Cover Page

Universiteit Leiden

Author: Raterink, Robert-Jan
Title: High-throughput profiling of small molecules using mass spectrometry
Issue Date: 2014-10-01

Chapter 5

Gas Pressure Assisted Micro-Liquid-Liquid Extraction coupled Online to Direct Infusion Mass Spectrometry: a New Automated Screening Platform for Bioanalysis.

ABSTRACT

In the field of bioanalysis there is an increasing demand for miniaturized, automated and robust sample pretreatment procedures which can be easily connected to direct-infusion mass spectrometry (DI-MS) in order to allow the high-throughput screening of drugs and/or their metabolites in complex body fluids like plasma. Liquid-Liquid extraction (LLE) is a common sample pretreatment technique often used for complex aqueous samples in bioanalysis. Despite significant developments that have been made in automated and miniaturized LLE procedures, fully-automated LLE techniques allowing high-throughput bio-analytical studies on small-volume samples using direct infusion mass spectrometry, have not been matured yet. Here, we introduce a new fully-automated micro-LLE technique based on gas-pressure assisted mixing followed by passive phase separation, coupled online to nanoelectrospray-DI-MS. Our method was characterized by varying the gas flow and its duration through the solvent mixture. For evaluation of the analytical performance four drugs were spiked to human plasma, resulting in highly acceptable precision (RSD down to 9%) and linearity (R^2 ranging from 0.990 to 0.998). We demonstrate that our new method does not only allow the reliable extraction of analytes from small sample volumes of a few microliters in an automated and high-throughput manner, but also performs comparable or better than conventional offline LLE in which the handling of small volumes remains challenging. Finally, we demonstrate the applicability of our method for drug screening on dried blood spots showing excellent linearity (R^2 of 0.998) and precision (RSD of 9%). In conclusion, we present the proof of principe of a high-throughput screening platform for bioanalysis based on a new automated micro-LLE method, coupled online to a commercially available nano-ESI-DI-MS.

INTRODUCTION

In the last decades there has been a strong demand for high-throughput MS-based technologies for bioanalytical and pharmaceutical screening studies such as in drug discovery and development[1]. Direct infusion-mass spectrometry (DI-MS) is an analytical technique which is in principle very suitable as a rapid and global high-throughput screening platform since it does not include often time consuming separation methods prior to MS detection[2]. However, as a consequence, DI-MS is susceptible to potential ion suppression effects due to interfering, co-eluting matrix components in the samples, especially when using electrospray ionization (ESI)[3]. By employing nano-electrospray (nanoESI) ionization instead of conventional ESI, the ionization efficiency can be somewhat improved as ion suppression is considerably reduced under low flow-rate conditions [4]. However, nano-ESI nozzles (with diameters of typically a few μm) are prone to clogging as a result of protein precipitation, salt crystallization and dust/impurities which asks for an efficient sample pretreatment prior to analysis. Moreover, when an effective sample pretreatment procedure is carried out, ion suppression may be further reduced, since the extract contains fewer molecules when co-introduced into the MS. Plasma is a complex sample due to the presence of proteins, salts and a wide range of (endogenous) metabolites with a chemistry which may be similar to the analytes of interest and/or causing ion suppression. Therefore, an efficient sample pretreatment procedure for the clean-up and selective extraction of the compounds of interest is often required for the reliable analysis of drugs in complex samples like plasma by DI-MS-based approaches.

With sample pretreatment being one of the major challenges in a typical (DI)-MS-based screening pipeline, significant progress has been made in the improvement of sample pretreatment procedures over the past few years[5]. New developments for sample pretreatment methods are often directed towards simplification, automation, miniaturization, integration in well-plate formats, more environment friendly and specificity enhancements of the clean-up and enrichment process[6][7]. Liquid-liquid extraction (LLE), next to solid-phase extraction (SPE), is a common sample pretreatment technique[8] which can be used to extract relatively apolar molecules from a complex aqueous biological sample phase into an immiscible organic phase. After the LLE procedure both fractions, containing polar and apolar compounds, can then be independently analyzed. Although LLE techniques are commonly used, their use in online systems is much less than e.g. SPE techniques. Moreover, most of the reported LLE systems are rather difficult to automate especially for small volume-samples of a few microliters due to the fact that conventional LLE requires a mixing and often a centrifugation step which are challenging to automate (e.g. in a multi-well format)[9]. Off-line sample pretreatment can be considered a limiting step for high-throughput bioanalysis of especially small sample volumes, indicating a clear need for on-line sample pretreatment procedures to shorten the total sample handling and analysis time and to reduce the possibilities for errors. In addition, when samples in a well plate are processed in a parallel manner prior to analysis, the handling time per sample is slow and variable and evaporation of volatile solvents and analyte degradation could be an issue.

An emerging trend in the field of biomedical and pharmaceutical analysis is to analyze drugs in limited sample amounts, such as dried blood spots (DBS) and plasma of mice (especially in the case of longitudinal studies the amount of plasma which can be collected from the same mouse, usually via the tail, is only a few microliters)[1]. Therefore, miniaturization and automation of the sample pretreatment procedure is needed in order to allow the analysis of drugs in these types of small samples in a very effective and high-throughput manner by MS. So

far, various miniaturized LLE strategies have been developed such as liquid-phase microextraction (LPME) approaches [7]. In LPME, extraction generally takes place from an aqueous sample phase into a small amount of a water-immiscible organic acceptor phase, and reducing the acceptor-to-donor ratio with typical enrichment factors between one and two orders of magnitude[10]. LPME has been developed in a variety of configurations such as single drop microextraction (SDME)[11][12][13], headspace LPME[14], hollow fiber (HF)-LPME[15][16], continuous flow LPME[17], solvent bar microextraction[18][19], dispersive liquid-liquid microextraction (DLLME)[20][21] and parallel artificial liquid membrane extraction (PALME) [22][23], among others. However, most of these LPME methods need a form of (off-line) agitation in order to get efficient extractions which will make it less straightforward to implement in a fully-automated high-throughput analytical platform. Interestingly, the online coupling of (micro)LLE procedures to nanoESI-DI-MS has, to our knowledge, not been explored yet.

Here, we present the proof principle of a new and simple approach to miniaturized and automated LLE, so-called gas pressure assisted micro liquid-liquid-extraction (GPA-μLLE) coupled online to nanoESI-DI-MS. Rapid extraction is realized by dispensing an aqueous sample phase into DCM after which both phases are mixed thoroughly by applying nitrogen gas pressure and flowing gas through the liquid phases. After passive settling of the phases under the mentioned conditions, 2 μL of DCM is aspired and consecutively analyzed with nanoESI-DI-MS. As a consequence, our method is easy to automate as it bypasses the need for off-line vortexing and centrifugation as is the current standard. Furthermore, as GPA-μLLE is performed in 384-well plate, it is compatible with handling small sample volumes. In addition, by the integration of GPA-μLLE in a commercially available nanoESI robot, GPA-μLLE is coupled on-line to DI-MS making it very suitable for a high-throughput screening platform. Moreover, in our method, every sample is sequentially processed which results in a short and constant handling time per sample which is also very suitable for volatile solvents such as DCM. The GPA-μLLE procedure is characterized by studying the effect of the gas pressure and its duration on the recovery of test analytes. Subsequently, the potential of micro-LLE coupled online to nanoESI-DI-MS for the extraction and screening of drugs from human plasma is demonstrated. A comparison between GPA-μLLE and conventional (off-line/manual) LLE is made, as well as with 'dilute and shoot' methods. Finally, the utility of GPA-μLLE for the screening of lidocaine in a DBS sample is shown.

EXPERIMENTAL SECTION

Chemicals and materials

Water (H_2O) and methanol was bought from Actu-All Chemicals (Randmeer, The Netherlands). Dichloromethane (DCM), crystal violet, fluorescein, lidocaine, propranolol, verapamil, loperamide were obtained from Sigma-Aldrich (Steinheim, Germany). Diltiazem and chlorpromazine were from Sigma-Aldrich (Steinheim, Germany) and used as internal standards. Diltiazem was used for normalizing lidocaine and chlorpromazine was used for normalizing propranolol, verapamil and loperamide. All solvents were HPLC grade. Human plasma (heparin) and whole blood (heparin) were obtained from adult healthy volunteers.

Chapter 5

GPA-μLLE

All the GPA-μLLE-DI-MS steps were programmed and performed in a Nanomate robot (Advion Triversa NanoMate, Ithaca, USA) in a fully automated manner, except of the prefilling of the well-plate. The steps used for GPA-μLLE are illustrated in Figure 1. The GPA-μLLE was performed according to the following procedure: in a 384-well plate, two adjacent wells were prefilled with 14 μL aqueous sample (blue), consisting of 14-fold diluted plasma dissolved in 71.5% methanol, and 10 μL DCM (orange) as an immiscible organic phase (Figure 1A). The aqueous sample phase consisted of 71.5% methanol which was based on a Bligh and Dyer[24] extraction in order to get an efficient extraction and protein precipitation. Moreover, after mixing, some of the methanol and H_2O was dissolved in the DCM, making it suitable for nanoESI (pure DCM could not be electrosprayed). The aqueous phase was dispensed into the DCM from the bottom of the well (Figure 1B). Thorough mixing of both phases was accomplished by applying a gas pressure of nitrogen (in our system typically 0.5 psi, or stated otherwise) on a pipette tip (inner diameter of 0.5 mm) inserted at the bottom of the well for a certain time duration (typically 20 sec, or stated otherwise), see Figure 1C. During a short period of time (90 seconds), both phases were allowed to settle and separate (Figure 1D) and, subsequently, 2 μL of the DCM phase was aspired into a new pipette tip (Figure 1E) and transferred for analysis (Figure 1F).

Comparison with 'dilute and shoot' methods

The 'dilute and shoot' experiment was carried out after spinning down the precipitated proteins of the aqueous phase. After centrifugation, the supernatant mixture was directly infused into the MS using nanoelectrospray ionization using the same MS settings as for the direct infusion of the DCM extract after GPA-μLLE.

Dried blood spots

20 μL of whole blood spiked with lidocaine was pipetted on standard blood spot cards obtained from RIVM (the Netherlands). The dried blood spots (DBS) were punched out (3 mm diameter) from the middle of the spot and inserted into the bottom of a 384-well plate, which was placed in the NanoMate. 20 μL of aqueous solvent (71.5% methanol) including 100 nM diltiazem as an internal standard was added to the DBS and 30 minutes of passive extraction was chosen prior to GPA-μLLE-DI-MS.

Mass spectrometry

The GPA-μLLE on-line coupled to DI-MS (positive mode) were carried out using an automated chip-based nanoESI (Advion Triversa NanoMate, Ithaca, USA) source coupled to a LTQ-Orbitrap XL (Thermo Fisher Scientific). In the NanoMate ChipSoft software (version 4.3.3.1108) the Advanced User Interface (AUI) was enabled in order to program and preform all the steps needed for μ-LLE. Finally, a 2 μL extract was aspired and infused with a back pressure of 0.4 psi and an electrospray voltage of 1.47 kV in the positive mode. The well-plate was set to a temperature of 4°C in order to reduce the evaporation rate of DCM and to ensure a fixed temperature. At this temperature no visible loss of DCM was observed within the experimental time of around 2 minutes. To further control evaporation, a well-plate cover foil could be used. The inlet capillary temperature was 120°C, the capillary voltage and the tube lens voltage was 30 and 100 V, respectively. The automatic gain control and injection waveforms were enabled. Mass spectra were recorded at a resolution of 100,000 using one microscan and 10-20 scans were averaged in order to further analyze the data. The response of the drug compounds analyzed was normalized with the appropriate internal standard by

Gas pressure assisted micro-liquid-liquid extraction coupled online to direct infusion mass spectrometry: a new automated screening platform for bioanalysis.

| 67

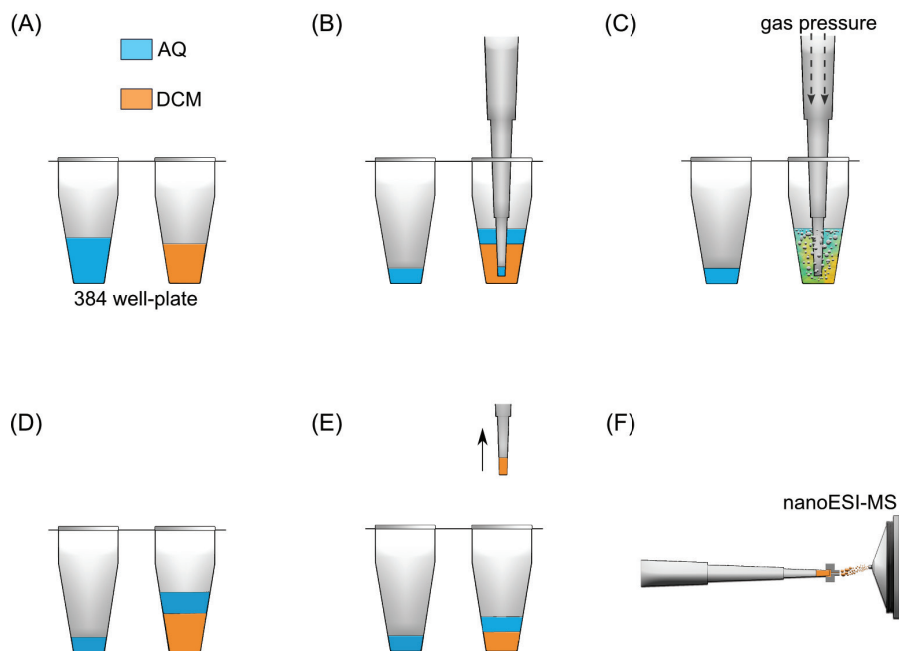calculating the ratio of intensity of the drug and intensity of the internal standard.

Figure 1: Schematic illustration of the individual steps of the GPA-µLLE method. (A) A 384 well plate is prefilled with an aqueous methanol-water (blue) sample and dichloromethane (DCM) (orange). (B) 14 µL of the AQ sample is pipetted into the bottom of the DCM layer. (C) Vigorous mixing is achieved by applying nitrogen gas pressure onto the pipette tip while the pipet is immersed in the liquid. (D) After mixing, both phases are allowed to settle and separate. (E) 2 µL of the DCM phase is pipetted and (F)directly infused into MS using nanoelectrospray ionization.

RESULTS AND DISCUSSION
In this section the proof of principle of GPA-µLLE is demonstrated followed by the characterization of two important parameters, i.e. the gas flow through the liquid mixture controlled by the applied gas pressure and its duration. Next, the performance of GPA-µLLE was evaluated and compared to conventional LLE and 'dilute and shoot' methods. Finally the utility of GPA-µLLE applied to DBS for drug screening was demonstrated.

Proof-of-principle of GPA-µLLE
The general working principle of GPA-µLLE is illustrated in Figure 1. For a visual proof of principle of the general operation, an experiment was conducted using a (green) mix of 59 µM crystal violet and 200 µM fluorescein as an aqueous sample. The results are shown in Figure 2. The aqueous sample was aspired from its well and dispensed at the bottom of the prefilled DCM well after which a bi-phasic system was generated (Figure 2B). After this step already some, but limited, extraction of crystal violet into the DCM could be observed, whereas, after 10 seconds of nitrogen gas-pressurized mixing (Figure 2C), the majority of the crystal violet was extracted. After 90 seconds the two phases were settled into a bi-layer system (Figure 2E).

During the mixing step the surface contact between both phases is enhanced and therefore the extraction efficiency is improved. From Figure 2D (taken 2 seconds after mixing) it can be seen that during mixing an emulsion of the organic phase and the aqueous phase was formed. Furthermore, the precipitated proteins are distributed in the aqueous phase, but not in the organic DCM phase, as can be observed in Figure 2F.
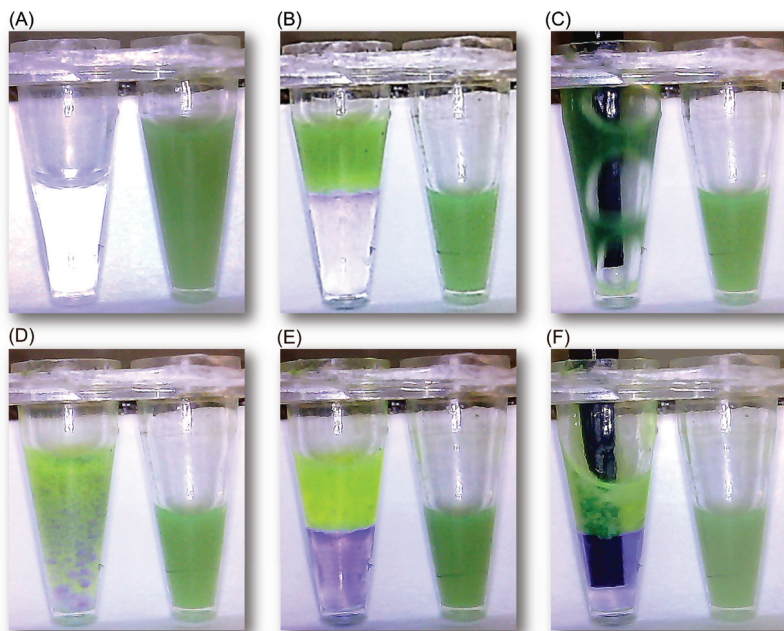

Figure 2 : Video stills showing the proof of principle of GPA-μLLE of a plasma sample: (A) 10 μL of DCM and 20uL of the aqueous sample are prefilled in adjacent 384-wells. (B) Bi-layer system after pipetting 14μL of the aqueous phase (consisting of 71.5% methanol containing a green mix of crystal violet and fluorescein and 14-fold diluted plasma) into the DCM. (C) Nitrogen Gas pressure assisted mixing of the phases. (D) After 2 second after mixing the created emulsion is clearly observed in which the crystal violet has been extracted from the aqueous phase into the organic phase, while fluorescein remains in the aqueous phase. (E) Bi-layer system after passive phase separation (typically around 90 seconds after the end of the mixing). (F) 2 μL is pipetted from the bottom of the DCM layer in order to be analysed by nanoESI-DI-MS. The precipitated proteins are visible in the aqueous phase.

Effect of gas pressure and its duration on extraction

To study the mixing due to the gas flow, the effect of gas pressure and its duration on the extraction efficiency during extraction was studied with four selected drugs as model analytes with varying size, pKa and log P (Supporting Table 1 and Figure 3). An equimolar mix of these drugs was spiked to human plasma (14-fold dilution in 71.5% methanol) at a concentration of 500 nM. Preliminary experiments using lower dilution factors resulted in less robust results (data not shown), probably due to the high protein content of human plasma.

To determine the extraction recovery, the steps after GPA-μLLE were modified: 2 μL of the DCM phase was mixed with 2 μL of DCM containing 1 μM of the internal standards and 2 μL of this mix was infused for DI-MS analysis. By adding the internal standards only after the extraction, we ensured that the internal standards could not be extracted into the aqueous phase during GPA-μLLE. As a consequence, we determined the relative recovery of the 4 drugs as the ratio of the intensity of drug and intensity of internal standard. Since methyl tert-butyl ether (MTBE) is a less toxic alternative to DCM[26], preliminary experiments with MTBE

instead of DCM were performed; however the infusion of MTBE extracts resulted in less stable nanoelectrospray currents (data not shown). Therefore, in all subsequent experiments DCM was used as an organic phase.

The gas flow through the tip was varied by choosing gas pressures of 0, 0.1, 0.3 and 0.5 psi for 2, 10 and 20 seconds (Figure 3). A practical limitation of the applied gas pressure with the used volumes is the sputtering of liquid out of the well, which can cause contamination of other wells. In our system at pressures higher than 0.5 psi, small amounts of liquid were sputtering
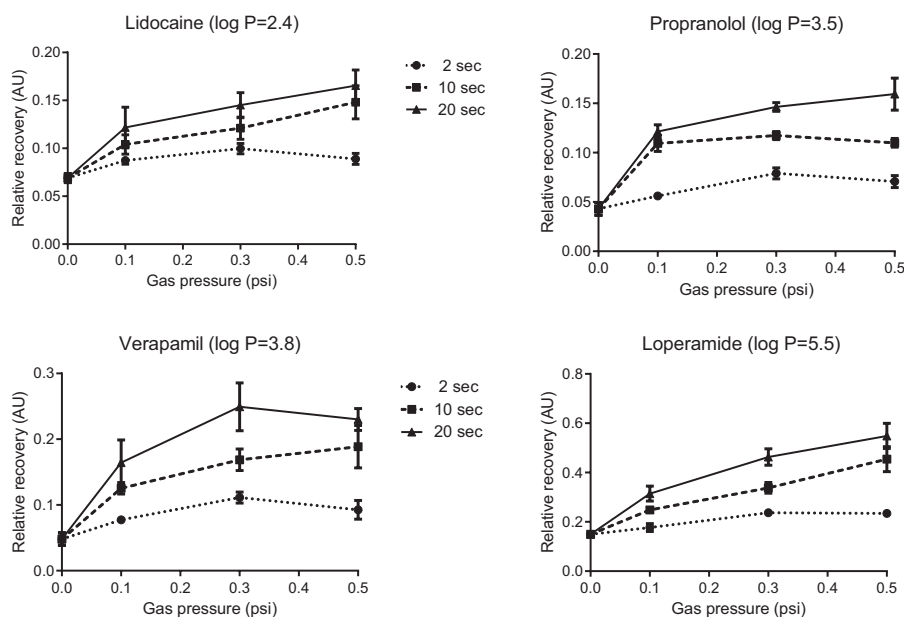

Figure 3 : Average of the relative recovery (ratio of intensity of drug and its internal standard) versus the gas pressure and its duration of the 4 drugs. Internal standards are added only after the extraction, see text; n=3, error bars are expressed as the S.E.M.

out of the well. Therefore 0.5 psi was the maximum pressure used in order to obtain reproducible results. As expected, by applying no pressure (0.0 psi) the relative recovery of the drugs was minimal (as already shown in Figure 2B). The fact that at 0 psi already some extraction was observed was caused by dispensing the sample at the bottom of the DCM layer (Figure 1B). From 0.3 psi upwards the recovery of the drugs did not seem to increase significantly, indicating that a higher gas flow at a higher pressure did not increase surface contact between both phases significantly any further. The duration of the applied gas pressure had a positive effect on the extraction: a duration of 2 seconds resulted in the lowest recovery, whereas a duration of 20 seconds provided the highest recovery. These results demonstrate that extraction of the drugs was improved significantly upon application of the gas pressure. Moreover, these results indicate that applying the gas pressure longer than 20 seconds would probably result in even higher recoveries. However, as there is a trade-off between speed and recovery we have chosen to minimize the time duration while still obtaining acceptable results.

Performance evaluation of GPA-µLLE

In the following experiments the analytical performance of GPA-µLLE was tested and compared with conventional off-line LLE, and direct infusion of the aqueous sample ('dilute and shoot') after spinning down the precipitated proteins. For the comparison of these methods the same nanoESI and MS settings were used. Calibration curves were obtained by spiking the four model drugs to 14-fold diluted plasma at the 0, 25, 50, 100, 250, 500 and 1000 nM level with diltiazem and chlorpromazine as an internal standard (500 nM). Again, after spiking the drugs, the aqueous sample phase consisted of 71.5% methanol. Subsequently, the aqueous samples were subjected to analysis by GPA-µLLE -DI-MS, conventional off-line LLE/DI-MS and 'dilute and shoot' DI-MS after protein spin-down of the aqueous sample. In order to compare GPA-µLLE with conventional LLE, the same aqueous: DCM solvent ratio as in GPA-µLLE was used, only the volumes were up-scaled in order to be able to perform the vortexing and centrifugation in 1.5 mL eppendorf tubes. After 30 seconds of vortexing followed by 10 minutes of centrifugation at 15 krcf, the DCM extract was carefully pipetted out of the tube and subsequently analyzed by DI-MS using the same settings as for GPA-µLLE. It should be mentioned that we also have tested a 'dilute and shoot' direct infusion of the aqueous phase without precipitation of proteins; however, as expected, we could not obtain a stable spray probably due to clogging of the nanoESI emitter caused by abundant plasma proteins.

For the 'dilute and shoot' method the same aqueous sample phase (after protein precipitation) was analyzed whereas for both GPA-µLLE and conventional off-line LLE the DCM phase was analyzed as described above. From Table 1 it can be seen that for the drugs tested, our new GPA-µLLE method performed comparable or better than the conventional off-line LLE in terms of repeatability, linearity and limit of detection. Linear behavior was observed by measuring the normalized response of the analyte versus their concentration up to 2-orders of magnitude. The R^2-values of the linear regression line of GPA-µLLE was good and ranged from 0.990 to 0.998 for the compounds tested. The precision of the GPA-µLLE measurements in plasma at 50 nM showed RSD values below 15%, except for lidocaine. This could be caused by a lower recovery due to lidocaine lowest log P and pKa, as extraction was carried out at neutral pH, (Supporting Table 1) and/or a lower ionization efficiency. The fact that the precision of our GPA-µLLE was improved over conventional LLE can be explained by the omission of the manual pipetting steps. The estimated LOD and LLOQ of GPA-µLLE seemed comparable with conventional, off-line LLE. The estimated LOD and LLOQ of loperamide were significantly higher than those of the other drugs. This is explained by the fact that a protonated ion with the same mass as loperamide was detected in the blank plasma, opposed to the other 3 drugs. In Figure 4, typical averaged mass spectra, zoomed in at the verapamil

Table 1: Analytical characteristics of GPA-µLLE of 14-fold diluted plasma compared with conventional off-line LLE; n=5.

analyte	precision(%) @ 50 nM[a]		linearity (25 - 1000 nM)		LOD (nM)[b]		LLOQ (nM)[c]	
	conv. LLE	GPA-µLLE	conv. LLE	GPA-µLLE	conv. LLE	GPA-µLLE	conv. LLE	GPA-µLLE
lidocaine	43	21	0.992	0.990	5	9	17	30
propranolol	11	9	0.990	0.996	3	4	10	13
verapamil	26	9	0.993	0.995	2	3	7	10
loperamide	20	12	0.989	0.998	25	30	80	99

[a] concentration in 14-fold diluted plasma. [b] LOD was estimated by $3(SD_{25nM} + SD_{blank}) / slope_{response\ curve}$. [c] LLOQ was estimated by 3.3LOD.

m/z range from blank and spiked human plasma, after GPA-µLLE-nanoESI-DI-MS analysis are shown. It can be observed that for verapamil no protonated ion or carry over was detected in the blank plasma. Moreover, at the lowest dilution of 25 nM a protonated ion was clearly detected. It should be mentioned that the GPA-µLLE method did not require a centrifugation after mixing in order to facilitate phase settling as is required for conventional LLE methods. This was the advantage of the miniaturization of the whole LLE procedure, since only 2 µL of DCM had to be phase-separated for successful nanoESI-DI-MS analysis, which was well-achieved within 90 seconds under our conditions.

Since we analyzed the aqueous phase with 'dilute and shoot' instead of the DCM in both LLE methods, we did not include these results in Table 1. However, with precision up to 62%, the 'dilute and shoot' method after protein precipitation performed significantly worse than both LLE methods, as expected. This demonstrates that effective fractionation/pretreatment is important for the analysis of complex samples using DI-MS. These findings demonstrate the potential of GPA-µLLE as a rapid, fully-automated screening platform for small molecules in bioanalysis with excellent performance comparable with conventional off-line LLE. Moreover, as GPA-µLLE was integrated in a 384-well plate, it was compatible with handling small volumes in order of 2 µL with good precision. In principle, GPA-µLLE can be downscaled even more when smaller well-dimensions are used. Next to the analysis of the organic DCM phase after GPA-µLLE, the aqueous phase could in principle also be analyzed, depending on the application and the used solvents. However, due to high salt concentrations in a typical biological sample, ion suppression effects and potential clogging could occur when analyzing
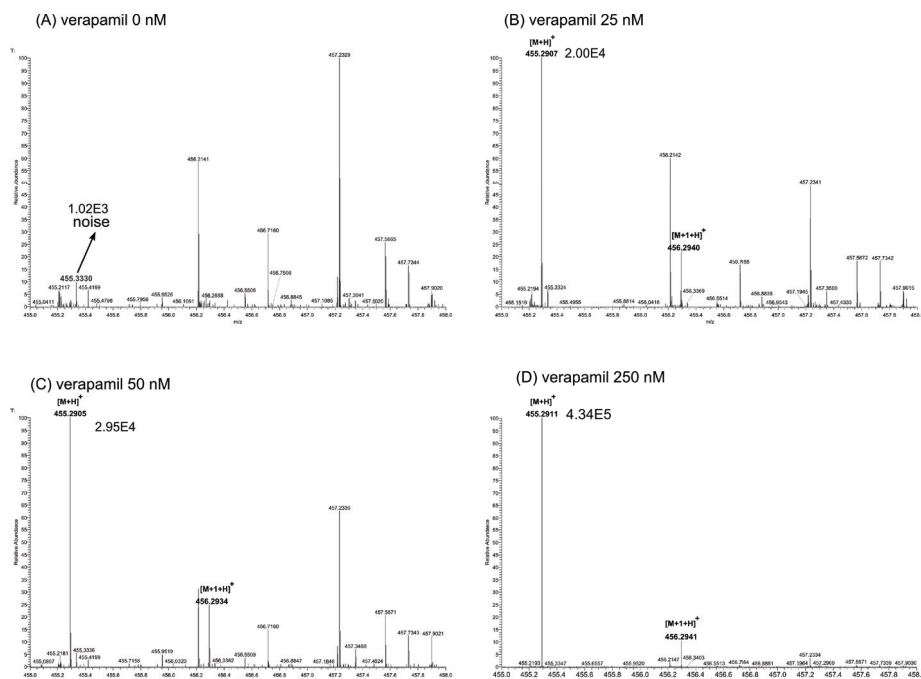

Figure 4: Typical averaged mass spectra zoomed in at verapamil (455-458 *m/z*) of blank and spiked human plasma analyzed with GPA-µLLE-nanoESI-DIMS. (A) In blank plasma, no protonated ion of verapamil was observed, while at (B) 25 nM concentration the protonated ion of verapamil as well as its isotopic ions can clearly be observed. As expected, at higher concentrations (C),(D) the intensities of the protonated and isotopic ions of verapamil is observed at higher levels.

Chapter 5

the aqueous phase, especially when using DI-MS.

After mixing and settling of the phases, the precipitated plasma proteins were present in the aqueous phase (as observed in Figure 1F). During the last step of μ-LLE, in which 2 μL of the bottom DCM-phase is aspired for consecutive DI-MS analysis, the pipette tip punched through the protein layer. Although this step could lead to protein contamination into the pipette tip, no indications of proteins in the DCM extract were observed: the resulting nanoESI spray was stable (around 10 nA) and moreover, in the 1000-2000 m/z range, no protein envelopes in the mass spectra could be observed.
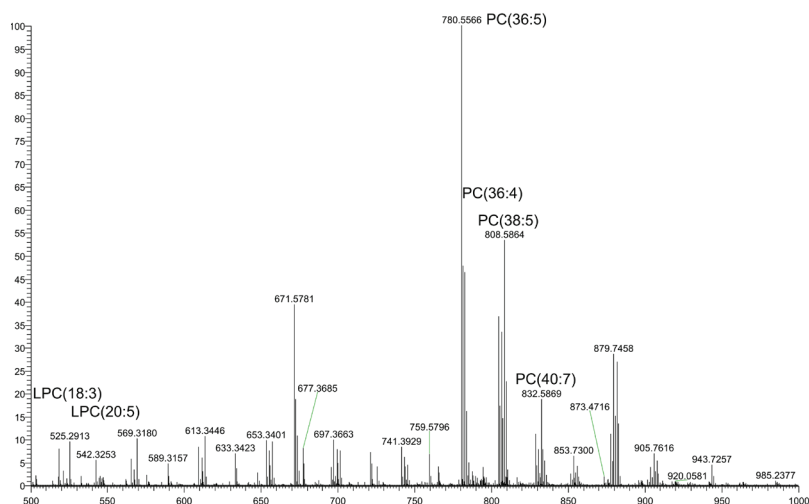

Figure 5: Typical averaged mass spectrum of the 500-1000 m/z mass range of (blank) human plasma analyzed with GPA-μLLE-nanoESI-DIMS. Several lipids were putatively annotated based on exact mass.

Interestingly and also expected, many other plasma components were observed in the MS spectra and by tentative identification (based on exact mass), e.g. several plasma lipids were identified in the higher mass range (500-1000 m/z) (Figure 5), suggesting that GPA-μLLE might also be suitable for lipid profiling. On the other hand, these observations suggest that ion suppression effects could occur during the DCM analysis on the tested drugs. In future, by fine-tuning the solvents used for extraction, an optimized method could be developed in order to minimize ion suppression effects in terms of sensitivity. Moreover, the use of stable isotopically labeled internal standards is expected to improve precision. In order to further verify the suitability of our method for lipid analysis, we calculated the precision of 4 typical plasma lipids: LPC (18:3), LPC (20:5), PC (38:5) and PC (40:7) from the blank plasma replicates with good RSD being 6, 9, 2 and 3%, respectively. Since no internal standard specifically for lipids was spiked, we normalized the 4 lipids with another endogenous abundant lipid, i.e. PC (36:5).

Since in GPA-μLLE the whole LLE procedure is automated, GPA-μLLE could be very suitable for rapid optimization of LLE procedures. Moreover, because in our method the samples are processed sequentially, the sample handling time is constant, making it also very suitable for extractive derivatization procedures. In this paper GPA-μLLE was integrated in an automated nanoESI-DI-MS robot and coupled online to MS. However, in the future GPA-μLLE could also be integrated into other platforms such as LC, CE and GC-MS, as long as the used auto-

Gas pressure assisted micro-liquid-liquid extraction coupled online to direct infusion mass spectrometry: a new automated screening platform for bioanalysis.

| 73

sampler/ sample preparation robot has the ability to apply a (controlled) gas flow through the liquid mixture.

Application of GPA-µLLE to dried blood spot analysis

The developed GPA-µLLE was also applied to dried blood spots (DBS) samples because of the simplicity of the sample preparation procedure. A calibration experiment was carried out by spiking whole blood with lidocaine (0, 25, 50, 100, 250, 500, 1000 and 2500 nM) and spotting these on a card and drying it for 7 days. In these experiments, the blood was not diluted. Linear behavior was observed by measuring the normalized response of lidocaine versus its concentration over 3-orders of magnitude (Supporting Figure 1). The R^2-value of 0.998 of the linear regression line of GPA-µLLE was excellent. The precision at 1000 nM was 9%, which is a favorable value to determine lidocaine in in a complex sample. Actually, the therapeutic window of lidocaine in plasma is at an even higher concentration range, i.e. between 6 to 25 µM[27], proving the suitability of our method for clinical use. The aliquot of blood of the DBS punch was only 2.8 µL, resulting in a 7-fold dilution after adding the aqueous solvent.

These results demonstrate that GPA-µLLE is also very promising for the rapid screening of drugs in DBS which could be useful in the pharmacokinetic screening in volume limited samples such as mouse plasma. However, it should be mentioned that the punching of the spot was performed manually and in an off-line manner. In future, further miniaturization of the punched spot size could be aimed for, which would enable more samples from one DBS, possibly for other analytical measurements, or for backup samples.

CONCLUSIONS AND OUTLOOK

In this paper, we have presented a miniaturized and fully-automated LLE method for profiling of small molecules based on gas flow mixing via a pipette through the solvent mixture followed by passive phase separation, coupled to nanoESI direct infusion mass spectrometry. We have shown that this method, which we called gas pressure assisted micro liquid-liquid extraction (GPA-µLLE), can be effectively used for the online clean-up of complex samples which was needed for reliable drug screening using nanoESI-DI-MS. The coupling of GPA-µLLE to nanoESI-DI-MS was found to be suitable for the rapid extraction and analysis of spiked drugs to human plasma samples and importantly, resulted in a stable protein-free nanoESI-MS signal. Moreover, compared to conventional LLE, GPA-µLLE showed an improved or comparable precision, linearity and sensitivity. Furthermore, the extraction can be optimized by varying the gas flow and its duration. Finally, the utility of the GPA-µLLE-nanoESI-DI-MS was demonstrated for the analysis of lidocaine in DBS samples. In future, research could be directed towards further improvements of GPA-µLLE in terms of speed. By adding ultrasonic transducers below the well plate, extraction and phase settling times will probably be shortened. In addition, by using salting-out effect by addition of salt, the LLE procedure might also be faster and more efficient, only in case the organic phase is to be analyzed. Furthermore, GPA-µLLE is very suitable for performing rapid LLE optimization studies in which typical LLE parameters could be tested. Next to coupling to DI-MS, GPA-µLLE can also be integrated in other applications such as LC-MS, GC-MS. Moreover, in principle both aqueous as organic phase could be analyzed, depending on the application and solvents used. When large amount of samples needs to be processed, the well plate can be covered with a foil and/or the organic phase could be pipetted from a stock, in order to further control possible evaporation of solvents. Thanks to its combination of simplicity of the fully-automated setup and the ability to downscale LLE volumes, GPA-µLLE holds the promise to be integrated in many sample pretreatment modules for high-throughput screening in bioanalysis.

Chapter 5

ACKNOWLEDGEMENTS

REFERENCES

[1]   P. D. Rainville, N. W. Smith, I. D. Wilson, J. K. Nicholson, and R. S. Plumb, "Addressing the challenge of limited sample volumes in in vitro studies with capillary-scale microfluidic LC-MS/MS.," Bioanalysis, vol. 3, no. 8, pp. 873–82, Apr. 2011.

[2]   R.-J. Raterink, F. M. Kloet, J. Li, N. A. Wattel, M. J. M. Schaaf, H. P. Spaink, R. Berger, R. J. Vreeken, and T. Hankemeier, "Rapid metabolic screening of early zebrafish embryogenesis based on direct infusion-nanoESI-FTMS," Metabolomics, vol. 9, no. 4, pp. 864–873, Jan. 2013.

[3]   A. Furey, M. Moriarty, V. Bane, B. Kinsella, and M. Lehane, "Ion suppression; a critical review on causes, evaluation, prevention and applications.," Talanta, vol. 115, pp. 104–22, Oct. 2013.

[4]   A. Schmidt, M. Karas, and T. Dülcks, "Effect of different solution flow rates on analyte ion s ignals in nano-ESI MS, or: when does ESI turn into nano-ESI?," J. Am. Soc. Mass Spectrom., vol. 14, no. 5, pp. 492–500, May 2003.

[5]   N. Y. Ashri and M. Abdel-Rehim, "Sample treatment based on extraction techniques in biological matrices.," Bioanalysis, vol. 3, no. 17, pp. 2003–18, Sep. 2011.

[6]   A. Spietelun, Ł. Marcinkowski, M. de la Guardia, and J. Namieśnik, "Recent developments and future trends in solid phase microextraction techniques towards green analytical chemistry.," J. Chromatogr. A, vol. 1321, pp. 1–13, Dec. 2013.

[7]   A. Sarafraz-Yazdi and A. Amiri, "Liquid-phase microextraction," TrAC Trends Anal. Chem., vol. 29, no. 1, pp. 1–14, 2010.

[8]   L. Nováková and H. Vlcková, "A review of current trends and advances in modern bio-analytical methods: chromatography and sample preparation.," Anal. Chim. Acta, vol. 656, no. 1–2, pp. 8–35, Dec. 2009.

[9]   J. Pan, C. Zhang, Z. Zhang, and G. Li, "Review of online coupling of sample preparation techniques with liquid chromatography.," Anal. Chim. Acta, vol. 815C, pp. 1–15, Mar. 2014.

[10]   D. A. Lambropoulou and T. A. Albanis, "Liquid-phase micro-extraction techniques in pesticide residue analysis.," J. Biochem. Biophys. Methods, vol. 70, no. 2, pp. 195–228, Mar. 2007.

[11]   M. A. Jeannot and F. F. Cantwell, "Solvent microextraction into a single drop.," Anal. Chem. , vol. 68, no. 13, pp. 2236–40, Jul. 1996.

[12]   R. Lucena, M. Cruz-Vera, S. Cárdenas, and M. Valcárcel, "Liquid-phase microextraction in bioanalytical sample preparation.," Bioanalysis, vol. 1, no. 1, pp. 135–49, Apr. 2009.

[13]   M. A. Jeannot, A. Przyjazny, and J. M. Kokosa, "Single drop microextraction--development, applications and future trends.," J. Chromatogr. A, vol. 1217, no. 16, pp. 2326–36, Apr. 2010.

[14]   A. Tankeviciute, R. Kazlauskas, and V. Vickackaite, "Headspace extraction of alcohols into a single drop," Analyst, vol. 126, no. 10, pp. 1674–1677, Oct. 2001.

[15]   S. Pedersen-Bjergaard and K. E. Rasmussen, "Liquid-liquid-liquid microextraction for sample preparation of biological fluids prior to capillary electrophoresis.," Anal. Chem., vol. 71, no. 14, pp. 2650–6, Jul. 1999.

[16]   G. Borijihan, Y. Li, J. Gao, and J. J. Bao, "Development of a novel 96-well format for liquid-liquid microextraction and its application in the HPLC analysis of biological samples.," J. Sep. Sci., vol. 37, no. 9–10, pp. 1155–61, May 2014.

[17]   W. Liu and H. Lee, "Continuous-flow microextraction exceeding 1000-fold concentration of dilute analytes," Anal. Chem., vol. 72, no. 18, pp. 4462–7, Sep. 2000.

Chapter 5

Gas pressure assisted micro-liquid-liquid extraction coupled online to direct infusion mass spectrometry: a new automated screening platform for bioanalysis.

| 75

[18]   X. Jiang and H. K. Lee, "Solvent bar microextraction.," Anal. Chem., vol. 76, no. 18, pp. 5591–6, Sep. 2004.

[19]   H. Ebrahimzadeh, F. Mirbabaei, A. A. Asgharinezhad, N. Shekari, and N. Mollazadeh, "Optimization of solvent bar microextraction combined with gas chromatography for preconcentration and determination of methadone in human urine and plasma samples.," J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci., vol. 947–948, pp. 75–82, Feb. 2014.

[20]   L. Guo and H. K. Lee, "Automated Dispersive Liquid-Liquid Microextraction–Gas Chromatography–Mass Spectrometry," Anal. Chem., p. 140325105025000, Mar. 2014.

[21]   M.-I. Leong, M.-R. Fuh, and S.-D. Huang, "Beyond dispersive liquid-liquid microextraction.," J. Chromatogr. A, vol. 1335, pp. 2–14, Mar. 2014.

[22]   A. Gjelstad, K. E. Rasmussen, M. P. Parmer, and S. Pedersen-Bjergaard, "Parallel artificial liquid membrane extraction: micro-scale liquid-liquid-liquid extraction in the 96-well format.," Bioanalysis, vol. 5, no. 11, pp. 1377–85, Jun. 2013.

[23]   A. Spietelun, L. Marcinkowski, M. de la Guardia, and J. Namieśnik, "Green aspects, developments and perspectives of liquid phase microextraction techniques." Talanta, vol. 119, pp. 34–45, Feb. 2014.

[24]   E. G. Bligh and W. J. Dyer, "A rapid method of total lipid extraction and purification.," Can. J. Biochem. Physiol., vol. 37, no. 8, pp. 911–7, Aug. 1959.

[25]   "DrugBank 4.0: shedding new light on drug metabolism.," Nucleic Acids Res., vol. 42, no. Database issue, pp. D1091–7, Jan. 2014.

[26]   V. Matyash, G. Liebisch, T. V Kurzchalia, A. Shevchenko, and D. Schwudke, "Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics," J. Lipid Res., vol. 49, no. 5, pp. 1137–46, May 2008.

[27]   K. A. Collinsworth, S. M. Kalman, and D. C. Harrison, "The Clinical Pharmacology of Lidocaine as an Antiarrhythmic Drug," Circulation, vol. 50, no. 6, pp. 1217–1230, Dec. 1974.

SUPPORTING INFORMATION

Supporting Table 1: Drugs with varying mass, log P and pKa values and the used internal standards. (* data from [25])

drug	Log P*	pKa*	MW
lidocaine	2.4	8.0	234.3
proppranolol	3.5	9.4	259.3
verapamil	3.8	8.9	454.6
loperamide	5.5	9.4	477.0
chlorpromazine	5.4	9.3	318.1
diltiazem	2.8	8.2	414.2
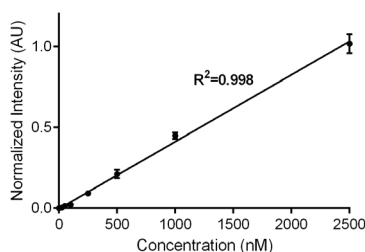
Supporting Figure 1 : Calibration curve of lidocaine spiked to whole blood (undiluted) and applied as DBS, analyzed with GPA-µLLE-DI-MS. A linear regression line is fitted through the average (n=4) of the normalized response; error bars are expressed as the S.E.M.