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ARTICLE *in* ANALYTICAL CHEMISTRY · MARCH 1997

Impact Factor: 5.64 · DOI: 10.1021/ac9606620 · Source: PubMed

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Microchip-Based Capillary Electrophoresis for Immunoassays: Analysis of Monoclonal Antibodies and Theophylline

Nghia Chiem and D. Jed Harrison*

Department of Chemistry, University of Alberta, Edmonton, Alberta, T6G 2G2, Canada

A microchip capillary electrophoresis device has been used to separate the reaction products of homogeneous, immunological reactions within ∼40 s. Determination of monoclonal mouse IgG in mouse ascites fluid, via a direct assay, and the drug theophylline in serum samples, via a competitive assay, was demonstrated on-chip. The mouse anti-bovine serum albumin IgG assay gave a linear calibration curve up to at least 135 μg/mL, with ±3% precision. The theophylline assay gave a threshold for detection of 1.25 ng/mL in diluted serum. A calibration curve of signal vs undiluted log[theophylline] is linear from 2.5 to 40 μg/mL, which includes the therapeutically useful range. Theophylline recoveries in spiked samples were 100%, within an experimental error of ±5%. A buffer system consisting of 0.05 M tricine adjusted to pH 8.0, 0.01% (w/v) Tween 20, and ∼40 mM NaCl was used. This buffer allowed for adequate separation (40 000 plates for theophylline; 1000 plates for theophylline−antibody complex and for human IgG) and gave reproducibility of migration times of 1−1.5% over 4-day periods, indicating minimal problems from adsorption in the uncoated chips.

Microfluidic devices etched in glass substrates provide a fluidic network in which chemical reactions, sample injection, and separation of reaction products can be achieved.[1−13] Electroosmotic pumping may be used to deliver reagents and samples on-chip,[2,5,8−11] and separation of the reaction products is performed using electrophoresis. This method pumps fluid at velocities up to 1 cm/s, while also controlling the direction of fluid flow at capillary intersections, without a need for valves or other moving parts. Electrophoretic effects lead to separation of ions in the applied fields, so that sample transport, reactions, and separations can be integrated within a single manifold of channels on-chip. The demonstration that these types of devices can be applied to commercially important analyses is of considerable significance for further development of this field, and in this paper we follow up our earlier brief report[11] on immunologically based assays on-chip.

Application of capillary electrophoresis (CE) to immunoassays is a newly developing field.[14−25] Immunologically based assays are a commonly used method for selective determination of many chemicals at low concentration. In immunosorbent assays, in which the reagents are adsorbed onto surfaces, there are many fluid handling steps involved. We expect[11] that the unique fluid delivery capabilities of the microelectrophoresis chip format will provide a novel, important new method of automating immunoassays in a manner that does not involve the conventional robotics used in clinical laboratories today. The chip format should also provide much more rapid presentation of results for samples in clinical and environmental applications, as was suggested by our preliminary study of on-chip immunoassay.[11a] Since that report, these potential advantages have also been recognized by Koutny et al.[25] One of the first key steps toward this technology is the separation of products and reactants resulting from homogeneous immunoassays. In this paper we present on-chip, clinically

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S0003-2700(96)00662-2 CCC: $14.00

relevant, immunological assays for proteins such as immunoglobulin G (IgG) and drugs such as theophylline.

Protein separations, particularly separations of antibodies (Ab) and complexes of antibodies with antigens (Ag), are made difficult by adsorption onto the capillary walls.[15,17,20−23] A number of approaches to reduce adsorption have been attempted, such as the use of zwitterionic buffers in the earliest Ab studies,[14,16] use of high pH and/or ionic strength,[15,18−20] cleavage of the antibodies into fragments to remove the heavy Fc chain,[15,17,20,24] and coating of the capillary walls with polymeric agents.[17,23,24] Of these methods we have targeted the use of zwitterionic buffers combined with solution-phase, surface-active components[22] to produce dynamic coating of the capillary walls. This is considerably simpler than dealing with coating the walls of planar glass chip devices and offers the potential advantage of being more reproducible. In addition, where polymer coatings tend to suppress electroosmotic flow, it is easier to maintain flow with dynamic coatings.[17,23] Since electroosmotic pumping provides a useful pumping mechanism in our system, it is preferable that it not be quenched.[1−6] The buffer solution we have utilized also provides for mobilization of antibody, antigen, and their reaction complex, in contrast to the recently reported methods employed for on-chip cortisol analysis.[25]

EXPERIMENTAL SECTION

Device Fabrication. Devices were fabricated in 4-in. square photomask glass (Agfa-Gevaert, Belgium) using microlithographic patterning and an HF/HNO_3 etchant, with a few modifications of the previously described method.[6,13] Devices consisted of two pieces of 1.6-mm-thick glass. The glass was first cleaned in HNO_3/H_2O_2 (3:1) for 30 min. Then a Cr/Au/Cr coating (25/100/25 nm thickness) was sputtered onto the glass substrates before spin coating with photoresist. The microchips used in this study were etched to a depth of 13−15 μm. The etched plate, and the top plate with 2-mm-diameter access holes drilled into it, were cleaned in a class 100 clean hood with a high-pressure cleaning station (MicroAutomation). The two plates were aligned before contact. Air trapped between the plates was squeezed out by manually applying pressure until the Newton's rings disappeared. The plates were separated and recleaned if any particulates prevented complete contact. Permanent bonding was done in an oven by warming at a rate of 10 °C/min and then maintaining at 440 °C for 2 h before cooling.

The chip layout is shown in Figure 1. The thick lines represent channels 240 μm wide, and the thin lines are regions that were 58 μm wide at the top and ∼25 μm wide at the bottom. To allow for easy calculation of electric field strengths in each channel, we express the lengths of all channel segments in terms of an equivalent length for a channel 58 μm wide at the top, as we described previously.[10] The channels connecting reservoirs 1 to 4 had a total equivalent length of 8.02 cm and the channel connecting reservoirs 2 to 3 had a total equivalent length of 1.41 cm. A double-T injector design was used,[4,5] with a center to center length between the two arms of the T of ∼150 μm, and a volume of ∼100 pL. The distance from the injector region to the separation channel waste reservoir (4) was 7.5 cm. The typical injector to detector distance was 5 cm.

Materials and Reagents. Human immunoglobulin G (H-IgG), bovine serum albumin (BSA), fluorescein-labeled bovine serum albumin (BSA*, 11.2:1 average fluorescein to BSA mole ratio), monoclonal anti-BSA in mouse ascites fluid, fluorescein,

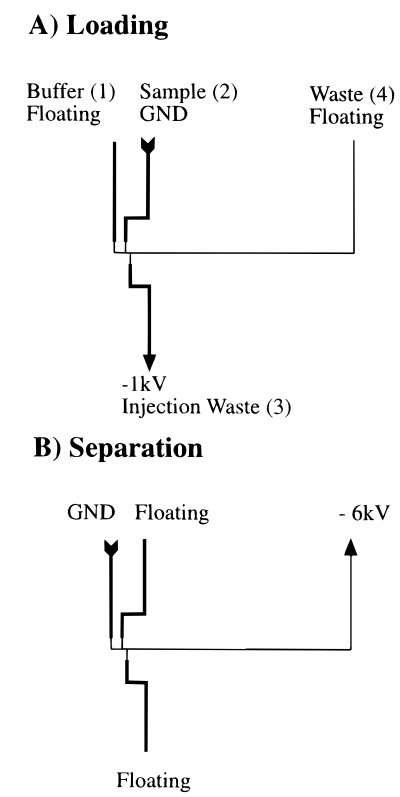

Figure 1. Schematic of chip layout showing (A) voltages applied for sample loading and (B) voltages applied during separation. Arrow heads and tails show the direction and source of fluid flow, respectively. Narrow lines are 58-μm-wide channels; wider lines are 240-μm-wide channels. Reservoir names and numbering schemes are also given in (A).

fluorescein isothiocyanate isomer I (FITC), theophylline, tricine, and tris(hydroxymethyl)aminomethane (Tris) were from Sigma (St. Louis, Mo). Fluorescein-labeled theophylline and antitheophylline were part of the Abbott TDx reagent set (from Sigma). Dimethyl sulfoxide (DMSO), hydroxylamine, NaOH, NaCl, and sodium bicarbonate were from BDH, while boric acid and sodium azide were from J. T. Baker (all via Fisher Scientific, Edmonton, Canada). Tween 20 was from Aldrich (Milwaukee, WI). Human serum was a gift from Bio-Rad (Hercules, CA). All chemicals were reagent grade; doubly distilled water was used for all solutions. Most solutions were filtered with 0.22-μm pore size filters (Millipore); small volumes were filtered with Micropure separators with 0.22-μm pores (Amicon, Oakville, Canada). Microconcentrators with various molecular weight cutoffs (MWCO) were from Amicon or Filtron (Northborough, MA).

The "tricine" buffer contained 50 mM tricine adjusted with sodium hydroxide to pH 8.0, 0.01% (w/v) Tween 20, and NaCl added to give a total ionic conductivity equivalent to a 40 mM NaCl solution. The amount of NaCl required was determined by adjusting the NaCl content of the buffer to obtain the desired current flow in the chip, when 6 kV was applied between reservoirs 1 and 4. A current of 30 μA was the target: the value measured for a 40 mM NaCl solution. Tricine buffer was filter sterilized (0.22-μm filter) and was used within 3 days for sample dilution and separation.

Labeling of H-IgG. H-IgG was labeled as previously described,[26] with some modifications. Purified H-IgG (3.4 mg) was dissolved in 1 mL of 0.1 M sodium bicarbonate (pH 9.0). While stirring, 50 μL of 1 mg/mL FITC in DMSO was slowly added in

5-μL aliquots. The mixture was left in the dark at 4 °C overnight, 150 μL of freshly prepared 1 M hydroxylamine (pH 8.0) was added, and the mixture incubated for 30 min to stop the reaction. The 1.2-mL solution was concentrated and dialyzed down to 150 μL by spinning a 10 000 MWCO Filtron Macrosep concentrator with a centrifugal force of 7000 at 4 °C. Then 1 mL of water was added to the mixture, and the dialysis process was repeated twice more. The final concentrated sample was reconstituted to 1 mL with antibody dilution buffer [25 mM Tris-boric acid, 10 mM NaCl (pH 9), 0.0025% Tween 20 (w/v), 0.0025% sodium azide (w/v)] and stored at 4 °C in the dark.

Direct Assay for Anti-BSA. Various volumes (0, 6, 12, 18, 24 μL) of mouse ascites fluid (Sigma, 2.8 mg/mL of monoclonal anti-BSA in 27 mg/mL total protein) were mixed with 25-μL aliquots of BSA* (1 mg/mL) in microcentrifuge tubes. Tricine buffer was added to each tube to give a total of 500 μL. The samples were incubated for 15 min at room temperature and then ~10 μL was introduced into the on-chip sample reservoir for analysis.

Direct Antitheophylline Assay. Solution T from the theophylline kit (fluorescein-labeled theophylline tracer) was used as received. To adjust the ionic strength, 150 μL of solution S (antitheophylline) was dialyzed through a 30 000 MWCO Amicon Microcon concentrator to ~10 μL, then reconstituted with 140 μL of tricine buffer, and stored at 4 °C. (There is ~5% loss of IgG in the dialyzing process.[27]) The antibody was diluted by a factor of 4 with tricine buffer before assays. For direct antitheophylline assay, 10 μL aliquots of solution T were mixed with 0-, 3-, 6-, 10-, and 40-μL portions of the 4× diluted antitheophylline solution and additional buffer to give a total of 80 μL in microcentrifuge tubes. These mixtures were vortexed for ~10 s and incubated for 15 min at room temperature before transferring 10 μL to the sample reservoir on-chip for analysis.

Competitive Theophylline Assay. For competitive theophylline assays, serum theophylline standards from the calibrator kit (0, 2.5, 5.0, 10, 20, 40 μg/mL) were used as received. Samples were made by spiking theophylline into human serum or buffer to give solutions 15 and 20 μg/mL in theophylline. Standard or sample solutions were then diluted 25× in tricine buffer. A 10-μL aliquot of solution T, 10 μL of the diluted standard or serum samples, and 50 μL of tricine buffer were mixed and vortexed for 3 s. Aliquots (10 μL) of 4× diluted antitheophylline solution were then added and vortexed for ~10 s; 10 μL was then transferred to the sample reservoir on-chip.

Instrumentation. The computer-controlled power supply and relay arrangement has been described previously.[3,6] Laser-induced fluorescence detection used a 488-nm argon ion laser operating at 3.2-mW output. The laser beam was focused with a 15-cm focal length lens parallel to the channel at an incident angle of 56°, measured from the plane of the chip. Emission was collected with a 25× Leitz Fluotar (0.35 NA) objective and then directed onto a Hamamatsu photomultiplier tube (PMT) after passing through an 800-μm pinhole (located at the image plane) and an optical bandpass filter (508−533 nm). The detector observes an 11-pL volume. The PMT signal was filtered through a six-pole Butterworth filter (Krohn-Hite Model 3342) with a 50-Hz low-pass cutoff frequency. The filtered signal was recorded simultaneously on a strip chart recorder and a computer. The A/D board sampling rate was 20 Hz.

Peak areas or height were analyzed using software written in Labview (Austin, TX). The peak heights are reported for anti-BSA assays. For theophylline assays, the peaks were integrated and normalized as discussed later. Signal to noise ratios (S/N) were obtained by calculating the mean signal of the baseline (over 100 points), and the standard deviation of the mean, using software written with Labview. The mean of several points at a peak was evaluated to obtain the peak signal. The S/N was calculated from (peak signal minus the baseline)/(standard deviation in the baseline). When the peaks were broad enough, the noise on the peak was also measured and was found to be the same as the baseline noise.

Mass spectra of BSA and fluorescein-labeled BSA were obtained from matrix-assisted laser desorption/ionization (Kratos Kompact MALDI) using a sinapinic acid sample matrix and 20-kV acceleration.

Chip Operation. Approximately 10 μL of sample was added to the sample reservoir (2) and buffer to the other reservoirs. Sample was injected using the double-T injector by applying −1 kV at the injection waste (3) and ground at the sample (2) as show in Figure 1A. Separation was effected by switching to −6 kV applied at the waste (4) and ground at the buffer (1) reservoirs, Figure 1B.

Fluorescein Detection Limit. Fluorescein samples of 0.1, 0.5, 1.0, 10 nM were prepared by serial dilution with 100 mM tris-boric acid buffer (pH 9). Electrokinetic injection of fluorescein samples was described above. Pump-driven injections were used to create sample plugs large enough that dispersion would not reduce the concentration for the majority of the plug length. A vacuum was applied to reservoir 3 to pull a 2 cm long plug of dye introduced via reservoir 4 toward the detector. Suction was then stopped while dye in reservoir 4 was removed and replaced with buffer; then the vacuum was turned on again to pull the plug of dye past the detector.

RESULTS AND DISCUSSION

Fluorescence Detection. Fluorescence detection provides a high degree of sensitivity, which is useful in microchip CE, where the sample volume is usually very small.[28] To perform the separations of immunological reactions reported here, it was necessary to substantially improve detection limits over our earlier reports.[3,13] Better limits were obtained by optimizing the glass wafer bonding process to reduce formation of light scattering centers on the surface of the glass devices. Careful alignment of the laser beam to eliminate scatter from the curved walls of the channels was also beneficial. Optimal alignment involved directing the beam along the axis of the channel instead of across it, with adjustment of the angle of beam incidence to minimize background. For the detection limit study, sample plugs were large enough that longitudinal diffusion did not decrease the concentration at the center of the plug over the time taken to reach the detector. S/N were measured across a range of 100−1000 pM fluorescein concentrations, and a plot of S/N vs concentration was linear (slope 0.144, intercept −1.393, $r^2 = 1.000$). This study allows us to estimate a detection limit (S/N = 3) within the

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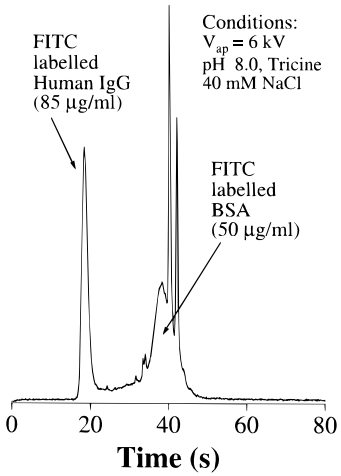

Figure 2. Separation of a mixture of fluorescein isothiocyanate (FITC)-labeled human IgG and BSA in a chip. A pH 8.0, 50 mM tricine/40 mM NaCl/0.01% Tween 20 buffer was used, with a 6-kV separation potential between reservoirs 1 and 4.

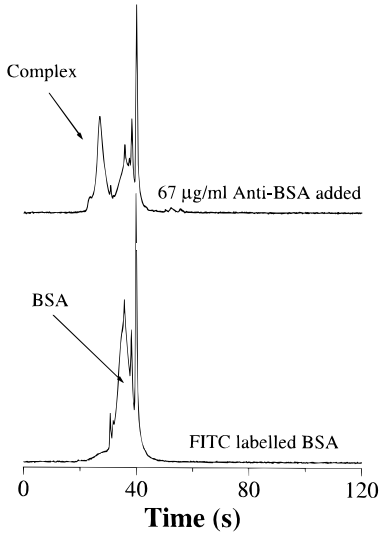

Figure 3. On-chip electropherograms of 50 μg/mL FITC-labeled BSA, and of a mixture containing 50 μg/mL labeled BSA and 67 μg/mL monoclonal anti-BSA in diluted mouse ascites fluid. Buffer and separation conditions were the same as for Figure 2.

detection volume of 30 pM for fluorescein at pH 9. (Formation of 120-pL plugs via the double-T injector resulted in ~3-fold lower signal for the same concentration and a 90 pM injected concentration detection limit, owing to dispersion arising from longitudinal diffusion.)[13] These results are a substantial improvement over the 2 nM limit we obtained previously[13] and are adequate for many immunoassay targets.

Buffer Composition. Adsorption of proteins leads to poor separations in capillary electrophoresis. The dynamic coating system we tested used the neutral surfactant Tween 20 to reduce protein adsorption, since it is commonly present in immunological reagent preparations and does not negatively affect the reagents or the immunoreaction.[29] Tricine buffer was selected from Good's listing[30] of biological buffers, as it has a pK_a of ~8, which is close to that used in the Abbott TDx theophylline kit discussed below. The ionic strength was adjusted with NaCl, since the presence of 40−100 mM salt has been reported to accelerate complex formation.[19] A value of 40 mM was chosen in order to minimize Joule heating effects.

The original antibody separation studies of Nielsen et al. utilized tricine as the buffer, with no other additives.[14] However, when only tricine was used within the glass chips, substantial tailing was seen and trace adsorption was evidenced by weak fluorescence from the walls of the channels. Additionally, formation and injection of sample plugs at the double-T injector was poorly reproducible, presumably due to adsorption-induced changes in the ζ potential in the sample channel. All of these phenomena were eliminated by the addition of Tween 20 and NaCl to the tricine buffer.

Figure 2 illustrates the separation of fluorescently labeled, polyclonal, human IgG and BSA. The IgG peak gave a clean, symmetrical peak with 750−1000 theoretical plates with 6 kV applied (750 V/cm), indicating the buffer/surfactant system selected is reasonably effective in uncoated chips. The BSA peak is well separated from IgG, so that we can anticipate an IgG−BSA complex, as discussed below, will lie between the two peaks in Figure 2 and should be resolvable. The large breadth of the BSA main peak and the presence of several other nearby peaks is due to the very heterogeneous character of the commercially labeled BSA. MALDI mass spectrometry showed the labeled BSA had a full width at half-maximum of 6000 Da, compared to the 1000 Da width of native BSA from the same commercial source.

Protein Assays. Homogeneous immunoassays are solution-phase reactions of an antibody and its target molecule, the antigen. By labeling either the antigen or the antibody with a fluorescent tag the complex produced will also be labeled.[15−17,20] Capillary electrophoresis can then be used to separate the reactants and products, since the complex will have a different charge to size ratio than either free Ab or Ag. Homogeneous assays can then be run in a number of modes and used to determine either the antibody or the antigen. Examples of both types of assays will be presented here.

Immunoassay with the moderate molecular weight protein BSA was performed in a direct assay mode designed to determine the concentration of anti-BSA produced in mouse ascites fluid. BSA* was used as the probe for monoclonal anti-BSA. Reaction of BSA* with anti-BSA results in decreased signal for BSA* and the appearance of an Ab−BSA* complex peak, as seen in Figure 3. As was expected, the complex migrates at a rate between that of labeled IgG and BSA* (cf. Figure 2). While the separation efficiencies observed are not high, it is important that the components are sufficiently well resolved to allow a high-quality assay of the anti-BSA.

The increase in complex peak height or area is proportional to the amount of anti-BSA present. Figure 4 shows a calibration curve for the assay of BSA antibody present in mouse ascites fluid. The measurement precision was ±3%, for four replicate injections. Assays could be run repetitively for weeks within the same chip with the same precision. Deliberate addition of up to 100 μg/mL polyclonal human IgG showed no complex formation, as illustrated in Figure 2, consistent with the known selectivity of most antibody reactions. The monoclonal IgG content of mouse ascites fluid is ~90% of the total IgG present, so the determination of anti-BSA can be successfully performed in the presence of a 10% content of other mouse antibodies.

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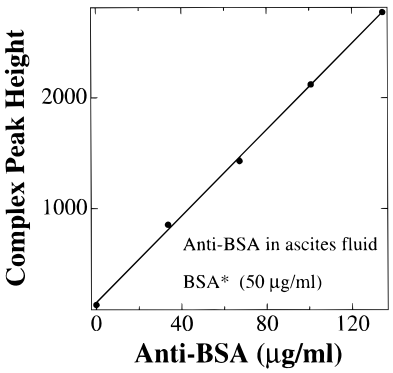

Figure 4. Calibration curve for anti-BSA in diluted mouse ascites fluid, showing peak height for the antibody complexed with 50 μg/mL labeled BSA as a function of antibody concentration. Buffer and separation conditions were the same as for Figure 2. Each point is the mean of four replicates. Standard deviations were equal to or smaller than the data points in the plots.

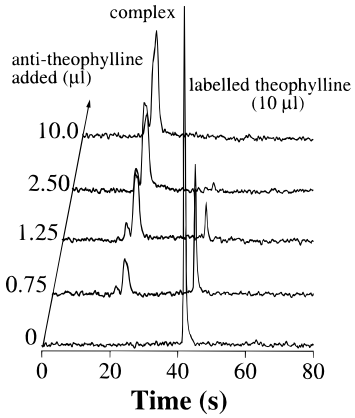

Figure 5. Series of on-chip electropherograms for 10 μL of labeled theophylline with added antibody solution, showing titration of Th* with the antibody. Buffer and separation conditions were the same as for Figure 2.

Ascites fluid is extracted directly from mice during monoclonal antibody harvesting. It is rich in a variety of proteins (27 mg/mL), of which only 10% is IgG. The fact that adequate separations and good, quantitative assays can be obtained over many days in this complex fluid illustrates the robustness of the microchip−CE system for these assays. This direct, on-chip assay is fast and simple and can potentially be used for rapid screening in hybridoma production of monoclonal antibodies to allow selection of suitable antibody-secreting cells. Hybridoma production is normally limited by the screening method,[26] so that rapid methods to determine whether a cell line is producing antibody with a useful binding constant for a particular target would be welcomed.

Hapten Assays. A large number of immunoassays target small molecules, known as haptens. A typical example is the analysis of a therapeutic drug for asthma treatment, theophylline (Th).[22] In order to get good separations by CE when haptens are used, it is necessary to label the hapten instead of the antibody.[20,22−25] Labeled Th (Th*) is thus mixed with a sample containing unlabeled Th, and the two are allowed to compete for a limited amount of antibody to Th, as illustrated.

$$\mathrm{Ab} + \mathrm{Th}^* \rightleftharpoons \mathrm{Ab{-}Th}^*$$

$$\mathrm{Th} + \mathrm{Ab} + \mathrm{Th}^* \rightleftharpoons \mathrm{Ab{-}Th} + \mathrm{Ab{-}Th}^*$$

A competitive assay leads to an increase in signal for the free, labeled Th* as the Th in the sample increases in concentration. There is a corresponding decrease in signal from the Ab−Th* complex. Figures 6 and 7 show a series of separations performed on-chip, in which varying amounts of Th, Th*, and Ab were mixed. The separations occur in less than 1 min and the complex is well resolved from free Th*. A theoretical plate number of 40 000 was measured for Th* (1000 plates/s, 10.7 plates/V), while the Ab−Th* complex gave ∼1000 plates. Variations in migration times (six replicates) were within ±0.6 and 0.9% for the complex and free Th*, respectively, over 1 day, and within ±1 and 1.5%, respectively, over a 4-day period. This stability illustrates the robustness of the chip when used with the tricine, Tween 20, and salt buffer combination, as extensive exposure to protein can cause large migration time variations if adsorption is not substantially prevented.

Figure 5 shows electropherograms of Th* and mixtures of Ab with Th* obtained at 6 kV. Addition of the Ab causes a decrease in the free Th* peak and formation of a new pair of peaks assigned to Ab and Th* complexes.[14,31] In order to perform a competitive assay, it is necessary to know the ratio of Ab and Th* that should be combined. The required ratio may differ from the 1:1 volume ratio used in the TDx instrument, owing to the different buffer conditions and measurement methods we have used. (Below, the Ab and Th* solution volumes are expressed in terms of the stock solutions of the Abbott reagent kit. Actual concentrations are not available.) Figure 5 shows the results of titration of 10 μL of Th* tracer solution with Ab. No free Th* is seen when 10 μL of Ab solution is used, while only 2.5 μL of Ab solution is required to reduce the free Th* peak to ∼5% of its original area. These results indicate the equilibrium binding constant in the tricine/Tween 20 buffer is not decreased relative to that seen in the Abbott buffer system. This result contrasts favorably with the decreased binding constant reported for the theophylline assay when SDS is used in micellar electrokinetic capillary chromatography.[22]

A competitive assay for Th was performed by mixing unlabeled Th with Ab and Th*. Separations for a series of theophylline standard solutions are shown in Figure 6, in which the Ab and Th* reagent kit stock solutions were mixed at a 1.5:10 volume ratio. The area of the two complex peaks can be summed to obtain the amount of complex, while the amount of free Th* may be determined from its peak area. The peak areas can then be normalized to the total amount of fluorescent Th injected with each standard, determined by adding the areas for all the peaks. This procedure was made possible by our ability to mobilize both the free Th* and its complex with the antibody and obviates the need for an additional internal standard.[23,25]

Figure 7 shows a calibration curve obtained with a set of calibrator kit standards and Ab and Th* reagent kit stock solutions mixed at a 2.5:10 volume ratio. A slightly higher Ab:Th* ratio was used in Figure 7 relative to Figure 6, which reduced the free Th* signal to near zero in the absence of added Th and improved the sensitivity at low Th concentrations. A plot of normalized peak area for Th* vs log[theophylline] was linear (slope 0.386, intercept 0.091) and allowed calculation of the solid line shown in the figure.

(31) The two peaks may be Ab-Th* and Ab-Th*$_2$, as suggested by ref 14, although a change in peak height ratios as the amount of Th increased would then be expected. Alternatively, the Abbot reagent kit may contain more than one form of antibody for Th.

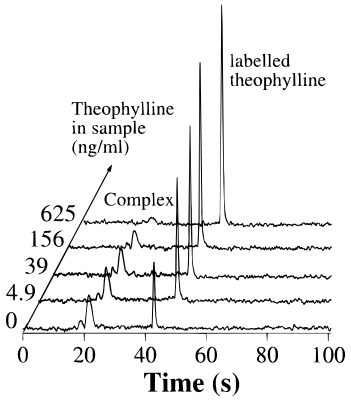

Figure 6. Series of on-chip electropherograms for a competitive Th assay using 10 μL of labeled theophylline, 1.5 μL of stock antitheophylline, and increasing amounts of unlabeled Th. Buffer and separation conditions were the same as for Figure 2.

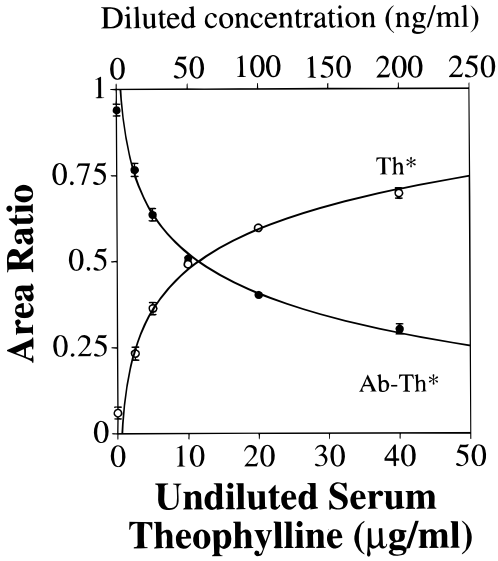

Figure 7. Calibration curve of normalized peak area (see text for procedure) vs concentration of Th in serum standards. Competitive assay used mixtures of 10 μL of labeled theophylline, 2.5 μL of stock antitheophylline, and increasing amounts of unlabeled Th. Lower axis shows Th concentrations in serum standards before dilution; upper axis shows actual concentrations during on-chip separation. Buffer and separation conditions were the same as for Figure 2. Each point is the mean of four replicates. Error bars show the standard deviations, where they were larger than the data points.

The lower axis of Figure 7 shows the original concentrations of Th in the serum standards, while the upper axis shows the actual concentrations in the measurement, following the buffer dilution steps. The calibration curve covers the important clinical range of 10−20 μg/mL for serum samples.

Spiked human serum samples were run at 15 and 20 μg/mL (four replicates). Use of the calibration curve to estimate their Th concentrations gave values of 16.2 ± 1.6 and 21.7 ± 2.7 μg/mL, respectively. To evaluate the role of the serum matrix, a 20 μg/mL Th sample was prepared in buffer. The serum-based standard curve provided an estimate of 20.1 ± 1.6 μg/mL for Th in the spiked buffer solution. These results demonstrate the calibrated device is accurate. The standard deviation measured from 12 replicate runs was ±5%, over both single and 4-day periods, confirming the robust response of the device. In addition, there is no apparent bias of the result introduced by the serum matrix, so that serum-based standards should not be required.

In order to determine the absolute threshold for detection of theophylline, we diluted a 2.5:10-μL mixture of Ab and Th* until the complex peak height had an S/N of 4.6, close to the detection limit of 3. Unlabeled Th was then mixed with Th* and Ab at this concentration until the S/N value for the free Th* peak increased to 7 from its initial value of 4. We have used a change of 3 in S/N as an estimate of the Th detection threshold. Defining the threshold in this way gave a value of 1.25 ng/mL, or 6.9 nM, in the diluted sample.

CONCLUSIONS

A key element in utilizing electrokinetically driven microfluidic devices for immunoassays is demonstration that separations of homogeneous immunological reaction products can be performed on-chip. The work presented here shows such separations can be performed on chip from a range of analytes with molecular weights between a few hundred and ~150 000 Da. When combined with our previous on-chip results,[11] those of Koutny et al.,[25] and those of many other authors for conventional capillaries,[14−24] it becomes clear that such separations will be readily achieved on-chip. The particular examples selected here also illustrate potential application areas in clinical analysis and the biotechnological production of proteins.

One of the difficult issues in CE separations of immunological reagents is associated with protein adsorption. Our results show we can obtain good, robust performance within microchips simply by using an appropriate buffer system, even with challenging sample matrices such as human serum or mouse ascites fluid. The buffer allowed us to mobilize and observe both the labeled antigen and the antibody−antigen complexes. The ability to see both components will provide for a higher level of precision and should obviate the need for additional internal standards. The ability to use antibodies without modification, and to work at near-neutral pH values, means that reagent kits presently in use can be directly adapted to the chips. The fact that coatings can be avoided may reduce the manufacturing cost of future devices substantially. In fact, the buffer system we have used may also prove quite useful when applying conventional fused-silica capillary electrophoresis to immunoassays.

ACKNOWLEDGMENT

We thank the Natural Sciences and Engineering Research Council of Canada, Defense Research Establishment Suffield, and the Alberta Microelectronic Centre (AMC) for support. N.C. thanks AMC for a Graduate Stipend. We also thank the staff at AMC for assistance in device fabrication, and R. Whittal for performing the MALDI studies.

Received for review July 8, 1996. Accepted November 4, 1996.[⊗]

AC9606620

[⊗] Abstract published in *Advance ACS Abstracts*, December 15, 1996.