Investigation of carrier systems containing antibiotics used in bone surgery

Ph.D. thesis

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Összefoglalás

Csontpótlás során a lokálisan ható antibiotikumok előnyösek a kórokozók elleni (ortopédiában leggyakoribbak a *Staphylococcus aureus* és *Staphylococcus epidermidis* törzsek) küzdelemben, akár osteomyelitises üregek kitöltésekor, akár fertőzött endoprotézisek revíziója során. A nemzetközi szakirodalom szerint, az ortopéd sebészetben a csontinfekció megelőzésére elsősorban gentamicint, ciprofloxacint, vancomycint alkalmaznak a csontcementre, illetve csontőrleményre impregnálva.

Vizsgálataink célja olyan technológia kifejlesztése és tanulmányozása, amely a revíziós műtéteknél a felhasználható csontgraftok és egyéb biokompatibilis anyagok antibiotikumokkal történő bevonását, impregnálását teszi lehetővé. Az antibiotikum csonthoz történő keverése, illetve a csontfelszín bevonása speciális technológiát igényel, mert a gyógyszer kiáramlását programozottan kell megvalósítani.

Doktori munkám során célul tűztem ki olyan specifikus hordozó rendszerek kifejlesztését, amelyek alkalmasak a csonthiányok pótlása során felhasznált csontgraftok antibiotikumokkal történő bevonására, és folyamatosan biztosítani tudják az alkalmazás helyén a hatékony koncentrációt.

Az előkísérletes vizsgálatok során különböző gélképző anyagok segítségével géleket állítottunk elő, melyeknek vizsgáltuk hatóanyagleadásuk és fizikai-kémiai tulajdonságaik tekintetében. A későbbiek folyamán viaszkeverékeket állítottunk elő. Vancomycint, gentamicint, ciprofloxacint és oxytetracyclint inkorporáltunk a különböző rendszerekbe. Ezen vizsgálati mintákat fizikai paramétereik, valamint hatóanyagleadásuk tekintetében vizsgáltuk *in vitro* körülmények között. Az *in vitro* kioldódási vizsgálatok értékelésére megfelelő analitikai módszereket alkalmaztunk, fejlesztettünk és validáltunk (HPLC, HPLC/MS).

Az alkalmas hordozórendszerek kiválasztásához mikrobiológiai értékmérést valamint *in vivo* állatkísérletes vizsgálatokat végeztünk, melyek eredményei alátámasztják az *in vitro* vizsgálati következtetéseket. Így lehetővé válik olyan hordozórendszerek kifejlesztése, melyek alkalmasak a hatóanyag több héten keresztüli leadására megfelelő koncentrációban.

Summary

INVESTIGATION OF CARRIER SYSTEMS CONTAINING ANTIBIOTICS USED IN BONE SURGERY

During bone substitution surgeries, local drug delivery systems can be favorable against bacteria (in orthopedic practice the most frequents strains are *Staphylococcus aureus* and *Staphylococcus epidermidis*), in case of the treatment of either osteomyelitis or the revision surgery of infectious prosthesis. According to international literature, in orthopedic surgery principally gentamicin, ciprofloxacin and vancomycin impregnated on bone cement or allograf are principally applied for the prevention of bone infection.

The aim of our studies was to develop and study a technology that enables the impregnation process of bone grafts or biocompatible materials that are used in revision surgery. The mixing of antibiotics with bone and the impregnation of bone surface requires special technology because the drug release has to be strictly controlled.

During my doctorial work, I set the aim to develop specific carrier systems that are able to impregnate the bone grafts during orthopedic surgeries and can assure the effective concentration at the site of action.

Gel systems were prepared in preformulation work using different gel forming agents. The gels were studied in physical and drug release aspects. Then waxy systems were prepared. Vancomycin, gentamicin, ciprofloxacin and oxytetracyclin were applied as model drugs. These samples have been investigated in physical and drug release aspects in *in vitro* circumstances. For the determination of samples drawn in drug release studies, appropriate analytical methods have been developed and validated (HPLC, HPLC/MS).

For the selection of proper composition microbiological investigations as well as in vivo studies have been performed. The results of these studies confirmed the results of the in vitro investigations. Thus the development of carrier systems that are releasing the active ingredient for weeks is becoming possible.

Introduction

In our days locomotor disorders belong to the most frequent attaches. The extended pain and disableness are usually related to skeletal and muscular disorders. Classical treatments as well as surgeries have great interest in their treatment.

The treatment of chronic osteomyelitis can be difficult since it often couples with the necrosis of the bone, and the danger of the infection of the surrounding tissues also exists. The oral and intravenous administration of antibiotics may cause few side effects, accordingly there is a great demand for the development new carrier systems which may release the antibiotic drug locally.

The implants that are in the body for long time may cause complications (technical, biological). The most common complication is the mechanical relaxation of the implanted prothesis which causes problems usually 10-15 years after the implantation. Then the prothesis leads to osteolysis, loss of bone. Other complication can be inflammation around the implanted prothesis, periprothetic infection. In this case, bacteria proliferate on the surface of prothesis and bone, secondary lead to osteolysis.

The orthopedic surgery, that has the aim of substitution of bone joint, is now the leading therapeutical solution for osteoarthrosis. In revision surgery, when the implant is renewed, different bone supplement methods are available. The most important is the use of bone grafts. The implanted bone does not have own circulation until osteointegration, when the implanted bone integrates to the body structurally and vasculary. Until this time (approximately 4 weeks), the bone graft is unprotected against microbes (exogen and endogen). In orthopedic practice, Staphylococcus aureus and Staphylococcus epidermidis cause periprothetic infections (infection around prothesis). Among these bacteria, multiresistant germs are frequent. The materials, which are used in orthopedic surgery, show different characteristics in the point of view of bacterial adherence.

In case of inflammation of bone or joint, there is often a great demand for bone replacement. Local antibiotic releasing systems may be used advantageously against microbes, or in case of filling the cavity caused by osteomyelitis, or infected prothesis replacement. The local antibiotic application achieves higher local concentration of the active ingredient without causing systemic side effects.

Department of Pharmaceutics and Department of Orthopedics (Semmelweis University) were concerned with the research of technology and special carriers that allow the impregnation of bone grafts and biocompatible materials with antibiotics (Hungarian

Scientific Research Fund, Grant No.: T049480). This might put the bone more resistant of microbes.

Research objectives

Periprothetic infections (inflammation around the prothesis), which are of great interest in orthopedic surgery, are mainly caused by Staphylococcus aureus and Staphylococcus epidermidis germs. These infections often lead to osteolysis, the loss of bone.

In drug formulation it is a fundamental requirement to prepare a drug carrier system that is able to carry the active ingredient to the site of act and assure there the appropriate concentration continuously.

In clinical practice there is no biodegradable, antibiotic containing system, available that can be applied by mixing or bonding to bone grafts. The objective of our research was to design and investigate a drug carrier system that is capable of releasing antibiotic locally after applying during orthopedic surgery. The aim was to develop such specific systems that are eligible to coat bone grafts with antibiotic agents. Hereby they can assure continuously –for few weeks- the effective concentration at the site of application. The controlled release of the antibiotics can significantly reduce the prevalence of septic complications of revision surgeries.

My objectives were:

1. preparation of drug delivery systems containing antibiotics, that are suitable to coat bone grafts, to mix with bone cubes, thus can assure the appropriate local antibiotic concentration,
2. investigate the drug release profiles, and the effect of different excipients on the drug release rate,
3. development and validation of analytical methods for the determination of the active ingredients in drug release studies,
4. investigation of the physicochemical properties of the prepared gels and carrier systems,
5. *analyze the connection of in vitro in vivo* investigations,
6. study the applicability and *in vivo* behavior.

Materials and methods

During my research work I have prepared gel systems containing vancomycin (VCM) and gentamicin (GEN) using different gel forming agents. For the further studies hydrophobic carrier systems have been produced in which VCM, GEN, ciprofloxacin (CPFX) and oxytetracyclin (OTC) were incorporated as active ingredient.

The physicochemical properties of the carrier systems have been examined. The melting point (Exstar 6000 DSC, Seiko Instruments Inc., TX, USA), dropping point (Ubbelohde-equipment), hardness (Erweka equipment), and contact angle (Sigma KSV 7010, KSV Instruments, Helsinki, Finland) have been determined.

The dissolution tests in case of gel systems were done in 100mL phosphate buffer (pH=7.5) tempered at 37 ºC without stirring.

For the dissolution testing of drug delivery systems, a new method was necessary, because the extremely long drug release. The in vitro drug delivery test of samples was done for 2 weeks. The dissolution medium was 100 mL. The pharmaceutical formulation was placed into the dissolution medium. Drawing of 5 mL samples was performed in every 24 hours. The dissolution medium was replaced by fresh medium every day. Purification of the samples was not necessary.

For the determination of the active ingredient from the samples drawn in dissolution tests (VCM, GEN) new analytical methods have been developed and validated, in case of CPFX a formerly developed method was used. In case of OTC a USP method was used.

The determination of VCM was performed with an Agilent Technologies (Palo Alto, CA, USA) LC MSD 1100 high-performance liquid chromatograph with HP Chemstation software revision A10.02 (Agilent, Waldbronn, Germany). The system consisted of a binary pump, on-line degasser, autosampler, column heater, and diode-array detector (DAD). Compounds were separated isocratically on a 2.1 mm x 150 mm, 5 µm particle, Zorbax Rx-C-18 column (Agilent, Waldbronn, Germany) with methanol–water–ammonium acetate (0.02 M, pH 9) 25:70:5 (v/v) as mobile phase at a flow rate of 0.3 mL/min. The column temperature was 30 ºC and the injection volume 20 µL. Chromatograms were recorded at 230 nm by use of the DAD.

The determination of GEN was carried out with a HP 1050 High Performance Liquid Chromatographic instrument (Agilent Technologies Inc. Palo Alto, CA, USA) with HP Chemstation software Rev. A10.02 (Agilent, Waldbronn, Germany). The system consisted of a binary pump, on-line degasser, autosampler, column heater, diode array detector (DAD). A Zorbax Rx-C-18 (2.1x150 mm, 5 µm) column (Agilent, Waldbronn, Germany) was used for

the separation. Column temperature was 30°C, the volume of injection was 20 µL and the flow rate was 0.3 mL/min. In the isocratic separation, the mobile phase consisted of methanol-water-ammonium acetate buffer (0.02M, adjusted with concentrate ammonia to pH=9): 35:60:5 v/v/v. The chromatograms were recorded at 280 nm according to the absorption maximum with DAD.

For the quantitative analysis external standard method has been used.

Statistical evaluation of linearity has been carried out by using Table Curve 2D Version 5.01 (Systat Software INC., Chicago, USA).

Previous to the animal tests of VCM containing drug carrier systems, in vitro microbiological studies have been performed. The aim of these researches was to examine the effectiveness and to determine the Minimal Inhibition Concentration (MIC).

The drug delivery system containing vancomycin was used in a rabbit osteomyelitis model in order to evaluate the antimicrobial efficacy of the antibiotic bone graft and applicability. The prepared system had been previously sterilized by gamma radiation. 40 adult New Zealand rabbits were used. The animals were randomized in three groups. In group I no osteomyelitis was induced, the drug delivery system was implanted in the rabbit's tibia to prove the biodegradability in non-infect tissues. In group II and III MRSA (Methicillin-resistant Staphylococcus aureus) induced osteomyelitis was carried out. In group II autologous cancellous bone and the drug delivery system with vancomycin was used. In group III plain cancellous bone was filled in. 6 weeks post op the animals were sacrificed, histological, laboratory and radiological evaluations were performed. The drug delivery system containing vancomycin was effective against MRSA induced osteomyelitis, and did not inhibit the osteointegration.

Results and discussion

- **Analytical studies**

I have developed a new HPLC-UV method for the quantitative determination of VCM and GEN during dissolution studies. These methods were validated in the concentration range 1-100 µg/mL. These methods appeared to selective as no interfering component was shown in the chromatograms. In case of VCM, the LOD was found to be 0.2 µg/mL and LOQ to be 0.6 µg/mL. In case of GEN, the LOD was found to be 0.1658 µg/mL, the LOQ to be 1.0 µg/mL. In this concentration range the correlation between the concentration and area under curve

was linear (r^2 was 0.9988 for VCM and 0.99908 for GEN). The results of validation process meet the requirements of international guidelines.

For the determination of CPFX a validated method has been applied. The chromatographic system was suit for the measurement of the samples drawn in dissolution tests.

- **Physicochemical properties**

On the basis of the physicochemical studies, I was led to the conclusion that the applied excipients affect the physicochemical properties of the drug carriers. Using Precirol® (melting point: 56 °C), the melting point, dropping point and the contact angle was decreased, while the hardness was increased. The change of these parameters has great influence on applicability also.

- **Dissolution studies**

During method development, my aim was to approach the physiological parameters. Accordingly, physiological salt solution was chosen as dissolution media without stirring and tempered at 37 °C. These dissolution tests were proved to be simple, economical, and the developed analytical methods are sufficient to determine the active ingredients from the samples drawn from these tests.

On the basis of physicochemical and dissolution studies, the most appropriate composition can be chosen. In case of VCM Cera alba: Precirol®: Vaselinum album (45:45:10) and Cera alba: Precirol® (50:50), in case of GEN Cera alba: Vaselinum album (90:10) and Cera alba: Precirol® (50:50), in case of CPFX Cera alba: Precirol®: Vaselinum album (45:45:10) and Cera alba: Precirol® (50:50) compositions were proved to be the best in point of melting-, dropping point, contact angle and dissolution.

In the course of bone surgeries the application of gels is more difficult that the carrier systems. It is difficult to get the gels on the surface of bone which can not be easily carried out in surgical circumstances. At the same time, the shape and size of the prepared systems can be varied according to demands, which make it possible to mix with the bone. The other way of application can be the coating of bone grafts.

- **Microbiological studies**

During microbiological studies, the size of developing inhibition zone was observed. These studies proved the effectiveness of the drug carrier systems by all the three methods used.

- **In vivo studies**

The animal studies confirmed that the prepared waxy systems together with bone grafts meet the requirements mentioned above. These are to apply even in surgical circumstances. The examined VCM containing carrier systems can be mixed with bone so thus can be immediately implanted. The coating of the graft with these systems is also possible but for this appropriate circumstances are required.

The studies showed that the antibiotic containing systems are competent for the treatment of osteomyelitis caused by Staphylococci, do not inhibit osteointegration, and do not cause the regression of inflammation. These systems are non-toxic thus it has antibiotic effect without causing damages.

Conclusions

In my PhD work I have developed antibiotic carrier systems for orthopedic use. These systems were investigated according to their drug release profile, physicochemical properties and effectiveness in animal tests.

The ideal antibiotic treatment is done on the base of correct indication, with the most appropriate active ingredient, using the appropriate dos for the appropriate time. In revision surgery periprothetic infections are mostly caused by *Staphylococcus epidermidis* and *Staphylococcus aureus*, but in many cases multiresistant microbes should be counted with.

According to the results of validation procedure, the liquid chromatographic method is eligible for the determination of the active ingredient (VCM, GEN) in the samples drawn in dissolution studies. These methods are robust and fast in contrast to the reported methods. The LOQ values permit to determine the antibiotics reproducibly and dependably. The applied validated method for the determination of CPFX proved to be appropriate for the quantitative analysis of the dissolution samples. A USP method was used for the determination of OTC.

The drug release profile depends on either the drug carrier system (composition, porosity, wettability), or the characteristics of active ingredient (solubility, molecular weight). The modification of these parameters would change the dissolution profile (time, dissolution rate, released active ingredient). Thus, the composition of gels and carrier systems must be optimized in accordance with the active substance.

The newly developed VCM containing system proved to be biodegradable in animal tests and it did not inhibit osteogenesis. In osteomyelitis rabbit infected by MRSA effective

local concentration was achieved. For the human application, further studies are necessary to be done.

New scientific results related to thesis

- During my work, I developed new drug carrier systems and their investigating methods for orthopedic use. In preformulation studies different gel systems were prepared. In the further development hydrophobic carrier systems have been produced. Vancomycin has been used as model drug. In further studies gentamicin, ciprofloxacin and oxytetracyclin were incorporated to the systems.
- Dissolution method has been developed for the investigation of extended drug release.
- An analytical method, for the determination of antibiotics in the dissolution media, has been developed and validated.
- The dissolution method is capable of monitoring the effect of different excipients on the dissolution rate. By changing the rate of the excipients in the composition, the extended dissolution rate can be assured.
- The long drug release and the in vitro and in vivo characteristic have been confirmed by in vitro microbiological assays and in vivo animal tests. The carrier systems proved to be biodegradable, six weeks after application.

Functional applicability of results

- Drug carrier systems have been developed that are able to release different antibiotics for long period, for weeks. Herewith it provides facility for the application profilactically in orthopedic surgery and therapeutic application of septic patients. The investigated hydrophobic carriers might be eligible for human development with necessary clinical studies. The in vivo results confirmed the in vitro outcomes. These developed systems were proved to be effective and biodegradable.
- The developed fast, simple analytical methods are suitable for the quantitative determination of the applied antibiotics. These methods might be able for the determination of these antibiotics in human studies with appropriate sample preparation.

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