

Department of Pharmaceutical Biochemistry¹, Faculty of Pharmacy, Medical University of Łódź; Institute of Biopolymers and Chemical Fibres²; Department of Applied Pharmacy³, Faculty of Pharmacy, Medical University of Łódź, Łódź, Poland

Release kinetics of basic fibroblast growth factor (bFGF) from certain biopolymers in the presence of ketoprofen

M. MICHALSKA¹, M. MIROWSKI¹, A. BODEK², K. H. BODEK³

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Assoc. Prof. Kazimiera Henryka Bodek, Department of Applied Pharmacy, Faculty of Pharmacy, Medical University of Łódź, Muszyńskiego 1, 90-151 Łódź, Poland.

kazimiera.bodek@umed.lodz.pl

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The aim of the present study was the evaluation of basic fibroblast growth factor (bFGF, FGF-2) release *in vitro* from four types of polymer bases (carriers), fibrin, microcrystalline chitosan (MCCh), fibrin and MCCh, as well as MCCh and methylcellulose (MC) in the presence or absence of ketoprofen (KTA). Amount of released basic fibroblast growth factor was measured immunoenzymatically using Elisa (R&D System). Ketoprofen concentration was determined spectrophotometrically at 255 nm, using an appropriate absorbance factor, $\alpha_{1\text{cm}}^{1\%} = 662$. The most significant influence of ketoprofen on bFGF release was seen in the case of microcrystalline chitosan carrier elution. Parameters of the equation which describe the amount of bFGF released from chitosan carrier with and without KTA are $y = 6.842 \pm 1.637 \ln(t) + 14.935 \pm 2.378$, determination coefficient, $R^2 = 0.9332$ and $y = 4.070 \pm 0.622 \ln(t) + 10.589 \pm 1.011$, determination coefficient, $R^2 = 0.9606$. The time after which 20% of bFGF was released ($t_{20\%}$) in the presence of ketoprofen was 2.1 h whereas it was significantly longer without ketoprofen (10.1 h). The amount of bFGF released from fibrin carrier was lower in the presence of ketoprofen. The time taken for 20% of bFGF to be released ($t_{20\%}$) was very long (41.7 h) in the presence of KTA and 16.3 h. without KTA. The other carriers (fibrin + MCCh and MCCh + MC) in the presence of ketoprofen appear to have an insignificant influence on the kinetics of basic fibroblast growth factor release. For the chitosan carrier ($p = 0.05$, and also $p = 0.01$, when $t_{theoret} = 2.921$), there is a statistically significant difference between the coefficients (a_1 and a_2) of the regression equation describing the process of basic fibroblast growth factor release from the base with and without ketoprofen. It was also found that the amount of ketoprofen released varied considerably according to the carrier. All results clearly indicate that the type of carrier not only has an impact on the amount of bFGF released, but also on the kinetics of ketoprofen release.

1. Introduction

The initiation of the bone regeneration process after dental implant surgery, muscle and tendon surgery, cardiac surgery and treatment for skin ulcer and eye damage depends on a number of angiogenic factors (Hasegawa et al. 1999; Kulimova et al. 2006). One of these angiogenic factors is bFGF (*basic fibroblast growth factor*) – a polypeptide with a molecular weight of 18 kDa, which is synthesised by fibroblasts, endothelial cells, glial cells, and smooth muscle cells. In addition, bFGF, TGF- β 1 (*transforming growth factor β*) and VEGF (*vascular endothelial growth factor*) are involved in the regeneration process. These factors lead to the formation of new blood vessels in graft tissue and they promote angiogenesis. TGF- β 1 initially stimulates fibroblasts, proliferates and promotes differentiation of mature osteoblasts, which produce the bone matrix (Tabata et al. 1998). Fibroblasts under the influence of TGF- β 1 induce collagen matrix formation and promote blood vessel proliferation, which become visible around three days after the initiation of regeneration. bFGF and VEGF factors, as well as bone tissue, stimulate nerve, skin, mus-

cle and blood vessel wall regeneration (Jeon et al. 2005; Asahara et al. 1995). PDGF (*platelet derived growth factor*) induces mitogenesis in stem cells and osteoblasts and usually occurs in graft tissue. The initiation of bone regeneration begins at the moment of PDGF, TGF- β 1 and IGF-1 (*insulin-like growth factor*) release from the platelet granular reticulum in graft tissue (Nurden et al. 2008).

Conventional treatment relies on systemic drug administration, which frequently results in side effects and low efficacy. The aim of local therapeutic action is to find an appropriate growth factor which would provide a long-term therapeutic concentration and remain free of side effects (Wong et al. 2003; Jackson et al. 1996). The choice of growth factor carrier depends on its structure. The effects of angiomorphologic fibroblast growth factor (bFGF) and vascular growth factor (VEGF) strictly depend on fibrin clot structure (Nehls and Hermann 1996) and they affect blood vessel formation (Gamble et al. 1993).

Knowledge about bone tissue healing, especially the role of growth factors, has enabled the development of new therapeutic methods in prosthodontics and implantology. The local

introduction of proangiogenic factors by means of carriers stimulates both regeneration and healing. Biological carriers can be helpful in the application of antibiotics or chemotherapeutics (Markman et al. 1995; Han et al. 2006).

The selection of an appropriate carrier (“scaffolding”) or adequate growth factor still remains a serious obstacle (Park et al. 2000). According to tissue engineering, carrier enrichment with appropriate growth factors is essential in guided tissue regeneration (GTR) and guided bone regeneration (GBR). The most frequently used carriers are collagen preparations (Côté et al. 2004), glycosaminoglycan (Ranney 2000) cellulose (Galgut 1990), chitosan (Berger 2004; Kim et al. 2003; Tan et al. 2001), fibrin gel (fibrin glue) (Michalska et al. 2008a), apatite (Belcarz et al. 2005) and other synthetic preparations (Cieslik et al. 2005). A good carrier of angiogenic growth factors PDGF-AB and TGF- β is a combination of alginate and microcrystalline chitosan (MCCh), natural polymers used in tissue engineering as “biological scaffolding” (Michalska et al. 2008b). MCCh existing as the new physical form of standard chitosan, derived from chitin commercially extracted from shellfish waste, is a special multifunctional polymeric biomaterial (Struszczyk 1987). In the available literature there are few reports on the application of MCCh in medicine, pharmacy and other areas (Struszczyk and Kivekäs 1990; Hoekstra et al. 1998; Bodek, 2004). Microcrystalline chitosan in the form of hydrogel at neutral pH was used in our studies. Fibrin glue is a widely-used adhesive preparation in plastic surgery and reconstructions. It increases the opportunity for proper healing (Curie et al. 2001). In collaboration with fibronectin, it affects keratinocyte and fibroblast growth, it might be used in plastic surgery to significantly accelerate wound regeneration.

Ketoprofen is a nonsteroidal, anti-inflammatory drug (NSAID). It is widely used as a analgesic and anti-inflammatory agent, and is one of the most potent inhibitors of prostaglandin synthesis (Goger et al. 1998; Ermiş and Tarimci 1995).

A great deal of research is still required to improve the choice of materials used in surgery or implantology. The ideal material must have proper sorption properties, must be an accurate therapeutic substance, with available angiogenic growth factors, and it must possess good immunity to proteolytic enzymes (Ma 2008). The application of an appropriate membrane in conjunction with growth factors accelerates bone and soft tissue regeneration (Bennett and Schultz 1993; Nissen et al. 1999). One of the best known haemostatic dressings in surgery with very good haemostatic – sorption properties is a gelatin sponge (Kang et al. 1999). Preparations that contain collagen with connection of fibrin-glue (Schiele et al. 1991) or chitosan are used as medications and growth factor carriers for tissue engineering purposes (Abarrategi et al. 2008).

Numerous reports indicate that some therapeutics may affect the degree of growth factor release from newly-formed polymer carriers. In an effort to confirm these reports, the aim of the present study is the evaluation of bFGF release *in vitro* in the presence or absence of ketoprofen from certain biodegradable biopolymers (fibrin, microcrystalline chitosan, and methylcellulose).

2. Investigations and results

2.1. Release kinetics of bFGF

Release grade basic fibroblast growth factor (bFGF) was conducted both in and without the presence of ketoprofen derived from four kinds of carriers: fibrin, microcrystalline chitosan, fibrin and chitosan, as well as chitosan and methylcellulose.

The kinetics of basic growth factor (bFGF) release from polymer carriers both with and without ketoprofen, is illustrated on Fig. 1,2,3,4 The equation $y = a \ln(t) + b$ gives the amount of

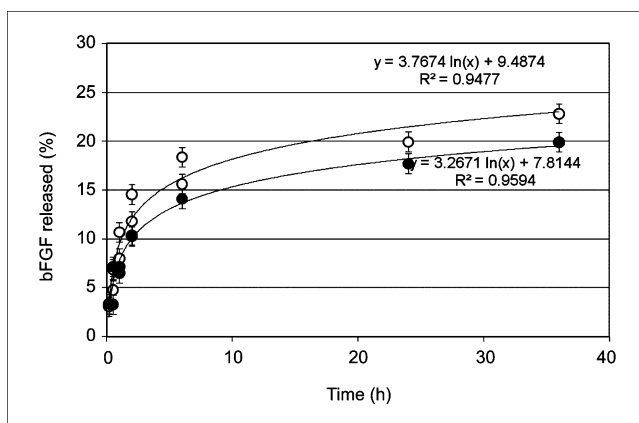


Fig. 1: Release profile of bFGF release from fibrin carrier (system I & II) without (o) and with (●) KTA

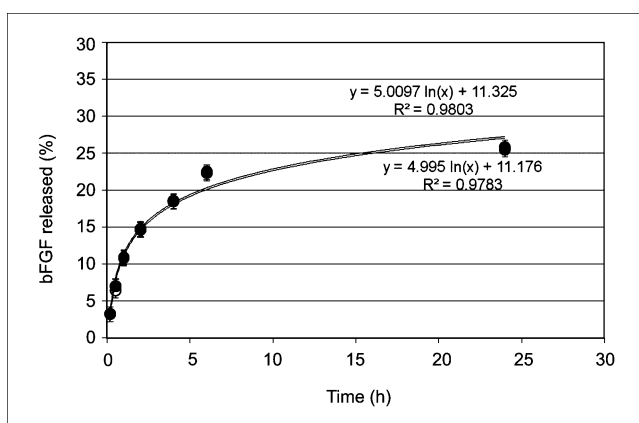


Fig. 2: Release profile of bFGF from fibrin+MCCh carrier (system III & IV) without (o) and with (●) KTA

released bFGF (y) at a certain point in time during the process (t), where a and b are parameters of this equation.

The kinetics of bFGF release from the fibrin carrier, both with and without ketoprofen, is illustrated in Fig. 1. The amount of bFGF released from fibrin carrier is lower in the presence of ketoprofen, as described by the equation $y = 3.267 \pm 0.507 \ln(t) + 7.814 \pm 0.897$, determination coefficient, $R^2 = 0.9594$, regression equation $y = 3.767 \pm 0.667 \ln(t) + 9.487 \pm 1.181$, determination coefficient, $R^2 = 0.9477$. The time taken for 20% of the growth factor to be released ($t_{20\%}$) was very long – 41.7 h in the presence of KTA and 16.3 h without KTA (Table 1).

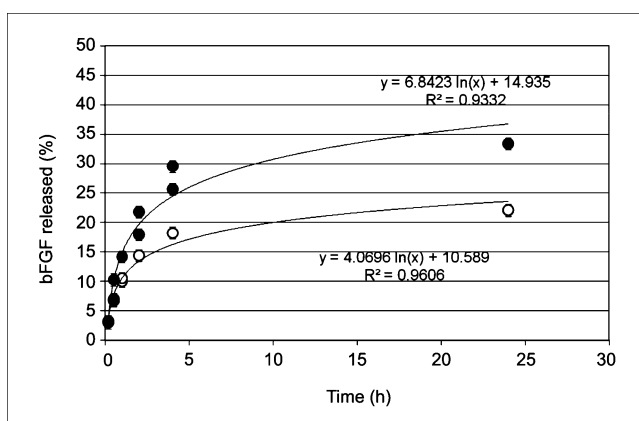


Fig. 3: Release profile of bFGF from MCCh carrier (system V & VI) without (o) and with (●) KTA

Table 1: The parameters of the equation $y = a \ln(t) + b$, which describe the amount of bFGF factor released from different polymer carriers

Basic carriers	System	KTA	Parameters of equation $y = a \ln(t) + b$			$t_{20\%}$, h
			$a \pm D$	$b \pm D$	R^2	
Fibrin	I	–	3.767 ± 0.667	9.487 ± 1.181	0.9477	16.3
	II	+	3.267 ± 0.507	7.814 ± 0.897	0.9594	41.7
Fibrin + MCCh	III	–	4.995 ± 0.560	11.176 ± 0.796	0.9783	5.9
	IV	+	5.010 ± 0.535	11.325 ± 0.762	0.9803	5.7
MCCh	V	–	4.070 ± 0.622	10.589 ± 1.011	0.9606	10.1
	VI*	+	6.842 ± 1.637	14.935 ± 2.378	0.9332	2.1
MCCh + MC	VII*	–	4.130 ± 0.790	10.982 ± 1.089	0.9562	8.9
	VIII*	+	4.063 ± 0.774	10.813 ± 1.066	0.9566	9.6

$t_{20\%}$ - the time taken for 20% of the growth factor to be released, D – (half confidence interval) $p=0.05$, $n=11$, * $n=9$. The results are the combined value of 3 independent measurements

Fig. 2 presents kinetic of bFGF release from a chitosan-fibrin carrier. The presence of ketoprofen appears to have little influence on the kinetics of bFGF release (Table 1). The time taken for 20% of the bFGF release ($t_{20\%}$) was 5.7 h in the presence of KTA and 5.9 h without KTA (Table 1).

The most significant influence of ketoprofen on bFGF release was obtained in the case of chitosan carrier elution (Fig. 3, Table 1). The parameters were found to be $y = 6.842 \pm 1.637 \ln(t) + 14.935 \pm 2.378$, determination coefficient, $R^2 = 0.9332$ and $y = 4.070 \pm 0.622 \ln(t) + 10.589 \pm 1.011$, determination coefficient, $R^2 = 0.9606$. A significant difference can be seen at the time for 20% of the growth factor to be released ($t_{20\%}$). The release took 2.1 h in the presence of ketoprofen compared with 10.1 h without ketoprofen (Table 1).

Ketoprofen appeared to have insignificant influence on the release of bFGF in case of the chitosan–methylcellulose carrier (Fig. 4, Table 1). The equation parameters are $y = 4.063 \pm 0.774 \ln(t) + 10.813 \pm 1.066$, determination coefficient, $R^2 = 0.9566$ and $y = 4.130 \pm 0.790 \ln(t) + 10.982 \pm 1.089$, determination coefficient, $R^2 = 0.9562$. The time taken for 20% of the growth factor to be released ($t_{20\%}$) was 9.6 h in presence of KTA and 8.9 h without KTA (Table 1).

To determine whether ketoprofen affects growth factor release from the bases examined, a hypothesis H_0 has been put forward that $a_1 = a_2$ for $H_1: a_1 \neq a_2$; where a_1, a_2 are coefficients of the regression equation describing the process of growth factor release from the base with/without the presence of ketoprofen (a_1/a_2) after linearization of the primary logarithmic function. The test verifying H_0 is based on the statistics of the Student's t-test calculated according to the formula(1):

$$t = (a_1 - a_2) / D(a_1 - a_2)$$

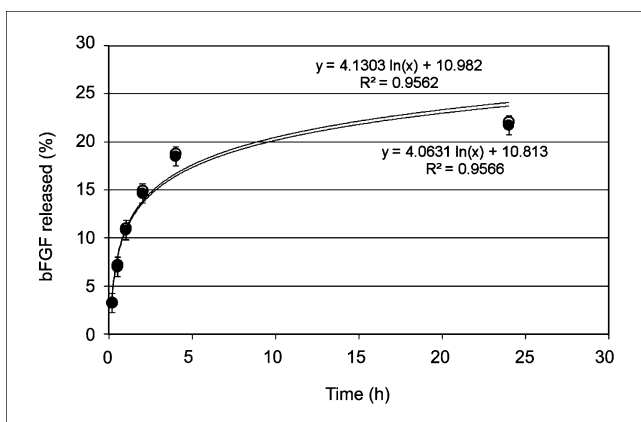


Fig. 4: Release profile of bFGF from MCCh+MC carrier (system VII & VIII) without (o) and with (●) KTA

where: t - statistics has the distribution of the Student's t-test with $n_1 + n_2 - 4$ degrees of freedom,

$D(a_1 - a_2)$ - a statistical function.

The results of calculations are presented in Table 2.

At a significance level of $p=0.05$, there is no basis for rejecting the hypothesis H_0 on lack of difference between regression equation coefficients. Ketoprofen does not exert a statistically significant influence on the speed of the growth factor release from the bases studied. However, the only significant difference between these coefficients was observed for microcrystalline chitosan, which indicates that ketoprofen present in the chitosan base affects the process of growth factor release from this carrier. This difference is also statistically significant at a significance level of $p=0.01$, when $t = 2.921$.

2.2. Release kinetics of ketoprofen (KTA)

The study enabled the investigation of ketoprofen release kinetics from different carriers (Fig. 5). Ketoprofen is released most effectively from fibrin gel. After 6 h, this amount reaches 25% and this concentration remains at this level for 24 h of the experiment.

Ketoprofen release from the fibrin + MCCh carrier reached the highest level during the first elution hour (25%) but later dropped down. Ketoprofen release from MCCh and MCCh + MC carrier is very similar.

After 20 min., elution with PBS buffer, only 7.5% of the ketoprofen was released from the MCCh carrier. In cases where MC was added to the chitosan carrier, about 15% was released. In both cases, the amount of medication released after 2 h remains constant at 4% during the 24 h of the experiment.

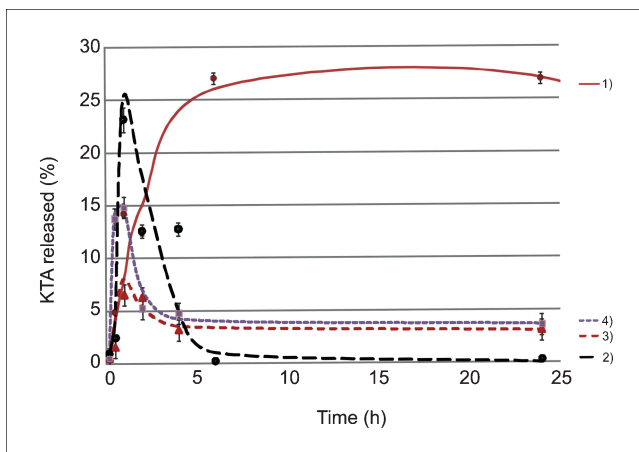


Fig. 5: Release profile of KTA from different carriers: 1 – fibrin (II), 2 – fibrin+MCCh (IV), 3 – MCCh (VI), 4 – MCCh+MC (VIII)

Table 2: The values of the Student's t-test parameter a for the regression equation

Basic carriers	System	KTA	n_1/n_2	Student's t -test	
				calculated	theoretical*
Fibrin	I	–	11/11	1.351	2.101
	II	+			
Fibrin + MCCh	III	–	11/11	0.032	2.101
	IV	+			
MCCh	V	–	11/9	3.113	2.120
	VI	+			2.921**
MCCh + MC	VII	–	9/9	0.932	2.145
	VIII	+			

* t values of the Student's t -test for $p=0.05$ and $n_1 + n_2 - 4$.

** t for $p=0.01$.

3. Discussion

In this study, the release of bFGF in the presence and absence of ketoprofen from fibrin carrier (I & II), fibrin + MCCh (III & IV), MCCh (V & VI), and MCCh+MC (VII & VIII) carrier was determined. Ketoprofen is a well-known and frequently-used analgesic drug. The presence of a carboxyl group in its structure allows for cross-linking through the creation of additional bindings with investigated polymers, chitosan and fibrin, through amine groups. Therefore, it seems to be important to investigate whether ketoprofen may have any influence on bFGF factor release from the above mentioned carriers during treatment.

However, based on the calculated $t_{20\%}$ coefficient, it was proved that 20% of bFGF is released after 16.3 h from pure fibrin carrier, but after 41.7 h in the presence of ketoprofen, which suggests not only an increased cross-link structure of the carrier but also the existence of additional cross-link bindings. A rapid release of bFGF factor was observed from MCCh in the presence of KTA ($t_{20\%} = 2.1$ h). Fibroblast growth factor (bFGF) release from fibrin+chitosan was slightly extended from 5.9 without to 5.7 h in the presence of ketoprofen ($t_{20\%} = 5.7$ h). In the absence of the drug, bFGF factor release from chitosan was significantly larger ($t_{20\%} = 10.1$ h) in contrast to the fibrin+chitosan carrier ($t_{20\%} = 5.9$ h). These findings suggest that the type of carrier affects the amount of release of a growth factor. For the chitosan carrier ($p=0.05$), there is a statistically significant difference between the coefficients (a_1 and a_2) of the regression equation describing the process of the growth factor release from the base with and without ketoprofen. This difference is also significant at $p=0.01$, when $t=2.921$. It can be stated that ketoprofen affects the bFGF release from the chitosan base to a considerable degree.

The percentage of ketoprofen released from the examined carriers was measured in the presence of bFGF (Fig. 5), which might have a significant meaning in analgesic therapy, surgery and implantology. The fastest release of ketoprofen was shown in the case of fibrin, subsequently remaining constant at 25% for the whole experiment (24 h). Carriers which include fibrin and chitosan, indicating an enhanced cross-link structure, have the best drug-release profile. Most of it is released during the first hours (reaching a value of 25% after 1 h) before falling decisively to zero. The amount of KTA released from MCCh and MCCh+MC carrier was 7.5% and 15.0% in the first hour of experiment respectively, than it remaining at a level of 4% (Fig. 5). All results clearly indicate that the type of carrier not only has an impact on the amount of bFGF released, but also on the kinetics of ketoprofen release.

Previous studies have shown that there is a significant influence from certain medications (such as heparin) which are added to

the gelatin carrier in order to facilitate cytokine release (bFGF, VEGF, PDGF) (Peattie et al. 2008) and in case of fibrin gel, the level of cross-link structure and incorporated peptides can also be a significant factor (Ichinose et al. 1983; Sakiyama-Elbert and Hubbel 2000). The ketoprofen carrier base, introduced by the author, was intended to explain the mutual influence on growth factor release and KTA from four different carrier bases (system II, IV, VI and VIII).

In conclusion, on the basis of this study, it can be concluded that the most rapid release of bFGF in the presence of ketoprofen was observed from the chitosan matrix. For the chitosan carrier ($p=0.05$, and also $p=0.01$, when $t_{theoret.} = 2.921$), there is a statistically significant difference between the coefficients (a_1 and a_2) of the regression equation describing the process of the growth factor release from the base with and without ketoprofen. The amount of bFGF released from fibrin carrier is lower in the presence of ketoprofen. The other carriers (fibrin+MCCh, MCCh+MC) in presence of ketoprofen exes an insignificant influence on the kinetics of growth factor release. It can be stated that ketoprofen affects considerably the basic bFGF release from the chitosan base. The amount of ketoprofen released varies considerably according to the carrier.

4. Experimental

4.1. Materials

Fibrinogen, fraction I, type I from human plasma (Sigma), thrombin, EC.3.4.4.13 (Biomed, Lublin), microcrystalline chitosan MCCh Fg-90 hydrogel (polymer content – 3.0%) (Institute of Biopolymers and Chemical Fibres, Lodz), methylcellulose MC (M-0262) viscosity of 2% aqueous solution at 20 °C, 400 centipoises (Sigma), recombinant human FGF basic rhbFGF and human FGF basic Quantikine ELISA Kit (R&D System, Minneapolis, MN, USA), factor XIII, fragment 92–97 (Sigma), ketoprofen KTA (K1751-5G, Sigma-Aldrich).

4.2. Preparation of polymer carriers

4.2.1. Fibrin

Fibrin was produced by dissolving fibrinogen (9.4 mg/ml) in PBS buffer (0.01 mol/l, pH 7.4) and adding 100 μ l human bFGF (2 ng/ μ l) and thrombin (8 NIH final concentration) to form the clot. After incubation (30 min.) clot was washed at 30 min. in PBS buffer (0.01 mol/l, pH 7.4) and eluted in the same buffer. Eluents were directly frozen and stored at –20 °C until subsequent analysis.

4.2.2. Chitosan

Microcrystalline chitosan MCCh in the form of 3% hydrogel was prepared by the aggregation of glucosamine macromolecules from an aqueous solution of organic acid (Struszczyk 1987).

4.2.3. Methylcellulose

Methylcellulose (MC) in the form of 3% hydrogel was used for preparation of complex carrier. To prepare of MCCh + MC carrier, a mixture of these polymers in the form of hydrogels was used

4.3. Methods

4.3.1. Determination of bFGF

Angiogenic fibroblast growth factor (bFGF) release was performed both in the presence of 100 μ l ketoprofen (0.01% final concentration) and without ketoprofen in eight system (Table 3). The amount of bFGF was measured immunoenzymatically using Elisa (R&D System). Absorbance was measured at 540 nm using an Elx800 Elisa Reader, BIO-TEK Instruments, Inc.

4.3.2. In vitro drug release studies

Ketoprofen (KTA) was released from the carriers into 1 ml of PBS buffer (0.01 mol/l, pH 7.4) at room temperature. Samples of 100 μ l were periodically used (after 5 and 30 min. and 1, 2, 4 and 24 h) for determination KTA.

Table 3: Component of Systems

Components	System							
	I	II	III	IV	V	VI	VII	VIII
100 µl (2.5 ng/µl) bFGF factor	+	+	+	+	+	+	+	+
1 ml of fibrinogen (9.4 mg/ml)	+	+	+	+				
20 µl (2 µg) fibrin stabilizing factor, FSF	+	+	+	+				
100 µl of thrombin solution (80 NIH/ml)	+	+	+	+				
1 ml chitosan (9.5 mg/ml)			+	+	+	+	+	+
0.5 ml methylcellulose (6.3 mg/ml)							+	+
100 µl of ketoprofen (0.01%) final concentration		+		+		+		+

* – 0.5 ml
All samples were incubated for 30 minutes at room temperature.

The whole set was eluted to PBS buffer (0.01 mol/l, pH 7.4).

Samples for bFGF and ketoprofen marking in the case I and II sets were taken after 5 and 30 minutes, and 1, 2, 4, 6, 24 and 36 hours. Measurements of bFGF level and ketoprofen concentration were carried out for the majority of sets after 5 and 30 minutes and 1, 2, 4 and 24 hours. The results are presented as the combined value of 3 independent readings.

Ketoprofen concentration was determined spectrophotometrically (Pharmacopoeia Polonica 2008) at 255 nm, using an appropriate absorbance factor, $\alpha^{1\%}_{1\text{cm}} = 662$, with a Spectromon 195 D spectrometer in small quartz cuvettes (Hellma, Light Path 10 mm).

4.3.3. Statistical analysis

Statistical analysis was performed using Students t-test with a Microsoft Excel Analysis Tool Pak in Microsoft Office Excel 2007.

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