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Recycling immunoaffinity chromatography for multiple analyte analysis in biological samples

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Abstract

The ability to isolate and measure multiple complex analytes in a single biological sample holds great potential in many biomedical fields, especially immunology and diagnostic clinical chemistry. We have developed a procedure involving recycling immunoaffinity chromatography for the simultaneous measurement of a number of analytes in a single sample. The procedure is based on the passage of a fluorochrome-labelled sample through a battery of small immunoaffinity columns, each column extracting a single analyte. Detection is achieved by acid elution of the bound analytes and laser-induced fluorescence. We have applied this system to a number of different biological fluids and found that it is capable of reliably isolating and measuring up to ten different cytokines in a 25-μl sample of human body fluid. © 1998 Published by Elsevier Science B.V.

Keywords: Recycling immunoaffinity chromatography; Multiple analyte analysis; Cytokines

1. Introduction

In both the biological and biomedical sciences there is a growing need for the development of techniques designed to measure a variety of different analytes within the same sample [1–3]. This is particularly true in the clinical setting where excessive sampling using existing techniques often presents a danger to patient health and care. Additionally, this need is often coupled with the need for miniaturisation in order to enable multiple sampling of a test subject over time. Such situations exist in clinical settings where multi-analyte analyses are required on post-natal and paediatric patients [4–6]. Similar situations occur when analysing valuable archival materials, low-density cell cultures, or samples from small experimental animals.

There are various chromatographic techniques available for the analysis of biological samples and many of them have been used for the recovery and measurement of clinically relevant analytes [7–10]. Immunoaffinity techniques, employing immobilised antibodies have been shown to be able to isolate and measure a number of different analytes in a variety of different biological matrices, including blood [11], plasma [11–13], urine [11], and cerebral–spinal fluid [11,14]. Immunoaffinity has the added advantage that most isolations are performed under physiological conditions, therefore preserving the biological activity of the analyte for further testing. Intact antibodies and their active digestion fragments can easily be immobilised onto a number of different surfaces

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suitable for use in immunoaffinity separations. Examples of such materials include the interior surfaces of small capillaries [15] and microchips [16], thus making them an ideal ligand for use in micro-scale systems.

In the present study, we describe a micro-scale system that employs a battery of small recycling immunoaffinity columns as the isolation step coupled with laser-induced (LIF) detection of the isolated analytes. The procedure described in this communication is capable of performing multiple separations on a single biological sample and has enabled us to isolate and measure ten different cytokines from a single 25-μl sample.

2. Experimental

2.1. Reagents

Streptavidin, *N*-(6-[biotinamido]hexyl)-3′-(2′-pyridyldithio) propionamide (biotin–HPDP), the ImmunoPure $F(Ab)_2'$ preparation kit, and Cleland's reagent were purchased from Pierce (Rockford, IL, USA). The streptavidin was purchased as a pure, lyophilised product and reconstituted in 50 m*M* carbonate buffer (pH 9.0). Recombinant human cytokines (interleukin (IL)-1, -2, -4, -5, -6, -10, -12, -13; tumour necrosis factor alpha (TNFα), and gamma interferon (γIFN)) and their corresponding anti-cytokine antibodies were obtained from R&D Systems (Minneapolis, MN, USA). All reagents were reconstituted to stock solutions of 1-μg ml^{-1} in 100 m*M* phosphate buffer, pH 7.4). Enzyme immunoassay kits, specific for each cytokine were purchased from R&D Systems. 3-Aminopropyltriethoxy-silane and 1,1′-carbonyldiimidazole were obtained from ICN Biomedicals (Costa Mesa, CA, USA). Analytical grade laboratory chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA). All solutions were passed through 0.2-μm nitrocellulose filters (Millipore, Bedford, MA, USA) prior to use.

Solid glass beads (~10 micron diameter) were obtained from Polysciences (Warrington, PA, USA). PEEK (Poly-ether-ether-ketone) biocompatible chromatography columns, column fittings, and chromatography tubing were purchased from Alltech Associates (Deerfield, IL, USA).

2.2. Instrumentation

Chromatography was performed with a modular HPLC system comprising two model 112 pumps and a dynamic gradient mixer (Beckman Instruments, Palo Alto, CA, USA). Ten immunoaffinity columns were connected to each other via a series of 4-port switching valves (Upchurch Scientific, Oak Harbour, WA, USA-model V100T) placed at the top and bottom of each column. Permanent plugs were inserted into port A of the top valves to columns 2–10 and port D of the top valve to column 1 (see Fig. 1A for port identification). Plugs were also inserted in the bottom valves; blocking ports C of the valves to columns 2–10 and both ports C and D on the port to column 1. This valve arrangement allowed for a continuous flow through all of the columns during the sample application phase and isolation of individual columns during the elution phase (see Section 2.7 for details). Samples were introduced into the system via a model 210 Altex injection port (Rainin Instrument Co., Woburn, MA, USA), equipped with a 25-μl sample loop.

The LIF detection unit was constructed in our laboratory and consisted of a 1 mW miniature diode laser module beam (650-nm-Edmund Scientific, Barrington, NJ, USA) filtered through a 10-nm spatial filter. The beam was focused at a 15-° angle onto the capillary via a 50-mm focal length 'plano' convex lens. The emission was collected and collimated using a parabolic reflector equipped with a scatter mask that positioned the flow-cell of the capillary at the reflector's point of focus [17]. The emission was passed through a 671 ± 10-nm interference filter prior to detection by a miniature fibre-optic spectrometer (Ocean Optics, Dunedin, FL, USA) and the signals relayed to an A/D conversion board in an IBM computer. Data was analysed using an MS-Windows-based software package supplied with the spectrometer (Ocean Optics), and compared to standard curves constructed from known amounts of pure recombinant cytokines.

2.3. Preparation of test samples

Samples of whole blood, plasma, urine, and saliva were obtained from a cohort of 25 normal, healthy volunteers (mean age 22 years) and spiked with known amounts of each recombinant cytokine. Prior

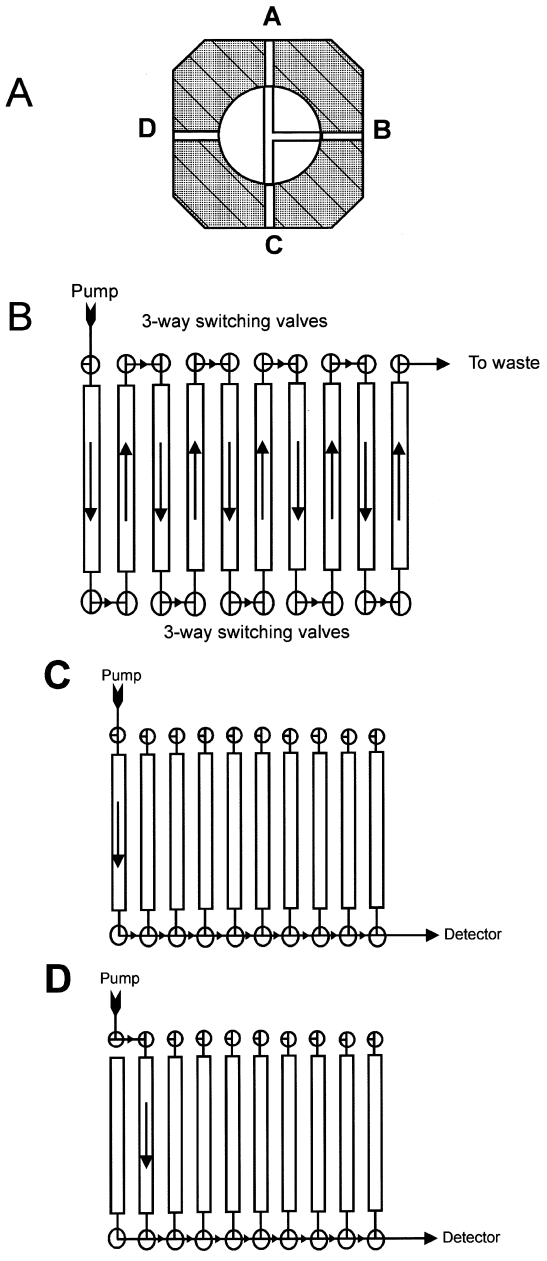

Fig. 1. Diagram of the recycling immunoaffinity system. A: Diagram of the switching valves indicating the positions of the different ports. B: Samples are passed through the battery of immunoaffinity columns as indicated by the arrows. The immobilised antibody in each column retains its specific analyte; non-reactive materials are pumped out of the system to waste. C: Elution of column 1 is achieved by isolating the column via combinations of the switching valves. The eluted analyte passes straight to the detector. D: Elution of column 2 showing the switching valve rearrangement. The same rearrangement was used to isolate each individual column.

to performing this procedure, each sample was tested to ensure that naturally occurring cytokine concentrations were within normal range (>20 pg ml^{-1}). The spiked whole blood samples were 'spotted' onto Whatman 3 mm filter paper, air-dried, and stored at 4°C prior to extraction and testing [18]. Areas equivalent to a 25-μl drop were punched from the filter paper and eluted in 25-μl of 0.01 *M* phosphate buffer, pH 7.4 prior to analysis. The protein content of each eluate was measured spectrophotometrically at 260/280 nm and the samples normalised to a standard protein content of 1 μg ml^{-1}.

Filter paper whole blood spots were also collected from 20 volunteers (mean age 26 years) who were suffering from fever due to influenza infections and a further 20 age-matched volunteers suffering from hayfever. These samples were treated in a similar fashion to that described for the whole blood samples and analysed for the presence of circulating cytokines.

2.4. Biotinylation of the anti-cytokine antibodies

$F(Ab)'_2$ fragments were prepared from each of the anti-cytokine antibodies using the Pierce Immuno-Pure $F(Ab)'_2$ preparation kit according to the manufacturer's instructions. These fragments were further reduced to monovalent FAb fragments by incubation with equal volumes of 200 m*M* Cleland's reagent for 30 min at 37°C [19]. The FAb fragments were biotinylated through their free thiol group by incubating them with biotin-HPDP [20]. Briefly, each FAb fragment was adjusted to 10 μg ml^{-1} in 0.5 *M* carbonate buffer, pH 9.0 and added to 10 μg of biotin-HPDP, dissolved in dimethyl formamide. The mixture was placed on an overhead mixer and incubated for 60 min at room temperature. Finally, the mixture was passed through a desalting column to remove the excess biotin.

2.5. Construction of the immunoaffinity columns

Antibody-coated glass beads were prepared as previously described [21]. Briefly, the surfaces of 0.1-g acid-washed beads were modified by refluxing them in a 10% solution of 3-aminopropyltriethoxysilane for 16 h followed by attachment of carbonyldiimidazole groups to the silanised bead surface. The beads were recovered, thoroughly washed in diox-

ane, air-dried and immediately coated with streptavidin by suspending 0.1-g beads in 1 ml of 50 m*M* carbonate buffer, pH 9.0, containing 0.1 g streptavidin. This mixture was incubated for 18 h at 4°C on an oscillating shaker, before recovering the beads and washing them five times in 0.01 *M* phosphate buffer, pH 7.0, by sedimentation. Finally, the beads were stored in 0.01 *M* phosphate, pH 7.0 at 4°C.

The biotinylated antibodies were immobilised onto the surface of the streptavidin-coated beads by incubating 50-μl of antibody (1 μg dissolved in 0.01 *M* phosphate buffer, pH 7.0) with 0.1 g of beads overnight at 4°C. The beads were then washed five times in 0.1 *M* phosphate buffer, pH 7.0, slurry-packed into 25×4.6 mm I.D. PEEK columns and attached to the HPLC system.

2.6. Pre-analysis labelling of samples

Each sample (25-μl) was mixed with an equal volume of a 1-μg ml^{-1} solution of the red light-emitting (670–675-nm) fluorochrome, Cy5 (Research Organics, Cleveland, OH, USA.), dissolved in 0.5 *M* carbonate buffer, pH 9.5. The mixture was placed on an overhead mixer for 30 min at room temperature and following this incubation, the mixture was further clarified by centrifugation at 10 000 *g* for 5 min prior to analysis [17].

2.7. Analysis by recycling immunoaffinity chromatography

The battery of immunoaffinity columns was assembled as shown in Fig. 1B. Prior to analysis, the valves placed at the top and bottom of each column were set to allow the sample to pass from one column to the next. This was achieved by setting the top valves to columns 1, 3, 5, 7, and 9 to the A/C/D configuration (Fig. 1B). The top valves to columns 2, 4, 6, 8, and 10 were then set to the A/B/C configuration. Meanwhile the bottom valves to columns 1, 3, 5, 7, and 9 were set to the A/B/C configuration and to columns 2, 4, 6, 8, and 10 to the A/C/D configuration. This ensured that the sample would pass in a serpentine fashion through the entire battery of columns.

Following injection of the sample, the system was isocratically developed in 0.01 *M* phosphate buffer, pH 7.0 to which 0.01% Brij 35 was added to minimise non-specific adsorption for 20 min at a flow-rate of 0.5 ml min^{-1}. During this phase, the immobilised antibody in each column captured its specific analyte, allowing the remainder of the sample to pass onto the next column. The top valve on the last column was set to allow all of the non-reactive materials to be washed out of the system and collected for further analysis.

Recovery of the bound materials involved acid elution of each column on an individual basis. This was achieved by sequentially changing the top and bottom valves on each column so that it became isolated from the rest of the battery and ensuring that the column effluent was directed to the detector (Fig. 1C and 1D). The lower valves to columns 2–10 were set in the A/B/D configuration and the bottom valve of column 1 to the A/B/C configuration. To elute column 1, the top valve was set to A/C/D thus allowing the elution buffer to pass through the column and onto the detector (Fig. 1C). Elution of column 2 was achieved by switching its top valve to A/C/D and switching the top valve of column 1 permanently to the A/B/D position. Following elution, the top valve of column 2 was set in the A/B/D position thus ensuring passage of the elution buffer through column 2 only (Fig. 1D). This process was repeated for all columns.

The running buffer was changed from pH 7.0 to pH 2.0 by the addition of 0.1 *M* citric acid [21] and passed through the first column. During this phase, the bound analyte was released and pumped to the detector. After 5 min, the switching valves were changed so that elution of the second column could be achieved. This process was repeated until the bound contents of all ten columns had been eluted. Throughout the procedure, the fluorescence detector constantly monitored the column effluent and the chromatogram recorded by the computer. To improve the working life of the system, the entire battery of columns were maintained in a temperature-controlled chamber at 4°C.

2.8. Comparison to conventional immunoassays

Commercially available enzyme-linked immunoassay measured the concentrations of each cytokine

in the immunoaffinity column effluents and the test samples. These assays were performed according to the manufacturer's instructions.

3. Results

3.1. Characterisation of the recycling immunoaffinity system

Analysis of over 250 batches of streptavidin-coated glass beads demonstrated that 0.1 g of beads can be coated with approximately 35 μg of streptavidin. Biochemical analysis of the immunoaffinity columns, used in this study, indicated that variable amounts of each anti-cytokine FAb fragment was present in the individual columns (Table 1). Antigen saturation studies were used to estimate the maximum binding capacity of each column and dilution studies were used to determine the lower limit of detection (LOD). The findings of these studies are summarised in Table 1. When the battery of columns were maintained at 4°C it was found that each column could be recycled approximately 200 times before a detectable loss in performance could be seen. However, this parameter was drastically shortened to approximately 50 cycles when the battery was operated at room temperature.

A typical immunoaffinity chromatogram is shown in Fig. 2. This chromatogram represents the elution profile from the first or anti-IL-1 column. A single peak is observed eluting at approximately 3.2 min. The elution profile from the entire battery of columns is represented as a series of peaks, each representing the release of an analyte specific to an immobilised antibody. Analytes are always eluted according to the order of the immunoaffinity columns and in the present study, this order was IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TNFα, and γIFN (Fig. 3).

Table 1
Characteristics of the immunoaffinity columns

Column	FAb concentration[a]	Max Binding[b]	LOD[c]
IL-1	4.2	38.1	2.3
IL-2	3.6	25.0	2.6
IL-4	3.8	31.3	2.8
IL-5	4.1	35.8	2.6
IL-6	3.9	31.4	3.0
IL-10	3.4	28.7	3.2
IL-12	3.8	31.5	2.4
IL-13	4.1	36.0	2.8
TNFα	3.4	25.6	2.5
γIFN	3.4	29.8	2.2

[a]Values expressed in μg column^{-1}.
[b]Values expressed in ng column^{-1}.
[c]Values expressed in pg column^{-1}.

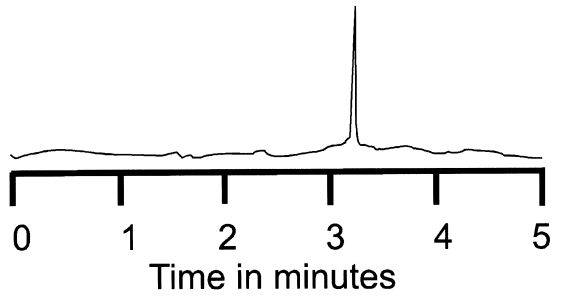

Fig. 2. Typical chromatogram produced by passing the 50 pg ml^{-1} IL-1-spiked plasma sample through a single immunoaffinity column containing immobilised anti-IL-1 FAb.

3.2. Precision and recovery parameters of the technique

The precision and recovery for each of the immunoaffinity columns was established by analysing ten duplicate samples of each body fluid spiked with a mixture of recombinant cytokines containing 100 pg ml^{-1} of each cytokine. Inter-assay precision was evaluated by running the same samples on five different days. For all columns, the inter-assay

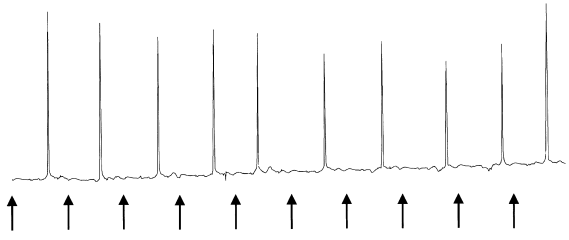

Fig. 3. A chromatogram obtained by passing a plasma sample, containing a mixture of cytokines through the battery of immunoaffinity columns. Peaks eluted in the order of IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13; TNFα, and γIFN according to the position of each column in the battery. Arrows indicate initiation of the elution phase for each column. The test mixture contained 100 pg ml^{-1} of each cytokine.

Table 2
Recovery of recombinant cytokines ($n=10$) from different spiked biological samples[a]

Column	Fluid			
	Whole blood	Plasma	Urine[b]	Saliva
IL-1	97.8±2.6[c]	98.1±2.8	91.9±3.5	88.6±4.5
IL-2	96.5±3.1	96.9±3.3	96.8±3.8	84.9±4.1
IL-4	97.1±3.5	97.5±3.8	96.1±2.9	85.7±4.8
IL-5	96.6±2.9	96.3±2.4	95.3±3.6	82.8±4.2
IL-6	98.4±2.2	98.9±2.4	94.9±3.4	86.6±3.8
IL-10	97.2±3.6	96.8±3.2	96.8±2.8	85.9±4.0
IL-12	95.9±2.5	96.2±2.9	95.4±3.9	85.2±3.6
IL-13	98.3±2.9	98.0±3.3	95.1±3.1	87.9±3.9
TNFα	96.8±3.1	97.1±3.2	98.2±3.4	88.3±4.3
γIFN	98.7±2.8	98.2±3.4	94.7±3.7	86.4±4.6

[a]Normal samples spiked with a mixture containing 100 pg ml^{-1} of each cytokine.
[b]Buffered urine sample.
[c]Value expressed in pg ml^{-1} as the mean±S.D..

coefficients of variation (C.V.) was less than 5.03±0.68. The recovery data given in Table 2 indicate that the addition of the Brij detergent greatly helped to reduce non-specific adsorption; a factor which greatly hinders all miniaturised recovery systems

Different biological matrices were shown to produce different effects. Recovery of all ten cytokines was shown to be optimal in both whole blood eluates and plasma, but less than acceptable in urine and saliva. This situation could be partially resolved when the urine samples were buffered to pH 7.0–7.2 with sodium carbonate. Following pH adjustment, the recoveries of all cytokines greatly improved, nearly reaching those obtained with whole blood and plasma. Despite several manipulations, immunoaffinity recovery of cytokines in saliva samples remained an unresolved problem. Table 2 summarises the recovery of the different immunoaffinity columns and the effects of the different biological fluids.

3.3. Comparison of immunoaffinity recovery with conventional immunoassays

The efficacy of the immunoaffinity isolations, to isolate cytokines from spiked samples, was compared with results obtained by using commercially available immunoassay kits. Analysis of the results obtained by the two different assay systems showed a close correlation that was within an acceptable range for monitoring clinical samples. Linear regression analysis of the results obtained by both techniques in spiked, normal samples demonstrated r^2 values in the range of 0.9214 to 0.9868. The poorest correlation was obtained with the saliva samples and the highest with plasma samples. Evaluation of eluted whole blood samples, demonstrating a close correlation second only to the plasma values.

3.4. Analysis of circulating cytokines in the different study groups

Analysis of the whole blood obtained from the subjects in the three study groups indicated the presence of different cytokine concentrations. Test subjects with clinical signs of fever due to influenza infection demonstrated marked differences in their cytokine expression when compared to healthy controls. The normal group demonstrated baseline concentrations of all ten cytokines as shown in Fig. 4A. These ten peaks present in the chromatogram representing cytokine concentrations in the range of 10–

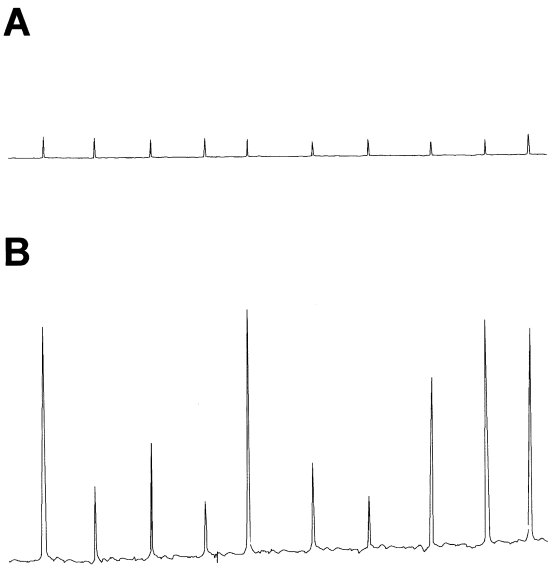

Fig. 4. Recycling chromatogram produced by passing eluted blood spot samples through the battery of immunoaffinity columns. A: A non-spiked, normal control sample. B: A sample obtained from an individual who was suffering from influenza-induced fever. Peaks developed in the order described in Fig. 3.

Table 3
Recycling immunoaffinity analysis of circulating cytokines in the different study groups

Cytokine	Test Groups		
	Normals[a]	Viral fever[a]	Hayfever[a]
IL-1	15±7	762±45	31±12
IL-2	20±10	41±15	42±18
IL-4	10±4	86±21	265±47
IL-5	12±6	29±10	321±56
IL-6	16±4	853±52	40±12
IL-10	10±3	39±14	24±10
IL-12	14±8	22±8	28±14
IL-13	12±4	62±21	26±8
TNFα	20±8	945±49	20±10
γIFN	15±8	231±40	17±7

[a]Values expressed in pg ml^{-1}. Mean±S.D..

35 pg ml^{-1}. However, examination of the infected group indicated significant alterations in the detectable concentrations of IL-1, IL-6, TNFα and γIFN. These cytokines are generally present during inflammation and its associated physiological changes, i.e. fever and drowsiness. Changes in other cytokines were also seen especially IL-13 which is associated with control of inflammatory processes. Small changes in IL-4 and IL-10 indicate the onset of immune reactivity resulting in the activation of B cells and the production of antibodies.

Examination of the samples obtained from the group with hayfever showed increases in the concentrations of IL-4 and IL-5. Both of these cytokines are associated with humoral immune activation, especially activation of IgE synthesis during allergic reactions. Table 3 summarises the cytokine concentrations measured by recycling immunoaffinity chromatography in the two groups.

4. Discussion

Measurement of multiple analytes in clinically-relevant samples is of great interest in the biomedical sciences. This interest stems partially from the need to examine and monitor paediatric and elderly patients, in whom multiple sampling is problematic. Such analyses are also of interest when examining large archival materials collected during epidemiological surveys. One of the most popular methods for collecting blood samples is by placing finger- or heel-stick blood onto filter paper and drying the spots prior to storage [22–25]. Although this technique is advantageous during the collection process, dried blood spots contain only minute amounts of materials that become in short supply during extensive analysis. Therefore the development of techniques which are capable of isolating and measuring a number of analytes within the same sample have merit.

The application of immunoaffinity to the analytical sciences has greatly expanded over the past five years. Immobilised antibodies can be successfully applied to the isolation of a number of different biological analytes, ranging from the purification of a specific protein from cell culture fluid [26] to recovery of materials from cell cytosol [27]. Immunoaffinity techniques have been applied to chromatographic isolation [28] and detection of a number of different analytes [11–15,17,29]. Although most workers use immunoaffinity procedures as a pre-analytical cleanup technique [30,31] prior to analysis by other techniques, immobilised antibodies can be used to measure the concentrations of specific analytes in biological fluids. Although most reports use such antibodies for single analyte analysis, mixtures of antibodies have been used to analyse a number of analytes in the same sample [32,33]. These procedures may involve techniques such as sequentially incubating the sample with a number of different immobilised or labelled antibodies in order to isolate and measure a number of different analytes. Using a sequential immunoassay system, Steffen and Eber-

sole [32] measured five cytokines in a 50-μl sample of gingival crevicular fluid. In the present study, we have been able to isolate and measure ten cytokines from a 25-μl samples of a number of different biological fluids. In our hands this system provided reliable and reproducible results in whole blood eluates, plasma, and urine. As previously reported, problems arose in the recovery of the analytes from saliva samples [11]. Although we have been unable to determine why immunoaffinity isolations prove to be difficult in this matrix, evidence is accumulating to show that the presence of excess mucin may be inhibitory to antibody–antigen interactions.

Recycling immunoaffinity chromatography is a versatile technique and can easily be adapted to automated or miniaturised systems. Using the system described in the present study, we have recently found that the non-reactive materials in the eluate can be further analysed. The non-reactive fraction following passage through the initial battery of columns can be reanalysed by a different battery of columns. This procedure appears to be possible for four or more cycles. Although the present study was performed in small chromatographic columns with acid elution of the bound analyte prior to detection, measurement of the analyte could take place in situ. It is possible to immobilise both intact and digests of antibodies directly onto derivatised silica surfaces. This opens the possibility for building immunosensors or micro detection units which not only capture the analyte of interest but also become the detector, itself [34]. Active antibody fragments or FAb fragments can easily be attached to the internal surface of fused-silica capillaries [15,17] thus providing an ideal detector cell. When pre-analytical fluorescence labelling is used it would be possible to perform LIF detection directly in the column itself. In this way, the immunoaffinity column, itself, can become a complete analytical unit, thus saving the time required for elution of the bound analyte. In a similar manner, enzyme assay detection can be performed in capillaries as demonstrated by Bao and Regnier [35]. This opens the possibility of extending the sensitivity of the detection system by analysing the bound analyte by performing an immunological detection in the detector cells, itself. In such a case, amplification of the end-product could easily be performed using chemiluminescence with a significant increase in sensitivity [36].

In conclusion, it was found that the procedure described in this study can be applied to the analysis of small biological samples, especially those that represent complex matrices, such as blood and plasma. The use of a series of immunoaffinity columns, each containing immobilised antibody specific for a single analyte enhances the selectivity and specificity of the system and allows for multi-analyte analysis is a reasonably short period of time.

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