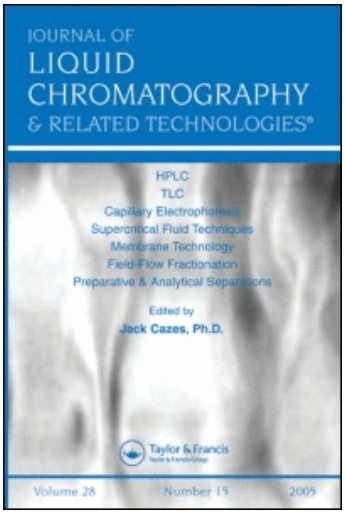

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Increasing Sample Preparation Throughput Using Monolithic Methacrylate Polymer as Packing Material for 96-Tip Robotic Device

Zeki Altun[a]; Lars G. Blomberg[a]; Mohamed Abdel-Rehim[b]
[a] Department of Chemistry, Karlstad University, Karlstad, Sweden [b] AstraZeneca R & D Södertälje, DMPK & BAC, Södertälje, Sweden

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Increasing Sample Preparation Throughput Using Monolithic Methacrylate Polymer as Packing Material for 96-Tip Robotic Device

Zeki Altun and Lars G. Blomberg
Department of Chemistry, Karlstad University, Karlstad, Sweden

Mohamed Abdel-Rehim
AstraZeneca R & D Södertälje, DMPK & BAC, Södertälje, Sweden

Abstract: In this work, a laboratory robot has been used to accomplish a system for high sample cleanup throughput. The robot operating in a 96-well format was furnished with 96 polypropylene tips packed with a chemically bonded monolithic methacrylate plug as sample adsorbent. Using this system, 96 samples could be handled in 2 minutes. Polypropylene tips were furnished with a chemically bonded monolithic methacrylate plug as sample adsorbent. Roscovitine and lidocaine in plasma samples were used as model substances. The validation of the methodology showed that the accuracy values of quality control samples (QC) were between +15%, and precision had a maximum deviation of 11%. The standard curve was obtained within the concentration range 14-5600 nM in both plasma and water samples. The regression correlation coefficients (R^2) for plasma and water samples were $\geq$0.999 for all runs.

Keywords: Packed 96-tips, Automated sample preparation, Monolithic methacrylate, Roscovitine, Lidocaine, Plasma, HPLC/MS/MS

INTRODUCTION

The measurement of drug levels in biological fluids is of crucial importance for drug discovery and development, as well as for pharmacological and

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Address correspondence to Professor Mohamed Abdel-Rehim, AstraZeneca R & D Södertälje, DMPK & BAC, SE-15185 Södertälje, Sweden. E-mail: mohamed.abdel-rehim@astrazeneca.com

pharmacodynamical studies and therapeutic drug monitoring. Sample preparation is often a limiting step to perform rapid bioanalysis; the introduction of high throughput sample pretreatment methods would greatly speed up the bioanalysis process. Further, as the number of samples increases, high throughput and fully automated analytical techniques become required. Current developments of sample handling techniques are directed toward automation on-line coupling of sample preparation units and detection systems. In addition, there is a trend toward development of more selective sorbents for sample clean up and enrichment. Recently, we introduced a new technique for miniaturised solid phase extraction that can be connected on line to GC or LC without any modifications, this new technique is called microextraction in packed syringe (MEPS).[1–4]

In this study, we introduce a new sample preparation technique. This is a 96-tip set packed with a short plug of a monolithic adsorbent; the set provides the possibilities to handle a 96-well plate in only two minutes. Methods for the preparation of porous polymer monoliths have been published.[5–7]

As an alternative to porous silica particles, monoliths have attracted considerable attention during the past years. Monolithic materials can be divided into two main categories, silica based and polymer based monoliths.[8] Monoliths consists of a continuous piece of support and they are attached to the walls of the chromatographic device, and, therefore, do not need frits. In most cases, the polymerization reaction mixture for the preparation of monoliths consists of monomers or dimers, cross-linker, porogenic solvent mixture, and initiator. Monoliths can easily be fabricated thermally or using ultraviolet light and can be made from a large variety of different monomers to obtain monoliths showing different selective interactions, such as ion-exchange, hydrophobic, hydrophilic, and affinity.[6,7,9,10]

When using conventional silica based packing materials, column efficiency is strongly affected by the size of the particles used. Usually, the efficiency is improved when the particle size is decreased. The efficiency of the polymeric stationary phases depends strongly on the resulted final pores properties. To obtain polymers having desired properties, a fine tuning of the polymerization mixture is needed. With such tuning, there are possibilities to adjust the porosity and pore diameter of the final monolith for different applications. The aim in this work was to achieve sorbents having low backpressure for high throughput analysis. However, low backpressure is generally obtained by application of material with large pores and, thereby, adsorption capacity is decreased. To compensate for this the amount of packing should be increased. The type and composition of the porogenic mixture is the tool often used to adjust the properties of the material. For instance, it has been shown that when decreasing pore size from 3,000 nm to 500 nm the plate height has decreased from 25 μm to 13 μm.[11] Moravcova et al. investigated the efficiency of copolymerization

of butyl methacrylate and ethylene dimethacrylate in capillary HPLC mode and compared them with that of capillary columns packed with 5 μm octadecyl silica. They obtained plate heights of about 30 μm for monolithic columns and about 15 μm for octadecyl silica columns.[12] They also found that a slightly changed composition of the porogenic solvent may have a large affect on the column characteristics. The efficiency of columns packed with particles smaller than 5 μm is still higher than that of columns packed with polymeric material.[13] But monolithic materials are still under development.

The UV initiated polymerization process is suitable for the preparation of monolithic materials *in situ* within microfluidic devices. For example, UV polymerization has been used for the fabrication of SPE microdevices,[14] capillary columns for micro HPLC,[15] and CEC.[16] Hsu et al.[17] have recently used UV polymerization 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.[18] 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 and the second step is the polymerization of the monolith.

Polyolefins such as polypropylenes are susceptible to photo initiated reactions.[19] Using UV as initiation source, the reaction proceeds through free radical mechanisms.[20] Grafting monomers, such as glycidyl methacrylate onto polypropylene has been described in a number of publications.[20–23]

The use of monolithic silica beds as the extraction plug in pipettes has also been described.[24] Further, pipette tips for manual sample clean up, with an immobilized bed of spherical silica beads fixed within a polymeric scaffold, such as ZipTip from Millipore Corporation (Bedford, MA, USA) and also Omix from Varian, Inc. (Palo Alto, CA, USA) are commercially available.

The aim of this work has been to develop a method for rapid and fully automated clean up of samples containing drugs in a plasma matrix. For that purpose, a Personal Pipettor robot has been used to handle 96-tips and a 96-well plate. Each tip has been provided with a small plug of monolithic support for the clean up procedure.

The methodology was validated using roscovitine and lidocaine in plasma samples as model substances. Roscovitine, (2-(R)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine), has been recently considered as a possible new chemopreventive and chemotherapeutic agent.[25,26] Lidocaine, an amide type local anaesthetic, has been used in anesthesiology for more than fifty years. Lidocaine also has antiarrhythmic effects and is used as a therapeutic agent in the treatment of cardiac disorders.[27–29] The present

sample preparation method is rapid and selective for studying lidocaine and roscovitine in biological fluids.

EXPERIMENTAL

Chemicals and Materials

Roscovitine and olomoucine (I.S) were supplied by Sigma-Aldrich (Stockholm, Sweden). Lidocaine and lidocaine-d_3 (I.S) were supplied by the Department of Medicinal Chemistry, AstraZeneca R&D (Södertälje, Sweden) as hydrochlorides (Fig. 1). Acetonitrile, methanol, formic acid, and ammonium hydroxide were obtained from Merck (Darmstadt, Germany). Glycidyl methacrylate (GMA), ethylene glycol dimethacrylate (EGDMA), butyl methacrylate (BMA), 1-dodecanol, and cyclohexanol were purchased from Sigma-Aldrich (Stockholm, Sweden). 2,2′-azobis(2-methylpropionitrile) (AIBN) was obtained from Acros Organics (New Jersey, USA) and acetone was from VWR International AB (Stockholm, Sweden). All the chemicals were used as received. The polypropylene (PP) pipette tips (550 μL) were obtained from Thermo Labsystems Oy (Vantaa, Finland). Olomoucine and lidocaine-d_3 were used as internal standards for roscovitine and lidocaine, respectively. Photographic images were taken with a Motic Moticam 480 from Motic Instruments Inc. (Richmond, Canada).

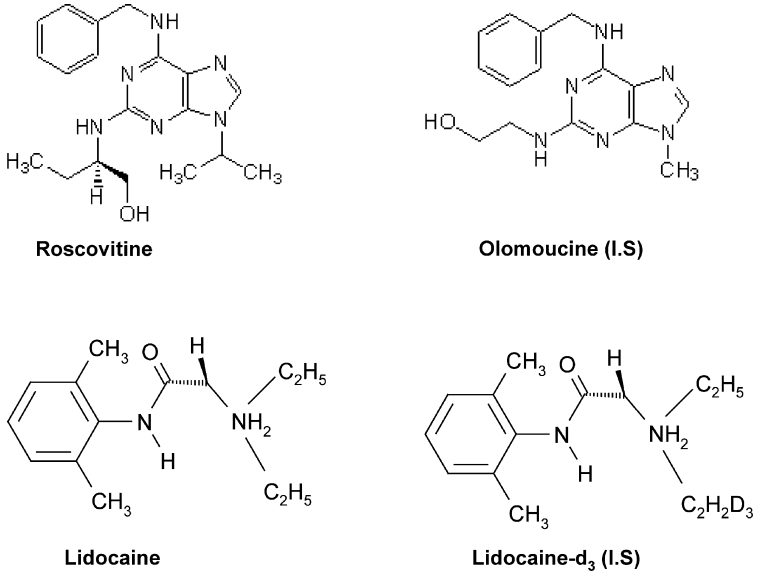

Figure 1. The structure of roscovitine, olomoucine (I.S), lidocaine, and lidocaine-d_3 (I.S).

Preparation of the Polymers

Poly(glycidyl methacrylate-ethylene glycol dimethacrylate-butyl methacrylate) monolith was prepared using a modified method originally suggested by Merhar et al.[30] Briefly, a solution containing GMA (20%), EGDMA (15.5%), BMA (3.5%), AIBN (1 wt% with respect to monomers), 1-dodecanol (30%), and cyclohexanol (30%) was vortexed for 10 min and purged with nitrogen for 10 min in order to remove oxygen.

The pipette tips (550 μL) were filled to about 8 mm by capillary action and placed vertically inside the polymerization apparatus, a Spectrolinker XL-1500 UV Crosslinker Spectronics Corporation (Westbury, NY, USA), calibrated at 254 nm UV light. The polymerization was allowed to proceed first for 60 min with the sharp end of the tip down and at a distance to the lamp of 15 cm, and then for 25 min with the sharp end up and at a distance of 5 cm to the lamp. Alternatively, the polymerization was performed in a gas chromatography oven at 57°C for 24 h. 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 monoliths were washed with elution solvent (Fig. 2). For photographic image (Fig. 2B) about a 3 mm long sample from the tip was cut with a razorblade, washed thoroughly with acetone, and dried under a stream of nitrogen.

Apparatus

The high performance liquid chromatography (HPLC) instrument included two pumps, Shimadzu (Kyoto, Japan), an autosampler, CTC-Pal Crelab (Knivsta, Sweden), and a 20 μL sample loop. A Zorbax (50 × 2.1 mm, SB-C8, 3.5 μm) column obtained from Agilent (CA, USA) was used as an analytical column connected to an Optiguard (C_8, 10 × 1 mm) as a guard column. A Valco C4W valve, Valco Instruments (Houston, USA) was used as a gate valve between the liquid chromatograph and the mass spectrometer. 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 mixer volume of 0.1 mL. Mobile Phase A was 0.1% formic acid in acetonitrile and water (10:90, v/v) and mobile phase B contained 0.1% formic acid in acetonitrile and water (80:20, v/v). The gradient started from 0% of phase B up to 80% from 1 to 5 min and then from 5 to 6 min isocratic at 80% of phase B and at 6.1 min phase B was set at 0% again. For system stability the next injection was performed after 8 min. The flow rate was 150 μL/min and sample volume was 20 μL.

All experiments were conducted using a triple quadrupole mass spectrometric instrument Micromass QII Z-spray (Manchester, UK) equipped with

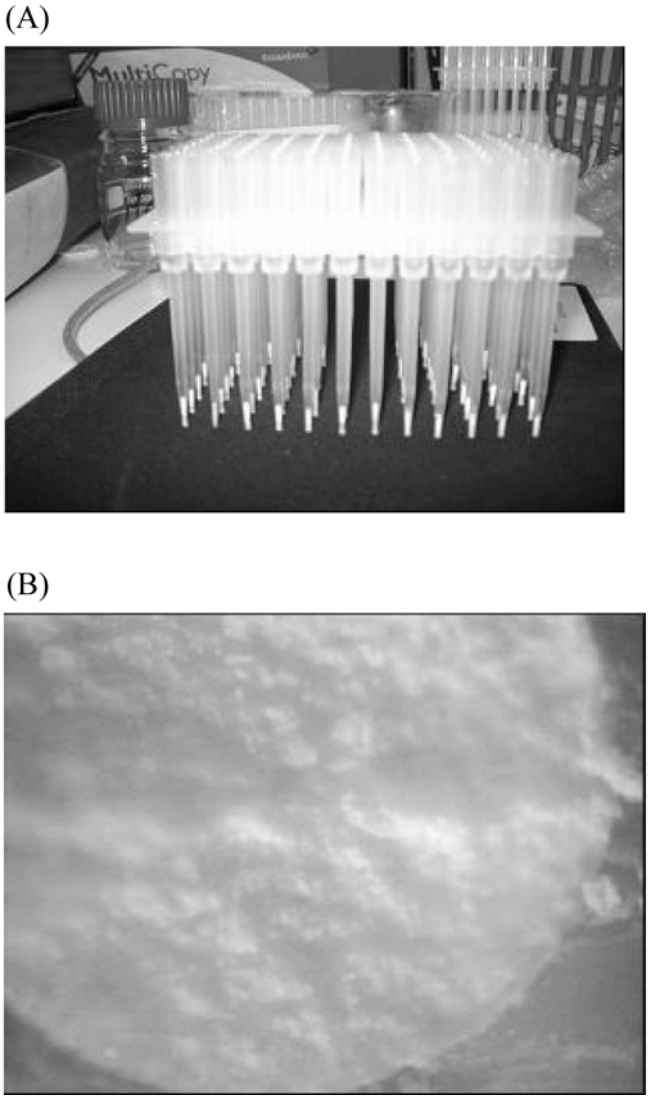

Figure 2. (A) UV-polymerized 96-tips packed with monolithic methacrylate polymer. (B) A photographing image of UV-polymerized porous polymer monolith inside a disposable pipette tip. The bed is seen from inside. The composition of the polymerization mixture is given in Preparation of Polymers section. Polymerization was allowed to proceed for 85 minutes using 254 nm light.

a Z-electrospray interface and operated in positive ion mode. The parameter settings used were: capillary voltage at 3.1 kV, cone voltage at 38 V, extractor at 5 V, RF lens at 0.2 V, source block and desolvation temperatures at 150°C and 300°C, respectively. Nitrogen was used both as drying (400 L/h),

and nebulizing gases (20 L/h), the vacuum was 2×10^{-5} in the mass analyzer and 2×10^{-3} in the collision cell. Argon was used as collision gas and collision energy was 25 eV. The gases were from AGA (Lidingö, Sweden).

The scan mode was multiple reaction monitoring (MRM) using a precursor ion at $(M + H)^+$ (m/z: 355, 299, 235, and 238) and after collisional dissociation the product ions 233, 91, 86, and 86 were used for quantification of roscovitine, olomoucine, lidocaine, and lidocaine-d_3, respectively. The data were collected and processed using MassLynx version 3.4, and all calculations were based on peak area ratios.

Sample Preparation

Plasma samples were stored at -20°C. Before use, the plasma was thawed at room temperature and centrifuged at 3500 rpm for 10 min. Stock solutions of roscovitine, lidocaine, olomoucine, and lidocaine-d_3 were dissolved in DMSO (roscovitine, olomoucine) and methanol (lidocaine, lidocaine-d_3). Spiked plasma samples were prepared by adding roscovitine (10–20 μL) to 200 μL of centrifuged plasma in 96-well plate format. Twenty μL of I.S (10 μM) were added to 200 μL plasma sample. The concentration range of the standard curve was between 14–5600 nM (14, 28, 140, 280, 560, 1400, 2440, 2800, and 5600 nM). A robot Personal Pipettor (PP-550 N-MS) obtained from Apricot Designs, Inc. (CA, USA) was used to handle the 96-Tips. One hundred μL plasma sample from a 96-well plate was sampled by the robot. When the plasma has passed through the monolith polymers the analytes were adsorbed to it. Then the solid phase was washed once by water (100 μL) to remove the proteins and other interferences. Finally, the analytes were eluted with 100 μL methanol directly into a 96-well plate.

Method Validation

Each calibration curve consisted of eleven calibration points covering the concentration range 14 to 5600 nM. Blank samples were run simultaneously. The plasma used was collected and pooled from different objects (6 pieces). The peak area ratios for analyte and internal standard were measured and a standard curve without zero concentration was constructed. The calibration curves were described by the equation:

$$y = ax^2 + bx + c$$

where y is peak area ratio, x is the concentration, a is the curvature, b is the slope, and c is the intercept. Due to the relatively low capacity of the monolithic polymer used here, the calibration curves were quadric and the weight was $1/x$. The quality control (QC) samples were prepared with the

concentrations of 70, 280, and 3300 nM. The accuracy and precision were calculated for the QC samples at three different assays. The method was validated at optimized conditions.

Accuracy was defined as the degree of deviation of the determined value and the nominal value: [(measure value − nominal value)/nominal value]∗100. Precision (%C.V.) was defined as the coefficient of variation and was determined as percentage of standard deviation of the observed values divided by their mean values: [(Standard deviation)/mean value]∗100.

RESULTS AND DISCUSSION

The porous monolith was synthesized directly inside disposable pipette tips by *in situ* UV light initiated polymerization or thermally initiated free radical polymerization. Figure 2B shows 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 monolith and the tip were observed. Despite, application of high pressure up to 4 bar (tested from both sides) the monolith did not slip out of the tip. This indicated that sufficient binding of the monolith to the plastic tube was achieved. Therefore, in our case, we find the modification of the surface of the PP tube prior to *in situ* preparation of the monolith given in the literature unnecessary.[18,31] 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 when testing with high pressure. Possibly, the degree of polymerization was insufficient in the center of the monolith. This conclusion is because the part of the monolith 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 uniform irradiation.

The system used combines a laboratory robot for 96-wells and 96 polypropylene tips containing a monolithic sorbent bed. This combination provided clean up of 96 samples in 2 minutes.

The method selectivity is defined as noninterference with the endogenous substances in the retention time windows of analyte and internal standard. LC–MS/MS analysis of the blank serum samples showed no significant interference peak with the quantification of roscovitine and lidocaine. Representative chromatograms of blank human plasma and plasma spiked with roscovitine and lidocaine are presented in Figs. 3A and 3B.

The constructed calibration curve consisted of eleven levels of human plasma samples in the concentration range 14–5600 nM. The regression correlation coefficients (R^2) for plasma samples were $\geq$0.999 for all runs of roscovitine and lidocaine, Table 1.

The intra-assay precisions (R.S.D.) at three different concentrations for quality control (QC) samples were about 3–8% (n = 6) for plasma samples.

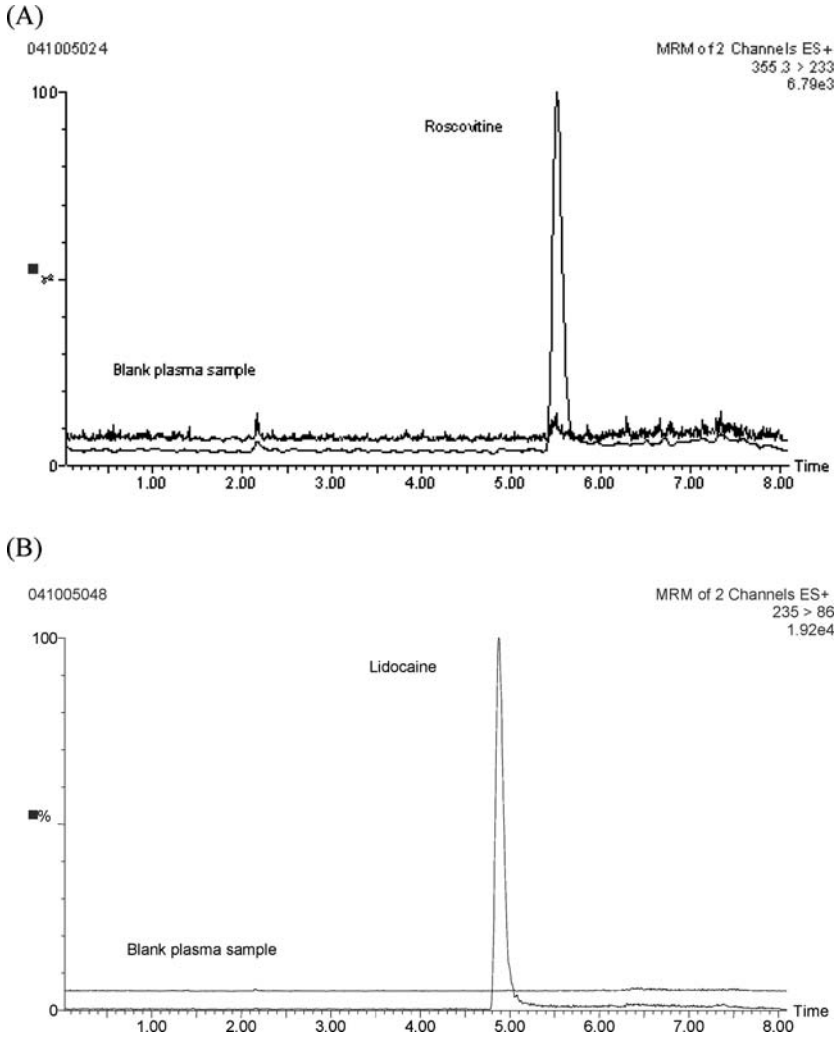

Figure 3. Chromatograms for blank plasma and spiked plasma sample (LLOQ) with roscovitine (A) and lidocaine (B).

The inter-assay precisions (R.S.D.) were at 3–11% (n = 24). The accuracy varied from 95% to 104% (n = 24). The accuracy and precision data are summarized in Table 2. The accuracy and precision of the method were within the internationally accepted limits.[32]

The lower limit of quantification (LLOQ) for the analytes studied was set to 14 nM. At this concentration, the accuracy of LOQ was between 90% and 110% and the precision had a maximum deviation of 10% (n = 6) for roscovitine and lidocaine (Fig. 3).

Table 1. Regression parameters for the calibration curve of roscovitine and lidocaine in plasma

	Curvature a (10^{-7})		Slope b		Intercept c		R^2	
	Rosco	Lido	Rosco	Lido	Rosco	Lido	Rosco	Lido
Assay 1	2.5	2.1	0.011	0.013	0.003	0.002	0.9990	0.9999
Assay 2	3.6	3.5	0.014	0.014	0.002	0.002	0.9996	0.9998
Assay 3	3.6	2.5	0.013	0.013	0.002	0.001	0.9998	0.9999

A comparison of the performance of poly(GMA-EGDMA-BMA) packed monolithic pipette tips with a commercially available microtip designed for manual sample clean up was done. The commercially available tip tested here contains an immobilized bed of spherical silica beads fixed within a polymeric scaffold. As was expected, the sample capacity of silica beads was higher than that of the tips containing similar amounts of monolith. The monolithic tips had about 25% of the sample capacity of silica based tips. But, as has been pointed out above, a larger quantity of monolithic material can easily be used to compensate for lower sample capacity in monolithic materials. This is without exceeding the pressure limit of the device. Using silica based packings this is generally not possible because of the relatively higher backpressure. A comparison of extraction capacity of monolithic based pipette tips containing about 2 mg packing and silica based pipette tips using lidocaine as test compound is shown in Figure 4. As can be seen, more sample is extracted using the monolithic tip and this is explained by the larger amount of polymer that was utilized.

CONCLUSIONS

Future requirements for sample preparation methods were discussed by Henion and coworkers.[33] Factors such as miniaturization, high sample

Table 2. Accuracy, intra- and inter-day precision for three concentrations of roscovitine and lidocaine (QC-samples) in plasma

			Precision			
	Accuracy (%) n = 24		Intra-day n = 6 (R.S.D%)		Inter-day n = 24 (R.S.D%)	
Concentration (nM)	Rosco	Lido	Rosco	Lido	Rosco	Lido
3300	99	102	5.0	3.0	6.0	5.0
280	104	104	7.0	5.0	5.0	3.0
70	95	103	8.0	6.0	11	5.0

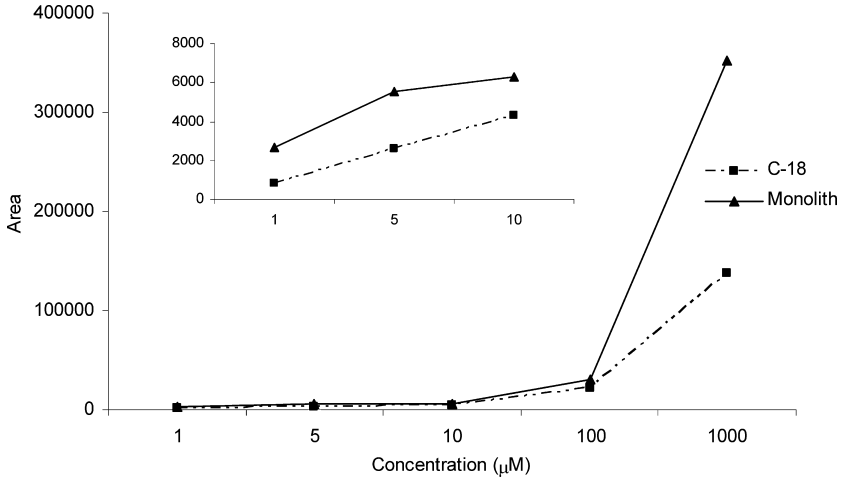

Figure 4. Poly(GMA-EGDMA-BMA) monolithic pipette tips are compared to silica based pipette tips. Lidocaine in water was used as sample compound. Sample volume 100 μL was extracted and washed with 100 μL water before eluting with 100 μL ACN/water 95:5 (v/v).

throughput, and the possibilities to run samples in parallel were emphasized. The present work should be considered as a step in the development of analytical systems to meet the demand for much higher sample throughput than is provided today. Using a laboratory robot in a 96-well plate format with 96 polypropylene tips containing a monolithic methacrylate bed as sorbent, 96 samples could be cleaned up in two minutes. The present method provides good accuracy and precision within the range of the calibration curve. Furthermore, the present method reduces the sample preparation time, which is of great importance in bioanalysis.

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