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New coated SPME fibers for extraction and fast HPLC determination of selected drugs in human blood

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ABSTRACT

Polythiophene (PTh) and polypyrrole (PPy) as sorbent phases for solid phase microextraction (SPME) were applied in order to extract the multi-resistant *Staphylococcus aureus* (MRSA) antibiotic drugs (linezolid and daptomycin) from whole blood followed by high performance liquid chromatography (HPLC) determination with UV detection. Relative standard deviations (RSDs) of *in vitro* and *pseudo in vivo* measurements performed in whole blood were in the range of 4.58–15.91% and 6.09–17.33% for linezolid and daptomycin, respectively. Determination coefficients (R^2) were in range of 0.9884–0.9945 and 0.9807–0.9818 for linezolid and daptomycin, respectively. This study proved better adsorption capacity of PTh SPME coating compared to PPy coating for both, linezolid and daptomycin.

1. Introduction

Antibiotics used in bacterial infections treatment belong to very important group of drugs [1–3]. Concentration range of antibiotics which have to be dosed to appropriate places in human body is usually very low and has to be very accurate since high concentration may cause side effects. This fact requires application of very sensitive and fast methods for direct concentration monitoring of antibiotics in human body fluids and tissues [4]. Methods which are the most often used in sample preparation is solid phase extraction (SPE) [5–7]. This technique is used for pre-cleaning samples from various complex environmental [8] and biological matrices including plants [9], urine [10], plasma, blood and tissue. Principle of this method is based on an affinity of analysing compounds to stationary phase and a solvent mixture which are passed through the column to remove all impurities and matrix components [11]. Since the nature of analytes have to be extracted, there is a possibility to use various kinds of stationary phases such as reverse phase, normal phase and ion exchange materials.

A new sample preparation method – solid phase microextraction (SPME) was separated from SPE technique at the beginning of 1990. This method, invented by Pawliszyn and Arthur [12] is supposed to be the most suitable method allowing fast direct sampling of drugs from blood vessel, human organs or tissue samples to the analytical compartment [13–16]. SPME relies on extraction of appropriate compound and further desorption of adsorbed molecules in optimized medium [17]. Desorption may be performed in dependence on kind of compound, for example, thermally (directly in gas chromatographic injector) or in liquid. Based on the nature of adsorbed molecules small amount of acidic or basic additives may increase desorption efficiency [12]. After optimizing a coating and adsorption–desorption process it is possible to reach very low detection limits [18–22].

In this work two antibiotics (linezolid and daptomycin) were taken under considerations. These drugs were investigated from standard solutions and whole blood by an *in vitro* and a *pseudo in vivo* using SPME followed by HPLC-UV measurements. *In vivo* experiments were performed by *Flow System Model* (Artificial Vein System/Heart–Lung Machine, HLM).

2. Experimental

2.1. Materials

Monomers: pyrrole (98%) and thiophene (99%) (both were freshly distilled before use), tetrabutylammonium perchlorate (purity ≥ 98.0% (T)) and tetrabutylammonium tetrafluoroborate (purity ≥ 98.0% (T)) were purchased from Sigma–Aldrich (Schnelldorf, Germany). Linezolid and daptomycin were supplied from Pharmacia GmbH (Karlsruhe, Germany) and Novartis Pharma

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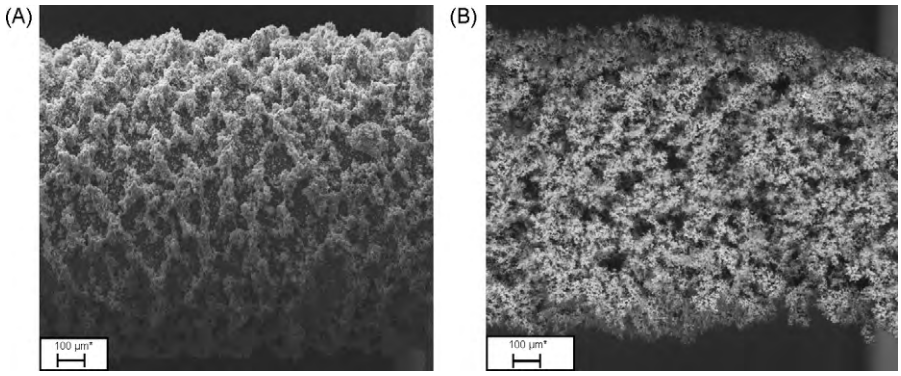
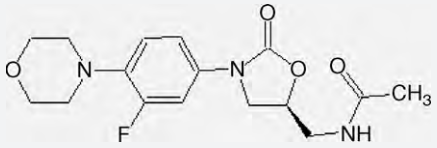
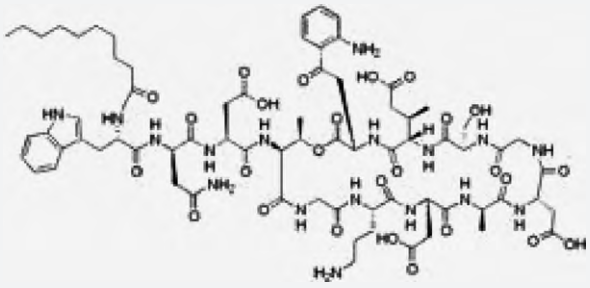


Fig. 1. SEM images of chosen polypyrrole (A) and polythiophene (B) fibers.

Table 1
Structure, molecular masses and protein binding of applied antibiotics drugs.

Drug (%)	Structure	Molecular mass (g/mol)	Protein binding
Linezolid		337.35	31
Daptomycin		1619.71	90–95

GmbH (Nuremberg, Germany), respectively. Linezolid was dissolved in buffer solution for infusion (Zyvoxid®) in concentration 2 mg/ml. Daptomycin was a powder (Cubicin). Their structures and molecular masses are presented in Table 1. Phosphate buffer saline (PBS) was diluted to reach pH = 7.4. Acetonitrile, methanol and water were purchased from Merck KGaA (Darmstadt, Germany). All these chemicals and reagents were of analytical grade and used without further purifications. Whole human blood (5 × 300 ml) was from healthy donors and collected in presence of anticoagulant (heparin).

2.2. SPME fibers

Two kinds of polypyrrole (PPy) and one polythiophene (PTh) SPME fibers were used in experiments. All fibers were prepared by using the electrochemical polymerization with a silver electrode as a reference electrode (ϕ = 500 μm), medical steel as a working electrode (ϕ = 750 μm) and platinum net as a counter electrode. Potentiodynamic conditions with constant boundary potential, different for polypyrrole (−0.4 to +2.0 V, 7 scans) and polythiophene (−0.4 to +2.2 V, 14 scans) were used. In all experiments constant polarization speed was set at 50 mV/s. Polypyrrole SPME coatings were prepared in two basic electrolytes (tetrabutylammonium perchlorate and tetrabutylammonium tetrafluoroborate). Polythiophene fibers were prepared in tetrabutylammonium perchlorate. Concentration of used electrolyte (in acetonitrile), thiophene and pyrrole were 0.1, 0.25 and 0.25 M, respectively [16]. Basic electrolyte, thickness and length of obtained materials are described in Table 2. High performance PGSTAT128N series Autolab model was used in all syntheses.

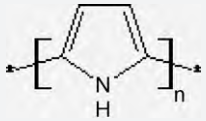
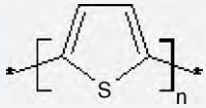
Thickness of SPME coatings was determined by Scanning Electron Microscopy (SEM) LEO 1430VP (LEO Electron Microscopy, Cambridge, UK). Example SEM micrographs of prepared fibers is shown in Fig. 1.

2.3. Chromatographic analysis of linezolid and daptomycin

The HPLC 1100 system (Agilent, Waldbronn, Germany) with binary pump, automatic sample injector and UV/DAD detector of 1200 Series was used for the measurement of linezolid concentrations. The column used in all measurements was Zorbax RX-C8, 150 × 2.1 mm with YMC Pack ODS-AQ, 10 × 2 μm guard column. Agilent ChemStation LC 3D systems software was used for data acquisition. The detection wavelength was set at λ = 251 nm. Isocratic conditions were applied: 20% ACN–80% water, the flow rate was 240 μl/min.

The HPLC system consisted of Shimadzu (Kyoto, Japan) HPLC 10A Series modules, degasser, two pumps, column oven, auto injector, sample cooler, UV-detector and system controller was applied for daptomycin determination. A guard column was Nucleosil C8 HD (100-5, 150 × 2.1 mm from Macherey-Nagel, Düren, Germany). The mobile phases composed of 0.08% formic acid in water (A) or acetonitrile (B) at a flow rate of 300 μl/min to gradient stages 0–13 min 66% A and 13–23 min 0% A were applied. Column temperature was 40 °C. Daptomycin was detected at λ = 219 nm.

Table 2
Kind of polymers used in experiments, applied basic electrolyte during synthesis, length and thickness of prepared fibers.

Kind of polymer	Structure of polymer	Basic electrolyte	Length (mm)	Thickness (μm)
Polypyrrole, PPy		Tetrabutylammonium perchlorate	15	90–95
Polypyrrole, PPy		Tetrabutylammonium tetrafluoroborate		90–95
Polythiophene, PTh		Tetrabutylammonium perchlorate		145–155

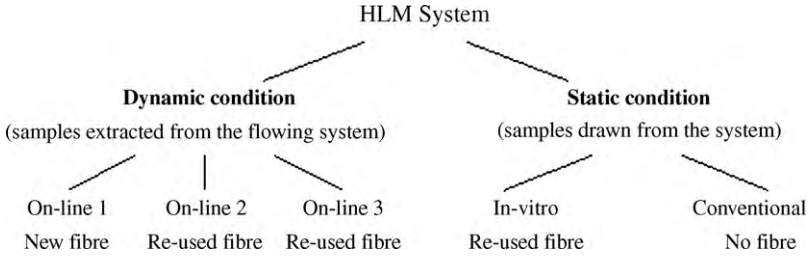


Fig. 2. Heart and Lung Machine (HLM) setup and sampling.

2.4. Method validation

Basic validation parameters such as limit of detection (LOD = $3 \times SD_{xy}/b$, where SD_{xy} is the standard deviation and b is the slope), limit of quantification (LOQ = $10 \times SD_{xy}/b$), determination coefficients (R^2) and relative standard deviations (RSDs) have been determined [23].

2.5. SPME experiments from standard solutions

Experiments were performed using standard solutions (phosphate buffer saline, PBS). For both drugs adsorption and desorption were performed in 1.5 ml solution at constant time: 10 min for adsorption and 5 min for desorption. Desorption was performed in pure methanol each time. After adsorption–desorption cycle fibers were conditioning in methanol/water (9:1) mixture for 10 min.

Time profiles for linezolid and daptomycin were analysed in standard solutions. Samples after extraction were evaporated and then dissolved in 50 μl of 20% and 35% ACN for linezolid and daptomycin, respectively. Analyses of resolubilized samples were performed according to procedure described in Section 2.3.

Two kinds of polypyrrole fibers prepared in perchlorate and tetrafluoroborate and polythiophene fibers prepared in perchlorate salt were compared. A linezolid in PBS solution at concentration 15 μg/ml was applied. During experiments three parallel measurements and 6 adsorption–desorption cycles with earlier conditioning of the fibers were performed.

2.6. Artificial vein system (Heart–Lung Machine, HLM)

Experiments were performed using artificial vein system [24] in whole blood for linezolid and daptomycin at six concentrations for each drug. The concentrations were adjusted to therapeutic range: 1, 3, 5, 8, 10, and 15 μg/ml for linezolid and 4, 12, 20, 32, 40, and 60 μg/ml for daptomycin. Experiments by using artificial vein system were performed at constant flow speed of blood equal 100 ml/min. Additionally, in two concentration, 5 and 15 μg/ml for linezolid and 20 and 60 μg/ml for daptomycin. The applied flow rates were 50, 250, 500 ml/min. HLM setup is briefly shown in Fig. 2.

Samples were extracted from the flow system utilizing new and re-used fibers. In these experiments extraction were performed in HLM system in three ports by using of polythiophene fibers. In port 1 new fiber was used each times. In port 2 re-used fibers were applied. Adsorption and desorption time in the circulating system was 5 min. After adsorption each fiber was washed gently with distilled water.

In vitro SPME experiments were performed by using HLM and polythiophene fibers prepared in tetrabutylammonium perchlorate. *In vitro* measurements were settled down to withdrawal spiked blood from HLM and then adsorption–desorption cycle was performed. For *in vitro* experiments re-used fibers were applied, it means that new fibers were taken each time at the start of measurements (1.5 ml of blood was removed from HLM for each concentration). Adsorption was performed for 10 min each time. After that fibers were gently washed with distilled water and the sample desorption was performed in methanol for 5 min. After conditioning in methanol/water (9:1) mixture for 10 min the same fiber was used again for the next experiment at different concentration. Each experiment was performed three times day after day. The same concentrations were analysed three times with use of three different fibers. After desorption samples were divided into two centrifuge tubes for linezolid and daptomycin determination. Samples were dried in SpeedVac, resolubilized in 50 μl of relevant eluents and analysed with HPLC.

2.7. Conventional analysis of linezolid and daptomycin

Conventional analysis was performed by withdrawal of 1.5 ml spiked blood from HLM and then divided it into two centrifuge tubes for linezolid and daptomycin analysis. Blood samples (1.5 ml) were centrifuged with speed 3500 rpm and then 120 and 175 μl of supernatant were moved to new centrifuge tubes for linezolid and daptomycin analysis, respectively.

Pre-cleaning of plasma samples for linezolid determination was performed by solid phase extraction (SPE) with C2 cartridges con-

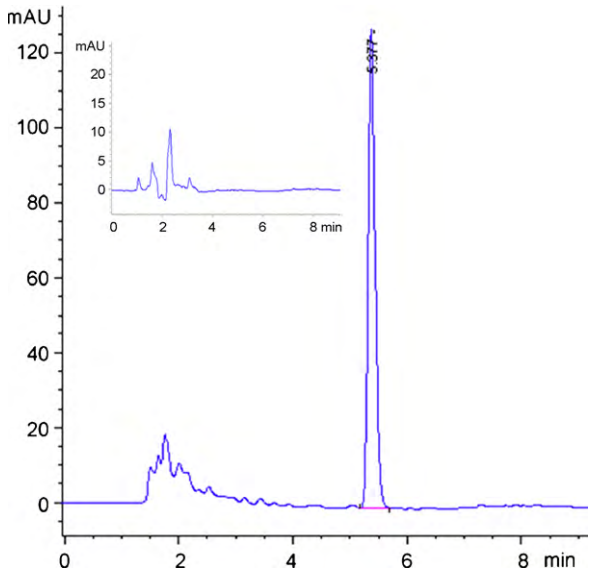


Fig. 3. Chromatogram of plasma samples spiked with linezolid at concentration 4 μg/ml.

taining 100 mg sorbent (Chromabond, Macherey & Nagel, Düren, Germany). The cartridge was conditioned two times with 1 ml of acetonitrile and water. After loading of 120 μl plasma samples sorbent was cleaned with 1 ml of water and 1 ml of 5% acetonitrile. Sample was eluted with 500 μl of methanol and afterwards evaporated, resolubilized in 50 μl of 20% ACN (mobile phase) and analysed with HPLC/UV.

Pre-cleaning of plasma samples for daptomycin determination was performed as described below:

To 175 μl plasma samples 825 μl of methanol was added and sample was centrifuged with speed 3500 rpm. After that supernatant was removed to new centrifuge tube, evaporated, resolubilized in 50 μl of 35% ACN (mobile phase) and analysed by HPLC/UV.

3. Results and discussion

3.1. Method validation

Analytical methods applied in HPLC analysis of linezolid and daptomycin were validated by the calculation of limit of detection (LOD) and limit of quantification (LOQ). These values were calculated according to procedure describe in experimental part. Limits of detection were 25 and 46 ng/ml for linezolid and daptomycin, respectively. Limit of quantification were 82.5 and 151.8 ng/ml for linezolid and daptomycin, respectively. The example shown in Fig. 3 represents the chromatogram of whole blood samples spiked with 4 μg/ml linezolid. The chromatogram of a blank sample as a reference is also shown in this figure.

3.2. Influence of applied counter ions and coatings for extraction efficiency of linezolid

In this experiment extraction abilities of two kinds of polypyrrole fibers prepared in perchlorate and tetrafluoroborate salts and polythiophene fiber prepared in perchlorate salt were compared. For comparison of SPME coatings, linezolid sample in phosphate buffer saline (PBS) at concentration 15 μg/ml was used. Measurements were performed parallel for three coatings by six adsorption–desorption cycles with earlier conditioning of fibers. In all cases first three cycles were characterized by increasing

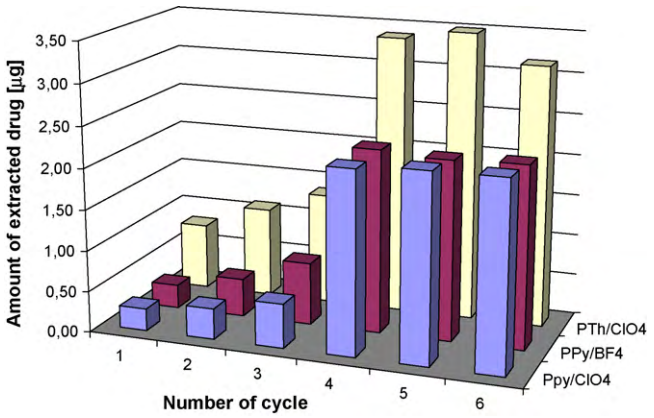


Fig. 4. Comparison of extraction efficiencies of polypyrrole fibers prepared in perchlorate (left) and tetrafluoroborate (right) salt (from PBS).

Table 3
Relative standard deviations and correlation coefficients for different fibers in six cycles.

Number of cycle	PPy/ClO_4		PPy/BF_4		PTh/BF_4	
	SD	RSD	SD	RSD	SD	RSD
1	0.5657	6.36	0.7071	7.44	0.9192	3.45
2	0.2121	1.72	6.7175	44.05	5.8690	16.19
3	0.3536	2.00	7.0711	28.28	4.7376	10.59
4	1.2728	1.74	1.4142	1.93	8.4146	7.56
5	1.4142	1.89	2.9698	4.14	6.6468	5.81
6	2.4749	3.30	2.8284	3.90	17.748	17.06

adsorption capabilities. From fourth cycle in each case amount of adsorbed drug became constant and from this cycle the investigations were started. In all further measurements preconditioning by three cycles in pure water and methanol were performed in order to avoid above-mentioned phenomenon.

On the basis of performed measurements one can notice that the counter ion does not affect the extraction efficiency (Fig. 4). In the case when anions with bigger difference in molecular size and properties were used the differences in extraction efficiencies have been reported [21,25].

The extraction capacity of polythiophene fibers prepared in perchlorate salt was about 50% higher than the capacity of polypyrrole fibers (Fig. 4). Based on this result polythiophene fibers were used in further experiments.

The relative standard deviations (RSDs) of extraction for linezolid and with all studied fibers were comparable (Table 3). After reach the equilibrium in fourth cycle the RSDs for both polypyrrole fibers were in range from 1.74 to 4.14. Relative standard deviations in polythiophene case were in range from 5.81 to 17.06.

3.3. Conventional analysis of linezolid and daptomycin

The results of conventional analysis performed without use of SPME fibers are shown in Fig. 5A and B for linezolid and daptomycin, respectively. RSD values calculated for linezolid and daptomycin were 12.02 and 4.04%, respectively.

3.4. Heart–Lung Machine experiments

Experiments with flow system (*FS*) were performed in three ports where in the first one (*FS1*) only new fibers were using each times. In port two (*FS2*) and three (*FS3*) re-used fibers were using as in *in vitro experiments*. Fig. 6 shows difference observed between new (*FS1*) and re-used (*FS2*) fiber for linezolid and daptomycin, respectively. Results from third port (*FS3*) exhibited less linear

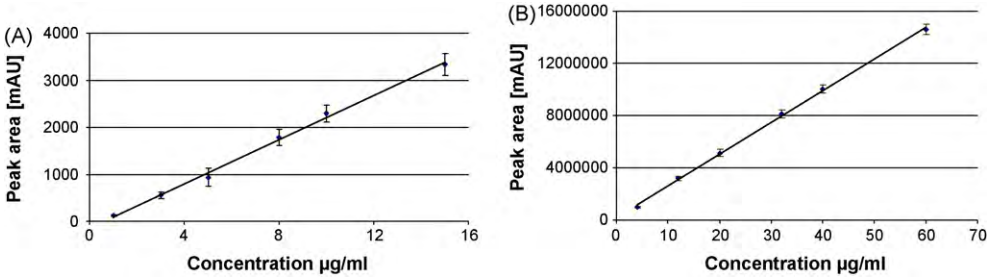


Fig. 5. Results of conventional analysis performed for linezolid (A) and daptomycin (B) in the HLM setting (from whole blood).

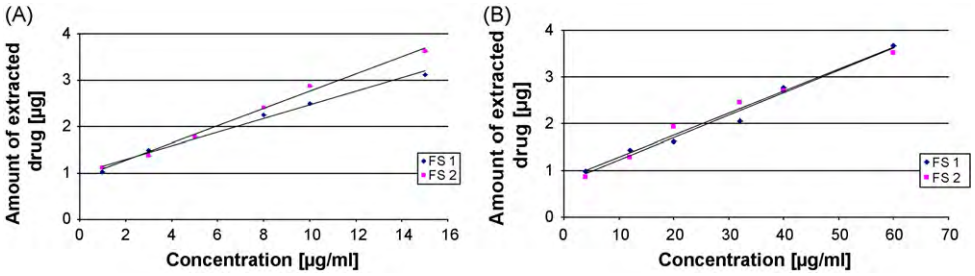


Fig. 6. Extraction efficiencies for linezolid (A) and daptomycin (B) with use of HLM (*FS1* and *FS2*) (from whole blood).

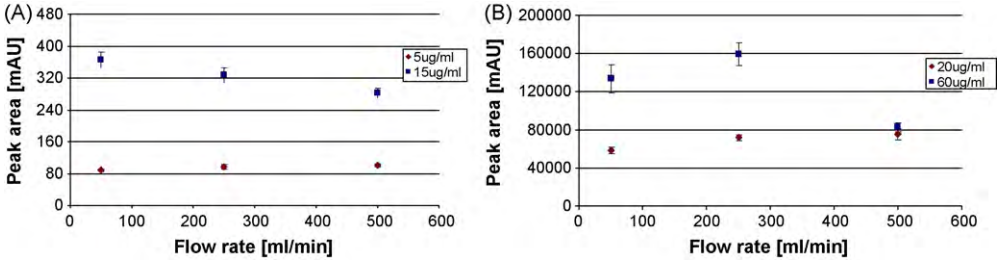


Fig. 7. Flow rate dependence for adsorption of (A) linezolid (from whole blood) and (B) daptomycin (from whole blood).

character. Probably, the major reason for this observation was insufficient flashing of inserted SPME fibers by circulated whole blood.

Absolute extraction efficiencies of linezolid and daptomycin were in the range of 20–30 and 5–10%, respectively. For linezolid determination low RSD were observed for both new and re-used fibers. In the case of daptomycin results are also promising but obtained correlation data are not as good as obtained for linezolid. Worse results in case of daptomycin might be caused by a high protein binding exhibited by this drug. Daptomycin is bounded by plasma protein ranging from 90 to 95%. In the case of linezolid protein binding is not as high (about 30%).

In vitro measurements (static condition) performed under the condition described in Section 2.6 with re-used fibers and port 3 did not allow obtaining of as good results as were obtained with the use of ports 1 and 2. The main reason for this could be the location of port 3. In case of ports 1 and 2 the blood flows through whole port. In the case of port 3, flowing blood did not flushed the whole port and hence also fiber.

Relative standard deviations and correlation coefficients values obtained for *FS* (all used ports) and *in vitro* experiments are shown in Table 4.

Flow rate dependence of adsorption efficiency was investigated for both drugs. In both cases similar dependences were observed (Fig. 7). In lower concentrations (5 μg/ml for linezolid and 20 μg/ml for daptomycin), only small changes in amount of adsorbed drugs were noted depending on the flow rate. Significant difference was observed for higher concentrations (15 μg/ml for linezolid and 60 μg/ml for daptomycin). These results are in a good agreement with the previous observations by Schubert et al. [18].

Table 4
Relative standard deviations and linear correlations for linezolid and daptomycin in *FS* and *in vitro* experiments.

	Linezolid		Daptomycin	
	RSD	R^2	RSD	R^2
FS1 (*new fiber*)	9.54	0.9878	11.80	0.9671
FS2 (*re-used*)	4.58	0.9968	6.09	0.9888
FS3 (*re-used*)	15.91	0.9951	17.33	0.8484
In vitro (*re-used*)	7.06	0.9830	12.29	0.9241

4. Conclusions

In this work two drugs were analysed with the use of solid phase microextraction as a sample preparation method. As an adsorbent two kinds of porous materials prepared in different basic electrolyte were applied. Measurements in standard solutions and whole blood were performed.

This study showed that it is necessary to perform at least three cycles in water to precondition each fiber to optimal adsorption capabilities. On the basis of investigations performed with the use of linezolid in PBS solutions it was proved that after proper precon-

ditioning polythiophene fibers are able to adsorb about 50% more tested drugs in comparison to polypyrrole fibers. Obtained results confirmed the potential of SPME with studied fibers as a fast and cheap near-bed directly sampling of drugs from human body.

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