



Monolithic packed 96-Tip robotic device for high throughput sample preparation and for handling of small sample volumes

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Abstract

In this study a rather new sample preparation technique is used; a 96-tip set packed with a short plug of a monolithic adsorbent. It is fully automated and has the possibilities to handle 96 samples in only 2 minutes

Monolithic materials can be divided into silica based and polymer based monoliths. In this study a polymer based monolith was prepared and was compared with commercial silica based tips.

The polymerization mixture consist of the monomers ethylene glycol dimethacrylate (EGDMA) (70%) and butyl methacrylate (BMA) (30%), the porogenic solvents 1-propanol (65%), 1,4-butanediol (25%) and water (10%) and the initiator benzoylperoxide (BPO) at a concentration of 1.5 wt% with relation to monomers.

The tips have a volume of 10 ml monolith mixture and polymerization was performed in situ within pipette tips that were surface modified with methanol/ benzophenone (BP) (5 wt.%).

Metoprolol and Pindolol are beta-blockers drugs that are widely used for many years. They were used for sampling in plasma and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was used for analyzing the samples.

The limit of quality control for Metoprolol and Pindolol was set to 1.0 nM after optimization with a 1.0 mm column. For quality control, the accuracy was $\pm 6\%$ and $\pm 18\%$, while the precision (RSD) was $\leq 14\%$ and $\leq 16\%$ for Metoprolol and Pindolol respectively.

For the commercial silica based tips, the accuracy was $\pm 7\%$ and ± 28 , while the precision (RSD) was $\leq 13\%$ and $\leq 16\%$ for Metoprolol and Pindolol respectively.

Calibration curves were in the range 5-5000nM and the regression correlation coefficients (R^2) were >0.99 for all the analytes in the calibration range studied.

96-tips sample preparation in blood sampling was investigated using the anti-cancer drug Cyclophosphamide and (LC-MS/MS) was used for analyzing the samples.

The limit of quality control for Cyclophosphamide was set to 10 nM. For quality control, the accuracy was $\pm 8\%$ and ± 2 , while the precision (RSD) was $\leq 16\%$ and $\leq 18\%$ for packed polymer 96-tips and the commercial silica 96-tips respectively.

Calibration curves were in the range 10-5000nM and the regression correlation coefficients (R^2) were >0.99 for all the analytes in the calibration range studied.

96-tips packed with a plug of polymer-based monoliths is a useful technique and can handle both blood and plasma samples. It provides an automated, easy, fast and low cost procedure.

This technique is on a discovery level and further optimizations are still necessary to improve the precision and accuracy.

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1 Introduction

The first appearance of the term “monolith” to describe a single piece of sponge used for protein separation emerged in 1993. A monolith is acting as a single, often rigid, uniform whole and the word monolith originates from Greek that combines two words; “mon” means single and “lith” means stone^[1].

Monolithic materials can be divided into two main categories; silica based (inorganic) and polymer based (organics) monoliths. Polymer based monoliths are ideal for high throughput application due to their high porous structure and have a wide pH range (pH 2-12) where silica based only are stable in pH 3-8 approximately. Monolithic materials are also easy to prepare thermally or using UV-light. Further, because monoliths are synthesized *in situ* and may be covalently attached to the walls of the chromatographic device, there is no need for frits to keep them in place.

The technology for the preparation of monolithic packing is simple, cheap and easy and was therefore introduced as an alternative to traditional separation materials.

1.1 Sample preparation

Sample preparation is an essential part of the analysis process to give a high recovery and a good reproducibility with a fast and easy procedure. Plasma, blood and urine are biological fluids with complex matrices consisting of salts, proteins and other organic compounds that may interfere with the analysis. Analytical methods for determining concentrations of drugs in biological fluids are performed in clinical studies. The drug may be present in very small concentrations (nM) and therefore it is important to eliminate interfering compounds in the sample preparation.

As the number of samples increase, sample preparation can be a limiting step to perform fast bioanalysis and development of new techniques for sample preparation is required. In this study a rather new technique is used; a 96-tip set packed with a short plug of a monolithic adsorbent. It is fully automated and has the possibilities to handle 96 samples in only 2 minutes.

1.2 Common sample preparation methods

Depending on the type of sample, there are various ways to prepare it in such a way that the sample is ready for measurement.

Liquid-liquid extraction is a method to separate compounds based on their solubility properties, usually one phase is a water-based solution and the other an organic solvent which is immiscible with water. It is an extraction of a substance from one liquid phase into another liquid phase. This method consumes large volumes of organic solvents and is difficult to automate.

Solid-phase extraction (SPE) is another method that uses a solid phase and a liquid phase to isolate an analyte from a solution. SPE is usually used to clean up and concentrate a sample before using a chromatographic or other analytical method. The sample passes through the solid phase and the compounds of interest and impurities adsorb onto it. The impurities are removed with a wash solution followed by elution of the analyte with a solvent appropriate for instrumental analysis ^[2]. SPE is in general more efficient than liquid/liquid extraction, is easily automated and uses less organic solvent. Solid-phase microextraction (SPME) treats smaller volumes than SPE and can be done without solvents.

Microextraction in a packed syringe (MEPS) is a miniaturized, solid-phase extraction technique that can be connected on-line to gas or liquid chromatography without any modifications ^[3]. 1 mg of the solid packing consisting of either silica or polymers is inserted into a syringe (100-250 μ L) as a plug. Sample preparation takes place on the packed phase.

Molecular imprinted solid-phase extraction (MISPE) is a method based on an attempt to mimic antibody-antigen interactions. The solid-phase consists of a polymer with recognition sites for the analyte and has been shown to be a highly selective method.

Micropipette tip-based sample preparation is another relatively new technique. The first commercially available micropipette tip (Zip Tip) was developed by Millipore (Bedford, Massachusetts, USA) in the late 1990s ^[4]. The original Zip Tip contained C18

chromatographic media embedded in a polymer. In this work, micropipette tips with a plug of methacrylate-based monoliths were used for sample preparation and also a test with present silica-based commercial tips.

1.2.1 96-tips sample preparation

96-tips sample preparation is a fast, high throughput and completely automated sample preparation method divided into three steps, extracting, washing and eluting.

Samples are prepared in a 96-well plate. In the first step the samples pass through the monoliths and adsorb to it. The next step cleans up the sample by washing the monolithic bed once by water, which removes hydrophilic interferences such as proteins and salts. In the final step the elution is performed in a 96-well plate containing elution solution. This 96-well plate is directly inserted to LC-MS-MS.

1.2.2 Commercial pipette tips

The company Varian manufactures OMIX pipette tips for micro extraction^[5]. The OMIX 96-tips contain a reversed-phase C18 functional group on a monolithic silica surface. The silica based tips will remove salts, detergents and hydrophilic contaminants that do not bind to the reversed-phase media. Rack of OMIX 96-tips can be used for automation on several robotic systems.

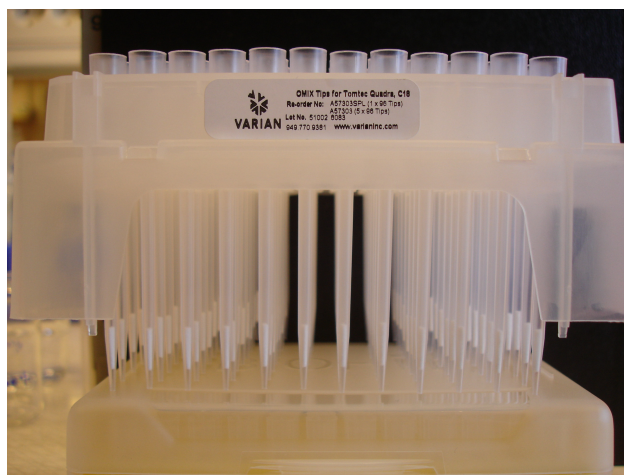


Figure 1. A photographic image of commercial OMIX pipette tips.

1.3 Preparation of the monolith and incorporation into pipette tips

In this study a methacrylate based organic monolith was prepared *in situ* in polypropylene based tips for high throughput sample preparation. Polypropylene is a saturated hydrocarbon polymer consisting of many monomers of propylene.

For monolithic pipette tips to be useful the substances must pass through the monolith and therefore it is important that the monolith has low backpressure for easy pumping of sample through and good attachment of the monolith to the wall.

1.4 Aim of the study

The aim of this study was to produce polymer-based 96-tips for sample preparation of biological fluids and then a test with commercial 96-tips application. The study was divided in three parts. The first part at Karlstad University describes the optimization of the monolith mixture and packing of the pipette tips. In part two applications of the packed polymer 96-tips and commercial 96-tips were made and validation using drugs in plasma. Part three handle test with whole blood application. All analyze in part two and three were performed at AstraZeneca, Södertälje.

Part I Manufacturing of pipette tips

2 Theory and background

2.1 Polypropylene (PP)

Polypropylene is a plastic polymer and due to their many applications and ability to be recycled polypropylene is used both in industry and in consumer produce. The melting point, 320° F (160°C) of polypropylene is very high compared to many other plastics, and makes it ideal for food containers that need to be dishwasher safe ^[6].

Polypropylene is also often used as a fiber in carpeting, such as the carpet around swimming pools because polypropylene does not soak up water.

Polypropylene is prepared from monomers of propylene ^[7]. Polymerization of propylene through the unsaturated two carbons will result in a growing chain that leads to the product polypropylene. The third carbon atom is participating in the reaction and producing a pendent methyl group. Polymerization of propylene through the unsaturated two carbons will result in a growing chain that leads to the product polypropylene.

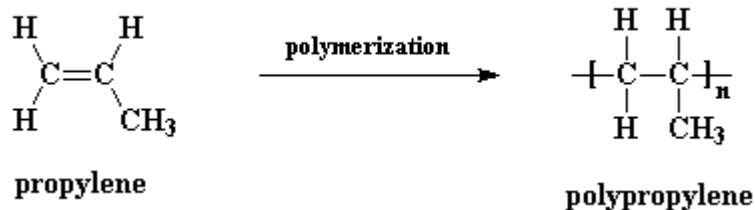


Figure 2. Polymerization of propylene.

2.2 Monolithic stationary phases

Monolithic phases can be classified in two main categories, silica-based monoliths (inorganic) and polymer-based monoliths (organic). Polymeric monoliths can be synthesized *in situ* and F. Svec et al. ^[8] introduced the *in situ* technique in the early 1990s. Polymer-based monoliths have high porous structure and are useful in a wide field of applications.

Polymer-based monoliths can be prepared with different functionalities depending on the character of the monomers used. Methacrylate-based monoliths are studied in this work and have attracted the most attention among organic monoliths ^[9, 10, 11, 12, 13].

2.2.1 Preparation of Methacrylate based monoliths

Acrylates are ester type monomers that contain vinyl groups and two carbon atoms double-bonded to each other. If a methyl group is attached to the α -carbon it is called a methacrylate.

The monolithic stationary phase is synthesized *in situ* in the pipette tips from a homogenous polymerization mixture containing porogenic solvent, photoinitiator, cross-linking agent and monomers. The monolith was prepared using a modified method suggested in prior work ^[9, 17].

2.2.2 Monomers

A monomer is a molecule that can react with other monomers or other compounds to form very large molecules (polymers).

The essential feature of monomer molecules is the ability to form chemical bonds with at least two other monomer molecules (polyfunctionality). Those able to react with two other can form chainlike polymers and monomers that can react with three or more can form cross-linked, network polymers.

In this work the monomer methacrylate is used. Methacrylates and acrylates are large groups of polymers that together form acrylic plastics used in many common products as hair sprays, glues, and plastic items and packaging.

2.2.3 Cross-linking agents

Cross linking is the formation of chemical links between molecular polymer chains to form a three-dimensional network of connected molecules. A cross-linking agent is a substance that promotes or regulates intermolecular covalent bonding between polymer chains and linking them together to create a more rigid structure.

In a polymerization mixture for monoliths the percentage of cross-linking agent influences the swelling and thereby the pore size and specific surface area of the material ^[14]. A high percentage of cross-linker in the polymerization mixture seems to result in an earlier phase separation that leads to a polymer with relatively small pore size. Small pore size materials are not generally permeable by liquids at reasonable pressure whereas larger pore size materials can be too delicate.

Ethylene glycol dimethacrylate (EGDMA) is a frequently used cross-linking agent for fabrication of methacrylate-based monoliths and is used in this work. A high percent of EGDMA results in a monolith with hydrophobic character.

2.2.4 Porogenic Solvents

Porogenic solvents used for the preparation of the monolith can be classified as “good” or “poor” solvents depending on the solvating capacity of the polymer.

The solvent for the polymerization should be miscible with monomers, but not dissolve the polymers that are formed. In “poor” solvents, polymer segments prefer each other above the solvent and in a “good” solvent the polymer segments favor contacts with the solvent. If a poor solvent is used the concentration of the monomers around polymer is high and leads to a decreased surface area of the polymer. As the surface area decrease the pore size increase ^[15].

Another approach to fabricate monoliths with different pore size is adjusting the ratio of porogenic solvent to monomers in the polymerization mixture. A monolith with maximum specific surface area that also results in a good flow through has been optimized to a ratio of approximately 4:6 of monomers to porogenic solvents ^[15, 16]. This ratio of monomers to porogenic solvent will be used in this work.

2.2.5 Free radical photoinitiators/thermal initiators

Polymerization reactions are most often initiated thermally or by photoinitiation using UV-light. A photoinitiator converts absorbed light energy, UV- or visible light, into chemical energy in the form of free radicals or other reactive intermediates.

UV-light polymerization is much faster than the thermal polymerization and is highly suitable for the fabrication of micro-fluidic devices ^[15].

Photoinitiators have different absorption spectra and it is important to choose a photoinitiator with excitation wavelength that lies in the emission spectrum of the UV-source ^[14]. The amount of polymerization initiators are also to be considered. When the amount of initiator increases, more polymer chains are transformed into individual globules which leads to smaller pore size.

Benzoylperoxide (BPO) is a polymerization initiator and is used in this work to initiate free-radical polymerization by UV light.

3 Experimental

3.1 Chemicals and materials

Methanol and benzophenone (BP) were obtained from Merck (Darmstadt, Germany). Ethylene glycol dimethacrylate (EGDMA), butyl methacrylate (BMA), methyl methacrylate (MMA), benzoylperoxide (BPO), 1,4-butanediol and ethanol were purchased from Sigma-Aldrich (Stockholm, Sweden). 1-propanol was purchased from KEBO Lab. (Stockholm, Sweden) and acetone was from VWR International AB (Stockholm, Sweden). All the chemicals were used as received. The polypropylene (PP) pipette tips (550 μ L) were obtained from Thermo LabSystems Oy (Vantaa, Finland).

3.2 Instruments

The modification and polymerization of the pipette tips were performed using a Spectrolinker XL-1500 UV crosslinker from Spectronics Corp. (Westbury, N.Y, USA), using 254 nm UV light. The instrument was equipped with 6 mercury lamps at 15 W each. The measurement of backpressure was performed using a micro-LC pump.

3.3 Methods

The pore size has a high impact on monoliths rigidity, permeability and sensitivity. Main focus of this part was to optimize the monolith to a highly porous structure with low backpressure. Parameters studied were the ratio of 1,4-butanediol in the polymerization mixture and packing of different volumes of the monolith in the pipette tips.

3.3.1 Packing of pipette tips

Plastic materials like polypropylene have a hydrophobic, chemically inert surface and the tips were for that reason surface modified using a solution with methanol and BP before use (5 wt%)^[17, 18]. Polypropylene based pipette tips (Finntips) with total volume of 550 mL and suitable for our liquid handling system were used for sampling.

Poly(ethylene glycol dimethacrylate-butyl methacrylate) monolith was prepared from a polymerization mixture composed of EGDMA and BMA as monomers, 1-propanol, 1,4-butanediol and water as porogenic solvents. Initiator BPO concentration was 1.5 wt% with respect to monomers.

The mixture was vortexed for about 10 minutes to dissolve the initiator and then purged with nitrogen for 10 minutes to remove oxygen. Using a micro syringe, the polymerization mixture was applied to the surface modified pipette tips. The tips were then sealed at their sharp end with parafilm. Modification was performed directly inside the pipettes in the above mentioned UV crosslinker under nitrogen atmosphere. Tips were first irradiated using 254 nm UV light with their sharp end down at a distance of 15 cm from the lamps. Irradiation time was 4800 s and then turned upside down, now at a distance of 5 cm from the lamp and further irradiated without parafilm for 3000 s.

After completion of polymerization, tips were washed with methanol to remove the porogenic solvents and other unpolymerized compounds in the monolith.

To investigate the flow through properties of the monolith the tips were connected to a micro-LC pump and the backpressure of methanol and water at different flow rates through the monolith was measured.

3.3.2 Adjusting the ratio of porogenic solvents

It was shown that the pore size could be influenced by adjusting the ratio of porogenic solvents, change in the hydrophobicity, in the polymerization mixture ^[19]. By varying the ratio 1,4-butanediol to 1-propanol in the mixture the back pressure with methanol through the monolith could be measured. Low back pressure indicates a monolith with highly porous structure and high back pressure a dense monolith with small porous structure.

1,4-butanediol concentrations were varied between 0-35 wt.% and each of the polymerization mixtures was applied in 6 pipette tips. The monolith was prepared as above. The tips were connected to a micro-LC pump and the backpressure was measured with a constant flow rate at 200 $\mu\text{L}/\text{min}$.

3.3.3 Different volumes of the monolith

In previous experiments ^[17] 10 mL of the polymerization mixture was used in packing of the tips but to see if the volume of the monolith is significant for a better flow through, three different volumes were packed into the tips. 5, 10 and 15 mL of the polymerizations mixture were applied to 6 tips each. The monolith was prepared as above and a study of the back pressure as above was made to investigate the flow through of the monolith.

4 Result and discussion

4.1 Validation of monolith preparation

Initial experiment was done varying the ratio of 1,4-butanediol to 1-propanol in the polymerization mixture and back pressure of the monolith with methanol was measured.

By adjusting the ratio 1,4-butanediol between 0-35 wt.% the pore size was changing. As is shown in diagram 1, best result with highest porosity occurs by 25 wt.% of 1,4-butanediol in the mixture and the pore size appears to decrease again when 1,4-butanediol content in the mixture was increased above 25 wt. %

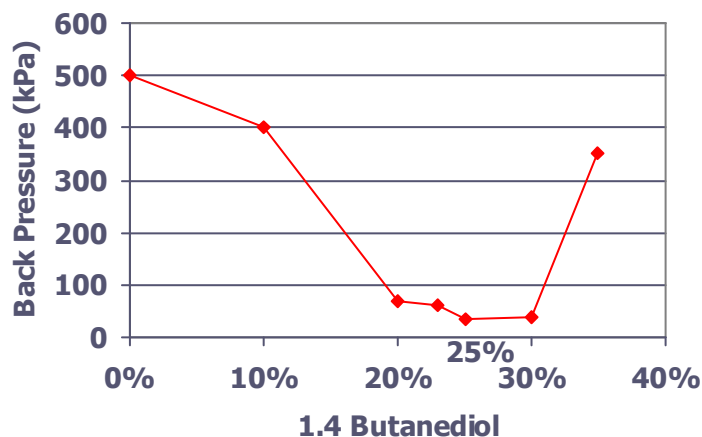


Diagram 1. Back pressures of the tips with methanol at different ratio of 1,4-butanediol.

Three different volumes were packed into the tips; 5, 10 and 15 mL of the polymerization mixture. A monolith with 5 mL polymerization mixture became very fragile and when washing with methanol the monolith fell apart. Monolith with 15 mL of the mixture was too dense and washing with methanol was impossible. So 10 mL seemed too be the best after all.

A polymerization mixture consisting of the monomers EGDMA (70%), BMA (30%) and the porogenic solvents 1-propanol (65%), 1,4-butanediol (25%) and water (10%) and the

initiator BPO at a concentration of 1.5 wt% with relation to monomers, seemed to be best. The tips have a volume of 10 mL monolith mixture and polymerization was performed *in situ* within pipette tips that were surface modified with methanol/BP (5 wt.%).

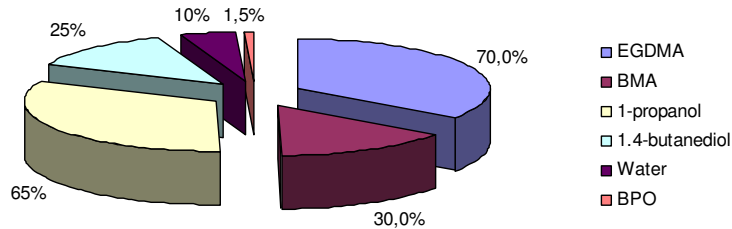


Figure 3. Composition of the monolith mixture.

To investigate the flow through properties of these monoliths in pipette tips, the tips were connected to a micro-LC pump and the backpressure of methanol and water at different flow rates through the monolith were measured. It can be seen in diagram 2, that the back pressure increases with increased flow rate, and back pressure of less viscous solvents, such as methanol, is lower than for the more viscous solvent water.

Since the application for this work was sample preparation of plasma and blood samples that are a lot more viscous than methanol, it was important to create a monolith that have good flow through properties.

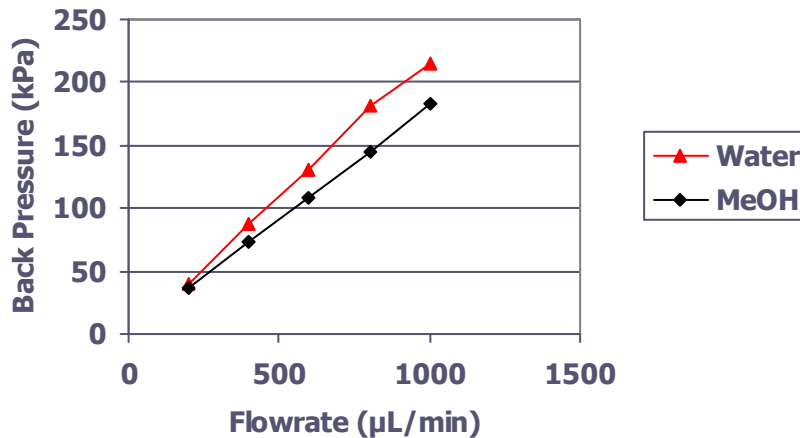


Diagram 2. Back pressure of the tips with methanol and water at different flow rates.

3 racks with 96-tips each (figure 4) were prepared to be used for sample preparation of drugs. These were first surface modified using a mixture of MeOH/BP and then filled with the optimized polymerization mixture.

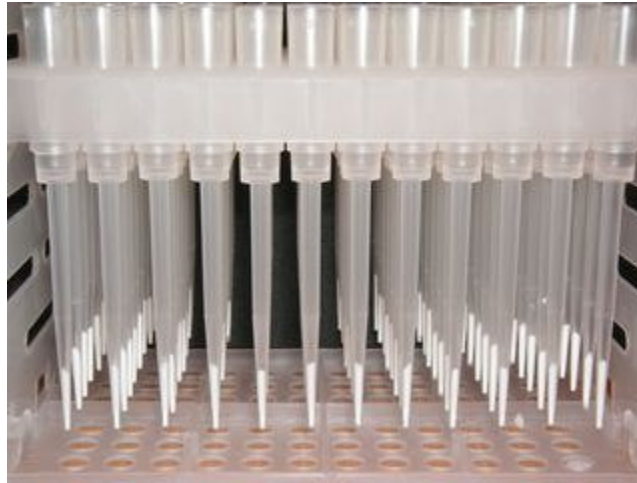


Figure 4. A photographic image of 96-tips packed with a plug of polymer-based monoliths for sample preparation.

5 Conclusion of part I

Main focus of this part was to optimize the monolith to a high porous structure with low backpressure for easy pumping of sample through the monolithic bed. Since the application for this work was sample preparation of plasma and blood samples that are a lot more viscous than methanol, it was important to find monoliths that have good flow-through properties.

Validation of monolith preparation was performed with a micro-LC pump and measurement of the back pressure was made using methanol. The monolith was optimized by varying the ratio of 1,4-butanediol to 1-propanol in the mixture and packing of different volumes of the monolith in the pipette tips. As is shown in diagram1, best

result with highest porosity occurs by 25 wt.% of 1,4-butanediol in the mixture and a monolith with 1 mL seemed to be the best, not too dense (15 mL) or fragile (5 mL). A polymerization mixture consisting of the monomers EGDMA (70%), BMA (30%) and the porogenic solvents 1-propanol (65%), 1,4-butanediol (25%) and water (10%) and the initiator BPO at a concentration of 1.5 wt% with relation to monomers, seemed to be best. The tips have a volume of 10 mL monolith mixture and polymerization was performed *in situ* within pipette tips that were surface modified with methanol/BP (5 wt.%). The irradiation time was totally 2 hours and 10 minutes for the optimized *in situ* polymerization.

This method results in a monolith packing with low backpressure for easy pumping of sample through and good attachment of the monolith to the walls of the tip. Further optimizations are still necessary and other experiments with more selective monolith phases (cation-anion-exchange/MIPs) are of interest.

The use of parafilm may decrease the transparency of UV-light and it is easy to damage the monolith when removing the parafilm so another way to make the tips without parafilm could improve the monolith.

Part II Analysis of drugs in biological fluids

6 Theory and background

6.1 Mass spectrometry (MS)

Mass spectrometry is a highly sensitive tool that is used to identify unknown compounds, quantify known compounds and provide information on the structure and chemical properties of molecules.

A mass spectrometer determines the molecular weight of chemical compounds by separating ions according to their mass-to-charge ratio (m/z). This is achieved by ionizing

the sample in an ion source. The separated ions are then detected and the results generate a mass spectrum.

6.1.1 Liquid chromatography and tandem mass spectrometry (LC-MS-MS)

LC-MS/MS is a technique that combines the solute separation power of HPLC, with the exquisite detection power of a mass spectrometer and results in a very high sensitivity. The HPLC system deals with analyte in the liquid-phase and gas phase ions must be generated before introduction to the MS. Therefore an interface for on line separation is needed. The interface is often an electro spray ion source (ESI), a mild ionization technique that results in a large amount of molecule ions ^[20]. ESI produces small charged droplets and the solvent is evaporated by the drying gas (N₂), leaving the sample molecule in the gas phase and ionized. The charged gaseous molecules are then led towards a cone on which an opposite electric charge is applied and further into the mass analyzer.

The molecule ion (parent ion) from the first separation in MS1 is taken into a collision cell where a collisions gas (Ar) is used to fragment the ions into so called daughter ions. The daughter ions fragments are then swept into a second MS (MS2) where they are separated.

6.2 Analytes

Pindolol and Metoprolol are beta-blockers (affects the heart and circulation) drugs that have been widely used for many years ^[21].

Metoprolol is used to treat angina (chest pain) and hypertension (high blood pressure). It is also used to treat or prevent heart attack. Metoprolol blocks the action of the sympathetic nervous system that stimulates the pace of the heart beat. By blocking the action of these nerves, Metoprolol reduces the heart rate and is useful in treating abnormally rapid heart rhythms. Metoprolol also reduces the force of heart muscle contraction and lowers blood pressure. By reducing the heart rate and the force of muscle

contraction, Metoprolol reduces heart muscle oxygen demand. Since angina occurs when oxygen demand of the heart exceeds supply, Metoprolol is helpful in treating angina.

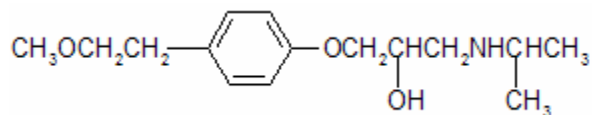


Figure 5. Metoprolol

Pindolol is a nonselective beta blocker which primarily affects heart (slows rate) and vascular musculature (decreases blood pressure). Mostly used as treatment of hypertension (high blood pressure).

Pindolol also shows membrane stabilizing effects like quinidine, possibly accounting for its antiarrhythmic effects. It acts on serotonin (5-HT_{1A}) receptors in the brain resulting in increased postsynaptic serotonin concentrations.

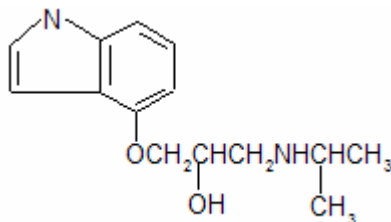


Figure 6. Pindolol.

7 Experimental

7.1 Chemicals and materials

Pindolol, Metoprolol, ²H₇-ropivacaine (IS) and Ropivaccain were supplied by the Department of Medicinal Chemistry, AstraZeneca (Södertälje, Sweden). Methanol, acetonitrile, ammonium acetate, acetone and formic acid were obtained from Merck (Darmstadt, Germany). All chemicals were of analytic grade.

7.2 Instrumentation

The high performance liquid chromatography HPLC instrument included two pumps, Shimadzu (Kyoto, Japan), an autosampler, CTC-Pal, Crelab (Knivsta, Sweden). The mass spectrometric instrument was a triple quadrupole Micromass Ultima equipped with an electrospray ionization operated in positive ion mode, figure 7. The source block and the desolvation temperatures were 150° C and 300° C, respectively and cone voltage was 25V. Nitrogen was used as both drying and nebulizing gas and argon was used as collision gas and collision energy was 20 eV.

A Zorbax SB-C8, 2.1 x 50 mm, 3.5 µ column was used as analytical column.

The Milli-Q water was obtained using a Reagent Grade Milli-Q Plus water purification system from Millipore Corporation (Bedford, USA). A centrifuge, Hettich Rotanta/AP (Tuttlingen, Germany), was used for plasma centrifugation

A gradient HPLC was used with mobile phase A 0.1% formic acid in 10% acetonitrile in water and mobile phase B 0.1% formic acid in 80% acetonitrile in water.

The gradient started from 0% of phase B from 0 to 1min and then up to 90% from 1 to 3 min and then from 3 to 4 min isocratic at 90% of phase B and at 4.1 to 6 min phase B was set at 0% again. The flow rate was 200 µL/min and sample volume (loading) was 20 µL.

The scan mode was multiple reaction monitoring (MRM) using precursor ion at (M+1) m/z (268, 249 and 282) and after collision the product ions 116, 116 and 133 were used for quantification of Metoprolol, Pindolol and internal standard ²H₇-ropivacaine respectively

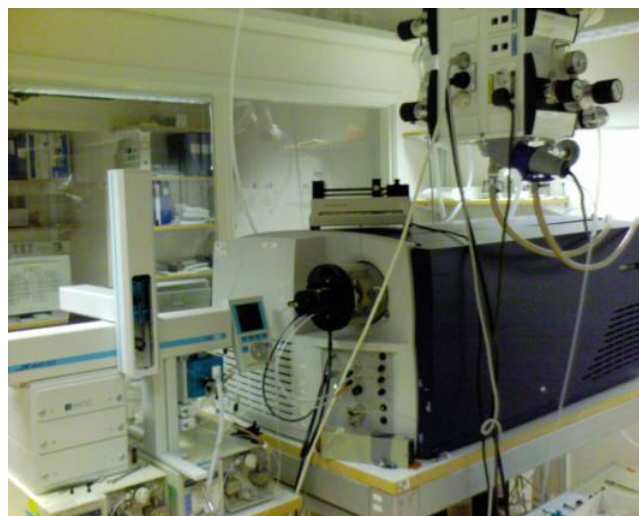


Figure 7. Micromass Ultima with electrospray ionization.

The data were collected and processed using MassLynx version 4.0, and all calculations were based on peak area ratios.

7.3 Liquid handling system

The liquid handling system used for sample preparation of 96-tips was a robot Personal Pipettor (PP-550N-MS) obtained from Apricot Designs, Inc (CA, USA).

Before use the 96-tips were thoroughly washed with water and MeOH. This was performed to remove possible porogenic solvents and impurities remained after the manufacturing of the pipette tips.



Figure 8. Automated liquid handling system used for 96-tips sample preparation.

The sample preparation began with the extraction step where the samples pass through the monolith and adsorb to it. The 96-well plate contained 100 μL sample in each well. The monolithic bed was then washed once by water to remove hydrophilic interferences such as proteins and salts that may interfere with the analysis. The analytes were then eluted with 150 μL elution solution (60% MeOH in water) directly into 96-well plate. This 96-well plate was directly inserted to LC-MS-MS.

Step	Air	Liquid (μL)	Repetitions
Washing before use with MeOH	300	150	1
Washing before use with water	300	150	1
Extraction (pause 10 s)	300	100	5
Washing with water	300	150	1
Elution (pause 10 s)	300	150	5

Table 1. Method for sample preparation with a robot Personal Pipettor (PP-550N-MS)

A pause for 10 seconds was introduced after the extraction and elution step to make sure all fluid had enough time to drip into the 96-well plate. If drops remain after this time they were wiped off with Kleenex. The aspiration of 300 μL air before any sample, washing solution or elution solution was drawn up, increases the pressure and makes it easier for the liquids to pass the monolith.

Elution solution containing different amounts of methanol in water was also investigated. Spiked plasma samples 600 nM of Ropivacain was prepared and treated as above and then analyzed. The best signal occurs with 100 % MeOH but the chromatography was much better with elution with 60% MeOH. Since there is no big difference in signal between them, 60% MeOH in water was used as elution solution.

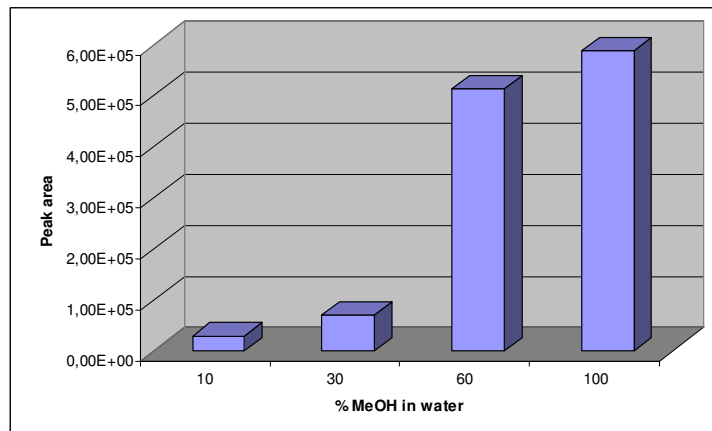


Table 2. Elution solutions containing different amounts of methanol in water.

7.4 Preparation of samples

Stock solutions 34 μM of Metoprolol, 38 μM of Pindolol in water and 900 μM of $^2\text{H}_7$ -ropivacaine (IS) in methanol were prepared. Spiked plasma samples were prepared by adding (50-1000 μL) Metoprolol and Pindolol to 5.0 mL of plasma. The plasma was diluted with water (1:5) and 0.1% HCOOH was added.

From the prepared samples 100 μL was added to a 96-well plate and further 10 μL of $^2\text{H}_7$ -ropivacaine (IS). The samples were vortexed before using the liquid handling system for the sample preparation in monolithic 96-tips. The concentration range of the standard curve was between 5 nM and 5000 nM.

7.5 Validation

Calibration standard curve with a concentration range of 5-5000 nM including the internal standard in plasma was prepared. The curve contained seven standard concentrations.

The peak area ratios of analyte and internal standard were measured and a standard curve was constructed. Calibration curves were typically described by the equation:

$$y = a x^2 + bx + c$$

Y is peak-area ratio, x is the concentration, a is the curvature, b is the slope and c is the intercept. The calibration curve was quadratic and the weight 1/x. Accuracy and precision were calculated for the three quality-control samples, 42 nM, 835 nM and 4175 nM for Metoprolol and 45 nM, 900 nM and 4500 nM for Pindolol. The quality-control (QC) samples were treated in the same way as the standards.

7.6 Test with Commercial pipette tips

A similar test was done with commercial OMIX pipette tips for a comparison with the packed polymer based 96-tips. The same spiked plasma samples of Metoprolol and Pindolol as above were used and the OMIX tips were treated in the same way as the packed 96-tips with the robot Personal Pipettor.

8 Result and discussion

8.1 Validation of packed 96-tips application

8.1.1 Calibrations

The constructed calibration curves consisted of seven standard samples with a concentration between 5 nM – 5000 nM in plasma for Metoprolol and Pindolol. The regression correlation coefficients (R^2) were >0.99 for all the analytes in the calibration range studied.

Compound name: Metoprolol
Coefficient of Determination: $R^2 = 0.998502$
Calibration curve: $-4.63193e-009 * x^2 + 0.000114095 * x + 0.00043823$
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: 2nd Order, Origin: Exclude, Weighting: 1/x, Axis trans: None

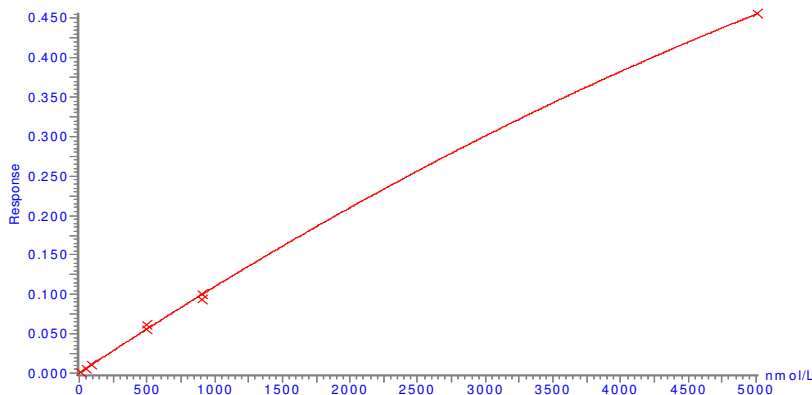


Figure 9. Calibration curve for Metoprolol

Compound name: Pindolol
Coefficient of Determination: $R^2 = 0.993741$
Calibration curve: $-3.63957e-010 * x^2 + 0.00015413 * x + 0.00134585$
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: 2nd Order, Origin: Exclude, Weighting: 1/x, Axis trans: None

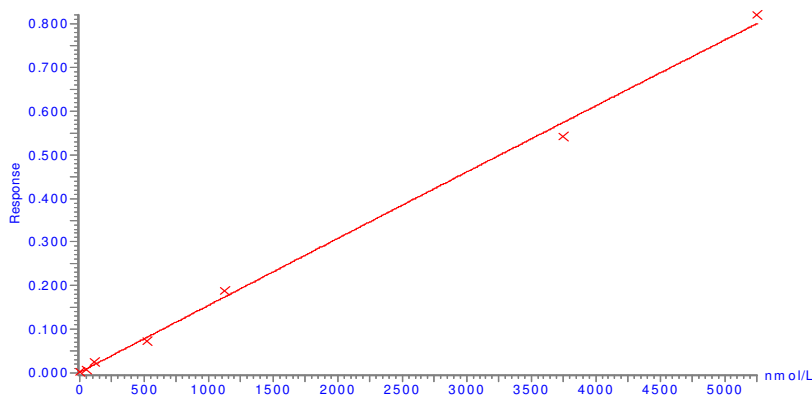


Figure 10. Calibration curve for Pindolol

8.1.2 Accuracy and precision

The accuracy was determined by the ratio of the found and the theoretical concentrations for human plasma control samples (QC) at three different levels (QCL, QCM and QCH). The precision is a measure of the random error and is determined by the percentage coefficient variation of the within- and between-day variations (intra- and inter-assays) at three different concentrations for quality control QC sample.

QC samples	Metoprolol		
	Accuracy (%)	Precision (RSD %)	
		Concentration (nM)	Intra assay n=3
	n = 6		
42	101	4	9
800	103	11	15
4200	102	6	8

Table 3. Accuracy and precision for Metoprolol in plasma

QC samples	Pindolol		
	Accuracy (%)	Precision (RSD %)	
		Concentration (nM)	Intra assay n=3
	n = 6		
45	114	5	11
900	105	10	13
4500	94	9	9

Table 4. Accuracy and precision for Pindolol in plasma

The accuracy for Metoprolol and Pindolol from the assays compiled in tables 3 and 4 were between 101% - 103% and 94% - 114% respectively.

Intra-assay precisions (RSD) varied between 4-11% for Metoprolol and between 5-10% for Pindolol. The inter-assay precisions (RSD) were at 8-15% for Metoprolol and 9-13% for Pindolol.

The accuracy and the precision of the method were both within the internationally accepted limits.

8.2 Validation of Commercial pipette tips

8.2.1 Calibrations

The constructed calibration curves consisted of seven standard samples with a concentration between 5 nM – 5000 nM in plasma for Metoprolol and Pindolol. The

regression correlation coefficients (R^2) were >0.99 for all the analytes in the calibration range studied.

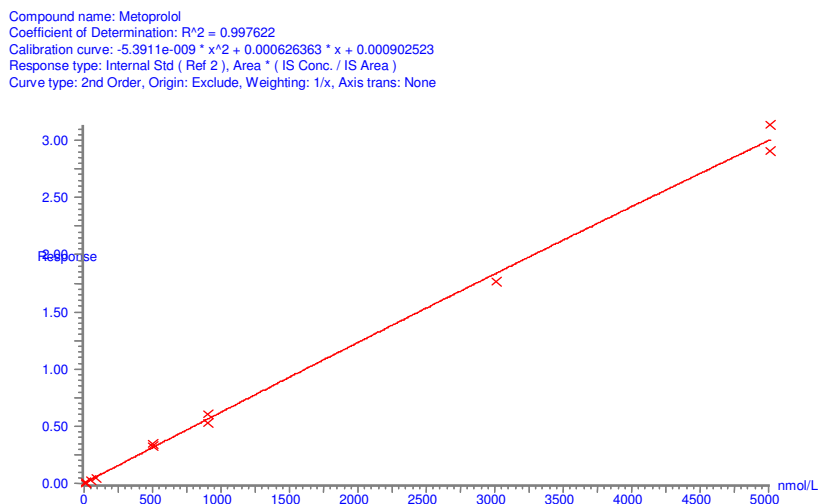


Figure 11. Calibration curve for Metoprolol.

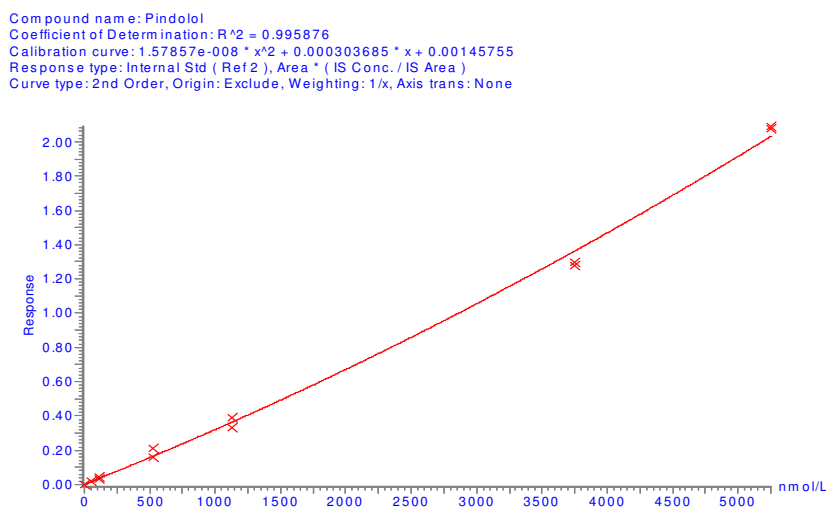


Figure 12. Calibration curve for Pindolol.

8.2.2 Accuracy and precision

The accuracy and precision was defined in the same way as above.

The accuracy for Metoprolol and Pindolol from the assays compiled in tables 5 and 6 were between 104% - 107% and 120% - 128% respectively.

Intra-assay precisions (RSD) varied between 5-9% for Metoprolol and between 5-9% for Pindolol. The inter-assay precisions (RSD) were at 10-13% for Metoprolol and 9-16% for Pindolol.

The accuracy of the commercial tips was higher for Pindolol, compared with the packed 96-tips.

QC samples	Metoprolol		
	Accuracy (%)	Precision (RSD %)	
Concentration (nM)	n = 6	Intra assay n=3	Inter assay n=6
42	105	9	13
800	107	7	13
4200	104	5	10

Table 5. Accuracy and precision for Metoprolol in plasma

QC samples	Pindolol		
	Accuracy (%)	Precision (RSD %)	
Concentration (nM)	n = 6	Intra assay n=3	Inter assay n=6
45	122	7	9
900	120	5	13
4500	128	9	16

Table 6. Accuracy and precision for Pindolol in plasma

8.3 Limit of quantification (LOQ)

The limit of quantification (LOQ) was set as the lowest measurable concentration with acceptable accuracy and precision. The LOQ for Pindolol and Metoprolol was set to 5.0 nM for both packed and commercial 96-tips. At this concentration the accuracy of LOQ was between 94% and 108% and the precision had a maximum deviation of 12% (n = 6).

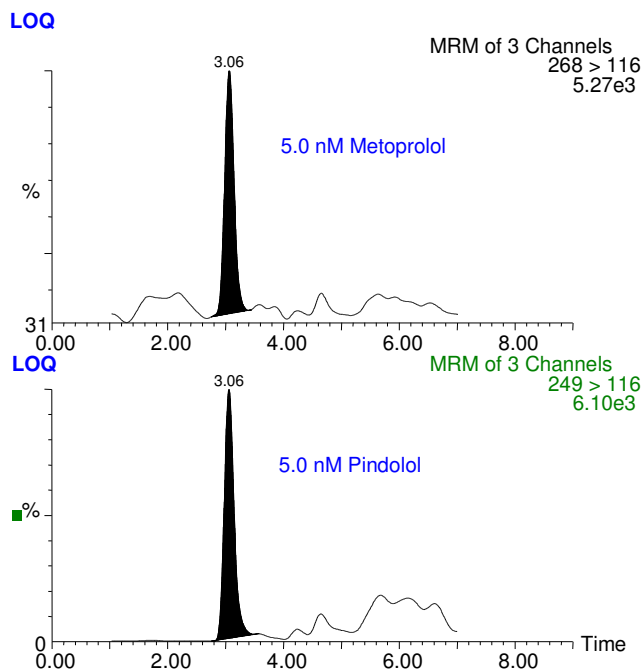


Figure 13. Chromatogram LOQ for Pindolol and Metoprolol in plasma.

9 Conclusion of part II

An LC/MS/MS method for the assay of Metoprolol and Pindolol in plasma samples using packed polymer 96-tips and commercial silica 96-tips has been developed and validated. The results showed both methods to be selective and accurate. The accuracy of the commercial tips was higher for Pindolol, compared with the packed 96-tips. Pindolol has a different structure than Metoprolol which maybe makes polymer based tips work better for Pindolol.

It was shown that only small sample volumes were required, solvent consumption was low and the procedure was fast. Sample preparation was done in only 2 minutes for 96 samples.

Part III Whole blood application

10 Theory and background

10.1 Cyclophosphamide

Searching for new chemical weapons during World War II led to the discovery of nitrogen mustard derivatives that have cytotoxic properties. Cyclophosphamide is a nitrogen mustard analogue and was introduced as an anti-tumor agent in 1958 [22]. Cyclophosphamide (CPA) is one of the most widely used anticancer agents in the treatment of hematological malignancies as well as solid tumors. CPA is also used to treat some autoimmune disorders, like rheumatoid arthritis and systemic lupus erythematosus.

It is a prodrug, the substance is administered in an inactive (or significantly less active) form and once administered, the drug is metabolised in vivo into the active compound.

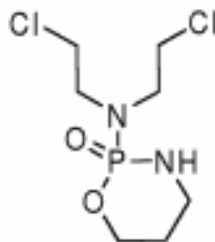


Figure 14. Cyclophosphamide

11 Experimental

11.1 Chemicals and materials

Cyclophosphamide and Ifosfamide (IS) was supplied by Huddinge University Hospital (Sweden). Methanol, acetonitrile, ammonium acetate, acetone and formic acid were obtained from Merck (Darmstadt, Germany). All chemicals were of analytic grade.

11.2 Instrumentation

The high performance liquid chromatography HPLC instrument included two pumps, Shimadzu (Kyoto, Japan), an autosampler, CTC-Pal, Crelab (Knivsta, Sweden). The mass spectrometric instrument was a triple quadrupole mass spectrometric instrument Micromass (Manchester, UK) equipped with a Z-electrospray interface (ESI) operated in positive ion mode, figure 15. The source block and the desolvation temperatures were 150° C and 300° C, respectively and cone voltage was 27 V. Nitrogen was used as both drying and nebulizing gas and argon was used as collision gas and collision energy was 25 eV.

A Symmetry (50x2.1 mm, C₈, 3.5 µm) column obtained from Waters (Massachusetts, USA) was used as analytical column

The Milli-Q water was obtained using a Reagent Grade Milli-Q Plus water purification system from Millipore Corporation (Bedford, USA). A centrifuge, Hettich Rotanta/AP (Tuttlingen, Germany), was used for plasma centrifugation.

A gradient HPLC was used with mobile phase A 0.1% formic acid in 10% acetonitrile in water and mobile phase B 0.1% formic acid in 80% acetonitrile in water.

The gradient started from 0% of phase B from 0 to 1min and then up to 80% from 1 to 4 min and then from 4 to 6 min isocratic at 80% of phase B and at 6.1 to 10 min phase B was set at 0% again. The flow rate was 150 µL/min and sample volume (loading) was 20 µL.

The scan mode was multiple reactions monitoring (MRM) using precursor ion at (M+1) m/z (both 261) and after collision the product ions 140 and 154 were used for quantification of Cyclophosphamide and internal standard.

The data were collected and processed using MassLynx version 3.4, and all calculations were based on peak area ratios.

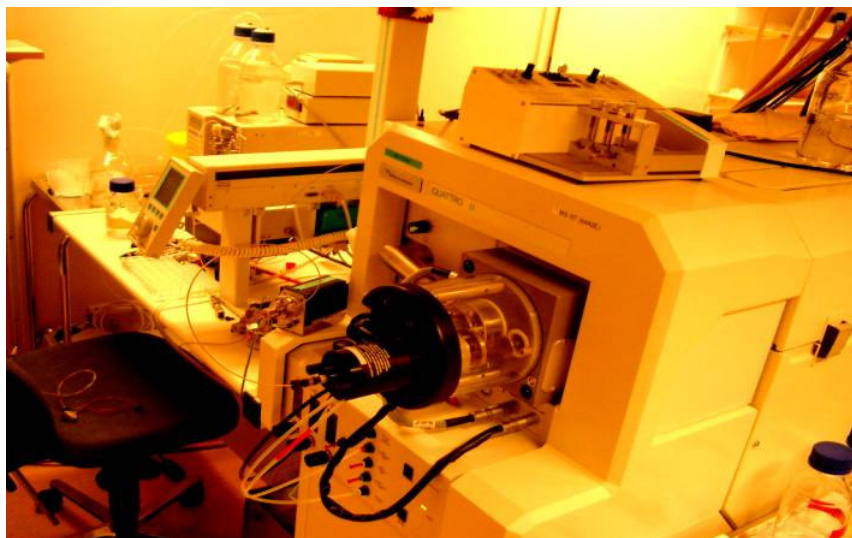


Figure 15. Micromass Quattro II with Z- spray.

11.3 Preparation of samples

Stock solution (20 μM) of Cyclophosphamide and Ifosfamide in water was prepared. Spiked blood samples were prepared by adding (50-1000 μL) Cyclophosphamide to 5.0 mL of blood diluted with water (1:25).

From the prepared samples 100 μL was added to a 96-well plate and further 10 μL of Ifosfamide (IS). The samples were vortexed before using the liquid handling system for the sample preparation in both packed monolithic 96-tips and commercial 96-tips. The concentration range of the standard curve was between 10 nM and 5000 nM.

11.4 Blood sampling for pharmacokinetic study

10 mg of Cyclophosphamide (powder) was dissolved in 10 mL water, so the concentration was 10 mg/mL. The dose was 150mg/kg and was calculated for each mouse according to its weight.

20 μL blood from tail vein was collected along with 80 μL EDTA (0.5M) in 8 time points after drug administration, intraperitoneal injection (IP).

The samples were diluted with water (1:25) and were treated as the previous Cyclophosphamide samples with commercial 96-tips and then analyzed.

12 Result and discussion

12.1 Calibrations

The constructed calibration curves consisted of seven standard samples with a concentration between 10 nM – 5000 nM in blood for Cyclophosphamide. The regression correlation coefficients (R^2) were >0.99 in the calibration range studied.

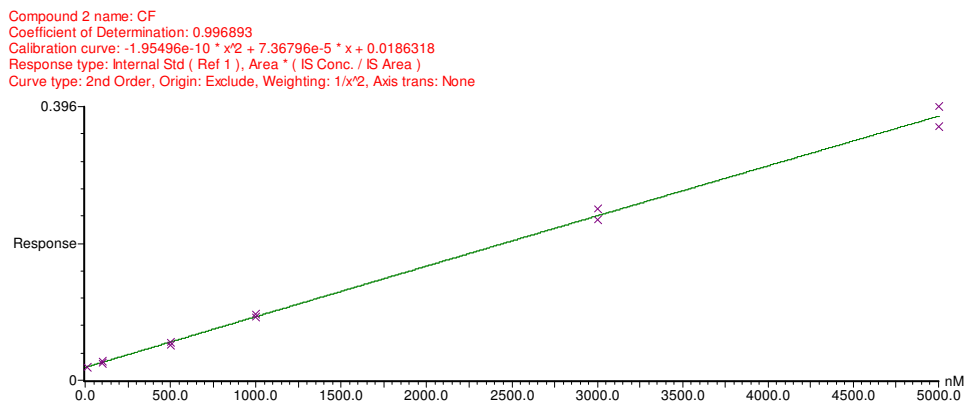


Figure 16. Calibration curve for Cyclophosphamide. Packed polymer 96-tips.

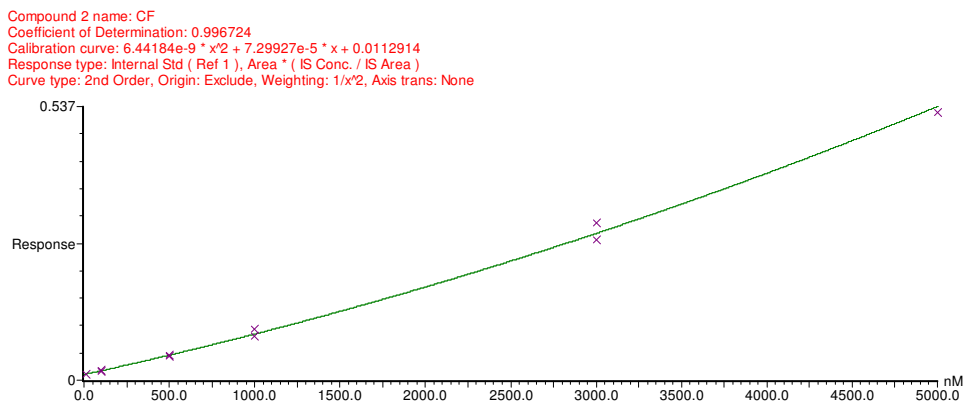


Figure 17. Calibration curve for Cyclophosphamide. Commercial silica 96-tips.

12.2 Accuracy and precision

The accuracy and precision were defined in the same way as above.

The accuracy for Cyclophosphamide from the assays compiled in table 7 were between 103% - 108% for the packed polymer 96-tips and 100% - 102% for the commercial silica 96-tips.

Intra-assay precisions (RSD) varied between 10-16% for packed tips and between 4-11% for commercial tips. The inter-assay precisions (RSD) were at 15-16% for packed tips and 7-18% for commercial 96-tips.

QC samples Concentration (nM)	Packed Tips			Commercial Tips		
	Accuracy (%)	Precision (RSD%)		Accuracy (%)	Precision (RSD%)	
	n=6	Intra assay n=3	Inter assay n=6	n=6	Intra assay n=3	Inter assay n=6
40	-	-	-	102	4	7
2000	103	10	15	101	11	18
4000	108	11	16	100	6	12

Table 7. Accuracy and precision for Cyclophosphamide in blood.

When analyzing 40 nM with packed 96-tips, the result became very high and accuracy and precision could not be measured at that concentration. The packed tips have been used many times and when washing the tips between uses, maybe some of the substance remains in the monolith.

12.3 Limit of quantification (LOQ)

LOQ for Cyclophosphamide was set to 10 nM. At this concentration the accuracy of LOQ was between 90% and 106% and the precision had a maximum deviation of 10% (n= 6).

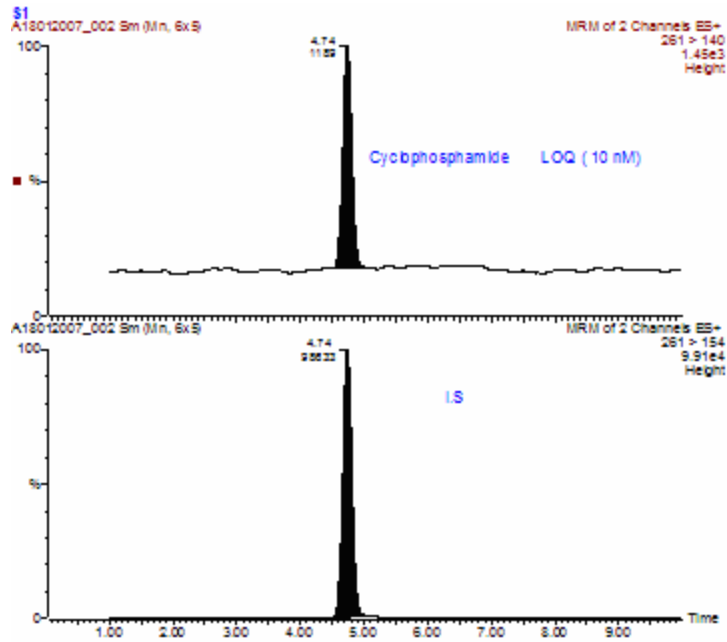


Figure 18. Chromatogram LOQ for Cyclophosphamide in blood. Commercial 96-tips.

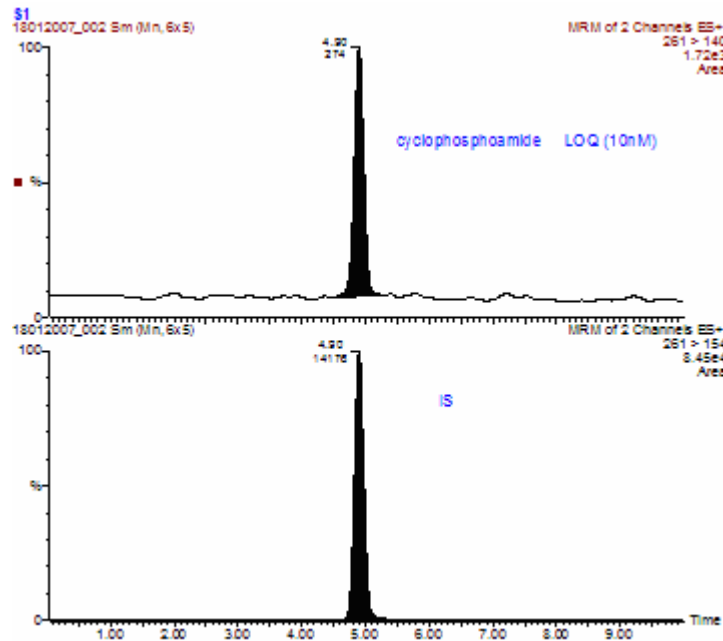


Figure 19. Chromatogram LOQ for Cyclophosphamide in blood. Packed 96-tips.

12.4 Validation of blood sampling for pharmacokinetic study

According to the calibration curve for Cyclophosphamide a calculation of concentration of the 8 time point was made. The highest concentration 57.9 μM of Cyclophosphamide occurs after 30 min. Predicted value (from Huddinge University Hospital) was 64.4 μM .

Time points	Conc. (μM)	Predicted
0	0.025	0
15 min	48.4	41.1
30 min	57.9	63.4
1 h	21.6	18.7
1 h 30 min	4.6	5.5
2 h	3.0	1.6
3 h	1.7	0.14
4 h	0.4	0.01

Table 8. Concentration of Cyclophosphamide

13 Conclusion of part III

Whole blood samples can be handled by 96-tips and both commercial and packed 96-tips gave good accuracy and precision.

When working with plasma samples the tips could be used several times and still get good results. Washing the tips after blood samples did not take away all the impurities and the substance remains in the monolith. Small amounts of the substance then become difficult to analyze.

96-tips sample preparation was successfully used in blood sampling for a pharmacokinetic study. The measured concentrations accord well with the predicted values.

14 Test of 1.0 mm LC column

Further optimization with a 1.0 mm column was made. 1.0x50mm, Hypersil gold, 5 μm was used and a pre-column 1x10 mm (C30). Hypersil gold columns are new highly pure silica based LC column and offering excellent peak shape ^[23].

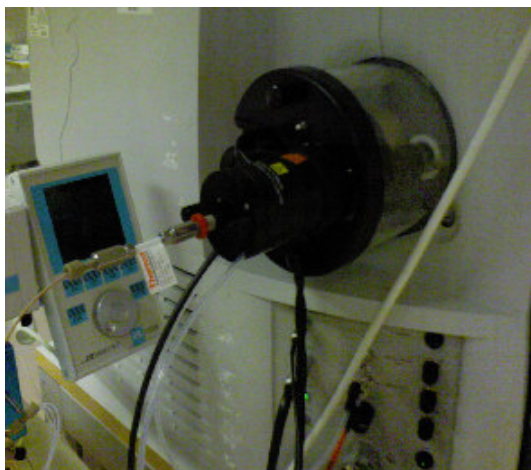


Figure 20. 1.0x50mm, Hypersil gold, 5 μm .

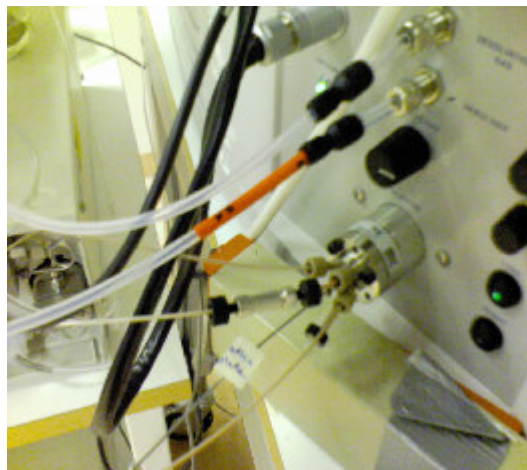
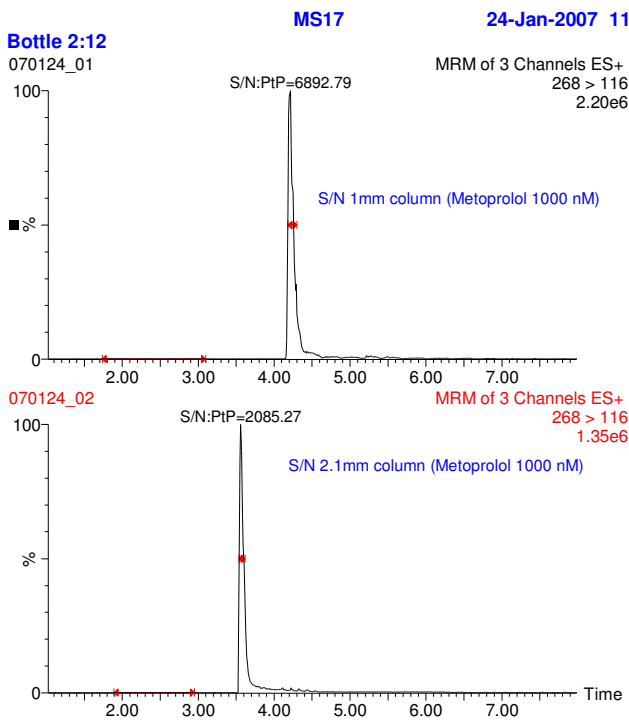


Figure 21. Pre-column 1x10 mm (C30)



A test was done with Metoprolol with a concentration of 1000 nM. Almost 3 times better signal occur when using 1.0 mm column.

Figure 22. Comparison between 1.0 and 2.1 mm column.

14.1 Calibrations

To make shore we have a robust system, calibration curves with same samples of Metoprolol and Pindolol as above was made with the 1.0 mm column. The packed polymer tips were used as sample preparation. The regression correlation coefficients (R^2) were 0.998 and 0.996 for the calibration range studied.

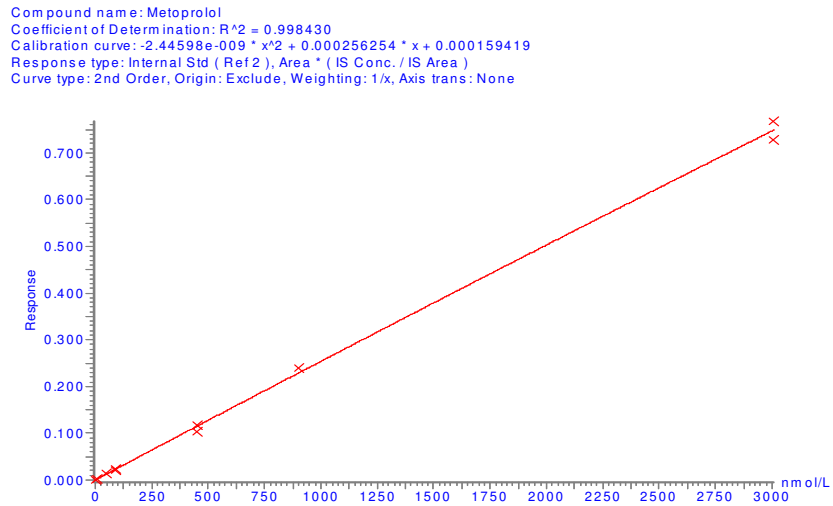


Figure 23. Calibration curve for Metoprolol.

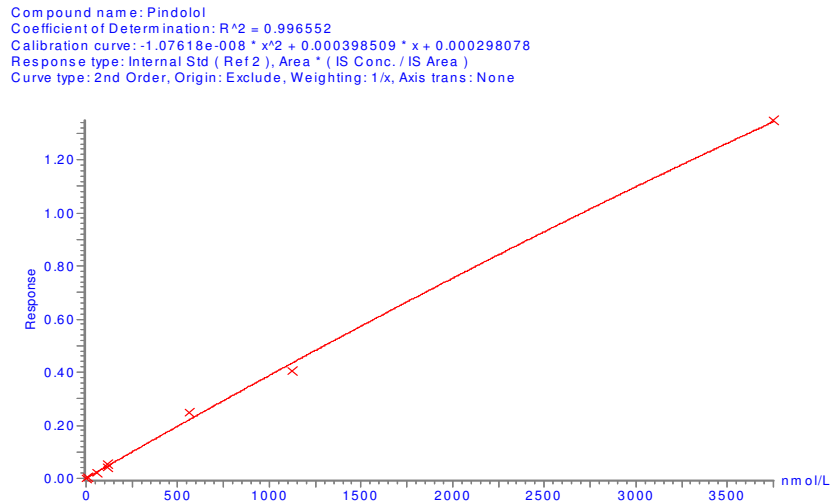


Figure 24. Calibration curve for Pindolol.

14.2 Accuracy and precision

The accuracy and precision was defined in the same way as above. The accuracy for Metoprolol and Pindolol from the assays compiled in table 9 was between 94% - 101% and 98% - 118% respectively.

Intra-assay precisions (RSD) varied between 4-11% for Metoprolol and between 3-8% for Pindolol. The inter-assay precisions (RSD) were at 4-14% for Metoprolol and 6-16% for Pindolol.

QC samples Concentration (nM)	Metoprolol			Pindolol		
	Accuracy (%)	Precision (RSD%)		Accuracy (%)	Precision(RSD%)	
	n = 6	Intra assay n=3	Inter assay n=6	n = 6	Intra assay n=3	Inter assay n=6
40	101	4	8	118	3	6
2000	101	4	4	98	4	6
4000	94	11	14	105	8	16

Table 9. Accuracy and precision for Metoprolol and Pindolol with 1.0 mm column.

14.3 Limit of quantification (LOQ)

The LOQ for Pindolol and Metoprolol and ²H₇-ropivacaine was set to 1.0 nM. At this concentration the accuracy of LOQ was between 95% and 105% and the precision had a maximum deviation of 9% (n = 6).

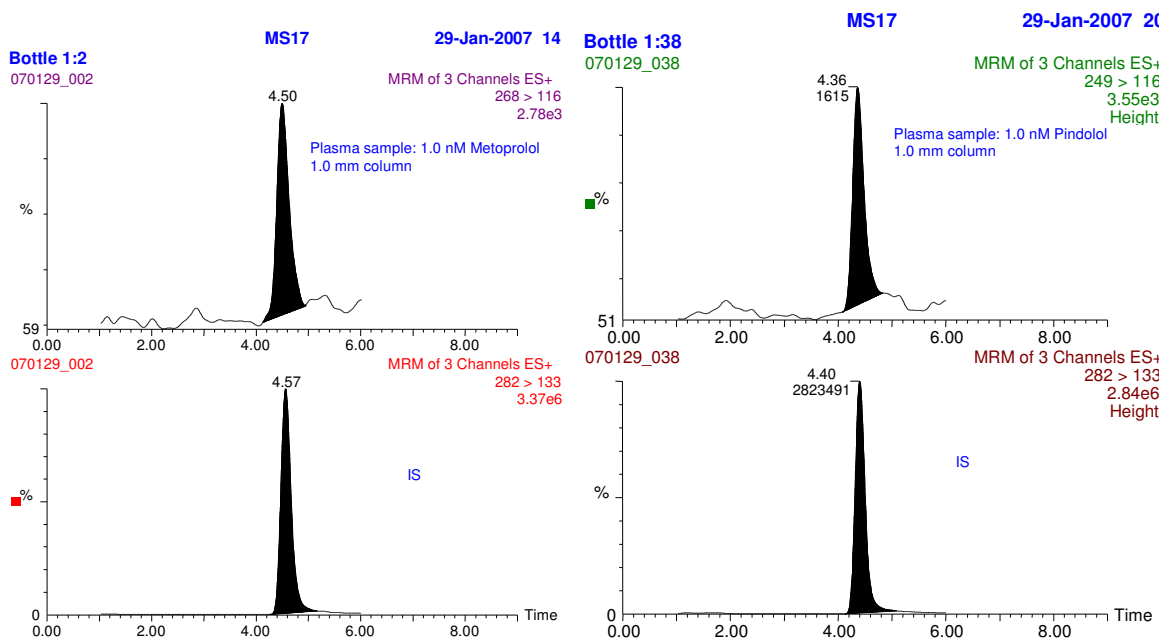


Figure 23. Chromatogram LOQ for Metoprolol and Pindolol in plasma samples for 1.0 mm column.

15 Overall conclusion

Using 96-tips packed with a plug of polymer-based monoliths is a useful technique and can handle both blood and plasma samples. It provides an automated, easy, fast and low cost procedure.

The results showed good accuracy and precision and the limit of quantification (LOQ) was after optimization with 1.0 mm column set to 1 nM for the test substances Metoprolol and Pindolol. LOQ for Cyclophosphamide was set to 10 nM.

The packed polymer tips could be used several times and still get good results but after blood samples the substance remain in the monolith and gave incorrect result. But observations with plasma samples indicate that it is possible to recycle them and this can be of great interest for further investigations. The commercial OMIX 96-tips were purposed for disposal usage and are not recommended to reuse to eliminate sample contamination problems.

This technique is on a research stage and further optimizations are still necessary to improve the precision and accuracy.

The monolith in this study adsorbed analytes due to hydrophobic interactions. To improve the selectivity other experiments with more selective monolith phases (cation-anion-exchange/MIPs) would be of interest.

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