable way, but often at the cost of sensitivity.

In order to be suitable as a process-monitoring technique, the assay needs to be rapid, reliable and automated. Speed in the assay normally means non-equilibrium conditions during binding. This places strong demands on reproducibility. We have previously reported on the use of flow-injection analysis as a rapid immunochemical method [1–3]. The present investigation was focused on an evaluation of a sequential competitive binding assay as a process-monitoring technique.

EXPERIMENTAL

Chemicals

Horseradish peroxidase type II and IV and alkaline phosphatase type XXX-L from calf intestinal mucosa were purchased from Sigma (St. Louis, MO, USA), concanavalin A (Con A)–Sepharose and protein A–Sepharose from Pharmacia LKB Biotechnology (Uppsala, Sweden), rabbit antibodies to human serum albumin from Dakopatts (Glostrup, Denmark) and human serum albumin (HSA) and human immunoglobulin G (IgG) from KabiVitrum (Stockholm, Sweden). All other chemicals were of analytical-reagent grade.

Isolation and immobilization of antibodies against human serum albumin

HSA was immobilized on Sepharose (Cl-4B using tresyl chloride activation [4]. HSA (110 mg) was used for coupling to 10 ml of activated gel. The gel was packed in a column and used for affinity puri-

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fication [5] of 10 mg of rabbit antibodies against HSA. The bound antibodies were eluted at a low pH (0.1 *M* glycine–HCl, pH 2.2). The purified antibodies were immobilized on Sepharose Cl-4B using cyanogen bromide activation [6].

Preparation of human serum albumin conjugates with horseradish peroxidase

The carbohydrate groups on horseradish peroxidase (HRP) were oxidized with $NaIO_4$ to form aldehyde groups which were used to react with primary amino groups on HSA [7]. HRP (4 mg) dissolved in 0.2 ml of 0.1 *M* $NaIO_4$ was mixed for 20 min at room temperature and then dialysed overnight at 4°C against 1 m*M* sodium acetate buffer (pH 4.4). To the activated HRP solution were added 20 μl of 0.2 *M* sodium carbonate buffer (pH 9.5), then mixed with 1 ml of 10 m*M* sodium carbonate buffer (pH 9.5) containing 3.4 mg of HSA and stirred for 2 h at room temperature. The solution was dialysed against 0.5 l of 150 m*M* sodium phosphate buffer (pH 7.4) with 150 m*M* NaCl (PBS) containing 0.5 g of $NaBH_4$ for 4 h at 4°C and then against pure phosphate-buffered saline (PBS) overnight at 4°C. The reaction mixture was separated by gel permeation chromatography using a column of Sephacryl S-200 (500 × 16 mm I.D.) equilibrated with 50 m*M* Tris–HCl (pH 7.4) in 0.5 *M* NaCl. Fractions were collected and those containing the conjugate were pooled and supplemented with NaN_3 (0.02% w/v).

Preparation of conjugates between human immunoglobulin G and alkaline phosphatase

The coupling was performed using a modified two-step glutaraldehyde procedure [8]. Alkaline phosphatase (2 mg) dissolved in 250 μl of distilled water was activated by addition of 250 μl of 2% (w/v) glutaraldehyde solution in 0.1 *M* sodium phosphate buffer (pH 6.8). The solution was stirred and then allowed to equilibrate for 18 h at room temperature, then the solution (0.5 ml) was passed through a 10-ml column filled with Sephadex G-25 fine equilibrated with 0.15 *M* NaCl. The fractions containing the activated protein were pooled and directly mixed with 1 ml of 0.15 *M* NaCl containing 3 mg of human IgG. Sodium carbonate buffer (0.2 *M*, pH 9.5) (0.4 ml) was added and coupling took place during 18 h at 4 °C. The coupling solution was separated on a column of Sephacryl S-200 (500 × 16 mm I.D.) equilibrated with 50 m*M* Tris HCl (pH 7.4) in 0.5 *M* NaCl. This step was used in order to separate the conjugate from unreacted protein. The fractions containing the conjugate protein were pooled and supplemented with NaN_3 (0.02% w/v).

Flow-injection system

A flow-injection analysis (FIA) system as shown schematically in Fig. 1 was used. The arrangement is very similar to that described previously [2]. However, a thermostated metal block replaced the water-bath for temperature control of the immunosorbent. The affinity column was kept at 28.5 ± 0.3°C.

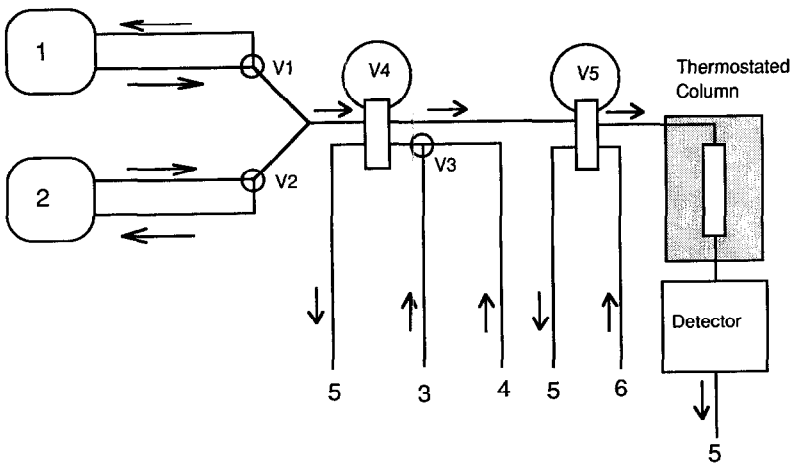

Fig. 1. Schematic diagram of the experimental set-up for competitive binding assays. 1 = Buffer (pH 7.4); 2 = buffer (pH 2.2); 3 = sample; 4 = substrate; 5 = waste; 6 = enzyme-labelled protein. VI–V3 are three-way valves and V4 and V5 are injection valves

The system consisted of two sample injection valves (Cheminert 414300) (Valco Instruments, Houston, TX, USA) and three three-way valves (Models 5301 and 5300) (Rheodyne, Cotati, CA, USA). All valves had pneumatic actuators. Substrate solution and conjugate solution were pumped to the injection loops by two pumps (Alitea C4) (Ventur Alitea, Uttran, Sweden). One pump (Minipuls 3) (Gilson, Villiers-le-Bel, France) supplied flows of running buffer, dissociating buffer and the sample solution. A spectrophotometer (SpectroMonitor III) (Laboratory Data Control, Riviera Beach, FL, USA) was used for detection of the enzyme reaction products. Pumps and valves are all controlled by a program written in Turbo Pascal. A Model 386 personal computer working at 25 MHz under MSDOS was used. The same program also collected the data from the detector and evaluated the results.

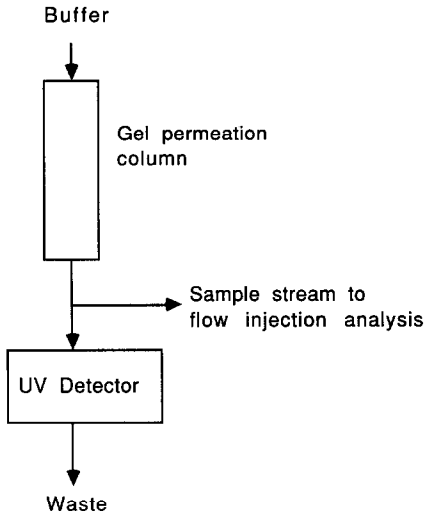

Fig. 2. Schematic diagram of the experimental set-up for monitoring gel permeation chromatography.

Experimental arrangement for monitoring the separation of protein mixtures in gel permeation chromatography

A 1-ml sample containing a mixture of proteins was passed through a column filled with Sephadex G-100 (600 × 16 mm I.D.) equilibrated with 50 m*M* Tris–HCl (pH 7.4)–0.5 *M* NaCl and eluted at a flow-rate of 0.6 ml/min. The adsorbance of the effluent from the column was measured continuously at 280 nm. Just in front of the detector a sample stream was withdrawn to the FIA system (Fig. 2). Hence the concentration of a specific protein could be measured simultaneously with the registration of the absorbance at 280 nm.

Measurements with the FIA system

The same set-up of valves and controls could be used in all the measurements. However, in this study both sequential competitive assays and direct binding assays were made. The connections between the valves had to be rearranged for the different modes of operation. The sequential competitive

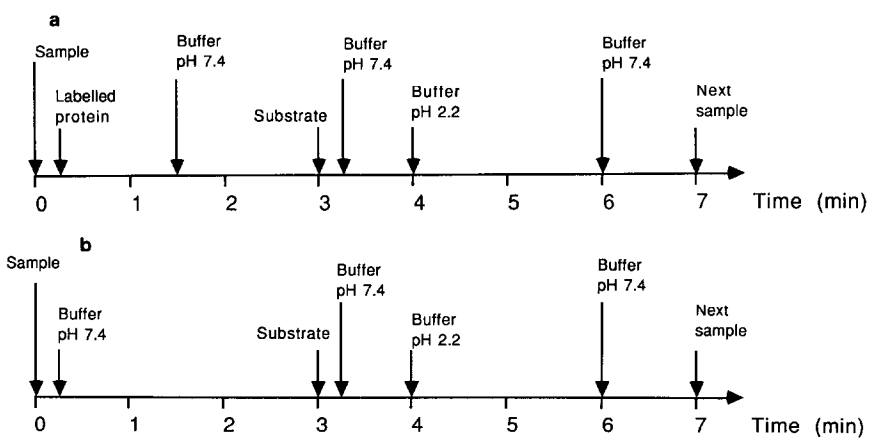

Fig. 3. Assay schemes for (a) competitive binding assays (*e.g.*; for human serum albumin and IgG) and (b) direct binding assay for horseradish peroxidase. Neither of the assay schemes is fully optimized. The direct binding assay could have been much shorter.

binding assay was used for all assays where an enzyme label was needed, whereas the direct binding assay may be used to monitor enzyme concentrations. The different assay schemes are shown in Fig. 3.

Sequential competitive binding assay

Measurement of human serum albumin concentrations. The immunosorbent, rabbit anti-human serum albumin antibodies covalently coupled to Sepharose Cl-4B, was packed in a column with a total bed volume of 100 μl. When a sample containing human serum albumin (HSA) was passed through the column an amount of HSA related to the total content in the sample was bound to the antibodies. Immediately after the HSA pulse a pulse with HSA conjugated with peroxidase was passed through and bound to the remaining available antigen binding sited on the gel. After washing the column with running buffer, the enzyme activity was measured by passing a 15 s pulse of substrate (14 m*M* phenol, 0.8 m*M* 4-aminoantipyrine and 9.3 m*M* hydrogen peroxide). The result was a level of enzyme activity that declined with increasing content of HSA in the sample (Fig. 4). To rinse the immunosorbent from bound antigens, a pulse of 0.1 *M* glycine–HCl (pH 2.2) was applied.

Measurement of human immunoglobulin concentrations. The same principle as was used in the assay with HSA was applied but in this instance the analyte was conjugated with alkaline phosphatase instead of peroxidase and the column contained a packed bed with a volume of 100 μl of a commercial protein A–Sepharose gel. Protein A has affinity to IgG from several species. The substrate solution used was 0.003 *M* *p*-nitrophenyl phosphate and the activity was detected spectrophotometrically at 410 nm. The calibration graph had the same general shape as with HSA (Fig. 5) and the same dissociation buffer could be used for regeneration of the column.

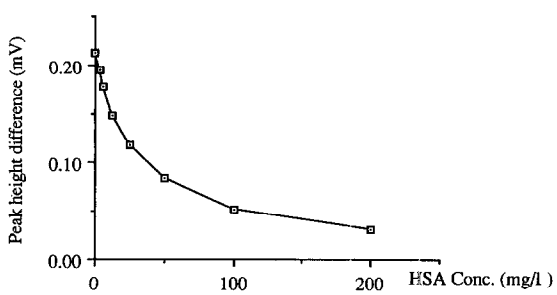

Fig. 4. Calibration graph for human serum albumin (HSA).

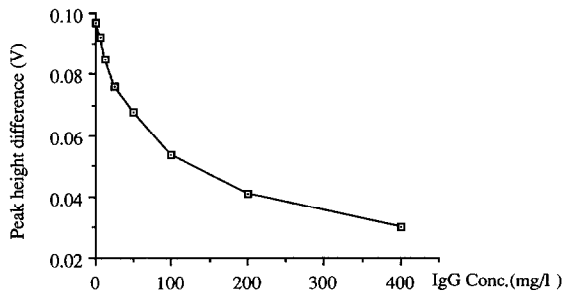

Fig. 5. Calibration graph for human IgG.

Direct binding assay

Measurement of horseradish peroxidase concentration. A 100-μl column was packed with a commercially available Con A–Sepharose gel and connected to the continuous buffer flow in the flow-injection system. Con A binds to the carbohydrate structures on HRP. When a sample is passed through the column most of the HRP is bound, thus HRP is affinity enriched on the support. After washing the column with buffer [50 m*M* *T*ris–HCl (pH 7.4) in 0.5 *M* NaCl] a 15 s pulse of substrate solution (14 m*M* phenol, 0.8 m*M* 4-aminoantipyrine and 9.3 m*M* hydrogen peroxide) was passed through the column. The reaction products from peroxidase activity were measured at 540 nm by means of a spectrophotometric detector at the outlet. The response to the enzyme concentration was linear (Fig. 6) but at very high enzymatic activity the reaction product may precipitate in the column, which may give erroneous results. After measurement, the enzyme was dissociated from the Con A column by a washing pulse of low-pH buffer [0.1 *M* glycine–HCl (pH 2.2)] and the system was then ready for a new sample.

Calculation of the sample analyte concentration. Calibration was made by measuring solutions with known analyte concentrations under the described assay conditions. The readings of bound enzyme label were stored in the computer. The peak-height absorbances in millivolts compensated for the peak

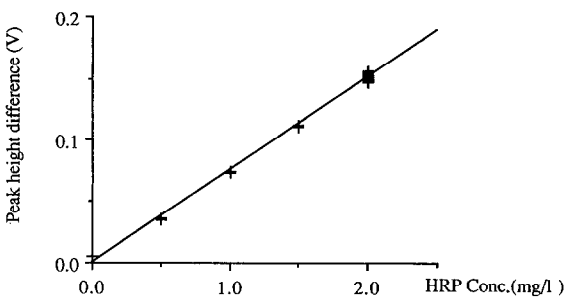

Fig. 6. Calibration graph for horseradish peroxidase.

height produced by the substrate itself were used as values. In direct binding measurements of HRP concentrations the calibration points could be fitted to a straight line. In the sequential competitive binding measurements the computer fitted straight lines between the mean values of adjacent concentrations. In both instances the column capacity declined slightly for subsequent samples owing to denaturation or loss of affinity binder. The computer compensated for this decline in capacity. In all the experiments the first point measured was a calibration point with a known content of analyte. This gave the computer information about the present column capacity. The operator had to calculate a presumed capacity decline for the first ten measurements. This value was fed to the computer. A new calibration point was measured after every ten samples and the computer used this value to calculate the expected column capacity decline for the subsequent samples. The time for one sample cycle was set at 7 min.

RESULTS AND DISCUSSION

The FIA system was used for measurement of three different proteins using three different experimental approaches. HRP was captured by a column with immobilized Con A and the enzyme activity in the column was measured. In a binding step HSA bound to immobilized antibodies. This was followed directly by a pulse of peroxidase conjugated HSA, which gave a binding of the two antigens which was to some extent competitive. IgG was also quantified in a sequential competitive manner. The sample was passed over a column with immobilized protein A and subsequently a pulse of alkaline

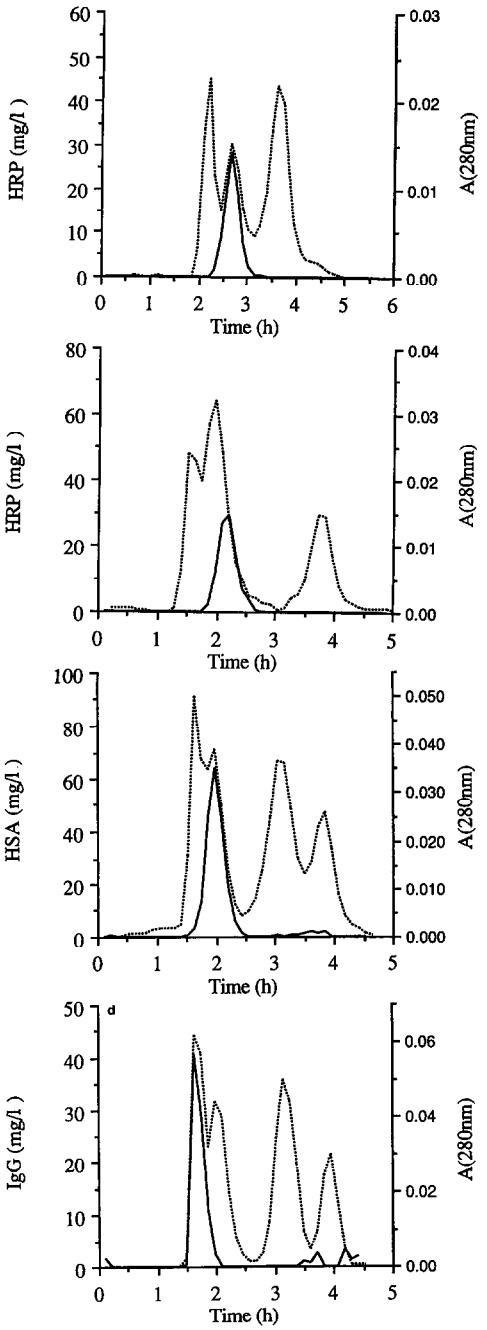

Fig. 7. Monitoring of separations using gel permeation chromatography. Dotted lines represent absorbance at 280 nm (right-hand ordinates) and solid lines are the results of flow-injection binding assays (left-hand ordinates). (a) Detection of HRP after separation of IgG, HRP and lysozyme; (b) detection of HRP after separation of IgG, HSA, HRP and N-acetyltryptophan; (c) detection of HSA after separation of IgG, HSA, lysozyme and N-acetyltryptophan; (d) detection of IgG after separation of IgG, HSA, lysozyme and N-acetyltryptophan.

phosphatase-conjugated IgG was introduced. Protein A is known to bind firmly to the F_c part of the immunoglobulin. The sample was then allowed to bind first to the column followed by a pulse of the enzyme-labelled IgG.

When evaluating the performance of flow enzyme-linked immunosorbent assay (ELISA) as a process-monitoring aid in downstream processing, gel permeation chromatography was studied. Protein mixtures were separated and the effluent from the chromatographic column was monitored. The same FIA system could be used for measurements of three different proteins by simply exchanging the column material and making some minor changes to the flow and injector loop sizes. The assay cycle time was 7 min but has not yet been fully optimized. A shorter time would give a better resolution but also a higher consumption of conjugated analyte in the cases with competitive assays. The resolution with a 7-min cycle time was considered adequate. Before more is known about the need for time constants in monitoring biotechnological operations it is not the first priority to strive for quicker analyses.

In the FIA system there is a time delay of 6.5 min between taking the sample at the sampling point in the outflow from the chromatographic column and reading of the result. Further, this is not optimized but it depends on the flow-rates and the volumes of the column and injector loops. In the Figures showing measured concentrations of a specific protein together with absorbance at 280 nm, the peak positions have been adjusted for this time difference.

In Fig. 7a the concentration of HRP was measured in a protein mixture where the gel filtration gave a good separation. In Fig. 7b the HRP peak was overlapped with a peak with HSA but the HRP peak could still be detected with the FIA system. Much lower HRP concentrations could have been monitored with the system but then nothing would have been detected by UV detection. An injection loop of only 25 μl was used to avoid overloading of the column. In Fig. 7c and d, where HSA and IgG concentrations were monitored, the situations are different. In Fig. 8 the registration on the chart recorder is shown when detecting HSA in the effluent from a chromatographic column. The baseline is not as straight as in the HRP assays as there are more factors affecting the level in a competitive than in a direct binding assay. In the competitive case the baseline represents maximum binding of enzyme label and it is greatly affected by the column capacity and temperature. Measurements of analyte concentrations with competitive binding assays are most accurate in the middle of a calibration graph not too close to zero and not at high concentrations where the curve levels out. The proper concentration interval is determined by the number of binding sites in the column and the sample injector loop size. In this FIA system, the same loop was used for injection of both the sample and the substrate solution. Hence making this loop larger would make the measurements more sensitive, but would also prolong the times for both the sample pulses and the substrate pulses. The enzyme–protein conjugate solutions were injected through the other loop. The amount of conjugate must be large enough to produce a good enzyme activity peak but still kept as low as possible to minimize the conjugate consumption.

It has been argued that chromatographic separa-

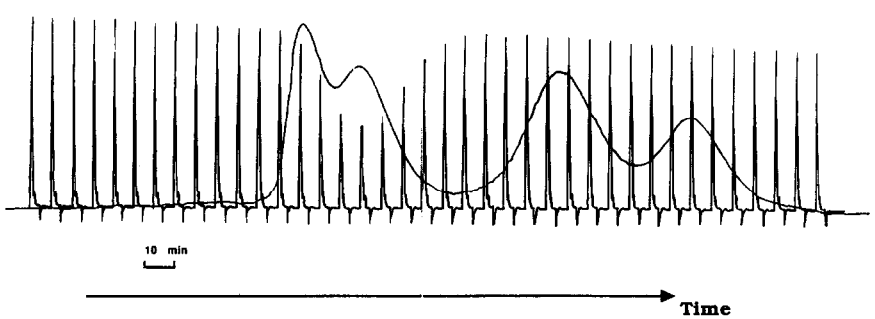

Fig. 8. Registrations on the chart recorder the absorbance at 280 nm of the effluent after separating IgG, HSA, lysozyme and *N*-acetyltryptophan on a gel permeation column. The content of HSA was monitored using the sequential competitive flow ELISA.

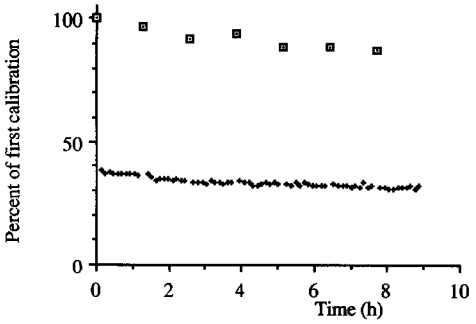

Fig. 9. Comparison of the peak heights for calibration points and sample points with the first obtained calibration point. (□) calibration; (+) 50 mg/l HSA. The peak heights registered decline as the column capacity declines but the relationship between the sample peak heights and the calibration peak heights remains approximately constant. This makes the calibration graph valid even though the column capacity decreases.

tion using an affinity sorbent and conventional absorbance detection at 280 nm should offer an alternative to immunological binding assays. This may be so under certain conditions, but it is worth noting that the concentration ranges in which the two techniques operate are different. Moreover, using a highly sensitive technique reduces the volume of sample to be analysed. It is important to note that the sequential mode of operation of the competitive assay as described here works completely satisfactorily in the systems studied. The time for passage of the sample pulse or of the conjugate pulse through the immunosorbent column in only *ca.* 10–12 s. This means that most systems are operating in a kinetic mode and are far from equilibrium.

The concept of operating with sequential competitive binding assay simplifies the handling and thus the automation of the process. A potential problem could arise if the two species, the native and the labelled antigen, were to show large differences in binding characteristics. This has not been observed, however, with any of the many antigens that we have studied so far. Displacement of the first bound antigen by the labelled antigen might be a possibility when operating with weak antibodies. In the system presented here, this would cause severe disturbances, but also offers opportunities for a displacement-based binding assay, especially for haptens [9]. As can be seen from Fig. 9, the stability of the response in repeated assays clearly indicates that the sequential competitive assay works as well as the traditional methods. The present study clearly illustrates that specific monitoring in downstream processing is a realistic possibility. It is then a matter to define when measurements are wanted or needed and how this new information will be utilized.

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REFERENCES

1 B. Mattiasson, M. Nilsson, P. Berdén and H. Håkanson, *Trends Anal. Chem.*, 9 (1990) 317–321.
2 M. Nilsson, H. Håkanson and B. Mattiasson, *Anal. Chim. Acta*, 249 (1991) 163–168.
3 M. Nilsson, H. Håkanson and B. Mattiasson, *Proc. Control. Qual.*, in press.
4 K. Nilsson and K. Mosbach, *Eur. J. Biochem.*, 112 (1980) 397–402.
5 C. Borrebaeck, B. Mattiasson and K. Svensson, in S. B. Pal (Editor), *Enzyme Labelled Immunoassays of Hormones and Drugs*, Walter de Gruyter, Berlin, New York, 1978.
6 R. Axén, J. Porath and S. Ernback, *Nature (London)*, 214 (1967) 1302–1304.
7 M. B. Wilson and P. K. Nakane, *Immunofluorescence and Related Staining Techniques*, Elsevier/North-Holland, Amsterdam, 1978, p. 215.
8 S. Avrameas, T. Ternynck and J.-L. Guesdon, *Scand. J. Immunol.*, 8, Suppl. 7 (1978) 7–23.
9 A. W. Kusterbeck, G.A. Wermhoff, P. T. Charles, D. A. Yager, R. Bredehorst, C.-V. Vogel and F. S. Ligler, *J. Immunol. Methods*, 135 (1990) 191–197.