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Short communication

On-line biological sample cleanup for electrospray mass spectrometry using sol–gel columns

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Abstract

Using a slight overpressure, a urine sample is loaded onto a monolithic photopolymerized sol–gel column that has been derivatized with hydrophobic carbon chains and then the complex urine matrix is washed with aqueous solution. A buffer containing organic solvent is used to elute the adsorbed peptides by an applied voltage and the sample is then introduced into a mass spectrometer by sheath flow electrospray. The importance of desalting this type of sample is demonstrated by an experiment that shows that the signal intensity of a test solution with neurotensin, sprayed directly into the mass spectrometer, decreased from 4.5×10^4 cps to no detectible signal when just 10% urine is added to the sample solution. We suggest that this procedure may find general application for desalting biological samples prior to mass spectrometric analysis.
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1. Introduction

Body fluids are well known to carry information about the condition of an organism. Direct infusion of crude biological samples is generally not fully compatible with separation techniques such as liquid chromatography (LC) because the viscosity of the sample is usually too high and column clogging becomes a significant risk. In capillary electrophoresis on the other hand, the columns are open and direct infusion of viscous samples can be accomplished. If the body fluid investigated is blood (serum/plasma), saliva or pleura fluid, there are often high concentrations of albumin, immunoglobulins, transferrin or hemoglobin that can mask other analytes in the analysis. In urine high concentrations of salts and urea may interfere with the analysis. These highly abundant substances often complicate the detection of the target analytes. In electrospray ionization mass spectrometry (ESI–MS) even low concentrations of electrolytes causes ion suppression that result in a lower sensitivity for the analytes of interest [1]. To be able to detect very low concentrations of species in the sample, salts and electrolytes need to be removed before the competitive ionization process. This removal can be done by a sample-preparation step prior to the separation technique. The sample cleanup is generally the most important step in the analytical chain. As important as isolating the analyte is the task of eliminating interfering substances.

In recent years several sample cleanup techniques have been developed for use in bioanalysis. Extraction of peptides and proteins can be performed by centrifugation, precipitation, solvent extraction, liquid–liquid extraction (LLE), solid-phase extraction (SPE), microdialysis and ultrafiltration [2]. These techniques are usually used off-line, and they involve multi-step methods that often give rise to losses of various sample components. The most common technique in sample cleanup is solid-phase extraction, which consists of a stationary phase that traps the desired species while the complex matrix is washed away [3]. The most effective on-line solid phase columns commonly require reversed flow desorption using a valving system and solvent gradients [4–7].

A novel approach to the challenge of analyzing complex biological samples is on-line sample cleanup combined with capillary electrophoresis (CE). The latter is an excellent technique that offers constant flow without any solvent gradient, fair

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robustness with low cost capillaries, compatibility with most buffers and analytes, convenient coupling to MS detection and finally a fast and high resolution separation. A drawback of CE is mainly the limited sample volume that can be loaded onto the column. To overcome this disadvantage, on-line preconcentration can be performed by an adsorptive solid-phase bed inside the separation column. Particle-packed segments have been used, but the fabrication is difficult and the frits holding the particles in place often give rise to bubble formation that is detrimental to the separation [8]. An alternative preconcentration method is the use of monolithic columns, i.e., a continuous bed that is polymerized from a solution inside the capillary. Two types of fabrication are commonly used, thermal or photoinitiated. Both methods are effective for preconcentration [5,9–11]. The photoinitiated sol–gel (PSG) columns created by Dulay et al. [12] are very useful because they give rise to a defined structure and are easy to fabricate. The preconcentration factor is as high as a thousand fold as shown by Quirino et al. [13,14]. The silica-based sol–gel is easily modified with silanes that contain alkoxy or chloride groups, which make it possible to change the physical properties of the monolith easily and quickly [15].

The aim of this paper is to show a fast and robust way to desalt a biological sample and to be able to detect low concentrations of analytes. By using a C18-derivatized sol–gel bed in a fused-silica column, hydrophobic peptides can be adsorbed while the hydrophilic urine components are washed away without backflushing [16]. The selected sample matrix of urine spiked with peptides in this work is merely for demonstration. Nevertheless, peptide analysis may well be of interest in clinical investigations of renal problems and dysfunctions in glomeruli [17–20].

2. Experimental

2.1. Chemicals and reagents

The reagent chemical 3-(trimethoxysilyl)propyl methacrylate, 99% was purchased from Sigma (St. Louis, MO, USA), Irgacure 1800 from Ciba (Tarrytown, NY, USA) and ethanol from Solveco, Chemicals AB (Täby, Sweden). Solvents and acids were purchased from MERCK (Darmstadt, Germany) and the water was filtered using a Milli-Q^+ system (Millipore Corp., Marlborough, MA, USA). The octadecyltrimethoxysilane, 90% for modification and peptides (Angiotensin II, Leucine-Enkephaline (Leu-Enk), Leutenizing Hormone-Releasing Hormone (LHRH), Neurotensin, Oxytocin) for sample solutions came from Sigma–Aldrich (Milwaukee, WI, USA). Fused-silica capillaries (75-μm ID × 360-μm OD) were purchased from Polymicro Technologies (Phoenix, AZ, USA).

2.2. Instrumental setup

A CE instrument from Hewlett-Packard, Germany, ^{3D}CE, was coupled to a mass spectrometer, MS. The MS used in these experiments was a LC/MSD TOF from Agilent Technologies, USA. The drying gas was maintained at 130 °C at a rate of 5 L min^{-1}. A standard sheath-flow electrospray ionization (ESI) source was used for all experiments. The sheath liquid consisted of 50/50 (v/v) methanol/water with 5 mM acetic acid and was introduced at a flow rate of approximately 1 μL min^{-1}. A voltage of +4.3 kV was applied to induce electrospray. A schematic picture of the setup is shown in Fig. 1.

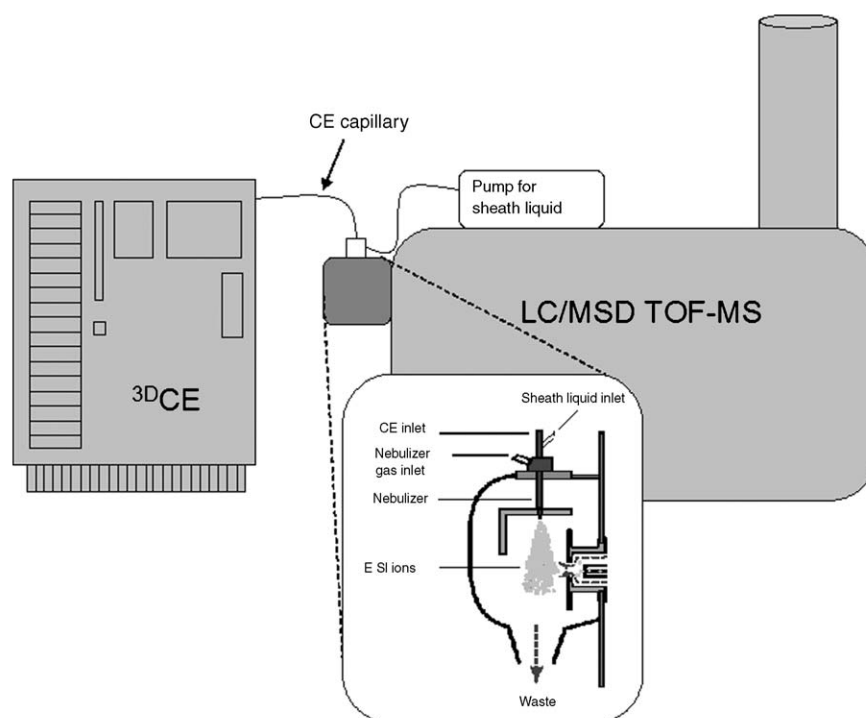

Fig. 1. Experimental setup.

2.3. Column preparation

To prepare the sol–gel solution, 575 μL of 3-(trimethoxy-silyl)propyl methacrylate and 100 μL of 0.12 M hydrochloric acid were mixed and stirred for 30 min at room temperature in the dark. Of that mixture, 120 μL was added to 480 μL of toluene (porogenic agent) and 60 mg of Irgacure 1800 and stirred with a magnetic bar for 5 min at room temperature in the dark. A 5-cm long exposure window for UV polymerization was created on a fused-silica capillary (75-μm ID; 360-μm OD) by removing a narrow strip of the polyimide coating. The removal procedure is accomplished with a razor blade as described by Dulay et al. [12]. The capillary was then filled with the sol–gel solution and plugged with parafilm at both ends before being irradiated at 365 nm for 4.5 min in a crosslinking apparatus (BIO-LINK® BLX, Marne-la-Vallée, France). To remove unpolymerized solution, the column was flushed with ethanol using a syringe pump. The total length of the column was 55 cm and the 5-cm sol–gel section was located approximately 13 cm from the injection end. The polymerized sol–gel network inside a fused-silica column is shown in Fig. 2. The photo is created by a Leo 1550 scanning electron microscope (SEM) (Thornwood, NY, USA) with a field emission gun yielding high resolving power. To avoid charging and heating of the monolith, the capillary was covered with a sputtered AuPd layer and a low acceleration voltage was used (3 kV). Before sample cleanup the column was equilibrated with 5 mM ammonium acetate, containing 60% acetonitrile, for approximately 30 min without voltage and 10 min with voltage 10 kV. The fabrication of PSG is well established and has shown to be very reproducible.

2.3.1. C18 modification

Derivatization with the C18 silane reagent was performed by flushing the column with neat octadecyltrimethoxysilane, $CH_3(CH_2)_{17}Si(OCH_3)_3$, solution for 60 min (approximately 2 μL min^{-1}) at 20 °C and then rinsing with ethanol for 30 min.

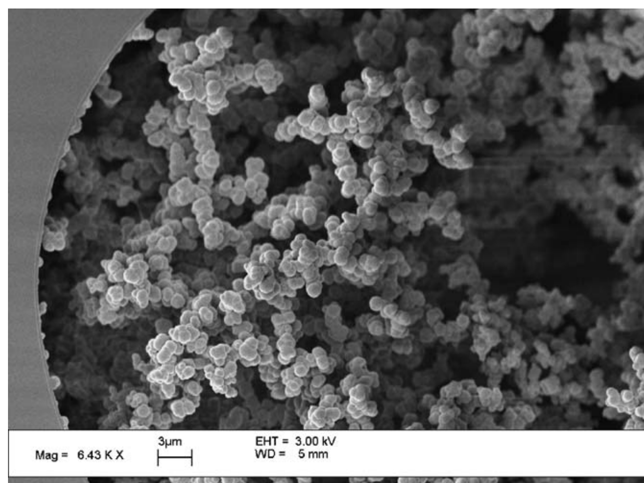

Fig. 2. SEM of the cross-section of a capillary containing photopolymerized sol–gel.

2.4. Sample and separation solutions

The samples were prepared by mixing different amounts of urine, water and peptide stock solution (15 μg peptide/mL water). The running buffer consisted of 5 mM ammonium acetate (pH 6.8) in 40% water and 60% acetonitrile. To rinse the column after urine injection, a washing buffer of 5 mM ammonium acetate, pH 6.8, in water was used. The urine was filtered using a Schleicher & Schuell (Dassel, Germany), FP 030/8, 0.2-μm pore-size filter, and stored at −20 °C. A freshly thawed sample was used each day so that each sample experienced only one freezing and thawing cycle before the time of analysis.

3. Results and discussion

3.1. Urine ion suppression

To evaluate the effect of urine on the MS signal, samples with different amounts of urine were electrosprayed by direct infusion using an untreated fused-silica capillary. The samples consisted of 2 μg mL^{-1} of neurotensin in 5 mM acetic acid. The signal intensity of neurotensin decreased from 4.5×10^4 cps to no signal (150 cps) when just 10% urine was present in the sample. As expected, the salts and electrolytes in the urine resulted in detrimental ion suppression and no signal was obtained for the analyte in a crude urine sample.

3.2. Sample cleanup

A separation column with a 5-cm sol–gel bed was conditioned with the washing buffer (which contained no organic solvent) for 5 min using slight over-pressure of 1 bar. Samples made up of urine from a healthy adult female and spiked with the peptides neurotensin, oxytocin, angiotensin II, Leu-Enk and LHRH (resulting in a sample containing 0.15 μg peptide mL^{-1} and 96.6% urine) were injected onto the column at 1 bar of pressure for 5 min, which is equivalent to 1.1 column volumes or 1.4 μL. To remove the salts and electrolytes present in the urine, the washing buffer was flowed through the column using a slight overpressure of 1 bar until the background urea peak had eluted from the column (approximately 15 min or 3.4 column volumes). The inlet of the column was then placed into a vial containing the running buffer (pH 6.8, 60% acetonitrile) and a separation voltage of +20 kV was applied. In Fig. 3 the desalting/preconcentration procedure is shown in greater detail. Neurotensin, LHRH and oxytocin elute in sharp peaks, approximately 10 min after the HV had been applied. Angiotensin II elutes somewhat later as a broader peak (see Fig. 4) and Leu-Enk elutes as a small broad peak at about 20 min (not shown). This last result is probably due to a combined effect of size and hydrophobicity. Leu-Enk is half the size of the other peptides used and can therefore penetrate more deeply into the porous monolith. Moreover, it is expected to be adsorbed more strongly because of its relatively high hydrophobicity. Under these conditions a preconcentration of about 70 times is achieved for all peptides in the PSG. The

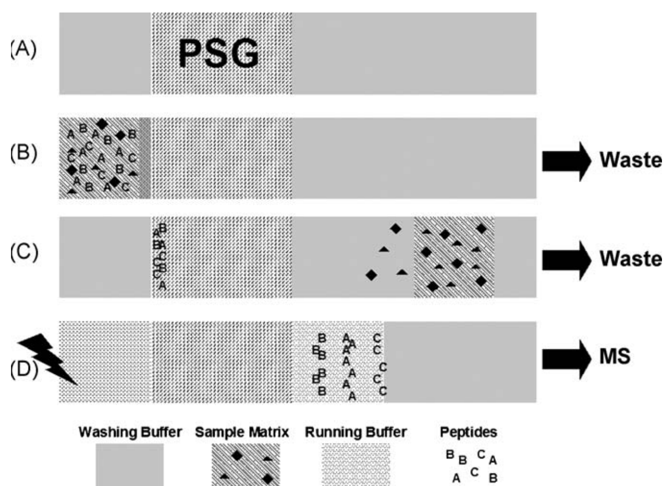

Fig. 3. Cleanup procedure: (A) the column is equilibrated with 5 mM ammonium acetate, pH 6.8; (B) the spiked urine sample is loaded onto the column by slight overpressure, 1 bar, for 5 min, and the peptides adsorb in the front part of the PSG; (C) the column is flushed with 5 mM ammonium acetate to wash away the urine matrix while the peptides remain adsorbed in the PSG; (D) the injection end is placed in 5 mM ammonium acetate, 60% acetonitrile and 20 kV is applied. The peptides desorb from the PSG when the organic solution reaches the monolith and they elute in highly concentrated plugs.

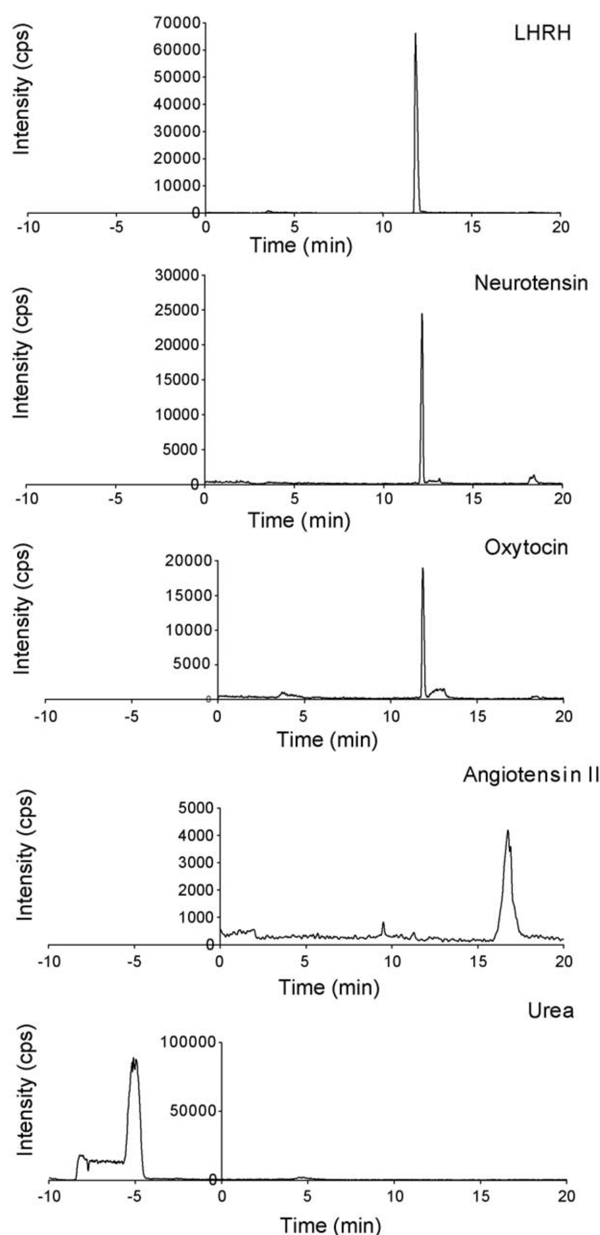

Fig. 4. Single-ion electropherograms for neurotensin (558.3 *m*/*z*), oxcytocin (504.2 *m*/*z*), LHRH (591.8 *m*/*z*), angiotensin II (523.8 *m*/*z*), and urea (61 *m*/*z*) during the preconcentration and desalting procedure.

life-time of an unused column has been interminable but when using crude urine samples the life-time is limited to about 30 runs.

The peptide affinity to the C18 modified sol–gel is strong when no organic solvent is included in the running buffer. The hydrophobic peptides adsorb in a narrow band immediately upon entering the monolith while the hydrophilic species such as urea and salt, pass through the column. The C18-modified sol–gel thus allows for selective preconcentration. As soon as the electrolyte solution, containing 60% acetonitrile, reaches the monolith, the peptides partition rapidly into the solution and elute as sharp peaks. There is thus no need to redirect the flow for desorption of analytes, in contrast to when packed particle beds are used. To avoid spraying the urine matrix into the mass spectrometer a metal shutter in front of the orifice could be used [21]. The spray current is then generated between the spray needle and the metal plate. After the urea and salt are eluted, this plate is removed and the elution buffer is sprayed into the MS.

3.3. Loading capacity and repeatability

The loading capacity of the PSG monolith was tested by loading different volumes of a urine sample spiked with a constant peptide concentration of 0.15 μg mL^{-1} onto the monolith and washing for 5 min before eluting the analytes electrokinetically. The peak area for each of the peptides increases up to approximately 2 μL injected sample volume, representing 0.3 ng of each peptide. Above 2 μL of injected volume, the peak areas only slightly increase as shown in Fig. 5. In addition to the target peptides, there are most likely several hydrophobic species (e.g., amines and steroids) in the urine sample that has affinity to the PSG. In a system free of interfering species, the more hydrophobic peptides would exhibit a larger loading capacity. These experiments show that all of the tested peptides reach their upper limit of detection at approximately the same injected volume, which suggests that other molecules present in the urine sample are occupying many of the available binding sites and saturating the monolith. Quirino et al. showed nice preconcentration with up to ~0.4 μg peptide loaded onto the column [13]. The repeatability of the migration times on a column is better than 1% and the peak area has a RSD of about 4% within day. Between days the RSD of migration time and peak area are approximately 1% and 5%, respectively.

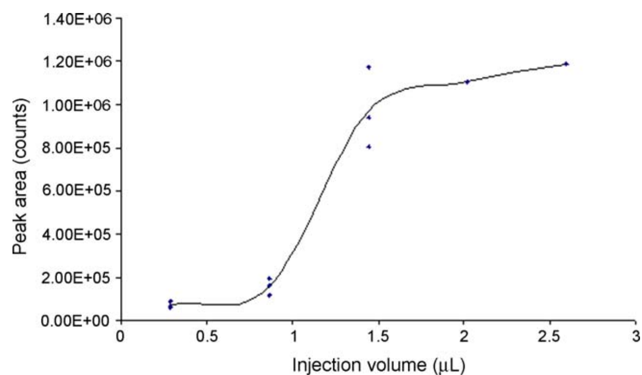

Fig. 5. Loading capacity, injection volume vs. peak area, for LHRH on the PSG column. The sample solution consists of 0.15 μg mL^{-1} LHRH in urine. A plateau of maximum peak areas is reached at approximately 2 μL (1.6 column volumes) injected sample under these conditions. The injected volume represents 0.3 ng of LHRH.

4. Conclusions and future prospects

Biological samples often contain species of high abundance that complicate the analysis of less abundant analytes. We have shown that when a urine sample spiked with a known peptide is introduced, by ESI, directly into a MS no signal can be detected for the added peptide. These experiments show a general method for the on-line desalting of biological samples and they also demonstrate the ability to selectively preconcentrate species of low abundance. The fact that the procedure is on-line greatly minimizes the loss of analytes during the cleanup process. Furthermore, the simplicity of this valve-free, unidirectional cleanup process provides both time saving and robustness to the method.

The production of the columns is fast and reproducible; also the surface has excellent derivatization possibilities to obtain different extraction abilities. Future applications can include ion exchange sites or an affinity-based trace-enrichment surface by incorporation of antibodies into the sol–gel structure.

Acknowledgments

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