Division for Chemistry
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New Techniques for Sample Preparation in Analytical Chemistry

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Abstract

Sample preparation is often a bottleneck in systems for chemical analysis. The aim of this work was to investigate and develop new techniques to address some of the shortcomings of current sample preparation methods. The goal has been to provide full automation, on-line coupling to detection systems, short sample preparation times and high-throughput.

A new technique for sample preparation that can be connected on-line to liquid chromatography (LC) and gas chromatography (GC) has been developed. Microextraction in packed syringe (MEPS) is a new solid-phase extraction (SPE) technique that is miniaturized and can be fully automated. In MEPS approximately 1 mg of sorbent material is inserted into a gas tight syringe (100-250 μL) as a plug. Sample preparation takes place on the packed bed. Evaluation of the technique was done by the determination of local anaesthetics in human plasma samples using MEPS on-line with LC and tandem mass spectrometry (MS-MS). MEPS connected to an autosampler was fully automated and clean-up of the samples took one minute. In addition, in the case of plasma samples the same plug of sorbent could be used for about 100 extractions before it was discarded.

A further aim of this work was to increase sample preparation throughput. To do that disposable pipette tips were packed with a plug of porous polymer monoliths as sample adsorbent and were then used in connection with 96-well plates and LC-MS-MS. When roscovitine in human plasma and water samples was used as model substance, a 96-plate was handled in two minutes.

List of Abbreviations

ACN	Acetonitrile
AIBN	Azobisisobutyronitrile
ATP	Adenosine Triphosphate
BMA	Butyl Methacrylate
C_2	Ethyl Silica
C_8	Octyl Silica
C_{18}	Octadecyl Silica
Cdks	Cyclin Dependent Kinases
CEC	Capillary Electrochromatography
DNA	Deoxyribonucleic Acid
EGDMA	Ethylene Glycol Dimethacrylate
ESI	Electrospray Ionization
GC	Gas Chromatography
GMA	Glycidyl Methacrylate
I.D.	Inner Diameter
I.S.	Internal Standard
k´	Retention Factor
LC	Liquid Chromatography
LLE	Liquid-Liquid Extraction
MEPS	Micro Extraction in Packed Syringe
MeOH	Methanol
MS	Mass Spectrometry
MS-MS	Tandem Mass Spectrometry
PDMS	Polydimethylsiloxane
RNA	Ribonucleic Acid
RP-LC	Reversed-Phase Liquid Chromatography
SBSE	Stir Bar Sorbtive Extraction
SPDE	Solid-Phase Dynamic Extraction
SPE	Solid-Phase Extraction
SPME	Solid-Phase Microextraction
SRM	Selected Reaction Monitoring
t_0	Retention Time Unretained Analyte
t_r	Retention Time Retained Analyte
UV	Ultra Violet

List of Papers

Paper I

Microextraction in Packed Syringe (MEPS) for Liquid and Gas Chromatography Applications. Part II - Determination of Ropivacaine and its Metabolites in Human Plasma Samples Using MEPS with Liquid Chromatography/Tandem Mass Spectrometry.
Mohamed Abdel-Rehim, Zeki Altun and Lars G. Blomberg, *J. Mass Spectrom.* 39 (2004) 1488-1493.

Paper II

New Trends in Sample Preparation: On-line Microextraction in Packed Syringe (MEPS) for LC and GC Applications. Part III - Determination and Validation of Local Anaesthetics in Human Plasma Samples Using a Cation-exchange Sorbent, and MEPS-LC-MS-MS.
Zeki Altun, Mohamed Abdel-Rehim and Lars G. Blomberg, *J. Chromatogr.* A 813 (2004) 129-135.

Paper III

Increasing Sample Preparation Throughput Using Monolithic Methacrylate Polymer as Packing Material for 96-Tips: 2 Minutes per 96-Well Plate.
Zeki Altun, Lars G. Blomberg and Mohamed Abdel-Rehim, Submitted to *J. Chromatogr. B* (2005).

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1 Introduction

1.1 Sample Preparation

In cases when the analytes of interest are present in complex matrices of, for example, environmental or biological origin, the sample is usually not suitable for direct introduction into the analytical instruments. Then sample preparation becomes an important step in the analytical process. Sample preparation is the series of steps required to transform a sample to a form suitable for analysis. The faster this procedure can be done, the more quickly the analysis will be completed. This procedure must be highly reproducible and without appreciable loss of the analytes.

An ideal sample preparation method should involve a minimum number of working steps, be easy to learn, be environmentally friendly and be economical [1]. Further, as the number of samples grows high-throughput and fully automated analytical techniques becomes required. Commonly used sample preparation methods include liquid-liquid extraction (LLE), solid-phase extraction (SPE) and solid-phase microextraction (SPME).

1.1.1 Solid-Phase Extraction (SPE)

Liquid-solid extraction or, as it is often called solid-phase extraction (SPE) is the method used for concentration and isolation of target analytes using a solid support. SPE is today the most commonly used sample preparation method in many areas of chemistry including clinical, environmental and pharmaceutical applications [2]. SPE was initially developed as complement or replacement for LLE. Some problems related to LLE are that it is labor intensive, difficult to automate and consumes relatively large volumes of high-purity solvents [2-4].

The first analytical application of SPE started to the best of my knowledge in the early 1950s [5]. Using an iron cylinder packed with 1200-1500 g of granular activated carbon, Braus et al. isolated organic material samples in six water plants on the Ohio River [6]. The study was performed for the determination of causes to tastes and odours in these waters. Since then an increased development of SPE has occurred with new formats and new phases with different chemistries [3]. The cartridge formats are today the most popular formats [2]. A typical construction of the cartridge device is shown in Figure 1.

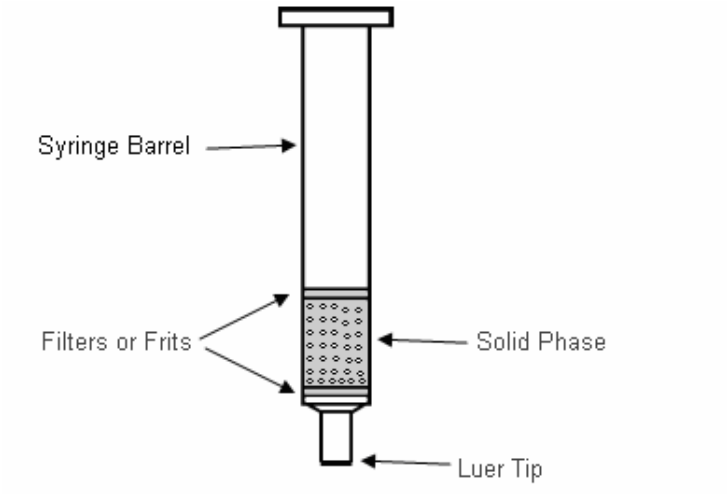


Figure 1. Schematic diagram showing a typical SPE cartridge.

The device consists of an open syringe barrel containing a sorbent packed between frits. SPE cartridges are available in sizes containing from 35 mg to 10 g of sorbent with the 100 mg to 500 mg sorbent cartridges most widely used. The most commonly used packing materials are silica-based with chemically bonded materials and highly cross-linked polymers such as styrene-divinylbenzene and polymethacrylates.
Further, to eliminate causes of memory effects the SPE cartridges are used only once, therefore the sorbent has to be cheap and thus of relatively low quality.

1.1.1.1 Sample Processing in SPE

A typical solid-phase extraction involves four processing steps, Figure 2. In the first step the sorbent is conditioned with 3-5 bed volumes of a solvent to remove impurities and ensure reproducible retention of analytes. The second step is application of the sample solution through the extraction device. This step is followed by rinsing and cleaning of the sorbent from interferences without losing the analytes. Finally, the analytes of interest are eluted from the sorbent using a strong solvent [2, 5, 7].

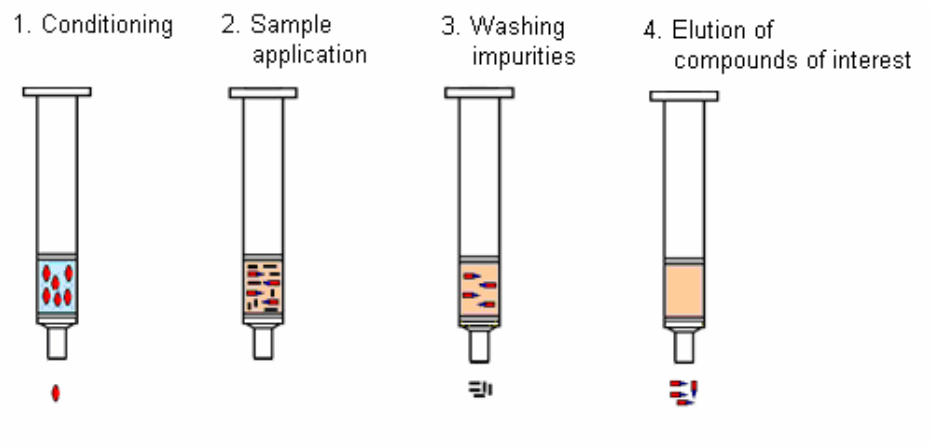


Figure 2. The four processing steps in the operation of SPE experiment.

The parameters describing the processing steps in SPE are according to the theoretical principles of liquid chromatography (LC) [3, 5, 7].

1.1.1.2 Compounds Retention in SPE

In SPE separation is based on the selective distribution of analytes between the solid packing material and liquid mobile phase. There are different SPE selectivities and the classification of these is based on the type of distribution applied in the extraction [2]. The dominating selectivity is reversed-phase SPE. Here, the stationary phase is usually silica spheres with chemically bonded alkyl and aryl functional groups onto the surface. C_{18} silica dominates but C_8 silica is also used extensively. The packing material in LC (see 1.5) and SPE are basically the same except that the spheres are larger in SPE. Most organic solvents will flow through the sorbent by gravity, but for aqueous and other viscous samples a slight vacuum is commonly employed. The retention of analytes onto these sorbents is due primarily to hydrophobic interactions. To elute analytes from these sorbents non-polar solvents are used.

For analytes that are charged when in solution ion exchange SPE can be used. The retention mechanism is primarily based on electrostatic attraction between charged analytes and charged functional groups on the surface of the sorbent. To elute analytes of interest, a solution having a pH that either neutralizes the functional group of the sorbent or neutralizes the charged analytes is used. Alternatively, an elution solvent with high ionic strength is used to elute the compounds.

1.2 Trends in Sample Preparation

Recent trends in the sample preparation area focus on how to miniaturize the process, increase the sample throughput, use selective sorbents and on-line couple the sample preparation units to detection systems [2, 5, 7-10].

The first attempts to miniaturize the process and provide high sample throughput were done with the introduction of new formats such as SPE disks [11], pipette tips [11-12], column switching systems [2, 13-16] and multi-well plates [17]. Further, miniaturization resulted in development of new microextraction techniques. Some examples of emerging new techniques will be given here.

Solid-phase microextraction (SPME) is presently the most commonly used microextraction technique [18-21]. In SPME a fused-silica fibre coated on the outside with an appropriate stationary phase is used for sampling. When the stationary phase is placed in contact with the sample matrix a partitioning of the analytes between the phases takes place. The fibre is then placed in the inlet of a GC injector for direct desorbtion. The extraction efficiency of SPME depends on a number of factors such as extraction time, agitation, sample pH, salt concentration and temperature. SPME enables extraction and pre-concentration of analytes from gaseous, liquid and solid samples.

Solid-phase dynamic extraction (SPDE) is a technique which utilizes a stainless steel needle of a gas tight syringe for sampling [22-23]. The inside wall of the needle is coated with polydimethylsiloxane (PDMA) and 10% activated carbon as stationary phase. The dynamic sampling is performed by pulling and pushing a fixed volume of the sample for an appropriate number of times. The adsorbed analytes are then recovered by heat desorption into a GC injector. The technique can be used for vapour and liquid samples.

Another technique that is used to extract analytes from liquid samples is stir bar sorptive extraction (SBSE) [24-25]. A magnetic stir bar coated with PDMS is used as stationary phase for sampling. Basically, the sample poured into a vial is stirred with stir bar for a certain time and then the stir bar is placed in an empty glass tube for thermal desorption.

The extraction principles of these techniques, SPME, SPDE and SBSE, are identical and they utilize the same extraction medium, PDMS, but the amount is different. SBSE uses 50 to 250 times more sorbent then SPME [25].

In addition to these emerging new techniques, a large number of non-selective and selective sorbents has been developed to compensate some of the drawbacks of silica based materials, e.g. some irreversible adsorption of basic analytes [1].

1.3 Microextraction in Packed Syringe (MEPS)

Microextraction in packed syringe (MEPS) is a new sample preparation technique that uses a gas tight syringe as extraction device [26-27]. In MEPS the sorbent material, about 1 mg, is either inserted into the syringe barrel as a plug with polyethylene filters on both sides, or between the syringe barrel and the needle, Figure 3.

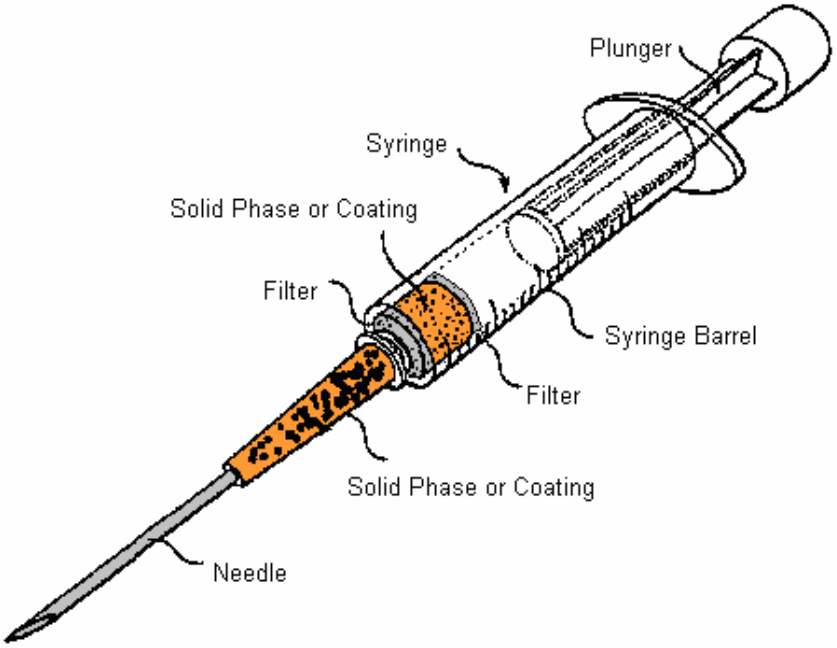


Figure 3. Schematic picture of microextraction in packed syringe (MEPS).

The sample processing steps in MEPS are similar to those of SPE. Basically, after conditioning of the sorbent with an appropriate solvent(s), the sample solution is drawn through the needle into the syringe up and down once or several times. This is followed by a washing step to remove interferences and, finally analytes of interest are extracted directly into the LC or GC injector. MEPS can be connected on-line to LC or GC without any modification of the instrument and the method can be fully automated.

In MEPS any sorbent material can be used either as packing bed or as coating. Compared to above mentioned microextrection techniques which use PDMS as sorbent in most of the cases, MEPS has a big advantage, because it can be used with a wide range of available sorbents.

1.4 Methacrylate Based Porous Polymer Monoliths

The preparation of porous polymer matrixes and their use in chromatographic separation was published for the first time by Kubín et al. cited in Ref. 28. Since then such monolithic supports have been used in many chromatographic areas including GC, LC, CEC, SPE and microfluidic devices.
Methacrylate monoliths possess several advantages that make them well-suited as separation media. They do not require frits, they can easily be fabricated thermally or using ultraviolet light and they can be made from a large number of different monomers. In addition, they have favourable hydrodynamic properties that allow high flow-rates under low pressures and they are stable under wide pH-ranges.

The polymerization mixture of methacrylate based monoliths consists of the monomers, a cross-linker and an initiator in the presence of a combination of porogenic solvents. The preparation procedure is simple and straightforward. Basically, after mixing, the polymerization mixture is degassed using nitrogen gas in order to remove oxygen and poured into the chromatographic device for polymerization, thermally or under UV-light.
Before use, the monolithic material is washed with an organic solvent to remove possible unreacted compounds.

There are a number of factors affecting the porous properties of the monoliths. The factors to be considered are the composition and amount of the porogenic solvents, the cross-linker, the initiator and the temperature or the intensity of the UV-light [29-32].

1.5 Liquid Chromatography (LC)

Liquid chromatography (LC) is presently the dominating separation technique in analytical chemistry [33]. The separation mechanisms and classification principles in LC and SPE are essentially the same (see 1.1.1.2). The technique was invented by Michail Semenovich Tswett (1872-1919) in his investigation of plant extracts [34]. The dominating LC technique is reversed-phase liquid chromatography (RP-LC). In this technique the stationary phase is usually silica spheres with hydrophobic surfaces, and the mobile phase is hydrophilic and is often prepared as a buffer for stable pH. The commonly used silica spheres have particle sizes of 3-10 μm, depending on the application. A basic LC apparatus is shown in Figure 4.

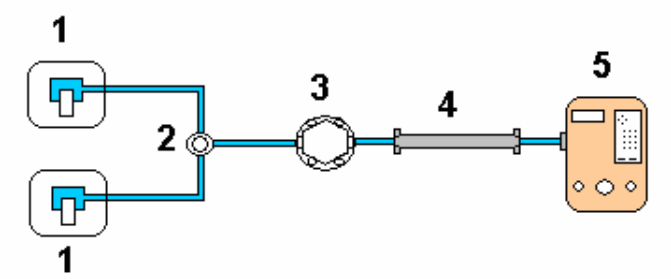


Figure 4. Schematic setup of an LC system. 1. Pump, 2. Mobile Phase Mixer, 3. Injector Valve, 4. Column and 5. Detector.

The sample is introduced on the top of the column by the injector. The pump(s) create a flow of mobile phase through the column. The analytes move with different rates through the column and elute one after another from the column. The average rate at which the analytes migrate, depends on the fraction of time spent in the stationary phase, and thus on the affinity of the analytes to the stationary phase. The retention is expressed by the retention factor k´:

$$k' = \frac{t_r - t_0}{t_0} \qquad (1)$$

where t_0 is the retention time for an unretained analyte and t_r is the retention time for a retained analyte.

1.6 Mass Spectrometry (MS)

Mass spectrometry (MS) was discovered by the physicist J. J. Thomson (1856-1940) [35]. MS is today one of the most important analytical instruments for molecular analysis. The basis in MS instruments is the production of ions and separation or filtration according to their mass-to-charge (m/z) ratios under vacuum [36]. From a mass spectrum qualitative as well as quantitative information can be obtained.

Combining LC instrument with MS was considered as an "unnatural marriage" [37]. LC operates in condensed phase while MS operates under vacuum. Because of this difference, care must be taken when integrating these two instruments. In order to maintain this integrity, the solvent molecules must be evaporated and targeted analytes must be transferred into the gas phase. The ionization technique used in this study was electrospray ionization (ESI), operated in positive ion mode. The solvent from the LC was passed along a stainless steel capillary tube, to the end of which a positive electrical potential (3-5 kV) was applied. The electrical field causes the solution to be vaporized into a spray of fine droplets. To assist this, a drying gas nitrogen (N_2) flows along and post the capillary. Before entering the mass spectrometer probe these

droplets passes through a slightly heated vacuum tube where the solvent evaporates and droplets get smaller. As the droplets get smaller the electrical charge density increases until such that neutral molecules are released from the surface. Finally, the remained sample ions enter the analyser where their mass to charge ratios can be measured [37-38].

In this study a triple quadrupole mass spectrometer (MS-MS) was used to obtain desired information about targeted analytes. As the targeted analytes from the chromatographic source enter the MS-MS, the first quadrupole (Q1) was focused on parent ions of the selected target analytes, Figure 5. In the second quadrupole (Q2), using argon as collision gas, these ions were fragmented into lower (m/z) ratio product ions. These product ions were then accelerated into the third quadrupole (Q3), where only one characteristic product ion from each targeted analyte was monitored. This technique is called selected reaction monitoring (SRM). Because each analyte has unique SRM pattern, each analyte can be quantitatively analysed without chromatographically being separated from each other [37-39]. This technique was necessary in quantitative analysis in this study, when no column was used.

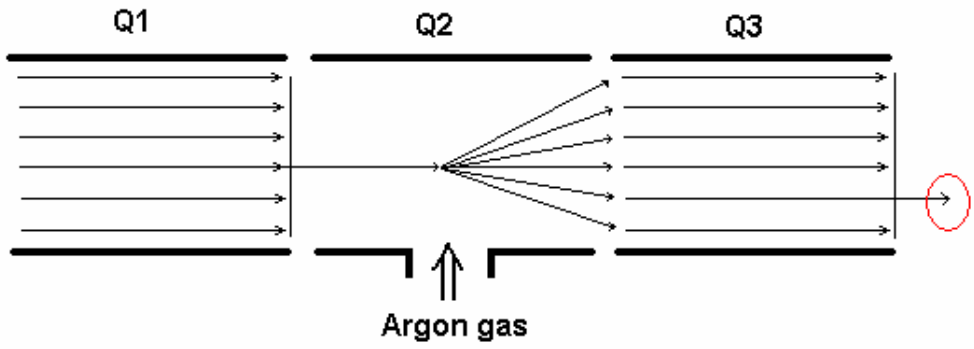


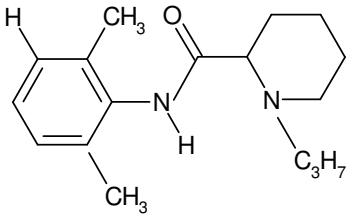
Figure 5. Schematic presentation of how SRM ion experiments in MS-MS are carried out.

1.7 Samples

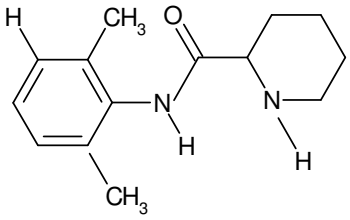
1.7.1 Local Anaesthetics

Ropivacaine, lidocaine and bupivacaine are basic, amide type local anaesthetic drugs, see Figure 6. Ropivacaine and bupivacaine are mainly used for surgery and for postoperative pain relief [40]. Unlike, bupivacaine which is used as racemic mixture, ropivacaine is exclusively the S-(-)-enantiomer. Ropivacaine has a lower central nervous and cardiotoxic potential than bupivacaine [41]. Lidocaine has antiarrhythmic effects and is used in the treatment of cardiac disorders [40].

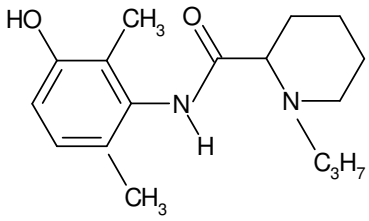
Ropivacaine is metabolized before being excreted, mainly in the liver [42]. The metabolic pathways include aromatic hydroxylation and N-dealkylation [43]. The major metabolites of ropivacaine are PPX and 3-OH-ropivacaine, Figure 6.



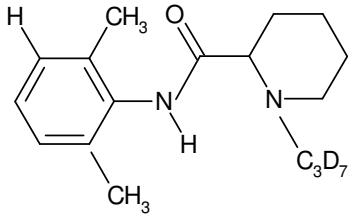
Ropivacaine, Mw 274 g/mol



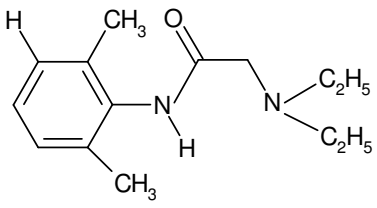
PPX, Mw 232 g/mol



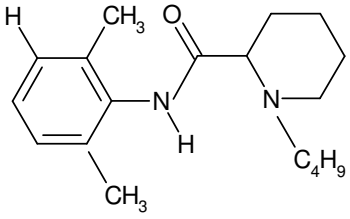
3-OH-ropivacaine, Mw 290 g/mol



$^{2}H_{7}$-ropivacaine, Mw 281 g/mol



Lidocaine, Mw 234 g/mol

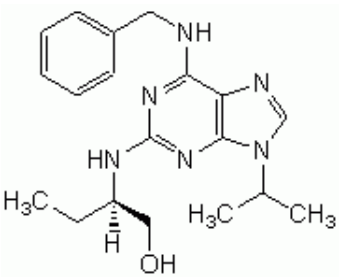


Bupivacaine, Mw 288 g/mol

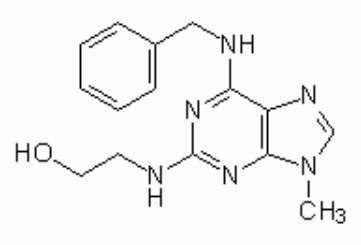
Figure 6. Structures and molecular weights of local anaesthetics utilized in this work.

1.7.2 Roscovitine and Olomoucine

Roscovitine and olomoucine, Figure 7, are purine derivatives considered as possible new anti-cancer drugs [44-45]. The drugs selectively inhibit cyclin-dependent kinases (Cdks), which are enzymes that play a crucial role in cell cycle regulation and several vital cell processes. The cellular effects of these drugs include inhibition of cell proliferation, induction of DNA fragmentation, inhibition of RNA and DNA synthesis, cell cycle arrest in S-phase, induction of apoptosis and as competitive inhibitor for ATP [45-50].



Roscovitine, Mw 354 g/mol



Olomoucine (I.S), Mw 298 g/mol

Figure 7. The structures and molecular weights of roscovitine and olomoucine (internal standard).

2 The Aim of This Work

Sample preparation is often a bottleneck in a system for chemical analysis. The aim of this work has been to investigate and develop new techniques to address this problem. The importance of these new techniques has been to provide full automation, on-line coupling to detection systems, short sample preparation time and high-throughput.
The aim has been to apply and evaluate microextraction in packed syringe (MEPS) as a new sample preparation technique. Evaluation of the technique has been done by the analysis of local anaesthetics. A further aim has been to prepare and evaluate pipette tips packed with porous monolithic polymers in connection with 96-well sample plates.

3 Results and Discussions

3.1 MEPS Considerations

In papers I and II MEPS was performed using a 250 µl gas tight syringe. The sorbent in the form of particles was weighted and inserted into the syringe barrel as a plug and tightened with a filter from both sides. Before using for the first time, the sorbent was manually conditioned with 50 µl methanol followed by 50 µl of water. After that, the syringe was connected to an autosampler and spiked plasma sample (25 µl) was drawn onto the syringe by the autosampler. It is important that plasma samples are drawn slowly (20 μLs^{-1}) and with caution to obtain good percolation between sample and solid support. The flow rate also can affect the retention of the analytes. The sorbent was then flushed with washing solvent to remove interferences. The targeted analytes were after that desorbed by an elution solvent directly into a gate valve, which was situated between the liquid chromatograph and the tandem mass spectrometer.

In MEPS, many extractions were performed with the same plug of sorbent. To be able to do that, the sorbent was flushed with 5x50 µl elution solvent followed by 5x50 µl of the washing solvent between every extraction. This step decreased memory effects, but also functioned as conditioning step before the next extraction. In the case of plasma samples the same plug of sorbent was used for about 100 extractions. After that the extraction efficiency and the recovery of the sorbent was reduced, and therefore it was discarded.

To measure analyte retention when using MEPS the recovery was defined. The recovery was measured as response of a processed spiked plasma samples as percentage of pure standard solution.

3.2 Method Development (Papers I and II)

To optimize the recovery for the selected analytes, some parameters affecting the recovery were determined. These were type and amount of sorbent, the composition and volume of the washing solution and composition and amount of the elution solution. Further, good understanding of the interactions between the analyte, the matrix and the sorbent was necessary for optimization of the extraction process.

3.2.1 The Sorbent

In the papers I and II the studied substances are amide type local anaesthetics, weakly basic, and with pK_a-values between 7.0 and 8.1. When these drugs are in plasma samples they may be protein-bounded which reduces the recovery. To disrupt these bindings the pH of the samples was shifted (pH~3) using 0.1% formic acid. In such a pH the studied analytes are positively charged. Thus, when the type of sorbent was selected both ion-exchange and hydrophobic interactions were considered. Sorbent containing strong cation-exchange and non-polar functional groups was targeted for the extraction of these analytes.

In the first paper different silicas (C_2, C_8 and C_{18}) and a hydroxylated polystyrene-divinylbenzene polymer (ENV+) were investigated, see Figure 8. The silica sorbents have particles with an average size of 50 μm and polymer particles were 90 μm [51]. In LC, it is well known that the hydrophobic retention depends on the length of the carbon chain as well as the number of carbon chains bonded at the surface of the silica spheres. An increase of both these factors increases the hydrophobicity. Many carbon chains bonded at the silica surface also increases the specific surface area of the sorbent. In addition, there are residual silanols at the surface of the utilized silicas in this work. Because these silanol groups were not totally shielded from the analytes, in addition to hydrophobic interactions, ionic interactions can occur when analytes are positively charged. As can be seen from Figure 8, different silicas (C_2, C_8 and C_{18}) provided higher sample recovery than the polymer (ENV+). Further, the recovery increased with decreased length of the carbon chain at the silica surface. The highest recovery was obtained when silica based C_2 was used as sorbent material. This, probably because the targeted analytes were slightly polar and were made positively charged in plasma matrices.

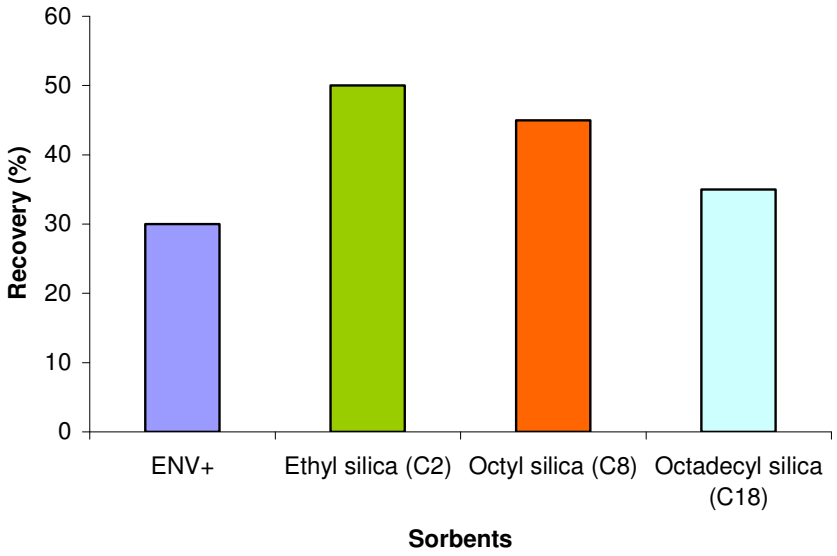


Figure 8. Effect of type of sorbents on the recovery of ropivacaine.

In paper II, a silica based benzenesulphonic acid was utilized as sorbent material. This sorbent is strongly acidic (pKa<1) and thereby charged over the entire pH range. The primary retention on this sorbent is due to strong cation exchange, but there are also other interactions such as non-polar interactions. When the pH of the sample matrix was low, the targeted local anaesthetics were positively charged and the sorbent material was negatively charged. In such case the analytes were adsorbed to the sulphonic functional groups at the surface of sorbent the material mainly due ionic interactions. To break these interactions the analytes were made neutral by using an elution solvent with high pH-value (pH~11).

To achieve acceptable recovery and eliminate carry-over (see 3.3), the amount of sorbent was optimized in relation to the nature and amount of sample, the washing solvent and elution solvent. As can be seen from Figures 9 (paper I) and 10 (paper II), using an amount of 0.5 mg sorbent material, recovery was lower than with 1 mg. This was probably due to insufficient adsorption capacity of the sorbent. Also when 2 mg of packing bed was used the recovery decreased. The reason for this could be that a larger volume of the elution solvent was needed for desorption of the analytes. The smallest amount sorbent at which acceptable recovery (~50 %) was obtained was 1 mg when 25 µL sample was extracted, Figures 9 and 10. This amount of sorbent was suitable for a concentration range of 2-5000 nM of the test analytes. For higher concentrations the amount of sorbent should be increased.

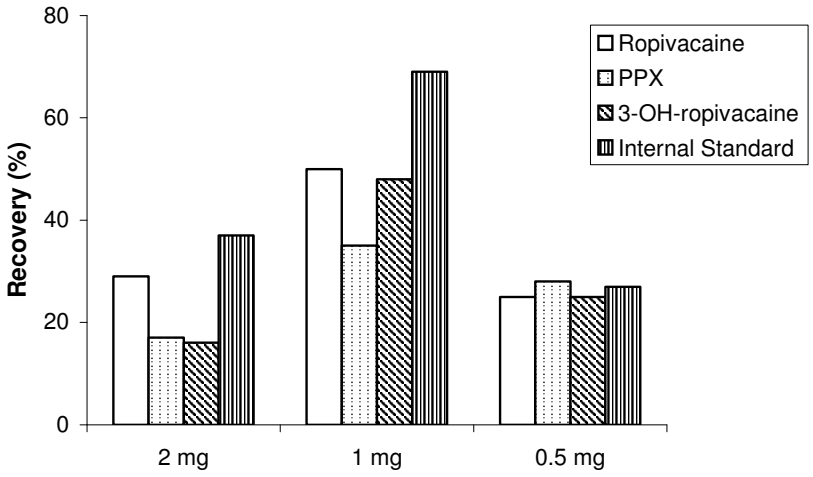


Figure 9. Effect of amount of sorbent, C_2, on the recovery of ropivacaine and its metabolites PPX and 3-OH-ropivacaine compared to direct injection of pure standard solutions.

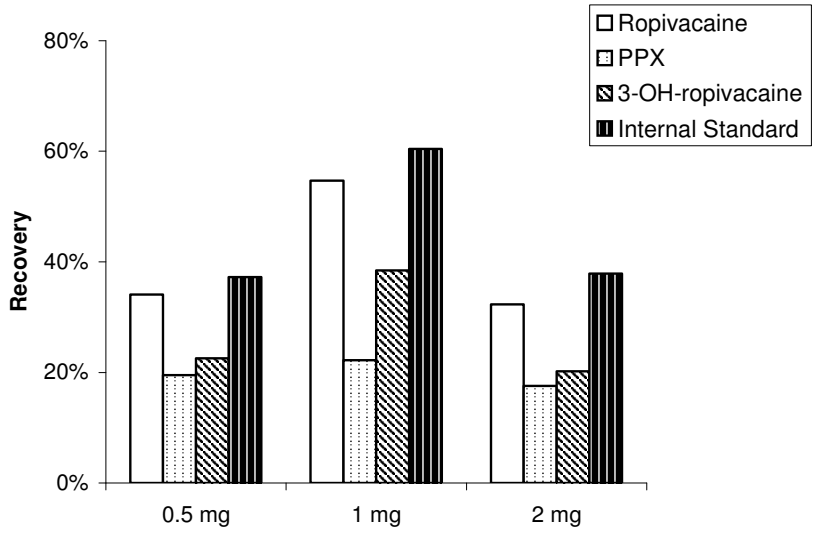


Figure 10. Effect of amount sorbent, benzenesulphonic acid, on the recovery of ropivacaine and its metabolites PPX and 3-OH-ropivacaine compared to direct injection of pure standard solutions.

3.2.2 The Washing Solvent

The purpose of the washing solvent in the MEPS process is to selectively remove unwanted compounds from the sorbent without losing the analytes. As mentioned above the analytes are weakly basic and their isolation from the plasma samples is based on ionic and non-polar interactions. In such a case, both the pH and the concentration of organic solvent will have effect on desired washing performance.

In paper I, water containing different concentration of organic solvents was tested to optimize the washing solvent. The volume of the washing solvent was 50 µL. As can be seen from Figure 11, increasing concentration of organic solvents in the washing solution decreased the recovery. The reason for this could be that the sorbent was silica based C_2, where the interactions are primarily hydrophobic. The use of 10% methanol decreased the recovery by about 10% compared to water alone.

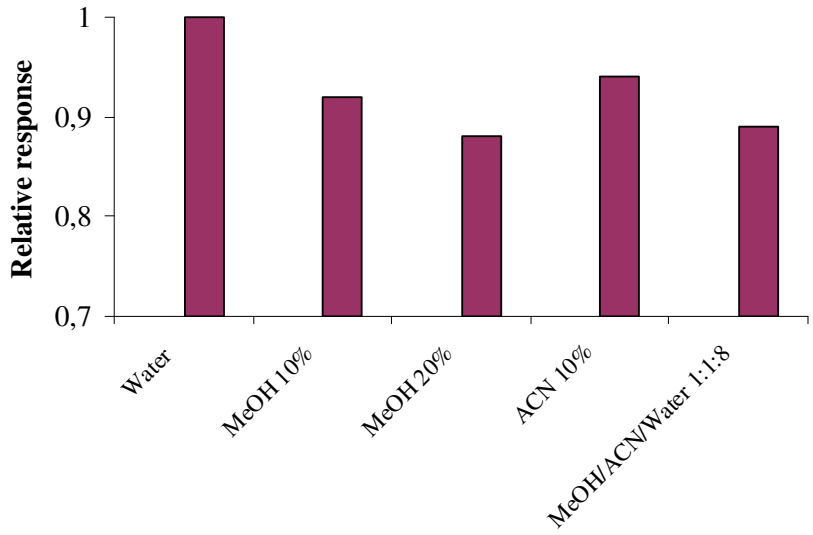


Figure 11. Effect of the washing solution on the response of ropivacaine.

In paper II, the isolation of the analytes was primarily due to ionic interactions. To prevent too high analyte losses, control of the pH, ionic strength of the washing solution as well as the concentration of organic solvent was necessary. Using a solution at low pH, the analytes remained positively charged and their interaction with the sorbent material was not interrupted. Different mixtures of water and methanol containing 0.1% formic acid were tested as washing solution. The lowest amount leakage (<0.2), with no detectable interferences and highest recovery was obtained when using 100 µL water containing 0.1% formic acid as washing solution.

3.2.3 The Elution Solvent

The elution solvent should be one which is able to displace targeted analytes from the sorbent in a minimum volume. If the retention is based on hydrophobic interactions only, a non-polar solvent would be enough to disrupt the forces that bind analytes to the sorbent. Further, in cases when there are ion exchange interactions, the pH of the elution solvent should be 2 pH units above pK_a values of the targeted analytes for their elution. In addition, in paper II the targeted analytes can be eluted by neutralization of the sorbent material or using a solution with high ionic strength. However, because ESI-MS-MS was used the later options were avoided because of the possibility for source contamination and interferences with targeted analytes when high salt concentrations are used [52].

In paper I, when silica based C_2 was used as sorbent material, besides the hydrophobic forces between the targeted analytes and the packing bed, there were also ionic interactions. Residual silanols were probably the reason for the later interactions. In paper II, the primary retention mechanism was ionic interactions and secondary interactions were hydrophobic. This means that the retention mechanisms in both cases were very similar. To elute the targeted analytes a solvent capable of breaking both hydrophobic and ionic forces was needed. As can be seen from Figure 12, to optimize the recovery different mixtures of water and methanol containing 0.25% ammonium hydroxide were tested as elution solvents. Keeping the concentration of ammonium hydroxide constant the recovery of ropivacaine increased as the concentration of methanol increased, Figure 12. Acceptable recovery (~50 %) was obtained when methanol/water 95:5 (v/v) containing 0.25% ammonium hydroxide was used. This elution solvent was used in both papers.

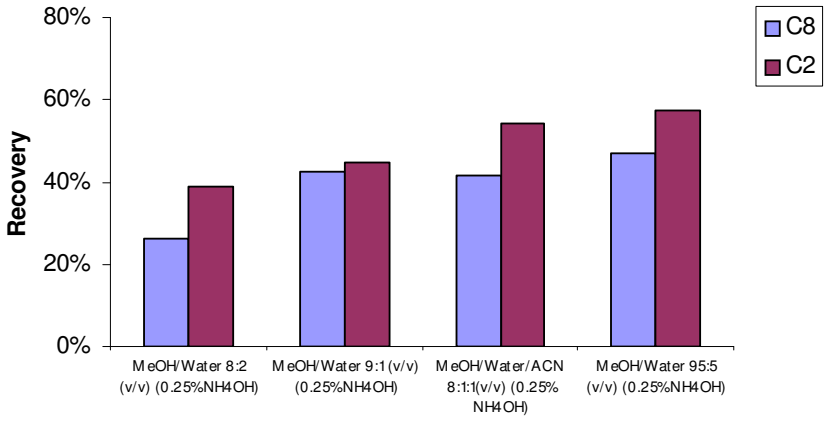


Figure 12. Effect of different elution solvents on the recovery of ropivacaine.

3.3 Carry-over

One of the limitations of automated systems is analyte carry-over. This effect depends on many factors including adsorption properties of the analytes, apparatus being employed and sensitivity of the method. At worse, carry-over can severely affect the precision and accuracy of the method. The smaller the carry-over the better the performance of the method will be [53].

In papers I and II carry-over was tested by injecting the elution solvent after the highest standard concentration (2000 nM) had been run. To eliminate carry-over the sorbent was washed between every extraction as described in section 3.1. Using this procedure less then 0.5% carry-over was observed and after an additional blank no carry over was observed. According to Rossi et al. carry-over ranging from 0.01 to 0.5% is typical for automated systems [53].

3.4 High Sample Throughput Using Monolithic Packed Tips (Paper III)

In paper III disposable pipette tips (550 μL) packed with methacrylate based monolithic sorbent in connection with 96-well plates were used for sampling. The 96-well plates were placed underneath the tips and the samples were eluted into them for further analysis by LC-MS-MS. In this way a 96-plate could be handled in two minutes.

Porous polymer monoliths as sorbent materials for high-throughput and on-line SPE has been utilized by Xie et al. [54]. Further, several groups have recently used different approaches for the fabrication of monolithic packed tips. For example, Hsu et al. [55] used photografting for the fabrication of disposable plastic pipette tips. In order to physically stabilize the adsorbent plug they inserted a 1 mm thick ring obtained from the sharp end of a pipette tip into another pipette tip in which the monolith was prepared. In this case, the polymer was not chemically bonded to the tip wall. Stachowiak et al. [56] used photografting to fabricate monoliths covalently attached to the walls of micropipette tips. The approach was made in two steps, where the first step was to modify the surface of the tip.
In contrast to above methods we developed a simple one step synthesis strategy for the fabrication of photopolymerized monolithic polymers attached to the walls of polypropylene pipette tips.

3.4.1 The Preparation of Porous Polymer Monoliths

The pipette tips were filled by capillary action to a height of about 8 mm (6-7 μL) with a polymerization mixture containing GMA (20%), EGDMA (15.5%), BMA (3.5%), AIBN (1 wt% with respect to monomers), 1-dodecanol (30%) and cyclohexanol (30%).
The pipette tips were then placed vertically inside the polymerization apparatus. The polymerization was allowed to proceed first for 60 min with the sharp end of the tip down and at a distance to the UV lamp of 15 cm, and then for 25 min with the sharp end up and at a distance of 5 cm to the UV lamp. After completion of polymerization the tips were removed, inspected under microscope for bubbles, and washed with acetone to remove the porogenic solvents and other compounds remaining in the monolith. Before use, the tips were washed with elution solvent.
For photographic image of the monolithic sorbent about 3 mm long sample from the tip was cut with a razorblade, washed thoroughly with acetone and dried with a stream of nitrogen.

3.4.2 Synthesis of Poly(GMA-EGDMA-BMA) Monoliths for Pipette Tips

The porous monolith was synthesized directly inside disposable pipette tips by *in situ* UV-light initiated polymerization. Figure 13 displays a photographic image of the monolithic structure inside a pipette tip indicating complete filling of the polymer across the tube. No significant differences and no voids between the polymer matrix and the tip can be seen. Despite, application of high pressure (up to 4 bar) the monolith did not slip out of the tip. This indicated that sufficient binding of the monolith to the plastic tube had been

achieved. Therefore, in our case, we find the modification of the surface of the tips before the polymerization given in the literature unnecessary [56]. However, we performed a set of experiments in which we used different irradiation times to examine the attachment of the monolith to the tube. We noted that when short irradiation times (less than 30 min) were used, the monolith may slip out of the tube. Possibly, there was not enough polymer in the center of the monolith. This conclusion is because the polymers attached to the walls of the tube remained inside the tube. Further, we noted that it is important to place the tips vertically inside the UV box to achieve equal irradiation.

Figure 13. A photographic image of UV-polymerized monolith inside a disposable micro pipette tip. The polymerization mixture contained GMA 20%, EGDMA 15.5%, BMA 3.5%, AIBN 1% (with respect to monomers), cyclohexanol 30% and 1-dodecanol 30%. Polymerization was allowed to proceed for 85 minutes using 254 nm light.

3.4.5 Sampling

To evaluate the performance of the monolithic tips, roscovitine in human plasma and water was used as model substance.
The manually prepared 96 pipette tips were handled by a robot, a Personal Pipettor (PP-550N-MS) obtained from Apricot Designs, Inc. (CA, USA), Figure 14. The spiked plasma or water samples (100 μL) from a 96-well plate were drawn up by the robot. When the samples were passed through the monolith polymers the analytes were adsorbed to it. Then the solid phase was washed once with water (100 μL) to remove the proteins and other interferences. Finally, the analytes were eluted with methanol (100 μL) or the LC mobile phase directly into a 96-well plate for further analysis by LC-MS-MS. Using this procedure, the cleaning of the samples from possible interferences in a 96-well plate was performed in two minutes; the speed of this part of the analytical procedure was thus greatly increased.

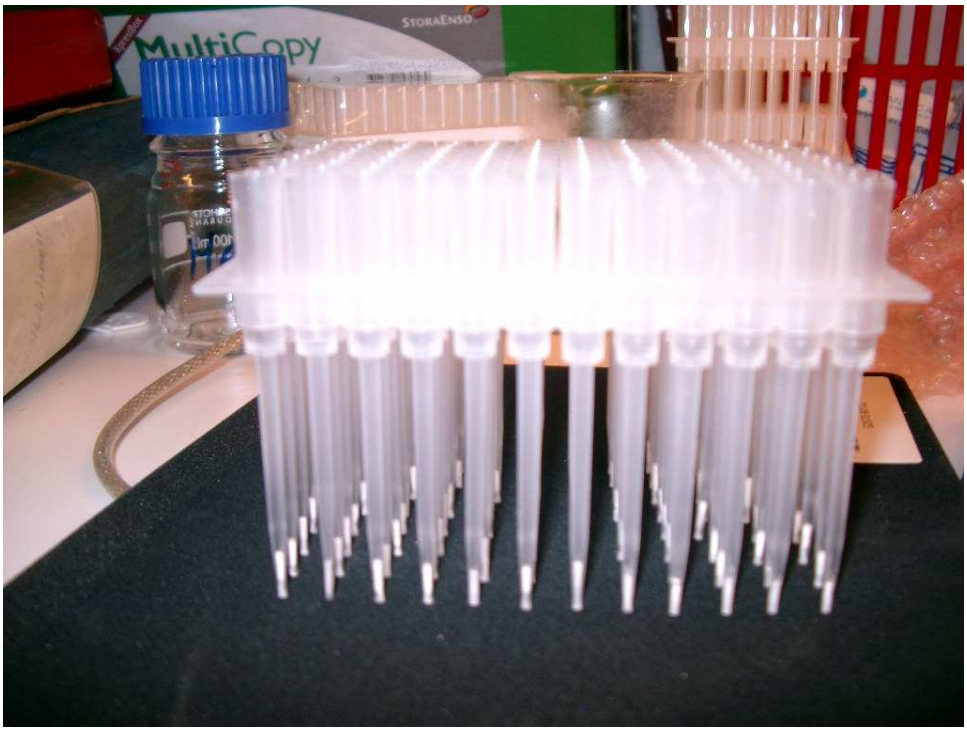


Figure 14. Packed 96-tips with monolithic methacrylate polymer.

3.4.6 Selectivity

The method selectivity is defined as non-interference with the endogenous substances in the regions of interest. Figures 15A and 15B show representative chromatograms of blank human plasma and human plasma spiked with roscovitine and olomoucine (I.S.). According to these chromatograms, in the LC-MS-MS analysis of roscovitine in human plasma using monolithic pipette tips, no interfering compounds were detected at the same retention time as the studied compounds. This indicates a good selectivity for the application of monolith containing tips as sample preparation method in the analysis of roscovitine.

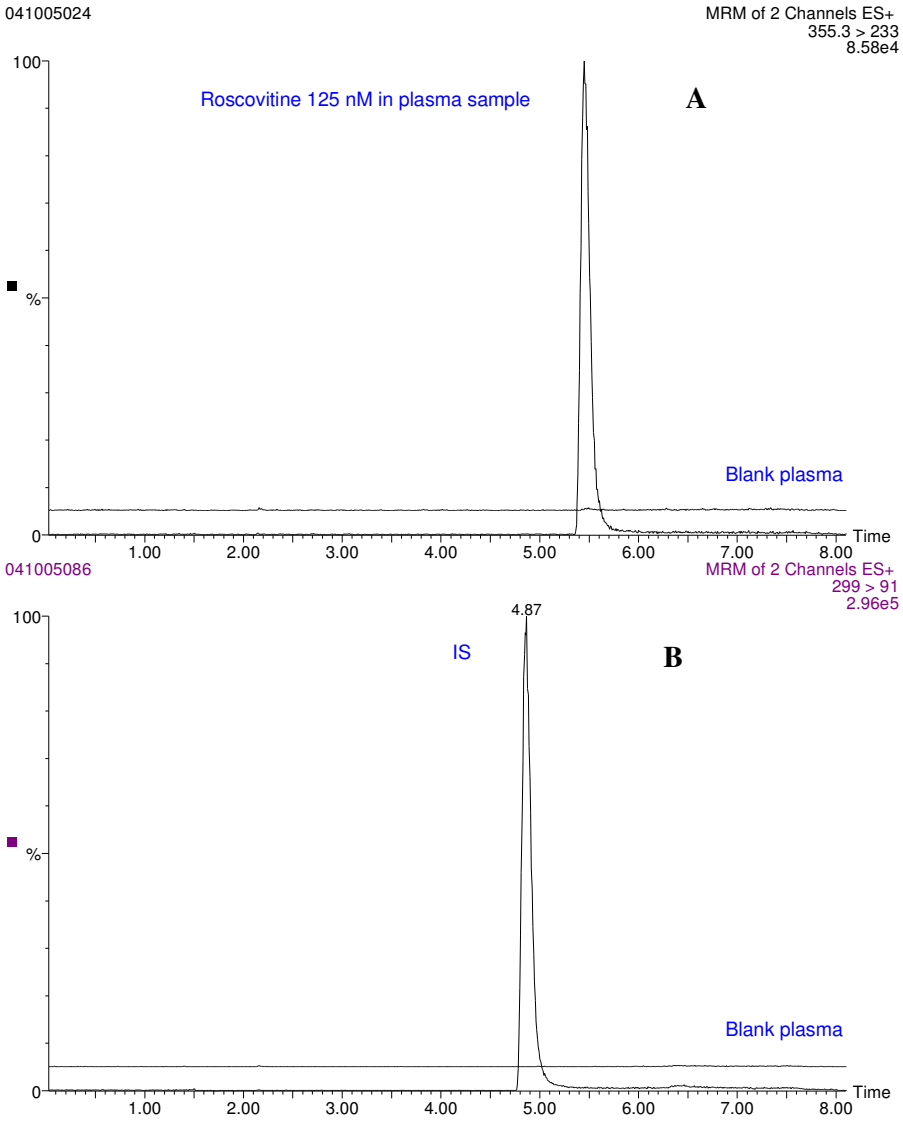


Figure 15. Chromatograms of (A) blank plasma and plasma spiked with 125 nM roscovitine and (B) blank plasma and plasma spiked with olomoucine (I.S.). Chromatographic conditions: column Zorbax 50 x 2.1 mm I.D., SB-C8, 3,5 µm connected to a guard column, an Optiguard 10 x 1 mm I.D., C8; mobile phase (A) 0.1 % formic acid in ACN-Water (10:90, v/v), (B) 0.1% formic acid in ACN-Water (80-20, v/v); linear gradient 0% B for 1 min, 0-80% B in 4 min, 80% B in 1 min; flow rate 150 µL/min.

4 Conclusions

Microextraction in packed syringe (MEPS) on-line with LC-MS-MS is an excellent sample preparation technique which was demonstrated for the determination of local anaesthetics in human plasma samples. Connected to an autosampler MEPS was fully automated and each sample took only one minute to extract. In application to plasma samples the same plug of sorbent could be used for about 100 extractions before its extraction efficiency and the recovery was reduced. Further, it was shown that MEPS can provide suitable selectivities, required small sample volumes, consumed low solvent volumes and could be used with different sorbent materials.

In addition, disposable pipette tips were packed with a plug of methacrylate based porous polymeric monoliths as sample adsorbent. Manually packed 96-tips were used in connection with a 96-well plate. Roscovitine in human plasma and in water samples was used as model substance. Using this system 96 samples were prepared for analysis in two minutes.

The present work should be considered as a step in the development of analytical systems to meet the demand for higher sample throughput than is provided today.

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New Techniques for Sample Preparation in Analytical Chemistry

Sample preparation is often a bottleneck in systems for chemical analysis. The aim of this work was to investigate and develop new techniques to address some of the shortcomings of current sample preparation methods. The goal has been to provide full automation, on-line coupling to detection systems, short sample preparation times and high-throughput.

A new technique for sample preparation that can be connected on-line to liquid chromatography (LC) and gas chromatography (GC) has been developed. Microextraction in packed syringe (MEPS) is a new solid-phase extraction (SPE) technique that is miniaturized and can be fully automated. In MEPS approximately 1 mg of sorbent material is inserted into a gas tight syringe (100-250 µL) as a plug. Sample preparation takes place on the packed bed. Evaluation of the technique was done by the determination of local anaesthetics in human plasma samples using MEPS on-line with LC and tandem mass spectrometry (MS-MS). MEPS connected to an autosampler was fully automated and clean-up of the samples took one minute. In addition, in the case of plasma samples the same plug of sorbent could be used for about 100 extractions before it was discarded.

A further aim of this work was to increase sample preparation throughput. To do that disposable pipette tips were packed with a plug of porous polymer monoliths as sample adsorbent and were then used in connection with 96-well plates and LC-MS-MS. When roscovitine in human plasma and water samples was used as model substance, a 96-plate was handled in two minutes.

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