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Standardised HPLC protocol adapted to drug eluting and ELISA protocol for the analysis of cytokine expression profiles under dynamic conditions

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Abstract

In ReBioStent special focus is laid on the reduction of animal experiments. For a detailed *in vitro* analysis of stent performance under dynamic conditions the biomechanical reactor will be facilitated. Here, protocols for the analysis of media samples taken from the reactor are presented. On the one hand drug elution profiles can be determined using the developed HPLC procedure. On the other hand potential inflammatory induction of endothelial cells is to be analysed using the described cytokine expression profiling.

This deliverable is of the nature “Other” and this document describes the protocols presented.

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Impressum

Reinforced Bioresorbable Biomaterials for Therapeutic Drug Eluting Stents

ReBioStent

WP8: *In vitro* assays under simple cell culture conditions and simulated *in vivo* studies

Task 8.4: Drug elution and cellular response

Deliverable D8.7: Standardised HPLC protocol adapted to drug eluting and ELISA protocol for the analysis of cytokine expression profiles under dynamic conditions

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Executive summary

Although today's stents are of great benefit to patients with coronary artery disease complications such as in-stent restenosis and thrombosis do occur in some cases. The stents developed during the ReBioStent project will address these issues and therefore techniques for the detailed *in vitro* and *in vivo* characterisation of these features will be needed. D8.7 describes two protocols for the *in vitro* analysis of such qualities.

The anti-proliferative drug rapamycin will be incorporated into the stent in order to prevent in-stent restenosis. Thus, the *in vitro* evaluation of the drug release kinetics is of great importance to achieve the best possible reduction of neointimal growth *in vivo*. Here, the protocol for the HPLC-assisted determination of drug amount is presented which can be used to analyse the drug release profile under dynamic conditions.

Thrombosis may be a result of incomplete endothelialisation which among others can occur if the material induces an inflammatory response. In this case inflammatory signalling molecules such as interleukins are upregulated in various blood cells and endothelial cells. Here, an enzyme-linked immunosorbent assay-based analysis of the expression of interleukins 6 and 8 by endothelial cells is described.

List of authors

Company	Author	Contribution
RUB	Dr. Jochen Salber	HPLC protocol development, WP management
RUB	Dr. Sandra Pacharra	Cytokine expression protocol development and evaluation, HPLC evaluation

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1 Introduction

Today, a wide variety of stents is used in percutaneous coronary intervention (PCI) in order to restore blood flow in patients with coronary artery disease (CAD). Extensive studies on existing stents have shown that complications may arise from stent placement the main issues being in-stent restenosis (ISR) and stent thrombosis (ST) [1]. It was found that restenosis can be reduced by the use of drug-eluting stents while early thrombosis is circumvented by dual anti-platelet therapy (DAPT).

1.1 In-stent restenosis and drug-eluting stents

The placement of a stent via PCI causes an injury to the arterial wall which in turn initiates a series of acute and long-term processes [2]. During the resulting inflammatory cascade various immune cells, endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) are activated. VSMC activation then leads to their migration and proliferation which may result in neointimal hyperplasia and finally in restenosis – the re-narrowing of the treated artery [3].

In order to overcome in-stent restenosis drug-eluting stents (DES) were developed their aim being to control or reduce VSMC migration and growth as well as to prevent the inflammatory response [4]. An advantage of this approach is that biologically active agents can be directly delivered to the target site, resulting in therapeutically effective drug concentrations in the surrounding tissues with minimal systemic release of the drug.

One of the commonly used drugs in coronary stents is rapamycin (sirolimus). It prevents the proliferation and migration of smooth muscle cells but also the proliferation of lymphocytes by blocking G1 to S cell cycle progression [5]. Thus, the risk for ISR is reduced by inhibiting VSMC growth and by decreasing the immune response in the surrounding tissue.

To ensure drug retention during stent deployment and optimal restenosis reduction afterwards it is important to have precise control of the drug release kinetics. VSMC proliferation starts one day after the injury resulting from PCI and lasts about two weeks [4]. Thus, it is believed that anti-restenotic drugs need to be delivered for at least 3 weeks to prevent smooth muscle cell migration and proliferation.

1.2 Stent thrombosis

Subacute thrombotic coronary artery occlusion observed after stent placement, although infrequent, remains a devastating and unpredictable event that has a significant mortality [3,6]. Early ST (occurring in the first 30 days after implantation) mainly induced by patient-specific factors or mechanical issues can be prevented by the use of dual antiplatelet therapy. In contrast, late and very late ST (up to and after 1 year after implantation) are especially associated with the use of DES. In addition to their anti-restenotic properties,

the incorporated drugs also delay the process of re-endothelialisation and inhibit vascular repair which is thought to facilitate thrombus formation [7].

1.3 Inflammation and endothelial cells

Endothelial cells are a central part in the arterial physiology. In their basal state ECs maintain a non-thrombogenic blood-tissue interface, regulate thrombosis, thrombolysis, vascular tone and blood flow [8]. In response to stimulation they produce a variety of pro-inflammatory cytokines, chemokines and growth factors that recruit and activate inflammatory cells, regulate thrombosis and influence VSMCs.

In addition to the injury caused by PCI and stent deployment also material-specific especially polymeric properties induce inflammation and therefore may lead to ST [9]. The use of biodegradable polymers is thought to reduce polymer induced inflammation, delayed arterial healing, ISR and ST.

1.4 Aim

In the ReBioStent project a stent will be developed that on the one hand enables easy endothelialisation and on the other hand prevents restenosis. To achieve both aims the luminal and abluminal surfaces will be modified differently. The luminal surface will be modified so that particularly the blood vessel endothelial cells attach to and proliferate on top of the surface thus preventing ST. In contrast to that the abluminal surface is supposed to inhibit the adhesion and proliferation of smooth muscle cells in order to prevent ISR. This will be achieved by incorporation of rapamycin or tacrolimus.

For the detailed *in vitro* analysis of the newly developed stents a biomechanical reactor (BMR) will be used. Here, drug elution under flow conditions will be analysed by taking media samples at different time points. Also, endothelialisation can be observed by pre-seeding of stents with primary human endothelial cells. Media samples from these experiments can then be analysed regarding inflammatory signals.

In this report the protocol for the HPLC-assisted determination of drug amount present in media samples is presented. In addition, the enzyme-linked immunosorbent assay (ELISA) based analysis of the inflammatory signalling molecules interleukin (IL) 6 and 8 is shown.

2 Methods

2.1 General cell culture

The primary cells human cardiac microvascular endothelial cells (HCMEC) were obtained from PromoCell.

Primary cells were cultured according to the manufacturer's protocol in endothelial cell growth medium MV on tissue culture-treated polystyrene (TCPS). Medium was changed every three days while subculturing was performed at 70 to 90% confluence using the detach kit from PromoCell (with seeding densities according to Table 1). An incubator with a humidified atmosphere was used at 37°C and 5% CO_2.

Table 1: Seeding numbers

Cells	Subculture	Tube/Stent	Cytokine test
HCMEC	$1\text{-}2\cdot10^4$ per cm^2	$2\text{-}2.5\cdot10^5$ per tube	$1\cdot10^4$ per Well

2.2 Protocol for HPLC analysis of rapamycin elution

2.2.1 Materials

HPLC grade methanol was purchased from Sigma-Aldrich (Schnelldorf, Germany). Water was prepared by a Millipore Milli-Q Plus Water Purification System (Merck KGaA, Darmstadt, Germany). Rapamycin (Sirolimus, VETRANAL™, analytical standard) was provided by Sigma-Aldrich (Schnelldorf, Germany). All reagents were of analytical grade.

2.2.2 Elution of rapamycin under flow conditions

Tubes or stent prototypes were transferred to the vascular graft chamber of the biomechanical reactor (described in detail in D8.5 [10]) and incubated under flow conditions. At different time points media samples were taken and analysed regarding drug elution.

2.2.3 HPLC system and conditions

Chromatographic analysis was performed on the RIGOL HPLC system (Techlab GmbH, Braunschweig, Germany), consisting of a low-pressure gradient pump with integrated in-line degasser (RIGOL L-3245, Beijing, China), UV-Vis detector (RIGOL L-3500, Beijing, China), and a column oven (RIGOL L-3400, Beijing, China). The system was equipped with a Rheodyne injection valve with a 20 µL sample loop (Techlab GmbH, Braunschweig, Germany). HPLC control, data acquisition and statistical analysis was carried out by using Clarity HPLC Data System (Laserchrom HPLC Laboratories Ltd., Rochester, UK). A

reversed phase C8 column was used for separation purchased from MZ Analysentechnik GmbH (100 x 4.6 mm ID, 5 µm particle size, Mainz, Germany). Mobile phase was a mixture of methanol:water (80:20, v/v) which was freshly prepared and degassed. Column temperature was set to 57°C, a flow rate of 0.5 ml/min was used and UV detection was carried out at a wavelength of 277 nm. This method was derived from the procedure described in [11] with minor modifications.

2.2.4 Determining the concentration of rapamycin

A stock solution of rapamycin with the concentration of 1 mg/mL was prepared by dissolving rapamycin in methanol. The obtained solution was stored at - 20°C. All other solutions were prepared by the dilution of the stock solution with appropriate amount of PBS.

For calibration a serial dilution of rapamycin from 250 to 15.6 µg/ml was prepared and analysed via the described HPLC protocol.

2.3 Protocol for the analysis of cellular cytokine expression

2.3.1 Materials

For the cytokine expression analysis the Novex^{TM} ELISA Kits for IL-6 and IL-8 from Invitrogen were used.

2.3.2 Cell culture under flow conditions

Tubes or stent prototypes were seeded with HCMECs under static conditions as described in D8.3 [12]. After 24 hours of static incubation to allow cell adherence the devices were transferred to the vascular graft chamber of the biomechanical reactor (described in detail in D8.5 [10]) and further incubated under flow conditions. At different time points media samples were taken and analysed regarding cytokine expression.

2.3.3 Cytokine ELISAs

The ELISAs were performed according to the manufacturer's protocol. In brief, standards and samples were added to the precoated wells of the ELISA plate. After addition of the appropriate Biotin Antibody Conjugate the plate was incubated at ambient temperature to allow binding. The plate was washed thoroughly and Streptavidin-HRP was added. After incubation at ambient temperature the wells were washed and Stabilized Chromogen was added. The plate was incubated for 30 minutes in the dark and Stop Solution was added. Finally, the absorbance at 450 nm was measured.

The standard curve was fitted using the four parameter algorithm.

2.3.4 Test of cytokine expression after stimulation with LPS

Lipopolysaccharides (LPS) from *Escherichia coli* 055:B5 (Sigma Aldrich) were used to stimulate the inflammatory reaction of HCMECs in order to prove their expression of IL-6 and IL-8.

HCMECs were seeded into the wells of a TCPS 96 well plate (Nunc). After 4 hours of incubation LPS was added to a final concentration of 10 ng/ml or 100 ng/ml while controls were left without the addition of LPS. After an overall incubation of 24 hours the cell supernatants were harvested and either used directly or frozen at -20°C.

Prior to use in ELISAs samples were mixed well, centrifuged to remove particular matter and LPS samples were diluted with Standard Diluent Buffer. The ELISA analyses for IL-6 and IL-8 were done according to the manufacturer's protocol.

2.4 Results and discussion

2.4.1 HPLC of rapamycin

In order to prove that the developed HPLC protocol works well for the detection of rapamycin a serial dilution from 250 to 15.6 µg per ml rapamycin was prepared in PBS. Each concentration was recorded (see Figure 1 for an exemplary HPLC profile) and the combined peak areas of all three rapamycin isomers (α, β and γ) were plotted against the rapamycin concentration to yield the calibration curve (see Figure 2).

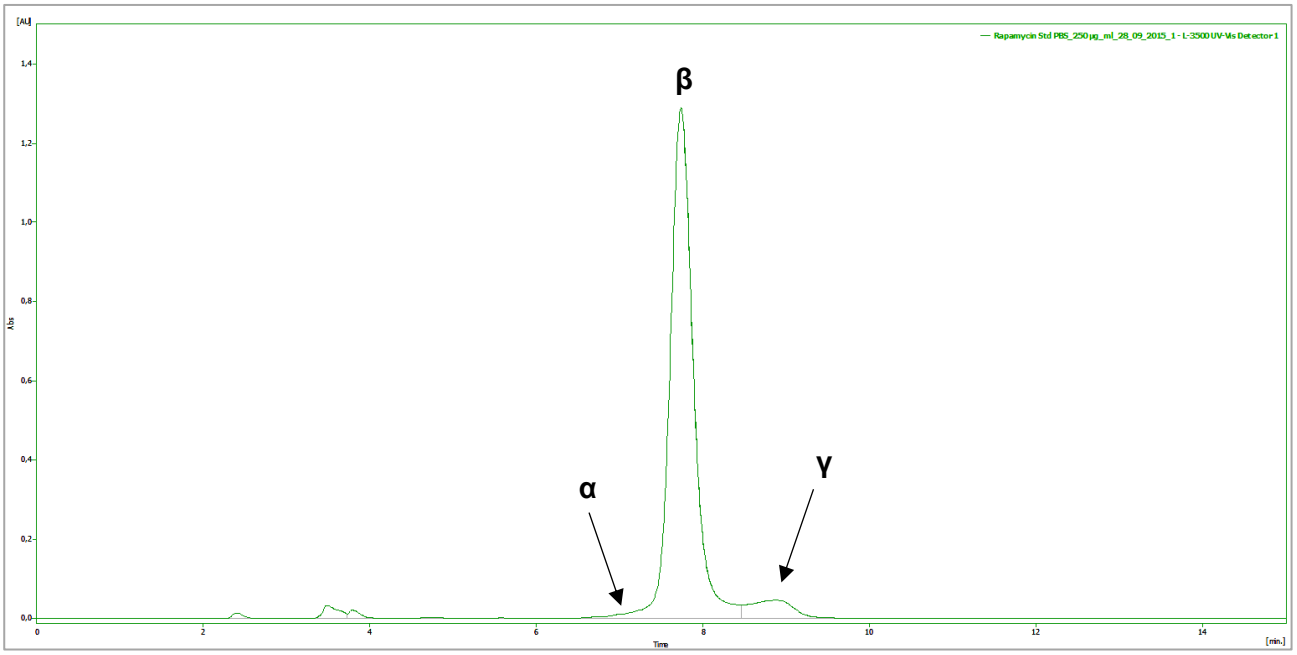

Figure 1: Typical HPLC chromatogram showing the separation of rapamycin isomers (α, β and γ) in a standard solution of rapamycin in PBS. A 250 µg/ml solution of rapamycin in PBS was submitted to the described HPLC procedure.

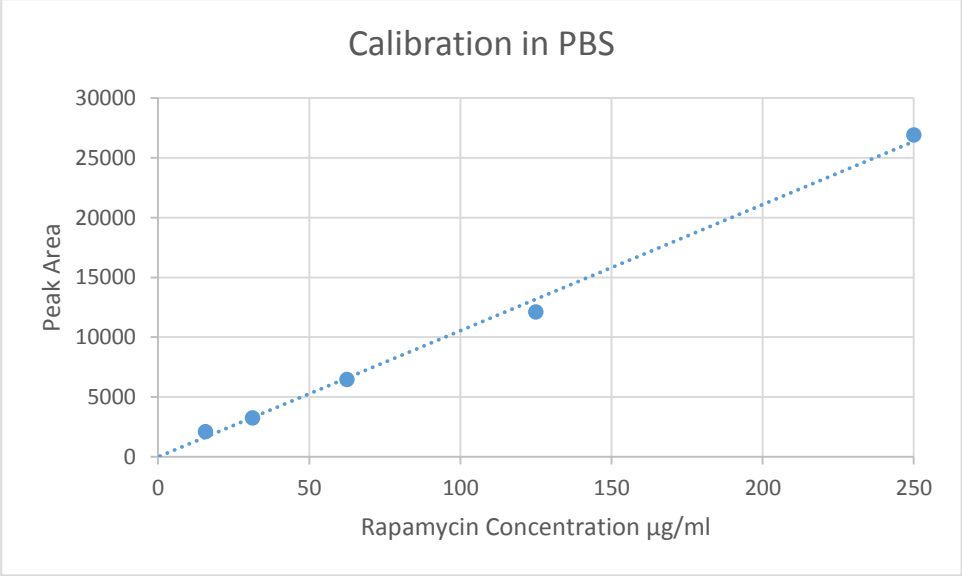

Figure 2: Calibration curve of rapamycin. A linear relationship is obtained between the analyte peak area and rapamycin concentrations over a range of 15.6-250 µg/ml.

The resulting HPLC chromatogram of rapamycin in PBS solution resembles the chromatograms observed by Sobhani et al. [11], where a solution of rapamycin in methanol was used, indicating that this procedure is also suitable for the detection of rapamycin in aqueous solution. A linear relationship between the peak area and the rapamycin concentration is observed in the analysed concentration range as can be seen in the calibration curve. Thus, the described protocol will be suitable to determine the rapamycin amount eluted from a stent prototype under dynamic conditions.

2.4.2 Cytokine expression of HCMECs

The expression of the cytokines IL-6 and IL-8 in HCMECs was validated using LPS stimulation. LPS which is a major component of the Gram-negative bacteria cell wall has been shown to induce the production of various cytokines in ECs [9].

As shown in Figure 3 both IL-6 and IL-8 are present in unstimulated cells in marginal amounts but are expressed in elevated concentrations depending on the concentration of LPS.

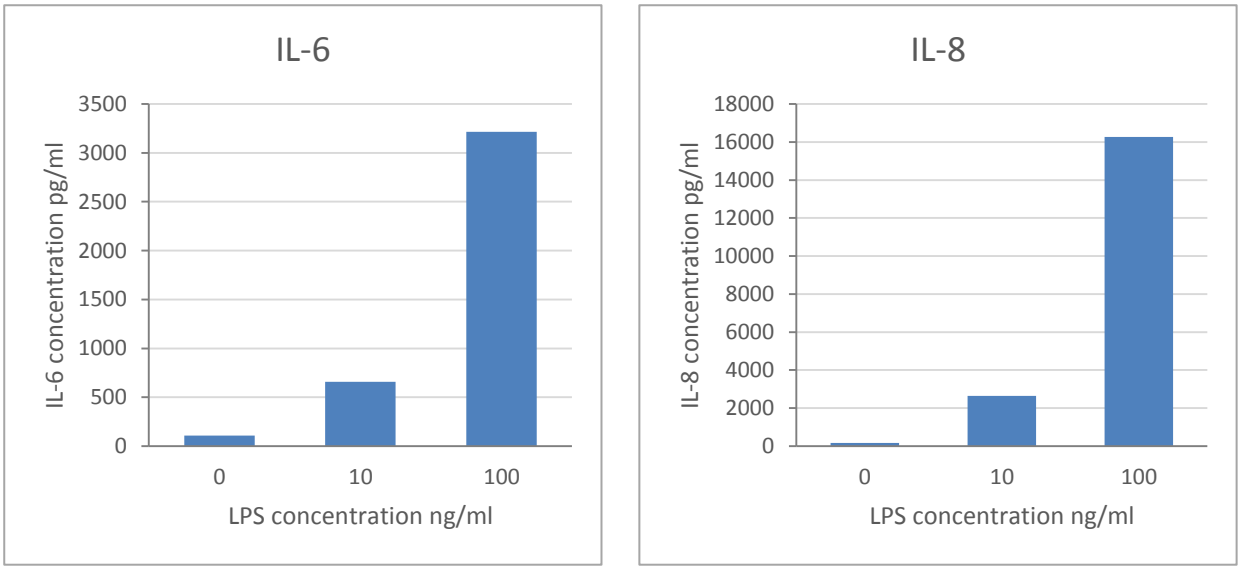

Figure 3: Interleukin concentrations as determined by ELISA. Interleukin 6 and interleukin 8 concentrations were determined from HCMEC culture supernatants without (0) or with stimulation by LPS (10 or 100 ng/ml).

These results prove that HCMECs are capable of IL-6 and IL-8 expression and this expression is upregulated upon inflammatory stimuli. Therefore, these tests are suitable to analyse if the developed stent prototypes initiate an inflammatory response in human endothelial cells under flow conditions.

3 Conclusion

In this study we have demonstrated that the newly developed protocols will be suitable for the in vitro analysis of the stent prototypes. The drug elution kinetics of rapamycin can be determined from media samples taken at different time points using the HPLC procedure. Also, possible inflammatory stimuli may be indicated on the basis of the shown cytokine expression analysis.

Altogether, these analyses will be able to expose possible risks of in-stent restenosis or stent thrombosis in the stent prototypes.

4 Abbreviations

CAD	Coronary Artery Disease
DAPT	Dual AntiPlatelet Therapy
DES	Drug-Eluting Stent
ECs	Endothelial Cells
ELISA	Enzyme-linked immunosorbent assay
HCMECs	Human cardiac microvascular endothelial cells
HPLC	High Performance Liquid Chromatography
HRP	HorseRadish Peroxidase
IL	Interleukin
ISR	In-Stent Restenosis
LPS	Lipopolysaccharides
PBS	Phosphate Buffered Saline
PCI	Percutaneous Coronary Intervention
ST	Stent Thrombosis
TCPS	Tissue culture-treated polystyrene
UV	Ultraviolet
Vis	Visible
VSMCs	Vascular Smooth Muscle Cells

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