Mass Spectrometry Hot Paper

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Direct Mass Spectrometry Analysis of Biofluid Samples Using Slug-Flow Microextraction Nano-Electrospray Ionization**

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Abstract: *Direct mass spectrometry (MS) analysis of biofluids with simple procedures represents a key step in the translation of MS techniques to clinical and point-of-care applications. The current study reports the development of a single-step method using slug-flow microextraction and nano-electrospray ionization for MS analysis of organic compounds in blood and urine. High sensitivity and quantitation precision have been achieved in the analysis of therapeutic and illicit drugs in 5 μL samples. Real-time chemical derivatization has been incorporated for analyzing anabolic steroids. The monitoring of enzymatic functions has also been demonstrated with cholinesterase in wet blood. The reported study encourages the future development of disposable cartridges, which function with simple operation to replace the traditional complex laboratory procedures for MS analysis of biological samples.*

Mass spectrometry (MS) has been demonstrated to be a powerful tool for chemical and biological analysis. The high specificity, high sensitivity, and high precision in quantitation are achieved traditionally in the laboratory by eliminating the matrix effect through sample extraction and chromatographic separation prior to the MS analysis. The development of ambient ionization,[1] especially with the recent demonstration of using a paper spray,[2] has indicated a promising future for direct MS analysis with high quantitation performance but with highly simplified procedures that consume ultrasmall amounts of samples. This would be extremely important for the translation of the MS analysis to field applications, especially point-of-care (POC) diagnosis. The underlying principle for a successful development along this direction is to minimize the sample consumption and to achieve high efficiency in an integrated process for analyte extraction and ionization. In this study, we developed a new method that uses slug-flow microextraction (SFME) and nanoESI (electrospray ionization) to perform a one-step analysis of biofluid samples. Excellent sensitivity and high quantitation precision have been obtained with blood and urine samples of only 5 μL. More importantly, we demonstrated how to incorporate a variety of different processes using a simple device, including liquid–liquid extraction, internal standard (IS) incorporation, chemical derivatization or even enzymatic reactions, which are necessary for a high performance mass analysis.

A disposable glass capillary of 0.8 mm i.d. (Figure 1 a) with a pulled tip for nanoESI was used to perform the entire sampling ionization process. Two adjacent liquid plugs were formed by sequentially injecting 5 μL organic solvent and 5 μL urine or blood sample into the capillary. Liquid–liquid extraction of the analytes from the biofluid into the organic solvent is expected, but at a fairly low efficiency because of the small interfacing area. However, the extraction speed could be significantly increased with the slug flows induced by the movements of the two liquid plugs, which can be facilitated by tilting the capillary (Figure 1 a) or by applying a push-and-pull force through air pressure (see Figure S1 in the Supporting Information). Due to friction with the

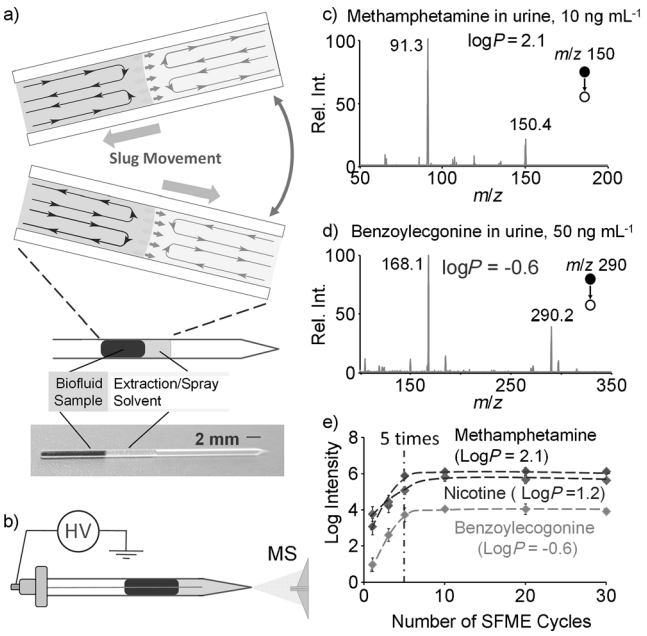

Figure 1. a) In-capillary sample extraction using slug-flow microextraction and b) the subsequent MS analysis with nanoESI. MS/MS spectra for c) 10 ng mL^{-1} methamphetamine in 5 μL urine and d) 50 ng mL^{-1} benzoylecgonine in 5 μL urine. e) Impact of the number of SFME cycles on the extraction of the analytes, intensities of the MS/MS product ions monitored for methamphetamine (*m*/*z* 150→91), nicotine (*m*/*z* 163→130), and benzoylecgonine (*m*/*z* 290→168), each at 50 ng mL^{-1} in urine samples. 2 kV used for nanoESI.

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capillary wall,[3] internal circulations are formed inside each plug (Figure 1 a) and transfer the analytes to and away from the liquid–liquid interface, therefore significantly improving the extraction efficiency. After the extraction process, the organic solvent plug can be simply pushed to the tip of the capillary, a stainless-steel wire then inserted through the biofluid sample to reach the organic solvent plug, and a high voltage applied to generate the nanoESI for MS analysis (Figure 1 b). The selection of the organic solvent is critical. It needs to be immiscible with the biofluid samples, have good solubility for the target analytes and be suitable for nanoESI. Several organic solvents were tested (see the Supporting Information) and ethyl acetate, which has a weak polarity, was found to provide the optimal performance for analyzing a broad range of chemical compounds in urine (Figure 1 c,d) and blood samples (see Figure S3 in the Supporting Information).

The extraction process with the slug flows has been shown to be very efficient, as demonstrated for extracting methamphetamine, nicotine, and benzoylecgonine (a main metabolite of cocaine) from urine samples. The equilibrium was reached after tilting the capillary five times (Figure 1 e). Limits of detection (LODs) as low as 0.05 ng mL^{-1} for verapamil have been obtained for whole blood samples using SFME-nanoESI (Table 1). Fewer extraction cycles were needed to reach equilibrium if the blood samples were diluted to reduce the viscosity. The distribution of the analyte between the sample and extraction phase can be relatively estimated by the partitioning coefficient (log*P*, see Equation S1 and derivation in the Supporting Information). The concentration of methamphetamine (log*P* value of 2.1) in the organic extraction solvent can be 100 times higher than in the urine sample after SFME, which certainly explains the good LOD of 0.03 ng mL^{-1} achieved with urine samples (Table 1). The log*P* value for benzoylecgonine is −0.6, which means it has higher solubility in urine than in organic solvents and the extraction into ethyl acetate was a dilution process; however, an LOD of 0.08 ng mL^{-1} was nevertheless achieved. This indicates that the limiting factor in the detection of the benzoylecgonine in raw urine samples might not be the absolute amount or concentration of the benzoylecgonine, but the interference by the matrix effects, such as the ionization suppression because of the high concentrations of salts in the urine sample.[4] An efficient separation of benzoylecgonine from the salts was achieved in the SFME process. Even with a lower benzoylecgonine concentration in the extraction phase, the ionization efficiency and the overall sensitivity of the analysis were improved significantly.

In addition to the sensitivity, adequate precision in quantitation is often mandatory for clinical and POC applications.[5] Simple means for accurate incorporation of internal standards are important[6] but can be challenging for samples of small volumes taken by minimally invasive methods. By using the SFME-nanoESI technique, the internal standard compounds could be spiked in the extraction phase (Figure 2, inset) and subsequently mixed with the analyte during the slug-flow extraction process. This method was tested for the quantitation of methamphetamine in bovine blood samples with [D_8]methamphetamine spiked in ethyl acetate at 2 ng mL^{-1} as the internal standard. The blood samples were diluted 10 times and then analyzed using the SFME-nanoESI and multiple reaction monitoring (MRM) analysis (transitions *m*/*z* 150 to 91 and *m*/*z* 158 to 94 for the analyte and internal standard, respectively; Figure 2 inset). Figure 2 shows a plot of the measured analyte-to-internal standard ratios (A/IS) as a function of the original analyte concentration in blood. A good linearity was obtained, which is governed by the partitioning process (see derivation in the Supporting Information). Relative standard deviations (RSDs) better than 10% were obtained for samples with concentrations higher than 10 ng mL^{-1}.

Chemical derivatization is an effective way of altering the properties of the target analytes to improve the efficiency of the separation or ionization for MS analysis.[7] For example, the steroids in urine or blood samples are expected to be extracted well into an organic phase using the SFME; however, the efficiency of the subsequent ionization by nanoESI would be low due to the low proton affinity of the steroid molecules. The reaction with hydroxylamine has

Table 1: Limits of detection (LODs) of analytes in urine and/or whole blood samples using SFME-nanoESI for MS analysis.

Analyte	Sample	Derivatization[a]	Sample volume [μL]	LOD [ng mL^{-1}]
methamphetamine	urine	NA	5	0.03
	blood	NA	5	0.1
benzoylecgonine	urine	NA	5	0.1
LOD	blood	NA	5	1
verapamil	blood	NA	5	0.05
amitriptyline	blood	NA	5	0.08
epitestosterone	urine	hydroxylamine	5	0.7
6-dehydrocholestenone	urine	hydroxylamine	5	0.6
5α-androstan-3β,17β-diol-16-one	urine	hydroxylamine	5	0.2
stigmastadienone	urine	hydroxylamine	5	0.8

[a] NA = not applied.

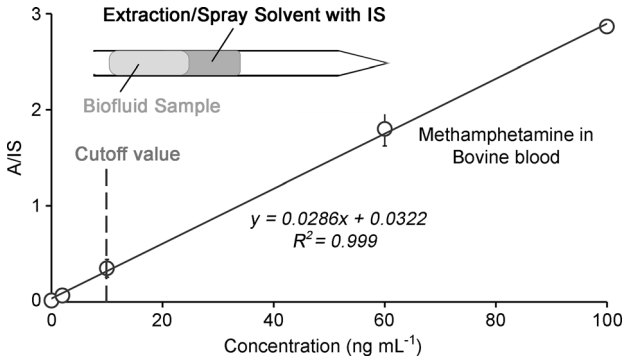

Figure 2. Quantitative analysis of whole blood spiked with methamphetamine (1–100 ng mL^{-1}). The blood samples were diluted 10 times to decrease the viscosity. [D_8]Methamphetamine (2 ng mL^{-1}) in ethyl acetate was used as the extraction solvent.

previously been proved to be effective in improving the ionization efficiency of the steroids,[8] and thereby was used in this study as an example. An additional liquid plug of 5 μL water containing 50 mM hydroxylamine was injected between the 5 μL ethyl acetate and 5 μL urine sample spiked with 200 ng mL^{-1} epitestosterone (Figure 3). With five SFME

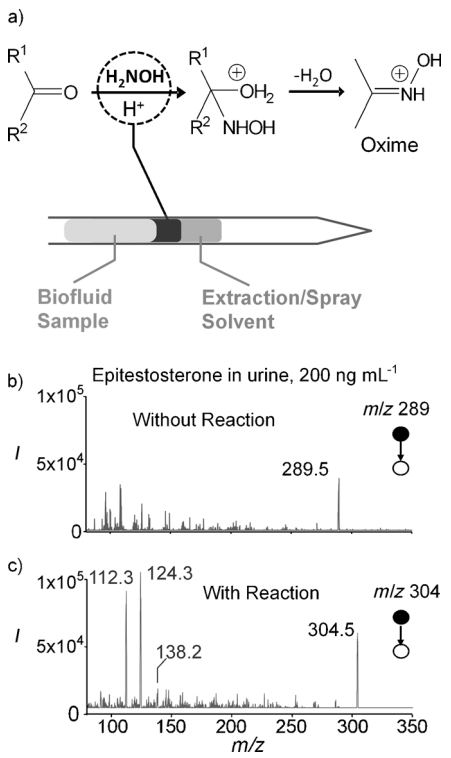

Figure 3. a) Reactive SFME-nanoESI with a reagent plug injected between the biofluid sample and the extraction solvent. MS/MS spectra from b) direct and c) reactive SFME-nanoESI analysis of 200 ng mL^{-1} epitestosterone in synthetic urine. 5 μL water containing 50 mM hydroxylamine was used as the liquid reagent plug.

cycles, the hydroxylamine solution mixed well with the urine sample. The MS/MS analysis of the reaction product *m/z* 304 produced spectra of significantly improved signal-to-noise ratios (S/N, Figure 3b,c). The reactive SFME-nanoESI technique was applied for analysis of a series of anabolic steroids in 5 μL urine samples, including epitestosterone, 6-dehydrocholestenone, 5α-androstan-3β,17β-diol-16-one, and stigmastadienone, with LODs of 0.7, 0.6, 0.2, and 0.8 ng mL^{-1} obtained, respectively (Table 1).

The use of the liquid–liquid extraction process with SFME allows the analysis to now be performed directly with wet blood samples. This provides an opportunity for probing the chemical and biological properties that only exist with the original liquid samples. For example, the enzymatic functions of the proteins are typically quenched in the dried blood spots or after the traditional laboratory procedure for sample extraction. In this study, we applied the SFME-nanoESI technique for monitoring the enzymatic activity of cholinesterase (ChE) in whole blood samples. The ChE facilitates the enzymatic conversion of acetylthiocholine (ATCh) into

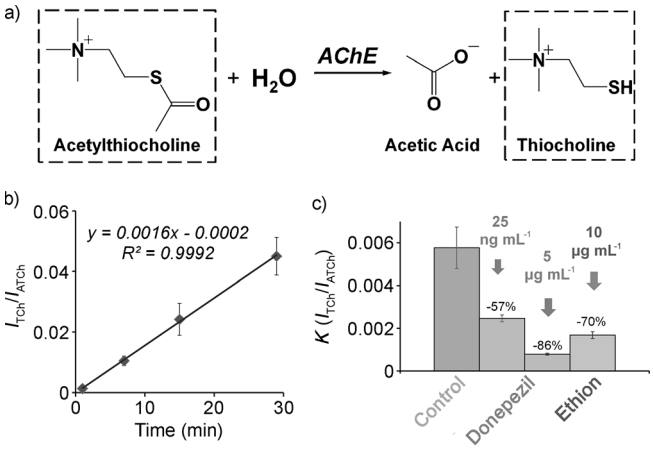

Figure 4. a) The reaction scheme of the enzymatic conversion of acetylthiocholine (ATCh) into thiocholine (TCh) catalyzed by cholinesterase (ChE). b) Progression curve of ATCh digestion determined by SFME-nanoESI. The incubation was for 30 min and catalyzed by blood cholinesterase (ChE) at room temperature. Acetylthiocholine iodide was added as the enzyme substrate into human whole blood at a final concentration of 1.8 mg mL^{-1} before incubation. The intensity ratios of product ions from thiocholine (*m/z* 102→61) and enzyme substrate (*m/z* 162→103) were monitored using MRM; c) Evaluation of blood ChE with different levels of enzyme inhibition. The ChE activity in the blood sample was determined by SFME-nanoESI after 5 min incubation.

thiocholine (TCh, Figure 4a). The blood sample was diluted 10 times to slow down the reaction rate as well as to facilitate the slug flows for SFME. The substrate acetylthiocholine iodine was added into the diluted blood sample at a concentration of 1.8 mg mL^{-1}, and then a 5 μL sample was taken immediately and injected into the capillary with a 5 μL extraction phase. The capillary with the sample and the extraction solvent was left at room temperature (25 °C) for incubation. The SFME-nanoESI could be performed repeatedly on the same sample and the ratio of the substrate ATCh and the reaction product TCh (see Figure S7 in the Supporting Information) could be monitored as a function of time to characterize the enzymatic activity of the ChE. A potential problem in this approach would be the organic solvent damaging the enzyme function. The impact of the organic extraction phase was investigated for ethyl acetate and other solvents such as chloroform with 5 min incubation. It was found that the reduction of ChE activity through contact with ethyl acetate was minimal, but much more severe (more than 60 % decrease) with chloroform (see the Supporting Information). A weakly polar solvent such as ethyl acetate can better preserve the enzyme structures.[9]

The SFME-nanoESI technique was performed repeatedly over 30 min using ethyl acetate as the extraction solvent, with 5 cycles for SFME and 5 s nanoESI at 1500 V for each analysis. The TCh/ACTh ratio is plotted as a function of time in Figure 4b, which is characteristic of the enzymatic activity of the ChE. An enzyme inihibition study was then carried out as a validation of this method. Two ChE inhibitors, donepezil (a therapeutic drug for Alzhimer's disease) and ethion (a neuron toxicant), were spiked seperately into blood samples,

thereby simulating enzyme inhibition to different degrees. The compromised enzyme activities were then determined uisng the SFME-nanoESI method with 5 min incubation. In comparion with the blood samples without adding the inhibitors, the defficiencies measured are resported in Figure 4c for blood samples treated with donpezil at 25 ng mL^{-1} and 5 μg mL^{-1}, and with ethion at 10 μg mL^{-1}. The percent decreases observed are consistent with the findings reported for previouse studies.[10]

In summary, the combination of slug-flow microextraction with nanoESI enabled a highly sensitive direct analysis of organic compounds in biofluids. Multiple types of processes for sample treatments, which traditionally require complex setups in the laboratory, can now be incorporated into a one-step analysis with an extremely simplified operation procedure. Since the biofluid samples are directly analyzed without being made into dried spots, an efficient liquid–liquid extraction can be designed based on the partitioning process. The chemical and biological properties of wet biofluids can also be retained and characterized thereby. The extraction process can be turned on and off by controlling the movements of the sample and extraction plugs. This allows an online monitoring of the chemical and biological reactions in a biofluid sample of only 5 μL. With the increasing interest in the translation of MS technologies to clinical applications, this development has a profound implication on designing disposable sample cartridges with adequate function for direct analysis. This could ultimately lead to an elimination of the traditional laboratory procedures that require complex setups and expertize. Its implementation with miniature mass spectrometers[11] would produce a powerful solution for POC diagnosis.

Experimental Section

All the experiments were carried out with a TSQ Quantum Access Max mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The bovine blood was purchased from Innovative Research Inv. (Novi, MI, USA). The human pooled blood for the enzymatic reaction study was purchased from BioreclamationIVT (Baltimore, MD, USA). The synthetic urine was purchased from CST Technologies (Great Neck, NY, USA). The steroids were purchased from Steraloids Inc. (Newport, RI, USA). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

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