Design and implementation of an automated liquid-phase microextraction-chip system coupled on-line with high performance liquid chromatography

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Abstract

An automated liquid-phase microextraction (LPME) device in a chip format has been developed and coupled directly to high performance liquid chromatography (HPLC). A 10-port 2-position switching valve was used to hyphenate the LPME-chip with the HPLC autosampler, and to collect the extracted analytes, which then were delivered to the HPLC column. The LPME-chip-HPLC system was completely automated and controlled by the software of the HPLC instrument. The performance of this system was demonstrated with five alkaloids i.e. morphine, codeine, thebaine, papaverine, and noscapine as model analytes. The composition of the supported liquid membrane (SLM) and carrier was optimized in order to achieve reasonable extraction performance of all the five alkaloids. With 1-octanol as SLM solvent and with 25 mM sodium octanoate as anionic carrier, extraction recoveries for the different opium alkaloids ranged between 17 and 45 %. The extraction provided high selectivity, and no interfering peaks in the chromatograms were observed when applied to human urine samples spiked with alkaloids. The detection limits using UV-detection were in the range of 1-21 ng/mL for the five opium alkaloids presented in water samples. The repeatability was within 5.0-10.8 % (RSD). The membrane liquid in the LPME-chip was regenerated automatically between every third injection. With this procedure the liquid membrane in the LPME-chip was stable in 3-7 days depending on the complexity of sample solutions with continuous operation. With this LPME-chip-HPLC system, series of samples were automatically injected, extracted, separated, and detected without any operator interaction.

1. Introduction

The high complexity of biological samples and low concentrations of target analytes are two of the main challenges for analytical detection and quantitation. Therefore, clean-up and enrichment procedures in order to resolve those analytical limitations are important, preferably in an automated way that is able to handle low sample volumes. For many years, liquid-liquid extraction (LLE), solid phase extraction (SPE), and solid-phase microextraction (SPME) have been the standard methods for sample preparation [1, 2]. In recent years, substantial interest has also been devoted to extractions across supported liquid membranes (SLM) where an organic liquid is immobilized in the pores of a porous hydrophobic membrane. Analytes of interest can be selectively extracted across the SLM driven by either a pH gradient as used in the format of liquid-phase microextraction (LPME) [3-6] or a voltage gradient termed electromembrane extraction (EME) [7]. With LPME or EME, membrane microextraction has demonstrated a significant potential in pharmaceutical analysis [8], environmental [9-11] and food analysis [12].

Due to the high versatility of SLM based extraction techniques, they are readily incorporated into different platforms and coupled directly with high performance analytical instruments such as liquid chromatography (LC) [13], gas chromatography (GC) [14], capillary electrophoresis (CE) [15], or flame atomic absorption spectrometry (AAS) [16]. Chip-based SLM- systems have been explored and coupled on-line with LC since the 1980s [17] due to their significant advantages in terms of miniaturization and automation [18]. Previous SLM-chip modules were made by packing a flat sheet membrane in between two grooved polymer holders, which were then clamped with bolts. The volume of the channels was generally in the range of 10-20 μL [19, 20]. The automated SLM-chip systems have been explored and applied for a wide range of biosamples, such as anaesthetics (SLM-GC) [21], bambuterol in human plasma (SLM-CE) [22], and peptides in spiked plasma (SLM-HPLC) [19].

Recently, SLM extraction has been successfully downscaled to a microfluidic chip for sample enrichment and clean-up [23-26]. The advantages of such microchip membrane extraction include minimal organic solvent consumption, the ability to handle a wide range of sample volumes, ease of use, potentially high enrichment factors from small sample volumes, and the ability to provide selective extraction of analytes depending on their polarity and charge. The chemical binding of flat sheet membranes into polymethyl methacrylate (PMMA) blocks was

developed in our group and high performance of this SLM-chip unit has been demonstrated by both EME [24, 25, 27] and LPME [23] work reported previously. In this microchip membrane extraction module, the sample solution was pumped into a 50 μm deep micro channel where the analytes were extracted through the SLM and into an acceptor channel located on the other side of the SLM. The driving force for the microchip membrane extraction was either a DC electrical potential [25], or a pH gradient [23]. With microchip membrane extraction, dynamic extraction was performed in which the samples were delivered continuously to the chip by a microsyringe pump. The enrichment factor (EF) was controlled by the ratio of the sample volume delivered to the device and the volume of the acceptor solution that could either be stationary (stopped flow) or delivered continuously [23, 24]. In addition, in the microchip EME system, the EF was also controlled by the applied extraction voltage [24]. Both the microchip EME and LPME systems have been used for online and real-time measurement of *in vitro* metabolism of drug substances by rat liver microsomes [23, 27].

The objective of this study was to integrate a microchip LPME system directly to a commercial high performance liquid chromatography (HPLC) system, and to fully automate the system. This report describes the design, construction, operation, and optimization of such a LPME-chip-HPLC system. The system was developed to automatically perform sample injection, LPME, SLM liquid regeneration, and fast HPLC separation. Different alkaloids were used as model analytes. The intention was not to develop an analytical method for the alkaloids, but rather to investigate fundamental aspects of the LPME-chip-HPLC system.

2. Experimental

2.1 Chemicals and sample solutions

Morphine (pK_a (base) = 8.2, pK_a (acid) = 9.7; log P = 0.89) was obtained from Nycomed DAK (Copenhagen, Denmark), codeine (pK_a = 8.2; log P = 1.19) and noscapine (pK_a = 6.3; log P = 1.5) were obtained from Nordisk Droge and Kemikalie (Copenhagen, Denmark), thebaine (pK_a = 8.4; log P = 2.0) was obtained from Nomeco (Copenhagen, Denmark), and papaverine (pK_a = 6.3; log P = 3.0) was obtained from Mecobenzon (Copenhagen, Denmark). All these substances were hydrochlorides and with purities >99 %. LC-MS grade formic acid, acetonitrile, tricaprylmethyl-ammonium chloride (Aliquat 336), and sodium octanoate were purchased from Sigma–Aldrich (St. Louis, MO, USA). 1-Octanol and 2-nitrophenyl octyl ether (NPOE) were

obtained from Fluka (Buchs, Switzerland). All water used was prepared with a Millipore Direct-Q3 UV system (Billerica, MA, USA).

Stock solutions containing 1 mg/mL of each model analyte were prepared in 10 % (v/v) acetonitrile in 100 mM HCOOH and stored protected from light at 277 K (4°C). Sample solutions of the compounds were prepared daily by adequate dilutions from the 1 mg/mL stock solutions by pure water or urine.

2.2 Instrumentation of the automated LPME-chip-HPLC

As show in Figure 1A, the integrated LPME-chip-HPLC consisted of three main parts: (1) an Agilent 1100 HPLC system (Agilent, Palo Alto, CA, USA) including an autosampler (model G1329A), a binary pump system (G1312A), and a UV detector (G1314A); (2) the home-built LPME-chip attached to a Valco Instrument (EHAM model, Houston, TX, USA) two position 10-port valve actuator control module; and (3) two microsyringe pumps (Kd Scientific, Holliston, MA). The HPLC software (Chemstation B.04.02) was applied for programming the sample injection, separation, and UV detection. The 10-port valve was used to synchronize the sample pretreatment, and to separate the low pressure of LPME-chip module from the high pressure of HPLC system by switching the positions between sample loading and injection. The automated operation of this 10-port valve was controlled by the remote control output (RS232 plug) on the HPLC system. The valve switching flow diagram for the LPME-chip-HPLC system is schematically illustrated in Figure 1B. In this setup, the two switch positions A and B in the 10-port valve were alternatively changed for microchip LPME sample pretreatment and on-line HPLC analysis, respectively. The two microsyringe pumps were used to deliver the sample carrier buffer solution and the acceptor phase through the LPME-chip for the dynamic extraction, respectively.

The construction of the LPME-chip was published recently and only a short description is given here [23, 27]. The porous polypropylene membrane (Celgard 2500 micro porous membrane; Celgard, Charlotte, NC, USA) with a 25 μm thickness (55% porosity, and 0.21 μm × 0.05 μm pores) used for the SLM was placed between two polymethyl methacrylate (PMMA) (53 mm × 53 mm × 2.1 mm) plates having 6 mm long channels with a depth of 50 μm and a width of 2.00 mm. The whole assembly was fixed by solvent-assisted bonding with ethanol and cured in a

343K (70°C) oven. At both ends of the channels, 1.6 mm I.D. holes were drilled through the plate to serve as inlet and outlet for the sample carrier liquid and the acceptor phase.

Prior to connection of the tubing to the chip, the supported liquid membrane was immobilized in the polypropylene membrane by filling approximately 0.2 μL of organic solvent (1-octanol or NPOE) into one end of the extraction channel using a micropipette. The solvent immediately immobilized into the polypropylene membrane by capillary forces, and this process was visually inspected as the appearance of the membrane changed from white to transparent during immobilization of membrane liquid. Subsequently, the tubings for the donor and acceptor flow were connected to the LPME-chip.

2.3 Procedure of carrier mediated LPME-chip-HPLC

Sample was loaded in the autosampler tray of the HPLC instrument in 2 mL LC vials (Microlab, Aarhus, Denmark), and extractions were normally carried out according to the following procedure; sample solution was prepared by the mixture of 500 μL analyte solution with 500 μL 50 mM sodium octanoate (ion-pair reagent) prepared in 25 mM pH 7.0 phosphate buffer. By means of the autosampler, 50 μL sample solution was draw into the injection needle and then directed back to the HPLC needle seat connected with the HPLC 6-port valve (all part of the Agilent® autosampler) (Figure 1A). The microsyringe on the donor side was connected with the 6-port valve of the autosampler, and was filled with 25 mM phosphate buffer (pH 7.0) used as the sample carrier liquid. With a flow rate of 5 μL/min, the sample plug was transferred to the LPME-chip and the analytes were extracted through the SLM. The tubings used for connecting the LPME-chip to the autosampler had a small dead volume in the order of 1 μL, also on the accepter side there was a small dead volume of approximately 0.5 μL for transferring the extracts to the 5 μL HPLC loop. The total extraction time was set for the requirement of total injected sample reached the chip, and also that the extract was transferred to the 5 μL sample loop. The time delay due to the dead volume was taken into account. Therefore, the on-chip membrane microextraction of 50 μL sample solution will take 15 minutes with a flow rate of 5 μL/min on the donor side. During the 15 minutes extraction, the analytes extracted into the accepter solution were continuously delivered to the 5 μL sample loop by the continuous flow of acceptor phase (50 mM HCl), which was pumped with a second microsyringe pump on the acceptor side at a flow rate of 0.5 μL/min.

Analysis of the extracts, collected by the loop, was performed by switching the 10-port valve to position B after 15 min extraction (Figure 1B). The software triggered the valve switching. In position B the mobile phase from the HPLC pump was directed to the 5 µL sample loop inserted on 10-port valve and thereby the enriched analytes was transferred into the HPLC column.

2.4 HPLC equipment

An Agilent 1100 series HPLC system (Agilent Technologies) was applied for the on-line LPME analysis as described in section 2.2. The LC separation was performed on a Zorbax Eclipse XDB-C18 column (Agilent Technologies) (4.6 × 50 mm, 1.8 µm particle size). The flow rate was 0.8 mL/min. Using a short column packed with 1.8 µm porous particles coupled with HPLC will shorten the analysis time without loss of the separation resolution compared to traditional 3-5 µm based columns [28]. Acidified water (100 mM HCOOH) and ACN were used as the mobile phases A and B, respectively. The solvent gradient adopted was as follows: 5% B at 0-2 min, 5-20% B at 2–4 min, 20% B at 4-10 min, 20–100% B at 10-12 min, followed by wash and equilibration. The analytes were detected using a UV detector at 282 nm. Baseline separation of the opium alkaloids was obtained in 10 min. The 10-port valve completely separated the low pressure of LPME-chip device from the HPLC system and the organic mobile phase never came in contact with the polymer chip. Acetonitrile in the mobile phase would otherwise have dissolved the PMMA and also modified the SLM used for the extraction.

2.5 Extraction efficiency

To determine the extraction efficiency of the model analytes, 50 µL of diluted standard solution with 25 mM sodium octanoate was extracted as described above. The same standard solution (5 µL unextracted) was also injected directly into the HPLC. Percentage recovery (R %) was calculated as follows:

$$R = \frac{V_a \cdot C_{a\,final}}{V_s \cdot C_{s\,initial}}$$

Where V_a is the volume of accepter solution (5 µL) injected into the HPLC having the concentration $C_{a\ final}$. V_s is the sample volume (50 µL) injected from the autosampler into the LPME device having the concentration $C_{s\ initial}$. Compared to the normal way of calculating the recovery for LPME, this equation also takes into account that not all the extracted compounds are

collected by the HPLC injection loop and the calculated recoveries reflect the amount of analytes collected and analysed by the HPLC.

The enrichment factor (*EF*) for the analyte was calculated according to the following equation:

$$EF = \frac{C_{a\,final}}{C_{s\,initial}}$$

Where $C_{a\ final}$ is the concentration of the enriched analyte solution injected into the HPLC and $C_{s\ initial}$ is the concentration of analytes in the untreated sample. Since only 50 μL of sample was injected into the LPME device and the accepter volume injected into the HPLC was 5 μL, the theoretical maximum enrichment factor was 10.

A standard curve using the LPME-chip–HPLC system was constructed for all analytes. For calculating the recovery, the standards prepared in 100 mM formic acid were injected directly into the 5 μL loop on the 10-port valve by filling the standard solution directly into the loop with a microsyringe.

3. Results and discussion

3.1 Principle of operation

The primary purpose of coupling the LPME-chip directly to the HPLC was to provide on-line clean-up, enrichment, and analysis in micro-scale without time-consuming off-line sample preparation. All autosamplers that allow the control of an external valve can be used in combination with the LPME-chip. In this work the entire system was controlled from the Agilent Chemstation software. The basic setup and a photo of the automated LPME-chip-HPLC system are illustrated in Figure 1. Initially the HPLC 6-port valve was in the bypass position and the donor phase pumped directly through the chip. Meanwhile, the 10-port valve was in position A, where HPLC mobile phase was passed directly through the HPLC column. When the LPME process was initiated, the HPLC 6-port valve was triggered by the injection program and switched to the mainpass position (Table 1). In this mainpass position, the donor phase was directed through the injection needle and delivered 50 μL sample solution directly towards the chip. The analytes were extracted across the SLM and into the acceptor phase. The acceptor phase was continuously pumped into a 5 μL sample loop with a flow rate of 0.5 μL/min. After 15 minutes of LPME, the 10-port valve was programmed to switch to the position B, and the mobile phase was switched to pass by the sample loop. Thus, sample injection, transportation, membrane

extraction, and HPLC analysis were carried out coherently by the program. The next sample extraction was initiated after HPLC analysis, and during this 15 minutes extraction the HPLC column had time to equilibrate. After every third run in the sample sequence, 0.5 μL 1-octanol was injected using the autosampler in order to regenerate the organic solvent of the membrane. This was important to maintain high repeatability, and relative standard deviations (RSD) were less than 10 % over 3 days' tests. A volume of 0.5 μL 1-octanol was found appropriate based on experimental experience.

3.2 Optimization of the extraction performance

In a series of experiments, the chemical compositions of the sample, SLM, and acceptor were optimized with primary focus on extraction recovery. First, the five alkaloids were extracted with pure 1-octanol and NPOE as the SLM. The solvents were selected based on earlier experience from conventional LPME [29, 30]. The pH in the sample was adjusted to 11.0, and the acceptor was 10 mM HCl. With 1-octanol and NPOE, the extraction system was not efficient, and recoveries were below 3-4 % for all the model analytes. For morphine, codeine, thebaine, and noscapine, log P-values are below 2.0, and these analytes were too polar to be extracted effectively in the LPME-system. Papaverine is less polar (log P = 3.0), and the reason for the low recovery for this compound was not clear.

In a subsequent set of experiments, carrier-mediated LPME was tested as an alternative extraction principle. Based on earlier experience, sodium octanoate was selected as carrier and was added to the sample solution, and 1-octanol was used as SLM [29-31]. The concentration of sodium octanoate in the sample was 25 mM. Concentrations above this were not used to avoid potential precipitation of the carrier. The sample was adjusted to pH 7.0 to ensure that both the carrier (acidic) and the alkaloids (basic) were ionized. With 10 mM HCl as acceptor, recoveries ranged between 8 and 38 % (Table 2). Clearly, carrier-mediated LPME was more efficient, and analyte molecules ion-paired with octanoate ions and were transferred across the SLM. To further optimize the carried-mediated LPME, the concentration of HCl in the acceptor was increased from 10 to 50 mM, and recoveries improved correspondingly to the range 17 to 45 % (Table 2). These extraction recoveries were comparable with earlier findings from carrier-mediated LPME in a traditional set-up [29-32], and were therefore not optimized further in this work.

3.3. Performance of LPME-chip-HPLC device

Calibration curves were established in the concentration range of 0.01-10 μg /mL for the five model alkaloids analysed with the LPME-HPLC system (Table 3). A linear relationship was obtained for all five opium alkaloids with R^2-values in the range 0.9959-0.9999. In addition, repeatability was tested based on five replicate experiments conducted with standard solutions of 5 μg/mL, and the RSD values were all below 11.0%.

In a final series of experiments, the LPME-chip was evaluated with human urine to test the compatibility of the system with a relevant biological matrix, and to indicate a potential application area for the future. In this experiment, the human urine spiked at the 2.5 μg/ml level with the five opium alkaloids where extracted for 15 min. In Figure 2, direct HPLC analysis of the spiked urine sample (Figure 2a) was compared with LPME-chip processed urine sample (Figure 2b). With direct HPLC analysis, the signals of the five opium alkaloids co-eluted with the urine matrix. But as seen in Figure 2b, the LPME-chip-HPLC system provided excellent sample clean-up from the urine matrix. This illustrated a great potential for sample clean-up with the LPME-chip-HPLC system.

In order to examine the potential of applying the LPME-chip-HPLC for larger sample series, eighteen injections of spiked urine containing 2.5 μg/mL of the alkaloids were introduced into the chip for extraction. The same membrane channel was used in 3 days to test repeatability and stability of measurements. As shown in Figure 3, the performance of the LPME-chip-HPLC system was stable and repeatable. The recovery of the five opium alkaloids in spiked urine sample were presented as follows: 12 % for morphine, 19 % for codeine, 36 % for thebaine, 28 % for papaverine and 22 % for noscapine with RSD values all below 10.0 %. The recoveries acquired here were slightly lower than from pure water samples as shown in Table 2. Minor matrix effect on the LPME extraction was probably attributed to the carrier-mediated extraction because of the formation of complexes of carrier and interfering ions in urine sample. The RSD values of peak areas for the 18 runs of spiked urine samples was 9.3% for morphine, 8.6% for codeine, 7.0% for thebaine, 8.0% for papaverine, and 9.9% for noscapine.

4. Conclusions

The present work has for the first time demonstrated coupling of a LPME-microchip device on-line to a HPLC instrument. This LPME-chip-HPLC system enabled automated injection,

extraction, separation, and detection of series of samples without any operator interaction. The LPME-chip effectively cleaned up samples and to some extent also pre-concentrated the analytes of interest. To avoid performance degradation of the supported liquid membrane, this was regularly regenerated as a part of the automated sequence by injection of a small volume of 1-octanol. The proof-of-principle of LPME-chip-HPLC system was evaluated with five opium alkaloids as model analytes, and demonstrated acceptable linearity and repeatability.

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Table 1

Inject program for LPME-chip-HPLC system using the Chemstation® software (Agilent technologies)

Step	Action*
1[a]	Draw default sample from sample
2[b]	Valve mainpass
3[c]	Wait 15 min
4[d]	Remote start request

[*] set action by using "Injector program" as injection mode

[a] default inject sample volume set to 50 µL

[b] switches the HPLC valve of the autosampler to connect the injected amount with the microsyringe carried donor phase (Fig. 1B)

[c] set injection time needed for LPME,

[d] stop extraction and trigger HPLC analysis (10 port valve switches to B position, whereby the extracted sample collected by the loop is injected onto the HPLC column)

Table 2

Recovery obtained with carrier (sodium octanoate) mediated extraction and influence from the concentration of HCl in the acceptor phase.

Acceptor	Recovery % (RSD%, n=5)				
	morphine	codeine	thebaine	papaverine	noscapine
10 mM HCl	8 (7.2)	14 (6.6)	38 (7.9)	32 (5.2)	18 (8.8)
50 mM HCl	17 (6.9)	23 (6.2)	45 (9.9)	38 (5.0)	24 (10.8)

SLM: 1-octanol

sample: five opiates each at 5 µg/mL containing 25 mM sodium octanoate, pH 7.0, injection volume 50 µL;

donor phase: 25 mM phosphate buffer (pH 7.0), 5 µL/min;

acceptor phase:, 10 mM HCl, 0.5µL/min;

extraction time: 15 min.

Table 3

Analytical performance of LPME-chip-HPLC system

Analyte	Calibration curve [a]	Linear range (μg/mL)	R^2	LOD (μg/mL)
morphine	y = 0.5005x + 0.174	0.1-10	0.9998	0.021
codeine	y = 0.8842x + 0.1847	0.1-10	0.9999	0.021
thebaine	y = 10.932x - 0.7379	0.05-5	0.9969	0.001
papaverine	y = 8.4735x + 0.785	0.01-5	0.9991	0.001
noscapine	y = 2.1787x - 0.2943	0.1-10	0.9959	0.021

[a] y: peak area (mAU), x: sample concentration (μg/mL)

SLM: 1-octanol, Injection volume: 50 μL.

Sample: 0.1-10 μg/mL of the five opiates prepared in 25 mM sodium octanoate, pH 7.0.

Donor phase: 5 μL/min, 25 mM phosphate buffer pH 7.0,

Acceptor phase: 0.5 μL/min, 50 mM HCl; extraction time: 15 min.

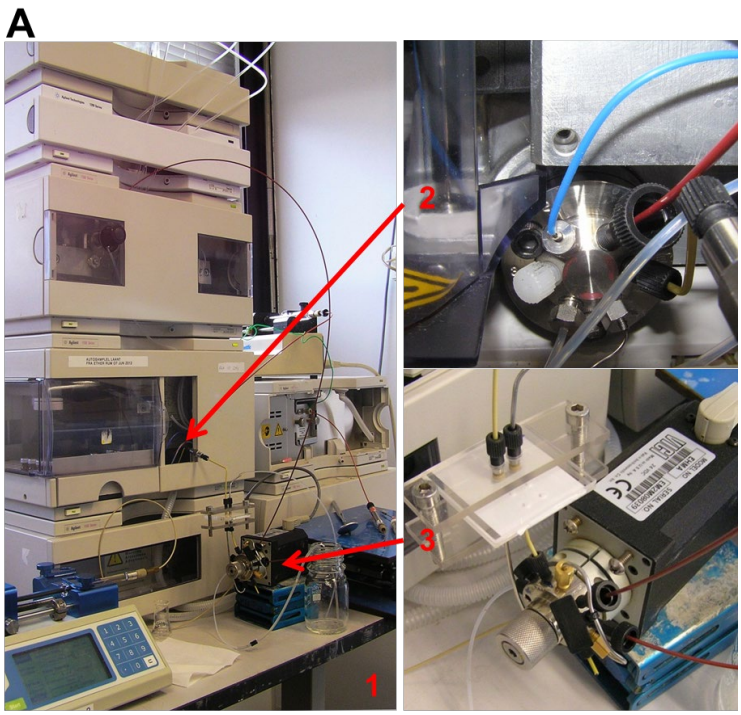
Figure caption

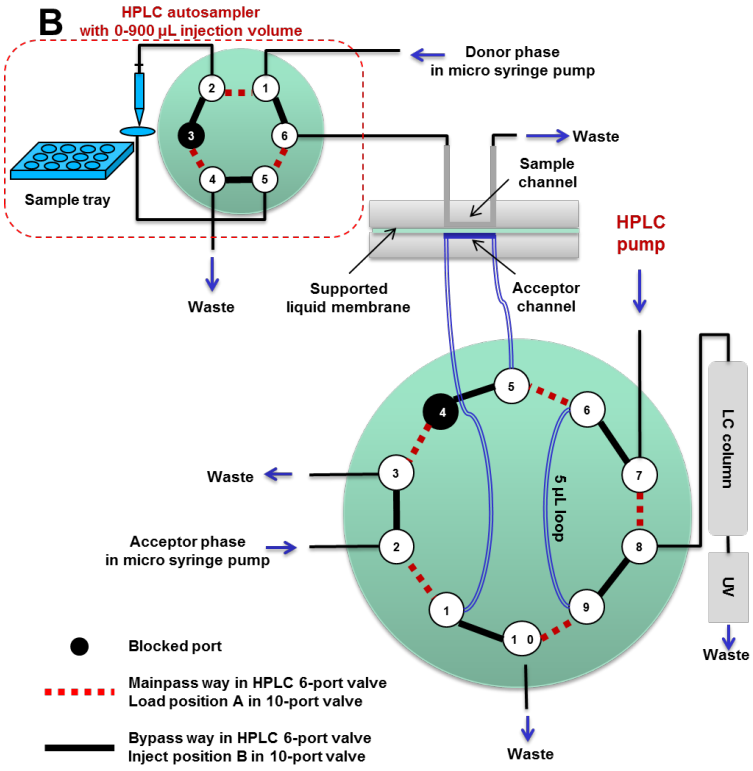
Figure 1 Photo (A) and schematic illustration (B) of automated LPME-chip-HPLC system. In Fig 1A, left panel (1) presented the overview of complete LPME-chip-HPLC device, and the right two panels showed the close-up view of the autosampler 6-port valve (2) as well as the chip system directly coupled to the external automated 10-port valve (3).

Figure 2 On LPME-chip-HPLC for spiked human urine. a) Direct HPLC analysis of spiked urine sample. b) Spiked urine sample after 15 min extraction on the LPME-chip-HPLC. Urine sample: spiked with 5 opiates at 5 μg/mL; SLM liquid: 1-octanol; injection volume 50 μL; donor phase:, 5 μL/min, 25 mM phosphate buffer (pH 7.0); acceptor phase: 0.5 μL/min, 50 mM HCl.

Figure 3 Repeatability tests of system with 18 runs were conducted in 3 days (6 runs per day) with the same channel cleaned with ethanol and dried after extraction every day. Urine sample: spiked with 5 opiates at 2.5 μg/mL; SLM liquid: 1-octanol; injection volume 50 μL; donor phase: 5 μL/min, 25 mM phosphate buffer (pH 7.0); acceptor phase: 0.5μL/min, 50 mM HCl; extraction time: 15 min.

400

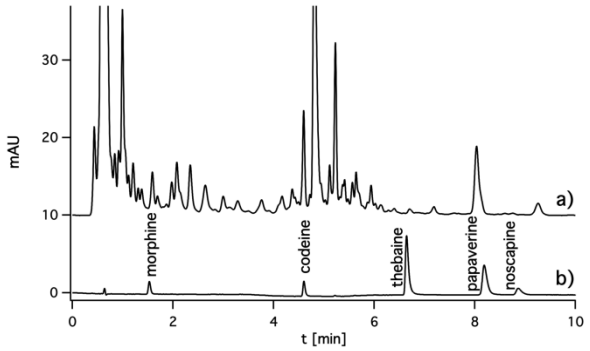




401

402 **Figure 1**

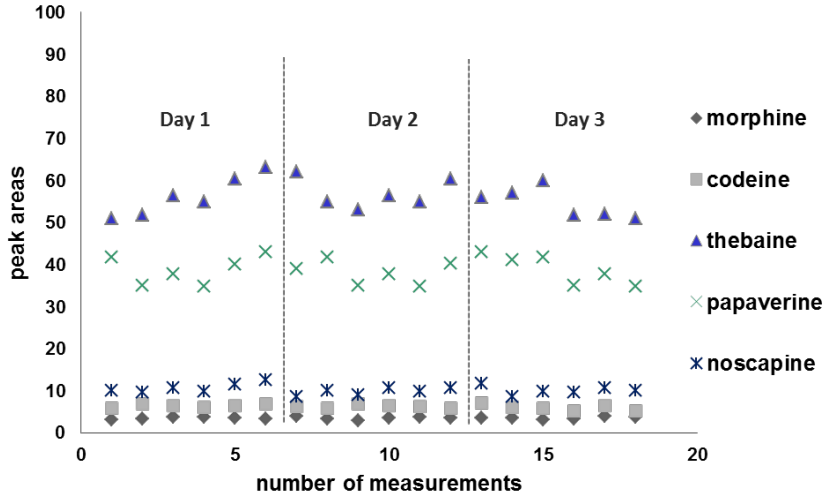
403



404

405 **Figure 2**

406



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408 **Figure 3**