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Dual microcolumn immunoassay applied to determination of insulin secretion from single islets of Langerhans and insulin in serum

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Abstract

A dual microcolumn immunoassay (DMIA) was developed and applied to determination of insulin in biological samples. The DMIA utilized a protein G capillary column (150 μm I.D.) with covalently attached anti-insulin to selectively capture and concentrate insulins in a sample. Insulins retained in the capillary immunoaffinity column were desorbed and injected onto a reversed-phase capillary column (150 μm I.D.) for further separation from interferences such as cross-reactive antigens and non-specifically adsorbed sample components. Bovine, porcine and rat insulin all cross-reacted with the antibody and could be determined simultaneously. Using a UV absorbance detector, the dual microcolumn system had a detection limit of 10 fmol or 20 p*M* for 500-μl sample volumes. The DMIA system was used to measure glucose-stimulated insulin secretion from single rat islets of Langerhans. Because of the separation in the second dimension, both rat I and rat II insulin could be independently determined. The system was also evaluated for determination of insulin in serum. Using microcolumns instead of conventional HPLC columns resulted in several advantages including use of less chromatographic material and improved mass detection limit.

Keywords: Insulin

1. Introduction

Immunoaffinity chromatography is an important tool for both purification and determination of trace substances in complex samples. In a typical immunoaffinity chromatography assay, analyte is selectively retained by the antibody while non-binding components of a sample are washed out of the column. The bound substances are eluted and detected following injection of an agent, such as low pH buffer, which disrupts the antibody–antigen interaction. In spite of the great selectivity possible with antibody stationary phases, interferences frequently degrade detection limits. For example, the desorbing agent can cause a baseline disturbance when using UV absorbance detectors. This disturbance can hinder observation of the analyte peak at low levels and worsen detection limits. Similarly, non-specifically adsorbed components or cross-reactive antigens can interfere with detection of analyte. In addition, slow desorption kinetics can have a detrimental effect on detection limits due to dilution of the analyte zone during elution [1].

To avoid the limitations of single column immunoaffinity assays, Regnier introduced the concept of dual column immunoassays (DCIAs) [1–3]. In this type of assay, a second chromatography column, typically reversed-phase, is placed in-line with the

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immunoaffinity column. The system collects the desorbed zone from the immunoaffinity column and injects it onto the second column where it is reconcentrated and separated from desorbing buffer, non-specifically adsorbed components, and cross-reactive analytes. The combination of reconcentration and added separation dimension result in significant improvement in selectivity and detection limit for DCIAs relative to one-column immunoassays [1]. DCIAs have proven to be versatile as well and have been used for determining antigens in a complex mixture, characterizing protein variants and determining antibody titers [1–3].

In this paper, we describe the development of a dual column assay that utilizes packed capillary columns which brings the advantages of microcolumns to DCIA. Using microcolumns instead of conventional HPLC columns gives higher mass sensitivity, better compatibility with mass spectrometry, and less consumption of reagents and stationary phases. Only one previous example of a dual column affinity assay that utilizes microcolumns has been described [4]. In that work, the affinity column was used to retain high concentration interfering substances in order to simplify the reversed-phase separation of the unretained components. In this work, we describe the development and application of a dual microcolumn immunoassay (DMIA) for insulin. It is found that the multiple stages of preconcentration allow low concentration detection limits for antigens, even with a UV absorbance detector in the microcolumn. In addition, the preconcentration steps simplify the instrumental requirements of the system.

As a demonstration, the assay is explored for determination of insulin in serum and insulin secretion from single rat islets of Langerhans. Islets are microorgans containing about 3000 cells each and are dispersed throughout the pancreas. Islets are responsible for secreting insulin and other hormones regulating glucose metabolism thus, the measurement of insulin secretion from islets is important in the study of diabetes and other metabolic diseases. It is often desirable to measure insulin release at the level of single islets since insulin secretion from islets may vary due to differences in islet size or position within the pancreas. In addition, islets are difficult to obtain and the ability to work at the single islet level saves considerable resources required to isolate islets. Typically, insulin secretion from islets is measured by radioimmunoassay (RIA) which can take over one day to complete. Therefore, more rapid and more sensitive assays for insulin may be of interest in diabetes research.

In our work, we utilize rat islets which benefit in another way from analysis by a dual column assay. Rats are unusual among mammals in that they synthesize and release two insulin variants (rat insulin I and II) with slightly different amino acid sequences [5]. Most secretion measurements by RIA do not distinguish between the forms and the functional significance of their existence has not been determined.

2. Experimental

2.1. Reagents

Rat and human insulin were obtained from Eli Lilly and Company (Indianapolis, IN, USA). Antibodies used in this work were monoclonals from Balb/c mice and were originally described by Schroer et al. [6]. Two different antibodies (designated antibody 1 and 2) were used and their properties are summarized in Table 1. The antibodies

Table 1
Properties of antibodies used

Antibody	Name[a]	Insulin immunogen	Isotype	Affinity constant K_0 $(L/M)^b$	Bovine I_{50}^c	Human I_{50}	Porcine I_{50}	RatI_{50}^d
1	DB9G8	Bovine	IgG_{2a}	5×10^7	1	1	0.5	3
2	AE9D6	Human	IgG_1	3×10^8	113	1	0.3	38

[a] Designation assigned by original developer of antibody in Ref. [6].
[b] Data from Ref. [6].
[c] I_{50} is the ratio of insulin variant to antigen required to inhibit binding of 50% of the immunogen to the antibody.
[d] No distinction was made between Rat I and Rat II insulin in this test.

were obtained in phosphate buffered saline following purification from ascites fluid using protein G affinity chromatography by the University of Florida Hybridoma Laboratory. Unless specified otherwise, all other reagents used were from Sigma. All solutions were filtered with 0.22-μm Nylon filters (MSI, Westboro, MA, USA) before use.

2.2. Preparation of capillary reversed-phase columns

Reversed-phase capillary columns were prepared using a previously described technique [7]. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA), 15 cm long with 150 μm I.D. and 360 μm (O.D.), were used as column blanks. Approximately 0.5 to 1 cm of the polyimide coating on one end of the capillaries was removed with a flame. Frits were installed by gently tapping the stripped end of the capillary into a pile of 15-μm glass beads (Duke Scientific, Palo Alto, CA, USA). This process was continued until a 0.1–0.2 mm section of the capillary was filled with beads. This section was then briefly heated with a match flame to sinter the beads in place.

Capillaries with frits installed were slurry packed at 1000 to 1500 p.s.i. from a high-pressure bomb equipped with a pneumatic amplifier pump (Alltech Model 1066). The slurry solvent was 50% acetonitrile–50% 20 m*M* phosphate buffer (pH 2.5) and used in a ratio of 100:1 (ml solvent/g packing materials). 5 μm particles with 300 Å pores modified with octyl stationary phase (Deltabond, Keystone Scientific, Bellafonte, PA, USA) were used for most of the work. For serum analysis, columns were packed with 10 μm Poros II R particles modified with octadecyl stationary phase (PerSeptive Biosystems, Cambridge, MA, USA).

2.3. Preparation of capillary immunoaffinity columns

Capillary immunoaffinity columns were packed in a fashion identical to that described above (Section 2.2) except the slurry solvent was 20 m*M* pH 2.5 phosphate buffer and the particles were 20 μm diameter perfusion particles modified with recombinant protein G (Poros 20 G, PerSeptive Biosystems). Antibody was loaded onto a protein G packed capillary by pumping a 60 μl slug of 0.375 mg/ml antibody (either antibody 1 or antibody 2 or a 1:1 mixture of the two) in 20 m*M* pH 6.5 phosphate buffer ("loading buffer") at 5 μl/min using a syringe pump (ISCO Model 100DM, ISCO, Lincoln, NE, USA) through the capillary. The antibody-loaded column was rinsed with several column volumes of loading buffer. Antibody was cross-linked to the protein G phase [8] by pumping 200 μl of 50 m*M* dimethylpimelimidate dihydrochloride (Pierce Chem., Rockford, IL, USA) in 0.2 *M* triethanolamine (pH 8.2) through the capillary column at 5 μl/min. The column was then rinsed with 0.2 *M* ethanolamine at 30 μl/min for 10 min. Several column volumes of desorbing buffer (20 m*M* phosphate at pH 2.5) were injected onto the column to remove unbound antibody. The column was stored in 10 m*M* pH 6.5 phosphate buffer containing 10 m*M* sodium azide at 4°C. Columns prepared in this fashion had an average binding capacity for insulin of 130±15 pmol (n=5).

2.4. Apparatus

Fig. 1 shows a block diagram of the DMIA instrument. The system was equipped with two six-port injection valves (C6W, Valco Instruments, Houston, TX, USA). Valve 1 was used to load samples onto the immunoaffinity column (injection loop volume depended on the experiment). The second valve (fitted with a 5-μl loop) was used to collect the elution peak from the immunoaffinity column and inject it onto the reversed-phase capillary column. Teflon tubing (Alltech, Deerfield, IL, USA) with 0.0625 in. (1 in.=2.54 cm) O.D. and 0.010 in. I.D. was used as a sleeve over the fused-silica tubing for connection to the injection valve ports. Flow through the immunoaffinity column was controlled by a ISCO 100DM syringe pump. Flow through the reversed-phase column was generated by a SSI 222D pump (Scientific Systems, State College, PA, USA). In order to maintain a smooth baseline, a post-pump mixing coil (stainless steel tubing 300 cm×1.0 mm I.D.) was used to enhance mixing of the mobile phase components. A splitter (10 cm×50 mm I.D. fused-silica capillary) was used between the gradient LC pump and the second injection valve to generate

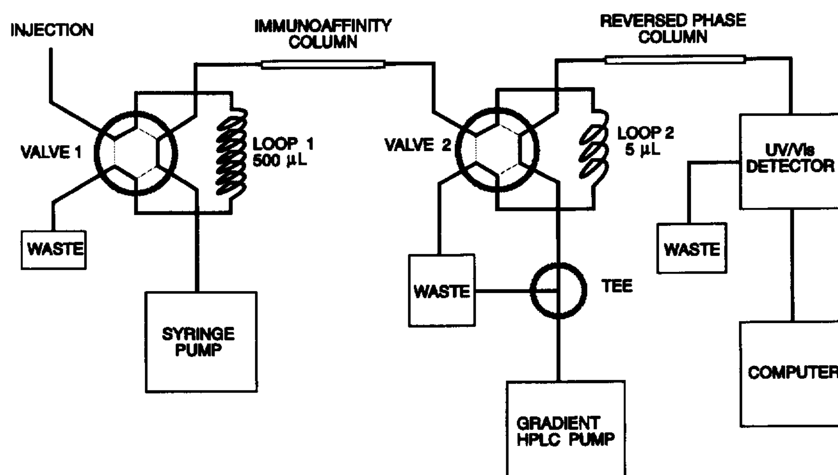

Fig. 1. Block diagram of DMIA instrument.

low flow-rates in the capillary reversed-phase column.

Detection was accomplished on-column using a Thermo Separation Products (Fremont, CA, USA) model Spectra 100 variable-wavelength UV absorbance detector set at 210 nm. The rise time of the detector was set at 1 s for all chromatograms. A computer (Gateway 2000 4DX2-66) equipped with a National Instruments data acquisition board (AT-MIO-16F-5) was used to collect data and analyze the chromatograms. For characterization of the immunoaffinity column, the detector was directly connected to the immunoaffinity column.

2.5. Procedure for DMIA

Samples up to 500 μl dissolved in loading buffer were loaded through an on-line filter frit into the loop of injection valve 1 and then injected. Unless stated otherwise, the sample was injected at 35 μl/min and the pump then adjusted to 5 μl/min. While the flow-rate was stabilizing (typically about 5 min was required), the immunoaffinity column was rinsed with loading buffer for over 10 column volumes. The immunoaffinity column was then rinsed with desorbing buffer at 5 μl/min. The desorbed zone was collected into the second sample loop by switching valve 2 at the appropriate time. For initial characterization studies using bovine insulin as a test analyte, the reversed-phase mobile phase was an 80:20 mixture of 0.1% trifluoroacetic acid–acetonitrile. For gradients, the mobile phase was stepped to 35% acetonitrile at 5.00 min. Conditions for the second separation for more complex mixtures of insulin are given for each sample.

2.6. Determination of insulin secretion from single islets

Islets, obtained from male Sprague–Dawley rats using a previously described procedure [9], were incubated at 37°C in the presence of 5% CO_2 for one to five days prior to use. For secretion experiments, a single rat islet was incubated in 70 μl of 3 m*M* glucose in Kreb's Ringer Buffer (KRB: 118 m*M* NaCl, 5.9 m*M* KCl, 2.54 m*M* $CaCl_2$, 1.19 m*M* $MgSO_4$, 1.19 m*M* KH_2PO_4 and 25 m*M* $NaHCO_3$) for 30 min. A 60-μl volume of the supernatant was removed for analysis. The same islet was then incubated with 70 μl of 8 m*M* glucose in KRB for another 15 min after which 60 μl was removed for analysis. The procedure was then repeated with 20 m*M* glucose on the same islet. The supernatants were injected directly onto the DMIA system and analyzed as described above except the sample loading was performed at 5 μl/min. The immunoaffinity column used for the insulin secretion measurement was loaded with a 1:1 mixture of both antibody 1 and 2. For the reversed-phase separation of rat insulins, solvent A was 20 m*M* phosphate buffer pH 6.5 and

solvent B was 40% solvent A with 60% acetonitrile. The initial mobile phase was 37% B. For the gradient, the mobile phase was increased to 55% B over 5 min, and then maintained at 55% B for 3 min.

2.7. Determination of insulin in serum

Serum samples were prepared by reconstituting lyophilized human serum as described by the supplier (Sigma). For calibration studies, insulin was spiked into the serum at appropriate concentrations. Reconstituted serum was directly injected onto the immunoaffinity column and assayed as described above with two modifications. First, the immunoaffinity column used for serum determinations was 15 cm long, but only 1.5 cm was packed. It was found that this short bed had sufficient capacity and much less non-specific adsorption than the fully packed capillary. Second, a larger rinsing volume (over 20 column volumes) was used after injection of serum onto the immunoaffinity column. For the reversed-phase separation, solvent A was pH 2.5 20 m*M* phosphate buffer and solvent B was 40% solvent A and 60% acetonitrile. The initial mobile phase was 30% B. For the gradient, the mobile phase was linearly increased to 60% B in 5 min then linearly increased to 100% B in 5 min.

3. Results and discussion

3.1. Characterization of DMIA

3.1.1. Effect of loading and desorbing conditions on insulin recovery

It is desirable to use high loading and desorbing flow-rates in order to minimize overall analysis times. The loading flow-rate is especially important when preconcentrating large volume samples (up to 500-μl samples were used in our experiments) on capillary columns. The loading flow-rate cannot be increased arbitrarily because: (1) the capture efficiency may decrease if insufficient time is given for binding and (2) the pressure limits on the packing material used. We found that recovery of insulin from injection of 20 μl of 1 m*M* insulin was ~100% over the loading flow-rate range of 1 to 35 μl/min. Higher flow-rates could not be used because of the pressure limits of the packing.

As with the loading flow-rate, high desorbing flow-rates are desirable in order to minimize analysis time. However, increasing the desorbing flow-rate causes greater dilution of the eluted antigen zone due to the relatively slow kinetics of desorption [10]. We measured desorbing peak volumes and time required for desorption using desorbing flow-rates of 1 to 10 μl/min as summarized in Table 2. As expected, the lower flow-rates yielded lower volume, and therefore more concentrated, antigen zones that required longer elute times. The longer elution times were compounded by a technical difficulty. Specifically, several minutes were required for the flow-rate to stabilize after changing from the loading flow-rate to a lower desorbing flow-rate. The stabilization time increased with decreasing desorbing flow-rate as illustrated in Table 2.

The effect of pH and ionic strength of loading buffer were also investigated for their effect on recovery. The pH could be increased to 7.4 and the ionic strength could be varied between 0.02 and 0.2 *M* (ionic strength was varied by addition of Na_2SO_4) with no decrease in recovery from our standard loading buffer. Increasing the ionic strength to 3 *M* and higher however, reduced loading efficiency by over 50%. Therefore, samples with extremely high

Table 2
Effect of desorbing flow-rate

Desorbing flow-rate (μl/min)	Desorbing peak volume (μl)	Desorbing time[a] (min)	Flow-rate to stabilizationtime[b] (min)
1.0	1.0	4.3	15.0
3.0	1.5	1.5	8.0
5.0	1.9	1.1	3.0
10.0	2.6	0.7	2.0

[a] Time needed from injection of desorbing buffer to the complete elution of antigen.
[b] Approximate time needed to reach the specified desorbing flow-rate from the loading flow-rate (35 μl/min).

ionic strength may need to be desalted before being used with this antibody.

3.1.2. Injection volume for reversed-phase column

As discussed above (Section 3.1.1), higher desorbing flow-rates result in dilution of the desorbed zone. The desorbed zone however, can be reconcentrated on the second column. Using the injection system described in experimental (Section 2.4), we found that up to 5 μl of insulin dissolved in the desorbing buffer could be injected onto the second column with only a 10% decrease in theoretical plates over that found for a 1-μl injection volume. The ability to inject this large volume is due to preconcentration by reversed-phase retention. Since desorbing buffers tend to be aqueous solvents, it is likely that many proteins could be similarly preconcentrated on reversed-phase columns in the second dimension.

Based on this experiment and the results in Table 3, we elected to use desorbing flow-rates of 5 μl/min. The elution volume of 1.9 μl at this flow-rate (see Table 3) was well within the tolerable injection volume range of the reversed-phase column. In addition, with the desorbed peak volume this small, it was straightforward to reliably capture the entire peak in a 5-μl loop for injection onto the reversed-phase column despite the dead volume of the valves and variation in the desorption time from the first column.

3.1.3. Detection limit and calibration

A goal of this work was to use the dual column system for quantitative analysis in biological samples. Therefore, we characterized the linearity of the peak area for bovine insulin obtained by DMIA following 500-μl injections of 0.1 to 5 n*M* bovine insulin dissolved in loading buffer. A chromatogram used in the calibration is illustrated in Fig. 2. The response was apparently linear (correlation coefficient of 0.992) with a slope of 0.026 n*M*/unit area and an intercept of −0.24 n*M*. Peak areas had relative standard deviaions of 5% for 500-μl injections. In general, the response was linear up to binding capacity of the immunoaffinity column.

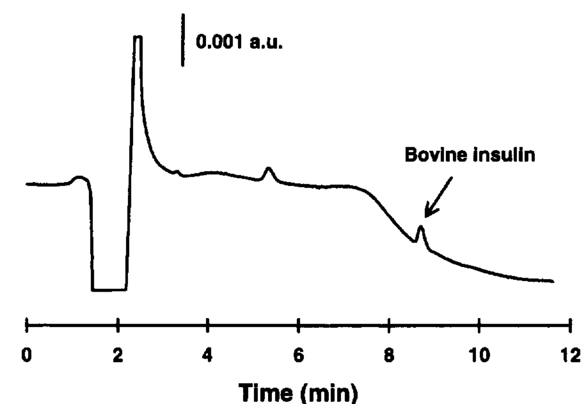

Fig. 2. DMIA chromatogram of 100 p*M* bovine insulin in 500 μl loading buffer. Mobile phase conditions are given in Section 2.5.

The detection limit, calculated as the concentration that would give a peak height three times the peak-to-peak noise was 20 p*M* for a 500-μl injection. This corresponds to a 10 fmol detection limit which is typical for UV detectors with capillary columns. The low concentration is directly attributable to the significant preconcentration possible with the two columns. In previous work, we had described a similar assay which utilized capillary electrophoresis in the second dimension [11]. That assay had a detection limit of about 25 n*M*, which is well above that described here. The improved detection limit is due to the ability to inject and concentrate the entire desorbed antigen zone on the reversed-phase column. In the CE case, on-line preconcentration in the second dimension was not readily accomplished and only a small portion of the antigen zone eluted from the affinity column could be injected.

Table 3
Single rat islet insulin secretion with different glucose stimulation levels[a]

Glucose (m*M*)	Insulin I (fmol)	Insulin II (fmol)	Total insulin (fmol)	Percentage of insulin I
3 (n=4)	96±19	19±2	114±21	83±3
8 (n=3)	196±29	30±6	226±35	88±3
20 (n=3)	389±119	70±40	460±154	85±3

[a] All data are reported as mean±one standard deviation.

3.1.4. Simultaneous detection of insulin variants

An important advantage of DCIA is the ability to simultaneously determine multiple, cross-reactive protein variants [1–3]. Fig. 3 illustrates a DMIA of 4 insulin variants, all of which cross-react with the antibodies used in the first column. In developing this assay, it was found that antibody 1 preferentially recovered rat insulin II. Therefore, we investigated antibody 2 for the immunoaffinity column which we found recovered both rat insulins equally, but gave lower recovery overall. Maximum recovery was obtained by immobilizing a 1:1 mixture of the two antibodies. Compared to a column that utilized just antibody 1, a column with a 1:1 mixture of antibody 1 and 2 had the same recovery for rat insulin II and a 40% increase for rat insulin I. The insulin from other species were unaffected by the use of a mixed antibody column.

3.2. Applications of DMIA for insulin

3.2.1. Determination of insulin secretion from single islets

Fig. 4 illustrates a DMIA for insulin secreted from an islet stimulated with 8 m*M* glucose. As shown, the two rat insulins were well-resolved and their peaks were above the detection limit allowing for quantitative measurements. A summary of the quantification of insulin secretion is given in Table 3. The total amounts of insulin released are in agree-

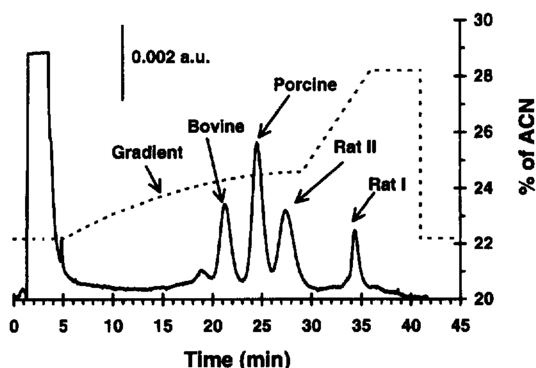

Fig. 3. DMIA separation of insulin variants. Sample injected was 60 μl bovine (30 n*M*), porcine (45 n*M*), rat I (100 n*M*) and rat II (100 n*M*) insulins in loading buffer. Mobile phase solvents A and B are the same as for separation of rat insulins (see experimental (Section 2.6)) and the gradient is indicated by the dashed line.

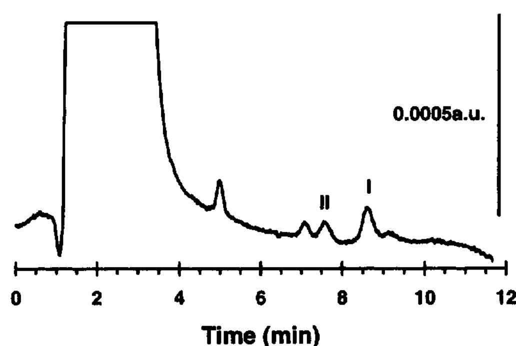

Fig. 4. DMIA chromatogram of supernatant from single rat islet stimulated with 8 m*M* glucose. Labeled peaks indicate rat insulin II and I as shown. Unlabelled peaks are either due to buffer or unknowns from the sample.

ment with previous studies of insulin secretion assayed by RIA coupled with HPLC from rat islets under similar conditions. For example, Ma reported that rat islets stimulated with 22 m*M* glucose secrete 0.83 pmol insulin/islet/30 min [12]. For comparison, the results in Table 3 for 20 m*M* glucose stimulation, expressed in the same units, would be 0.92 pmol insulin/islet/30 min (recall that a 15-min stimulation was used in our case). Finally, insulin quantified by both the DMIA and a capillary electrophoresis-based immunoassay [9] gave results that varied by 3.2% ($n=4$).

The results in Table 3 indicate that rat insulin I and II are released differentially, but at a constant ratio regardless of the glucose concentration. This result is also in agreement with previous studies. For example, in experiments that utilized 40 to 150 islets pooled together, Gishizky et al. showed that 85% [13] of the insulin released was rat I compared to 83% to 88% for our results. The agreement with previous results illustrates that the DMIA provides quantitative information that is consistent with RIAs. The previous measurements were made by separating the insulins by HPLC, collecting the fractions, and assaying them for insulin by RIA. This is a laborious, time consuming process (the RIAs required 8 h). By comparison, the assays used here required 45 min and the analysis could easily be automated. Further improvement in the analysis time could be obtained by using a more sensitive detection scheme (requiring less sample to inject) or by improving the separation speed. Furthermore, the assay could readi-

ly be adapted to other islet hormones by using multiple antibodies on the affinity column.

3.2.2. DMIA for insulin in serum

Another application that we explored with the dual column system was determination of insulin in serum. The complexity of serum samples presents a challenge to the selectivity of the dual column method. This is illustrated by the DMIA chromatograms of serum samples in Fig. 5. Fig. 5(A) shows a serum blank (i.e., a serum sample with no insulin added). As seen, a number of peaks appear in the reversed-phase chromatogram suggesting non-specific adsorption to the immunoaffinity column. In order to decrease this effect, the column length was shortened to 1/10 of the length used for the islet studies. This was done to reduce the non-specific adsorption sites while maintaining a sufficient insulin capacity. The shorter column reduced the overall background peaks to the level seen in Fig. 5(A). The height of the largest serum blank peak (the largest peak is not shown in the chromatograms in Fig. 5) decreased from 0.16 absorbance units (a.u.) with a 15 cm packed bed to 0.02 a.u. with the 1.5 cm packed bed used for Fig. 5(A). We attempted to further reduce the number of interfering peaks by several

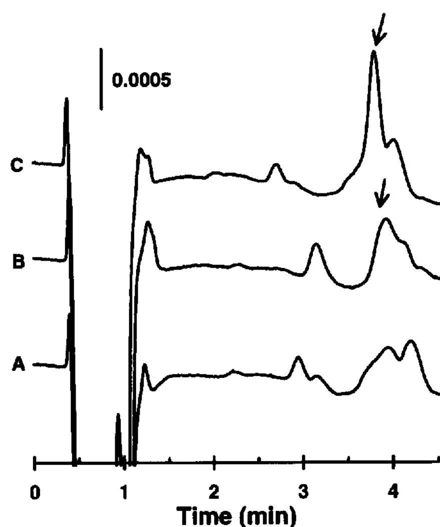

Fig. 5. DMIA of 500 ml serum samples. (A) Serum blank (no insulin added), (B) serum with 200 p*M* human insulin added and (C) serum sample with 1 n*M* human insulin added. The arrows indicate the human insulin peak.

sample treatments prior to injection onto the immunoaffinity columns. Sample pretreatments examined include: (1) extraction of lipids with 1,1,2-trichlorotriflouroethane, (2) removal of immunoglobulins by solid-phase extraction with Protein G particles, and (3) reduction of non-specific adsorption by adding 0.2% Tween 20 to the sample and loading buffer. These treatments decreased some of the peaks that appeared later in the chromatogram, but they did not reduce those in the range of 4 to 5 min, which is the expected retention time of insulin. Thus, for the further experiments we used untreated serum.

In spite of the interfering peaks at the insulin retention time, it was still possible to clearly observe a peak for insulin at 200 p*M* as illustrated by comparing Fig. 5(A) and Fig. 5(B). The detection limit in the serum was set by the variability of the co-eluting peak. We calculated a detection limit of 100 p*M* as the insulin concentration that would give a signal three times the standard deviation of the co-eluting interfering peak. This detection limit is nearly sufficient to reliably measure basal levels of insulin in serum which can be between 47 and 150 p*M*. A calibration performed by spiking insulin at concentrations of 0.2 to 3.0 n*M* into serum samples was linear with a slope of 6.27 n*M*/peak height and intercept of −0.10 n*M* with a linear correlation coefficient of 0.996.

It was found that the amount of insulin recovered on the reversed-phase column when insulin was dissolved in serum was 50±9.8% (n=4) of that found for insulin dissolved in loading buffer which resulted in a decrease in sensitivity. This decrease in recovery is possibly due to competition for binding sites with large amounts of non-specifically adsorbed material. It was also found that the immunoaffinity column capacity for insulin loading decreased by 18% after 14 serum sample loadings. The decrease in loading capacity is attributed to blocking of antibody binding sites by high concentration proteins or peptides found in the serum.

It is apparent that use of the system for real serum analysis would require some improvements. One possibility would be to use the standard addition method to determine the insulin concentration in the presence of the matrix peaks. A preferable choice would be to completely eliminate the co-eluting

interference peak by optimizing the mobile phase gradient or eliminating the non-specific adsorption on the immunoaffinity column with an appropriate pretreatment. (The coeluting interfering peak may include some native insulin, however attempts to reduce it by treating the sample anti-insulin modified particles did not reduce the peak significantly.) In addition, the use of more selective detection, such as fluorescence (perhaps with post-column derivatization) would allow for more freedom from interferences and improved detection limits. These results suggest that the dual microcolumn assay may be used for samples as complex as serum; however, trace-level substances, such as insulin and other hormones, will be difficult to determine without effort spent to optimize the separation and/or detection.

4. Conclusions

We have developed a DMIA system which utilizes a capillary immunoaffinity column coupled with a capillary reversed-phase column. The system can be used for many of the applications that have already been described for dual column assays such as characterization of protein variants, determination in complex samples, determination of multiple antigens and antibody titers [1–3]. In addition, several advantages of this system compared to conventional DCIAs were apparent. For example, the microcolumn system uses less materials. This is especially significant for expensive chromatographic packings like the protein G-modified particles. The system also has good mass sensitivity which allows insulin release from single islets to be readily measured. The preconcentration capability of the DMIA allowed islets to be manipulated in convenient volumes (incubation volumes were about 70 μl) without concerns about excessive dilution of the analyte. These advantages are achieved without sacrifice in convenience of operation. Typically microcolumn systems require extensive modifications to conventional HPLC equipment. In this case, the use of preconcentration allowed conventional injection valves to be used. Relatively high flow-rates in the immunoaffinity column allowed a readily available syringe pump to be used. The main modifications that were still required were a on-column detector cell and a splitter for the mobile phase flow in the reversed-phase column.

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