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Quantitative evaluation of sparfloxacin binding to urological catheter surfaces

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Our aim was to apply high-performance liquid chromatography method for quantitative evaluation of the total amount of sparfloxacin (SPA) immobilized on the surface of the antimicrobial urological catheters. The amounts of SPA bound to catheter were determined indirectly on the basis of the differences in SPA concentrations before and after the immobilization process (they have been shown to vary from 0.11 to 5.66 mg/g of catheter). We estimated the immobilization yield, which altered from 14% to 70% depending on the SPA concentration used. As *in vitro* release studies show, the antibiotic binds to the catheter matrix in two modes: relatively stable covalent bond and weak non-covalent bond. Antibacterial activity of the modified catheter samples with SPA was controlled by using the zone of inhibition test against gram positive and gram negative bacteria.

1. Introduction

Urinary tract drainage using catheters represents a fundamental aspect of urological practice. Putting these medical devices into a patient's bladder can lead to serious problems that limit their function, such as urinary tract infections, incrustation, patient discomfort and difficulties associated with catheter removal.

Fluoroquinolone antibiotics, administered orally, are widely used in the treatment of urinary tract infections which are predominantly caused by *Escherichia coli* and other Enterobacteriaceae, as well as by Staphylococci, Enterococci and non-fermenting bacteria such as *Pseudomonas* spp. These antibiotics are highly potent, broad–spectrum agents which penetrate bacterial cell walls and inhibit bacterial DNA gyrase activity, rapidly killing bacteria or preventing their growth (Kuhlmann et al. 1998; Sharma et al. 2009). However, clinical and *in vitro* data suggest that certain antibiotics including fluoroquinolones eradicate the planktonic microorganisms from the urine but do not eradicate the biofilms that adhere to devices**.** In complicated catheter-associated urinary tract infections, the susceptibility of the pathogens is several times lower when compared to the planktonic cells or pure culture cells. Therefore, it is necessary to use several times higher concentration of antibiotics to effectively overcome biofilm infections (Pascual 2002; Tenke et al. 2006; Reid 1999; Choong et al. 2001; Nicolle 2005; Yassien et al. 1995).

Despite the current lack of a completely biofilm-resistant catheters, the ongoing research for new antibacterial devices is promising. In several *in-vitro* studies, Stickler and associates (Jones et al. 2006) have shown that the filling of the retention balloons of urinary catheters with the antibacterial agent triclosan prevents the biofilm formation and incrustation processes.

Antimicrobial activity of the catheters has been achieved also by covering the catheter surface with silver and other metals (Johnson et al. 1990; Roe1 et al. 2008; Sekiguchi et al. 2007; Yao et al. 2008) or by controlled release antimicrobial agents or antiseptics incorporated into the catheter material (Baveja et al. 2004; Reid et al. 1994; DiTizio et al. 1998; Raad et al. 1996; Chaiban et al. 2005; Hachem et al. 2009).

We have previously reported on the development of antimicrobial urological catheters using the mixed, covalent and non-covalent binding of fluoroquinolone antibiotic, sparfloxacin, to heparin film applied on its silicone-coated latex surface (Kowalczuk et al. 2010).

The purpose of the present study was to evaluate the application of HPLC method as a tool for the quantitative evaluation of the total amount of sparfloxacin immobilized on the surface of the developed antimicrobial urological catheters, as well as for the evaluation of the *in-vitro* release of SPA from the modified catheter surface while controlling the antimicrobial activity of the tested catheter samples.

2. Investigations, results and discussion

The presence of heparin layer with sparfloxacin on the catheter surface was confirmed using the scanning electron microscopy and ATR-FTIR analysis (Kowalczuk et al. 2010). The catheter surface changes during the surface modification using two methods, named as *method 1* and *method 2* (Ginalska and Kowalczuk 2011), were also observed under ultraviolet light at 366 nm. The untreated latex samples and SPA-treated samples were visible as green fluorescent surfaces, but in the case of samples with SPA the fluorescence observed was more intensive. The samples coated with heparin appeared as fluorescence-quenched surfaces.

The HPLC method (Kowalczuk et al. 2011) with small modifications was adopted for the quantitative assessment of SPA binding to the HP-coated catheter surface modified using *method 1* and

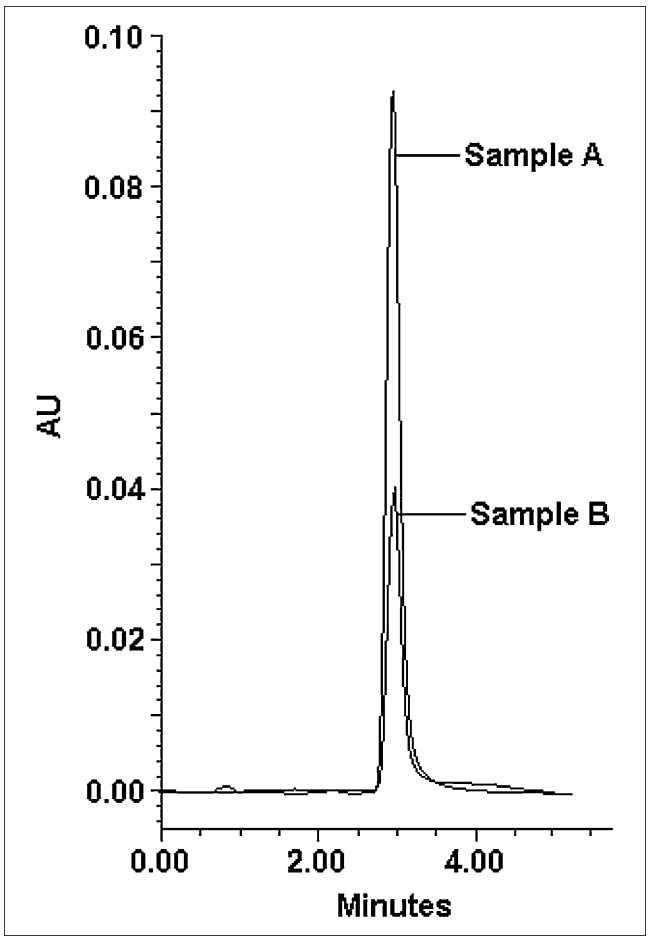

Fig. 1: Typical chromatograms obtained for the solutions before (sample A) and after (sample B) immobilization at initial SPA concentration of 1.0 mg/ml.

method 2. The total amount of SPA attached to the catheter surface via relatively stable covalent imine bond and other weak non-covalent bonds was determined using the HPLC method. This method allowed to estimate the release of SPA from the modified catheter surface as well.

The linear calibration curve (Mandel's linearity test: $TV = 1.01 < F_{99\%,\ (1,15)} = 8.68$) was constructed by plotting the peak areas of SPA standard solutions versus the concentrations in the range of 20.0–0.625 μg/ml and was expressed by the following equation: y = 96999.7 (± 1236.9) x + 10571.1 (± 11660.2) with correlation coefficient of 0.999.

The total amount of SPA bound to the modified catheter surface via the mixed-type bonds were calculated on the basis of the differences in SPA concentrations in the solutions prepared for immobilization and obtained after this process. The exemplary overlapped chromatograms obtained for the solutions before and after immobilization are illustrated in Fig. 1. The results achieved during optimization of the immobilization process are summarized in Table 1. The bound amounts of SPA as a function of the initial SPA concentrations is shown in Fig. 2. As it can be observed (Table 1, Fig. 2), the quantities of SPA attached to the catheter changed concurrently with the changes in the antibiotic concentrations in solutions used for immobilization. The immobilization of SPA increased progressively with the initial SPA concentration. The saturation was finally obtained at SPA concentration of 1.0 mg/ml, with the attachment of approximately 5.66 mg SPA per 1 g of catheter (*method 1*). In case of *method 2*, the immobilization of SPA increased as the initial SPA concentration increased in the range from 0.1 to 2.0 mg/ml with tendency for the saturation at SPA concentration above 1.0 mg/ml (a attachment of approximately 2.5 mg SPA per 1 g of

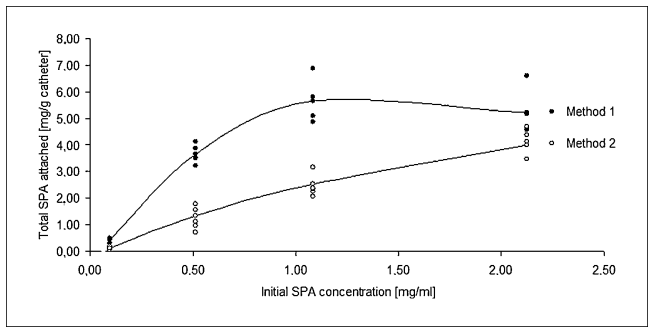

Fig. 2: Influence of SPA concentration in the solutions used for immobilization on the SPA amount attached to the catheter matrix for both immobilization methods.

catheter). The immobilization yield (Table 1, Fig. 3), expressed as percentage of SPA associated with the matrix compared to initial SPA concentration of 1.0 mg/ml, was found to be at an average of 56% for *method 1* and 31% for *method 2*.

The immobilization methods allowed to create covalent bonds between SPA and the modified surface of catheter. The non-covalent interactions of SPA with the heparinized surface such as physical adsorption, ionic interactions, hydrogen bond, turned out to be possible under the immobilization conditions applied. The affinity of SPA to the heparinized catheter depends on the matrix hydrophilicity which is related to the functional groups capable of specific interactions with this antibiotic. In case of heparin, which contains acidic sulphate groups and hydroxyl groups in its polysaccharide chain, ionic interactions and hydrogen bond interactions can take place. Via its amino groups, SPA can create acid-base interactions with the heparinized catheter.

The release of SPA incorporated into the catheter matrix was tested by carrying out washings with numerous portions of different solvents (water, sodium chloride solution, phosphate buffers at pH 3.2 and at pH 8.5). The amount of SPA released intra-day gradually decreased with each successive washing, finally reaching the detection limit of the HPLC method (LD = 0.02 μg/ml). The smallest quantity of the drug was released to water (0.85–1.25%), while its greatest quantity was released to the basic medium (4.58–6.85%) (Table 2). The amount of SPA remaining on the modified catheter surface after washing was calculated from the difference between the total amount of SPA immobilized into the matrix and the total amount of the antibiotic determined in the washings (Table 2). The inter-day release of SPA (every other day, for more than month) proceeded in the similar way, but with greater deviations

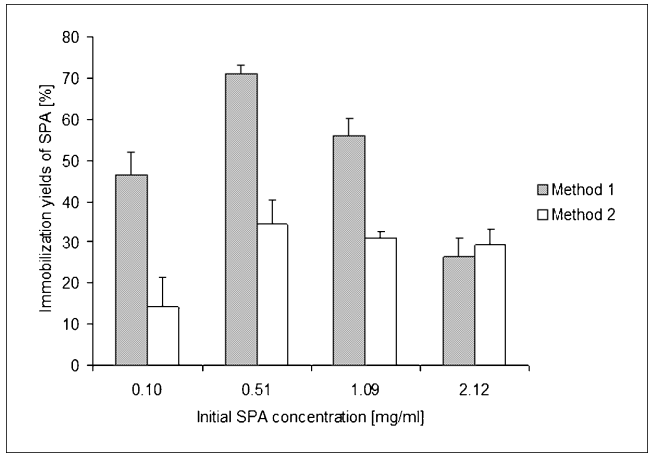

Fig. 3: The yields of immobilization process obtained for *method 1* and *method 2* versus initial SPA concentrations. Data are representative of means ± SD of five independent tests.

Table 1: Evaluation of the immobilization of sparfloxacin (SPA) at various concentrations according to *method 1* and *method 2* with use of HPLC method; solution before (solution A) and after (solution B) immobilization

Samples	Solution A (mg/ml)	Solution B (mg/ml)	SPA amount (mg/piece)	attached to catheter (mg/g catheter)	Immobilization yield (%)
Method 1					
S1	2.1249	1.5614	$0.5635 \pm 16.08^{*}$	5.2233	26.52
S2	1.0854	0.4778	$0.6076 \pm 7.62^{*}$	5.6600	55.98
S3	0.5123	0.1488	$0.3635 \pm 2.95^{*}$	3.6419	70.96
S4	0.0969	0.0518	$0.0451 \pm 11.63^{*}$	0.4437	46.57
Method 2					
S1	2.1249	1.5030	$0.6219 \pm 13.18^{*}$	4.4142	29.27
S2	1.0854	0.7510	$0.3345 \pm 5.69^{*}$	2.5356	30.81
S3	0.5123	0.3370	$0.1753 \pm 18.08^{*}$	1.3590	34.21
S4	0.0969	0.0830	$0.0139 \pm 28.65^{*}$	0.1126	14.39

* average value ± RSD% for n = 10

Table 2: Intra-day release study of sparfloxacin (SPA), immobilized on the catheter according to *method 1* (*M1*) and *method 2* (*M2*), to the different media by HPLC method

Medium		Total amount of SPA attached to catheter (mg)	Amount of SPA released from catheter (mg)	(%)	Amount of SPA remained on catheter (mg)	(%)
Water	*M1*	0.6254	0.0078	1.25	0.6176	98.72
	M2	0.3029	0.0026	0.85	0.3003	99.15
NaCl	*M1*	0.5283	0.0082	1.55	0.5201	98.45
	M2	0.3458	0.0030	0.87	0.3428	99.13
Phosphate	*M1*	0.6254	0.0424	6.85	0.5829	93.15
buffer pH 8.5	*M2*	0.3387	0.0152	4.58	0.3235	95.42
Phosphate	*M1*	0.5943	0.0119	2.01	0.5823	97.99
buffer pH 3.2	*M2*	0.3552	0.0137	3.79	0.3416	96.21

from the trend line (Fig. 4). It was found that the total amount of SPA released to washing solvent, after 40 days, varied from 63.0 to 166.1 μg (that is 6.86–18.08%), depending on the washing solvent used, immobilization method or size of catheter sample. The biggest quantity of the drug was released in the first washing and the significant part of the drug was released in the initial five washings. The release study showed that a relatively inconsiderable fraction of the total amount of the initially attached SPA was released, hence it can be concluded that SPA attachment to the modified surface of catheter is relatively stable.

The HP-coated SPA-attached catheter can be categorized as an antibiotic carrier. Taking this fact into account, the release profile was tested using the phosphate buffer solution at pH 5.5 (pH of urine) as the dissolution medium. As shown in Fig. 5, the release of SPA from the carrier increased progressively, showing a tendency for the stabilization. It was found that the average amount of SPA released to 1 ml of phosphate buffer, after 3 h, was 0.282 μg (it is 0.035%) for *method 1* and 0.054 μg (it is 0.011%) for *method 2*. Similarly as in the test described above, a relatively small fraction of the total amount of the initially attached SPA has been released.

Antibacterial activity of the test catheter samples against *E. coli*, *S. epidermidis* and *S. aureus* strains at each stage of its preparation and during release was examined using plate antimicrobial test. The obtained effects are shown in Fig. 5 (A, B and C, respectively). The zones of inhibition, on Mueller Hinton agar, around the catheters modified in a solution of 0.1 mg SPA/ml (samples S4, containing an average of 0.05 mg of SPA) were 18–36 mm. In the case of the catheters modified in a solution of 1.0 mg SPA/ml (samples S2, containing an average of 0.6 mg of SPA), the inhibition zones were 32–42 mm (the similar results were obtained previously, Kowalczuk et al. 2010). Catheters without

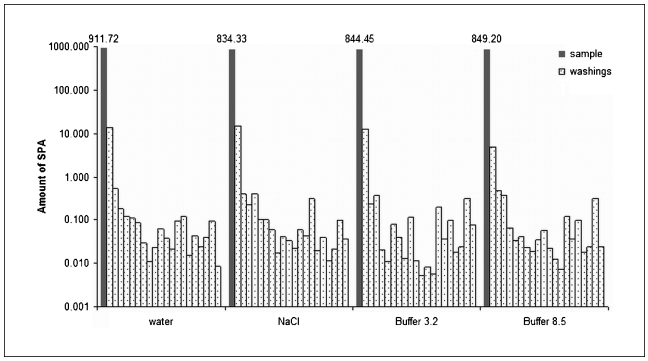

Fig. 4: The inter-day release of SPA from the modified catheter to the different washing solutions (μg SPA/ml of washing solution); sample = the modified catheter piece with SPA; logarithmic scale.

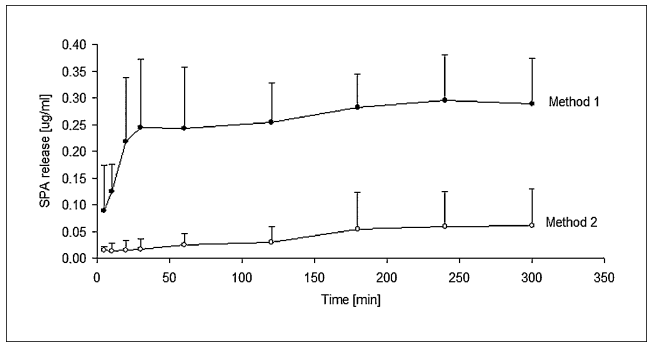

Fig. 5: *In vitro* release of SPA as a function of time at pH 5.5 observed for both immobilization methods.

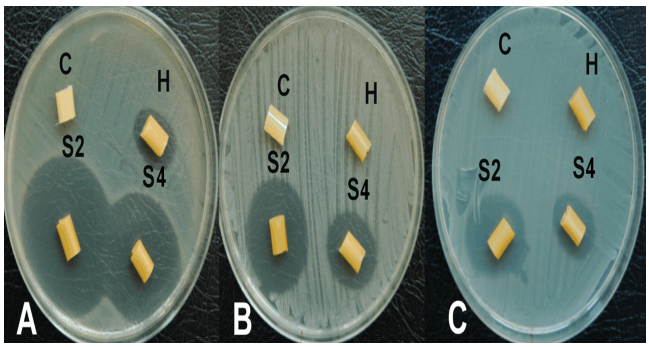

Fig. 6: Inhibition zones around the test samples as their effect against *E. coli* (A), *S. epidermidis* (B) and *S. aureus* (C) strains; SPA-HP samples obtained after immobilization in SPA solutions of 1 mg/ml (S2) and 0.1 mg/ml (S4), HP test samples (H), the untreated samples as control (C).

antibiotic showed no zone of inhibition against all tested strains, similarly to the catheters coated with heparin and oxidized (without antibiotic), with the only exception of small inhibition zone against *E. coli* strain. Judging from the dimensions of inhibition zones of bacterial growth around antibiotic-containing catheter samples, it can be concluded that the release of SPA depends on the total amount of the SPA attached as well as on the strength of the antibiotic-catheter binding. Results demonstrate also a slightly higher activity of SPA against the gram-negative *E. coli* than against the gram-positive *S. aureus* and *S. epidermidis*.

In conclusion, the HPLC method was applied for the quantitative evaluation of the total amount of SPA attached to the matrix of the developed antimicrobial urological catheter via mixed, covalent and non-covalent bonds. The studies of the SPA release from HP-coated SPA-attached surface proved that the binding of SPA is relatively stable. The SPA release from the modified catheter proceeded gradually while maintaining antimicrobial activity against *E. coli*, *S. aureus* and *S. epidermidis*. Despite intensive washing, the significant part of SPA immobilized via covalent bonds remained on the catheter. This characteristics of the drug release guarantees the effective protection against infections.

3. Experimental

3.1. Materials and chemicals

Pure sparfloxacin (SPA) was obtained from Rhône-Poulenc Rorer. Heparin sodium salt (HP) and all other reagents used for the immobilization process were obtained from Sigma-Aldrich Co. Foley urinary catheters of a natural latex coated with silicone fluid (Tyco Healthcare/Kendall, UK) were obtained from a local medical supplier (Skamex, Poland). Methanol, acetonitrile (both of HPLC grade) and all other reagents and solvents of analytical grade were obtained from POCh (Poland). Mueller-Hinton agar from Oxoid (UK). The water used in the experiments was double–distilled. Phosphate buffer solution (0.1 M) was prepared by dissolving 13.6 g of potassium dihydrogen phosphate in 500 ml water, adjusting the pH to 3.2 with phosphoric acid, or to 5.5 and 8.5 with sodium hydroxide and making up to 1000 ml with water. Sodium chloride solution (0.1 M) was prepared by dissolving 5.8 g of sodium chloride in 1000 ml of water.

3.2. Preparation of the test catheter samples

The test catheter samples were prepared according to the procedure described in a Polish Patent (Ginalska and Kowalczuk 2011). Briefly, the catheter segments of about 0.5, 1.0 or 2.0 cm in length were coated with heparin and then modified using two immobilization methods. In the *method 1*, the heparin-coated surfaces were directly oxidized and coupled with SPA in organic medium to form a Schiff base. In the *method 2*, the heparin-coated surfaces were activated with glycidol to introduce the diol groups, then oxidized and linked with SPA in organic medium to form a Schiff base. In order to optimize the immobilization process, HP-coated and oxidized samples were subjected to impact of SPA solutions in different concentrations of 2.0 mg/ml (S1), 1.0 mg/ml (S2), 0.5 mg/ml (S3), 0.1 mg/ml (S4).

3.3. HPLC determination of total amount of the immobilized sparfloxacin

Chromatographic analysis was carried out at λ=300 nm (spectrophotometric (UV) detection) and at λ_{ex} 290 nm/λ_{em} 530 nm (fluorescence (FL) detection) on a Waters HPLC system (USA) equipped with a Nova-Pak® RP-18 (3.9 mm × 150 mm, 5 μm) column, using a mixture of acetonitrile-methanol-water-85% phosphoric acid (25 : 25 : 50 : 0.025, v/v/v/v) as mobile phase at a flow rate of 1.0 ml/min.

The calibration solutions in concentrations of 0.625 μg/ml, 1.25 μg/ml, 2.5 μg/ml, 5.0 μg/ml, 10.0 μg/ml i 20.0 μg/ml were prepared by appropriate diluting the stock SPA with mobile phase in concentration of 100 μg/ml. The SPA solutions prepared for the immobilization process (S1–S4) and the SPA solutions obtained after the immobilization process were diluted 1:100 with the mobile phase and were subjected to HPLC analysis using UV-detection, and FL-detection for the control. The concentrations of SPA in the tested solutions were calculated from the calibration equation after multiplying by the dilution factor.

3.4. Release study in vitro

The studies of SPA release from the HP-coated SPA-attached surface were carried out through washing, under vigorous stirring, in various washing solutions and evaluating the amount of the released antibiotic in the washing solutions by UV-FL HPLC.

Intra-day release study. The SPA-treated catheter segments were immersed in 2 ml of a washing solution (distilled water, sodium chloride solution, phosphate buffer solution at pH 3.2, or phosphate buffer solution at pH 8.5) and were shaken repeatedly every 30 min with new portion of the washing solution. The drug released to washing solution was determined directly (without diluting). The experiment was conducted three times.

Inter-day release study. The SPA-treated catheter segments were immersed in 10 ml of washing solution (distilled water, sodium chloride solution, phosphate buffer solution at pH 3.2, or phosphate buffer solution at pH 8.5) and were repeatedly shaken every second day, for 40 days, with the washing solution changed after each shaking. The drug released to washing solution was determined directly (without diluting). The experiment was repeated three times.

3.5. Release profile

The drug release study was carried out using a paddle apparatus (Distek, USA) at pH 5.5, at the temperature of 37 °C and at rotation speed of 75 rpm. The modified catheter segments with SPA were immersed in 500 ml of the phosphate buffer solution as the dissolution medium. Samples (1 ml) were withdrawn from each recipient at definite time intervals and replaced with the same volume of fresh phosphate buffer solution previously heated to 37 °C for compensating the sampling. The dissolution study was carried out for three samples. The amount of SPA in solution samples was determined chromatographically.

3.6. Microbiological test

Antibacterial activity of the test catheter samples (S1–S4) against *Echerichia coli* (ATCC 25992), *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 12228) strains at every stage of its preparation were controlled using procedure described previously (Kowalczuk et al. 2010). Briefly, the samples were placed on the agar plates containing the appropriate bacterial test strain (1.5×10^8 CFU/ml) and then incubated at 37 °C for 24 h. Thereafter, the inhibition zones of microbial growth produced around the catheter segments were observed.

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