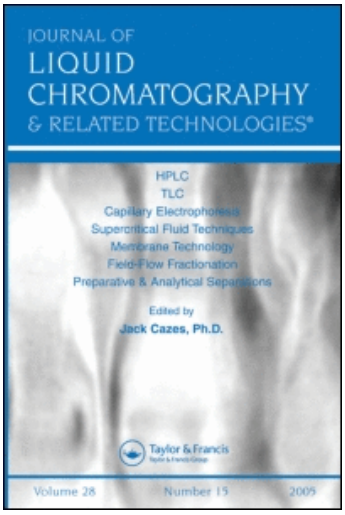

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IMMUNOAFFINITY ANALYSIS OF SUBSTANCE P IN COMPLEX BIOLOGICAL FLUIDS: ANALYSIS OF SUB-MICROLITER SAMPLES

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ABSTRACT

There is a growing need for systems capable of measuring specific analytes in biological samples, especially complex biological fluids. Additionally, this need extends to applying such analyses to precious clinical or research samples, for many of which only micro quantities are still in existence. Immunoaffinity separations provide a useful approach to isolating specific analytes from complex matrices, as well as being capable of miniaturization. In the present communication, we have designed an immunoaffinity system capable of measuring a single analyte in 50 nL samples. We have chosen the clinically important neuropeptide, substance P (SP), as our model analyte and applied it to studying the ability of a micro-chromatography system to measure SP in a number of

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different biological matrices. The system is capable of isolating SP from biological fluids, including cell cytosols, with reasonable efficiency and reproducibility. The incorporation of laser-induced fluorescence detection enabled the system to achieve a lower limit of detection around 500 femtograms/mL and could isolate the specific analyte in under 5 min, thus, making it potentially useful for high throughput clinical studies.

INTRODUCTION

There is an increasing demand in the biosciences for the development of relatively simple ultramicro techniques capable of analyzing extremely small samples, such as biopsy tissue lysates, single cell secretions, and certain neonatal clinical samples. Such analyses must be sensitive enough to handle sub-microliter samples measuring analytes in the picogram (pg) to femtogram (fg) range of concentrations. To this end, the emerging world of microfabrication offers potential answers to these problems, but the availability of such technology is not universally available, and often requires specialized equipment in order to perform the assays.[1–3] Capillary electrophoresis (CE) and its allied technique, capillary electrochromatography (CEC), are ideal approaches to microanalysis but in some areas, especially the biomedical sciences, these techniques have been slow to be introduced.[4] Both CE and CEC can be run in chip-based formats, making them extremely suitable for analyzing extremely small samples.[5–8] However, these techniques require relatively expensive equipment, making them unavailable to many laboratories. Chromatographic procedures, especially those coupled with affinity or immunoaffinity separations, are reasonably well accepted in both the biological and biomedical sciences,[9,10] making this approach a good candidate for developing ultramicro analytical techniques suitable for measuring biological analytes at the sub-microliter level.

Neuropeptides play important roles in many different cellular pathways, including pain induction, inflammation, and immune regulation.[11] Measurement of the neuropeptide, substance P (SP), in biological matrices is important in many clinical and research endeavors, including diagnostic procedures associated with monitoring allergy and inflammation. Further, SP is a relatively small molecule making it hard to analyze by conventional immunoassays, especially in relatively small biological samples. In this communication, we describe a simple ultramicro immunoaffinity system, constructed from commercially available components, that is capable of reproducibly analyzing SP in sub-microliter samples from a number of different complex biological matrices. Additionally, the system is sensitive, versatile, and relatively cheap, making microanalysis more available to clinical and research laboratories.

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EXPERIMENTAL

Reagents and Materials

Streptavidin and long-chain hydrazine biotin were purchased from Pierce (Rockford, IL). The streptavidin was purchased as a pure, lyophilized product and reconstituted in 50 mM carbonate buffer (pH 9.0). Recombinant SP, its corresponding anti-neuropeptide antibody, and a conventional immunoassay kit were obtained from Bachem Bioscience, Inc. (King of Prussia, PA) and reconstituted to stock solutions of 1 μg/mL in 100 mM phosphate buffer, pH 7.4. The glass modifying reagents, 3-aminopropyltriethoxysilane and 1,1′-carbonyldiimidazole, as well as all other analytical grade laboratory chemicals, were obtained from Acros Chemicals, Inc. (Fisher Scientific, Pitsburgh, PA). All solutions were passed through 0.2 μm nitrocellulose filters (Millipore, Bedford, MA) prior to use.

Solid glass beads (~10 micron diameter) were obtained from Polysciences (Warrington, PA). Poly-ether-ether-ketone (PEEK) capillary tubing, fused capillary tubing, and all PEEK connectors were purchased from Upchurch Scientific (Oak Harbor, WA).

Instrumentation

The chromatography system was constructed using a CMA102 microdialysis syringe pump (CMA Microdialysis, Chelmsford, MA), equipped with twin 1 mL glass syringes. The syringe pumps were connected to a micro gradient mixer (NanoMixer—Upchurch Scientific) via 2 cm lengths of 50 μm i.d. PEEK capillary tubing. A fifty nanoliter (nL) sample was introduced into the system through a micro-injection valve (Upchurch Scientific) equipped with a calibrated 50 nL PEEK capillary loop, and placed between the NanoMixer and the inlet of a micro-column assembly.

Micro-columns (12 mm in length) were prepared from 50 μm i.d. fused capillary tubing by loosely packing one end with 2 mm of dry silica particles. These particles were then heat-annealed using a micro-forge (World Precision Instruments, Inc, Sarasota FL) to form a porous glass frit used to retain the column-packing material. Antibody-coated glass beads were prepared as previously described.[12,13] Briefly, the surfaces of acid-washed beads were silanized with 3-aminopropyltriethoxysilane, further modified by chemically condensing carbonyldiimidazole groups onto the silane surface, and attaching a streptavidin coat. The anti-neuropeptide antibodies were biotinylated using hydrazine biotin via carbohydrate moieties present in their Fc portion.[12,13] The biotinylated antibodies were then immobilized to the surface of the streptavidin-coated beads. This was

achieved by incubating the beads in the antibody solution overnight at 4°C on an overhead mixer. The beads were extensively washed in l00 mM phosphate, pH 7.4 and slurry-packed into a column. The column was mounted into a commercially available capillary column assembly (Upchurch Scientific), the inlet of which was connected to the micro-injector and the outlet connected to a 50 μm i.d. glass capillary flow-cell incorporated into a laboratory-built laser-induced fluoreascence (LIF) detector.

The LIF detector consisted of a 633 nm 10 mW helium neon laser (Melles Griot, Irvine, CA) brought to focus by a 4× microscope objective through the sidewall of a 50 μm i.d. fused silica capillary flowcell. Orthogonal to the helium-neon excitation beam, a 400 μm flat-cleaved fused silica optical fiber was placed in close proximity to the focal point, using standard optical positioners (Newport Corporation, Irvine, CA) to ensure accurate and stable alignment of the flowcell, objective, and optical fiber. Fluorescent emission was collected through the optical fiber, filtered by a l0 nm bandpass interference filter centered at 650 nm (Oriel Corporation, Stanford, CT), and the signal processed by an S2000-FL Ocean Optics fiber optic spectrometer (Ocean Optics, Dunedin, FL) to separate the remaining scattered excitation light from the signal at 650 nm, which was captured by a l000 MHz A/D board (Ocean Optics) mounted in a PC-based computer. Figure 1 is a schematic of the micro-chromatography system and LIF detector.

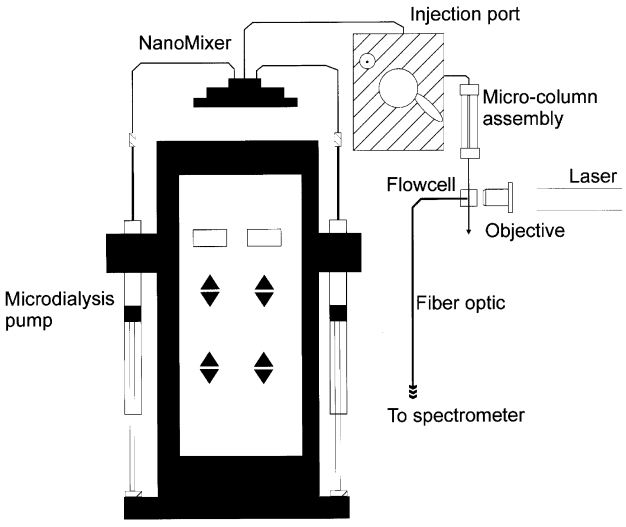

Figure 1. Schematic of the chromatographic system used for performing the ultramicro immunoaffinity analyses.

Preparation of Test Samples

Samples of serum, plasma, urine, saliva, cerebral spinal fluid (CSF), and tissue lymph were obtained from discarded clinical samples with the patients' consent. Cell lysates were made from lymphoid and microglial cell lines obtained from the American Type Culture Collection (Manassas, VA). Each sample was split into two aliquots. One aliquot was mixed 1 : 1 (vol/vol) with a 5 μg/mL solution of AlexaFluor 633 (a 650 nm-emitting fluorochrome from Molecular Probes, Eugene, OR), dissolved in 0.5 M carbonate (pH 9.5). This mixture was placed on an overhead mixer for 15 min at room temperature and clarified by centrifugation at 10,000*g* for 5 min prior to analysis. The other aliquot was used to determine the concentrations of SP by conventional immunoassay.

Immunoaffinity Analysis

Following injection, the sample was pumped through the micro-column using l00 mM phosphate buffer (pH 7.0) as the mobile phase. During this phase, the immobilized antibody captured its specific analyte and retained it, allowing the non-reactive materials to pass through to the collector. The column was washed for 1 min in running buffer, before introducing an acid elution gradient (pH 7.0–2.0) by the addition of glycine (0.5 M) to the running buffer. The elution gradient was applied for l.5 min before regenerating the column by returning it to pH 7.0 ready for the next sample injection. During this procedure, the LIF detector constantly monitored the column effluent and recorded the chromatogram, which was visually displayed using the spectrometer's software package.

RESULTS

Column Parameters

Analysis of 10 different columns demonstrated that the streptavidin-coated glass beads had a capacity to bind between 0.l–0.13 ng of anti-SP antibody per column, thus, having a potential capacity (due to the bi-valent nature of the immobilized antibodies) to isolate between 200–260 pg of analyte. However, experimentally, the columns demonstrated a maximum analyte binding capacity of 140–210 pg/column. Further, the lower limit of detection of the LIF detector used in this system was found to be 460 fg. Optimal SP recovery was found to be $98.25 \pm 0.8\%$, at a concentration of 50 pg/mL spiked in plasma with inter- and intra-assay coefficients of variation

(C.V.) of 5.66 ± 0.41 and 5.05 ± 0.36, respectively. Comparison of the efficiency of the micro-column to a conventional competition immunoassay, showed a close correlation between the two assays when analyzed by least-squares linear regression, using GraphPad Prism software (version 3.0—GraphPad Software, San Diego, CA) giving a regression line with a slope of 1.031 ± 0.044 and an r^2 value of 0.9586.

Temperature and flow rate both affected column performance. The former lowering both analyte binding capacity and bioactivity; columns stored and run at standard room temperature (22–24°C) could be regenerated up to 20 times before an appreciable loss (great than 5%) in bioactivity was noted. This situation greatly improved by storage of the columns at 4°C and operation in an ice bath; this procedure was found to increase column regeneration up to 200 times before a loss in either bioactivity or efficiency was observed. Likewise, flow rate greatly affected the column efficiency. Flow rates above 6.0 μL/min resulted in poor recovery, whereas the optimal analyte recovery was observed at flow rates of 1.0 and 2.0 μL/min (Table 1). To reduce assay time and prolong the useful life of the column, it was decided to adopt the flow rate of 2.0 μL/min and an operational temperature of 4°C for studying the efficiency of the micro-chromatography system in determining the concentrations of SP in 50 nL samples of different biological matrices. Using these parameters, the chromatographic system produced chromatograms typical of that illustrated in Fig. 2.

Table 1. Binding Efficiency of SP to the Immunoaffinity Matrix Under Varying Flow Conditions

Flow (μL/min)	SP Concentration (pg/mL)	Recovery (pg/mL)	Recovery (%)
1.0	10	9.83	98.3
	50	49.25	98.5
2.0	10	9.84	98.4
	50	49.15	98.3
3.0	10	9.71	97.1
	50	48.70	97.4
4.0	10	9.53	95.3
	50	47.95	95.9
5.0	10	9.41	94.1
	50	47.25	94.5
6.0	10	9.22	92.2
	50	45.30	90.6
7.0	10	8.74	87.4
	50	43.40	86.8

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Measurement of Substance P in Different Biofluids

The recovery of both spiked and naturally occurring SP in the micro-samples of biological fluids differed according to the fluid tested. Studies on samples spiked with 50 pg/mL of SP demonstrated that plasma and serum provided the most suitable matrix for immunoaffinity recovery, although recovery from plasma was marginally better. Tissue lymph, cell cytosol extracted from two different cell lines, and CSF also provided good recovery, although not as good as plasma or serum. Urine had to be neutralized by adding sodium carbonate prior to analysis, but this pre-treatment gave reasonable results. Recovery of SP from saliva proved disappointing. Only 41.3 ± 3.7 pg/mL could be recovered with a wide variation over the ten samples tested. This situation could not be resolved by chemical or filtration pre-treatment. The results of these studies are summarized in Table 2.

In an attempt to study the usefulness of the system in clinical and cell biology, we analyzed concentrations of SP in tissue lymph from a normal and injured subject. Fluid was extracted from a small (less than 0.5 cm diameter) skin blister, from an otherwise healthy individual, and subjected to immunoaffinity analysis. Substance P could be detected at l.8 pg/mL concentration [Fig. 3(A)], in the 50 nL sample extracted, which differed greatly from the 231 pg/mL recovered from tissue lymph exudates obtained from a traumatic injury subject accompanied by neurogenic inflammation [Fig. 3(B)]. Further, we investigated the ability of the system to analyze SP in cell cytosols. Antigen-sensitized lymphocytes were analyzed at rest [Fig. 4(A)] and following antigenic stimulation [Fig. 4(B)]. Initially, the cell cytosol expressed a SP concentration of 72.1 ± 5.97 pg/mL, but 1 hour following exposure to their specific antigen, these cells were capable of producing 199.4 ± 12.7 pg/mL. These studies

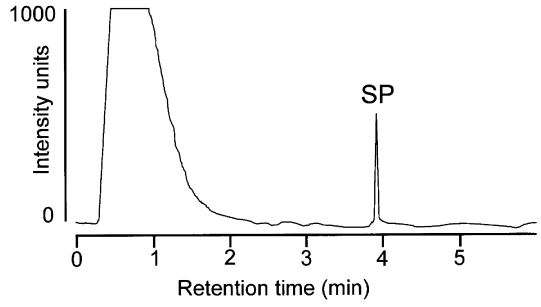

Figure 2. Typical immunoaffinity chromatogram of a sample of human serum spiked with 50 pg/mL of SP. The primary peak is the non-reactive material and the sharp second peak (labeled SP) is the immunoaffimty purified SP. Running conditions described in text.

Table 2. Recovery of SP from 50 nL Spiked[a] Samples of Different Human Biofluids

Biofluid	N	SP Recovered (pg/mL)
Serum	10	49.01 ± 1.1
Plasma	10	49.25 ± 0.8
Urine[b]	10	46.5 ± 2.2
Saliva	10	41.3 ± 3.7
CSF	10	47.1 ± 2.0
Tissue lymph	10	48.5 ± 1.9
Lymphocyte cell cytosol	5	47.9 ± 2.3
Glial cell cytosol	5	48.3 ± 1.8

[a]All biofluids spiked with 50 pg/mL of SP.
[b]pH adjusted to 7.0.

demonstrate the potential of this system for studying both clinical and research samples in a timely manner without excessive sample needs.

DISCUSSION

The potential of miniature chromatography systems in biomedical research is growing, especially with the introduction of nanotechnology and the ability to fabricate microfluidics devices on the micrometer and nanometer scale.[1–3] The merger of biotechnology with microfabrication techniques arising from the electronics sector has made the production of analytical microfluidics systems more common. The introduction of such instruments addresses the need in biotechnology and the biomedical sciences for analytical processes requiring sub-microliter sample volumes. Micro-analytical techniques also allow for analysis of samples previously too small for conventional analytical techniques. These samples range from archival research and clinical samples, to secretions from single cells or remainder materials in tissue and cell banks.

In the field of neonatology, the need for clinically oriented microanalysis is premium, mainly due to the limited sample volumes available. This need has led to the development of a variety of micro-analytical procedures and devices, including the "lab-on-a chip" concept.[14–15] Although the development of these micro-devices is appealing, one of the major drawbacks to this technology is the need for specialized fabrication facilities and specialized equipment required to perform the analyses. Further, miniaturization imposes restriction on the system's detection system, requiring higher sensitivity in order to measure analytes on a micro-scale. In most biological matrices, the analytes of interest are in relatively low

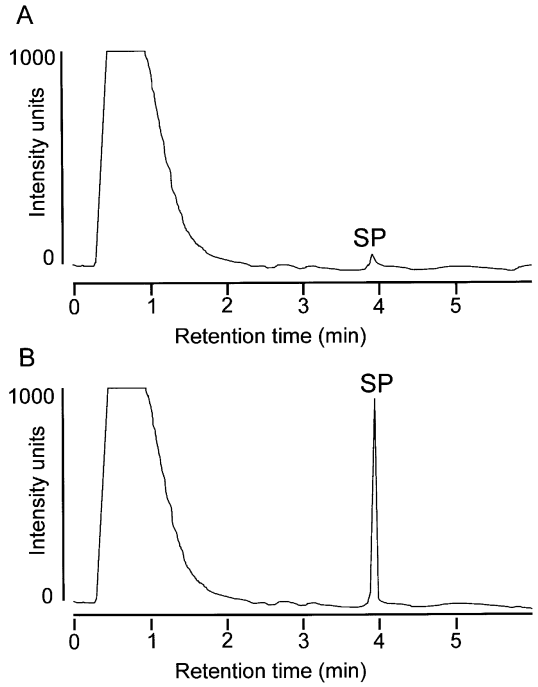

Figure 3. Immunoaffinity chromatogram of naturally occurring SP isolated from tissue lymph. (A) SP isolated from a small skin blister and (B) SP isolated from fluid accumulating at the site of a traumatic injury from an individual with localized neurogenic inflammation. Substance P indicates the immunoaffinity-purified peak. Running conditions described in text.

concentrations and submerged in complex fluids containing a wide variety of unrelated molecules. This poses two problems for the analyst, (a) isolation of the molecules of interest without undue contamination, and (b) detection of the isolated material. The former problem can be addressed in a manner similar to that described in this communication, namely immunoaffinity isolation. This approach has been highly successful in many areas of the biomedical sciences[9,10,16,17] and can be adapted to most analytical situations and scales. Micro-column immunoaffinity analysis has been used to measure either single or multiple analytes in a number of different biological fluids including CSF, saliva, dried blood spots, and urine.[18,19] The advantage of this approach is that immobilized antibodies can be used as selective ligands to capture and isolate specific analytes from relatively small samples of complex biological fluids. Additionally, immunoaffinity separations enable the investigator to measure the isolated analyte using the appropriate

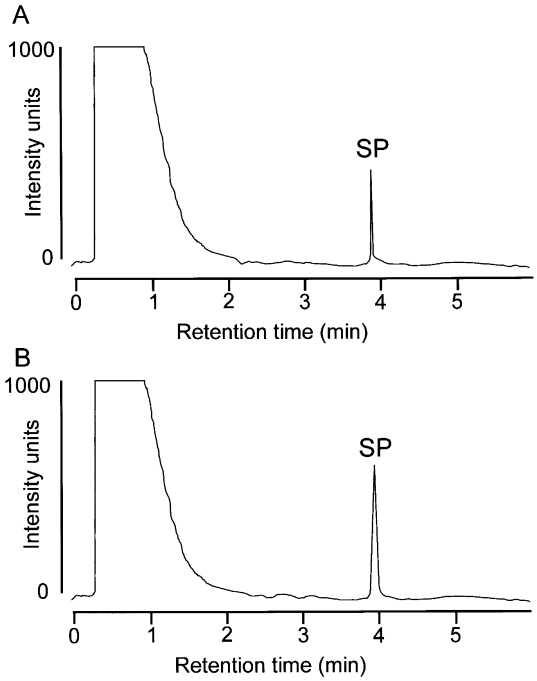

Figure 4. Immunoaffinity chromatogram of SP isolated from lymphocyte cell cytosol. (A) unstimulated or resting state and (B) l hour following antigenic stimulation. Substance P indicates the immunoaffinity-purified peak. Running conditions described in text.

detector. The application of laser-induced fluorescence detection is a logical approach to overcoming difficulties in measuring minute quantities of analyte in micro-samples, especially sub-microliter samples. We have applied LIF in our system with reasonable success—demonstrating that such a detector was able to detect less than 0.5 pg/mL in a 50 nL sample. Additionally, previous studies from our laboratory have demonstrated that immunoaffinity systems, coupled with LIF, can be used to detect secreted products from single lymphocytes maintained in specialized chambers.[20] This degree of sensitivity is well suited to detection in micro-devices and the level of sensitivity reported in the present study is well within the criteria required for clinical studies but may still require refinement for certain research experiments, especially analysis of single cell organelles.

The commercial availability of the components used in this report places micro-chromatographic analysis within the reach of most laboratories. These components allow the investigator to design several different configurations, including a multi-column system, by adding a 7-port manifold between the

injector and the column inlets. Additionally, the LIF detector was built from readily available components, further making this instrument easily obtainable without undue cost.

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