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Bone Regeneration: Freeze-Dried CMC/PLGA Microsphere Matrix of rhBMP-2

Dudley RH and Johnson KE

Northern Centre for Healthcare Research, Department of Clinical Pharmacology, University of Groningen, The Netherlands

***Corresponding Author:** Johnson KE, Northern Centre for Healthcare Research, Department of Clinical Pharmacology, University of Groningen, The Netherlands

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Abstract

The hypothesis of this research was that implants of poly(lactide-co-glycolide) (PLGA) microspheres loaded with bone morphogenetic protein-2 (rhBMP-2) and distributed in a freeze-dried carboxymethylcellulose (CMC) matrix would produce more new bone than would matrix implants of non-protein-loaded microspheres or matrix implants of only CMC. To test this hypothesis it was necessary to fashion microsphere-loaded CMC implants that were simple to insert, fit precisely into a defect, and would not elicit swelling. Microspheres were produced via a water-in-oil-in-water double-emulsion system and were loaded with rhBMP-2 by soaking them in a buffered solution of the protein at a concentration of 5.4 mg protein per gram of PLGA. Following recovery of the loaded microspheres by lyophilization, matrices for implantation were prepared by lyophilizing a suspension of the microspheres in 2% CMC in flat-bottom tissue culture plates. Similar matrices were made with 2% CMC and with 2% CMC containing blank microspheres. A full-thickness calvarial defect model in New Zealand white rabbits was used to assess bone growth. Implants fit the defect well, allowing for direct application. Six weeks postsurgery, defects were collected and processed for undecalcified histology. In vitro, 60% of the loaded rhBMP-2 released from devices or microspheres in 5 to 7 days, with the unembedded microspheres releasing faster than those embedded in CMC. In vivo, the rhBMP-2 microspheres greatly enhanced bone healing, whereas nonloaded PLGA microspheres in the CMC implants had little effect. The results showed that a lyophilized device of rhBMP-2/PLGA microspheres in CMC was an effective implantable protein-delivery system for use in bone repair.

INTRODUCTION

Until the mid-1980s, research on the newly cloned proteins of pharmaceutical importance was difficult because of the scarcity of the factors for study. Biotechnology changed the situation, and now many factors are in clinical studies. Although the Food and Drug Administration has approved a number of protein drugs, the drugs are usually not effective with oral administration because of low bioavailability. This stems from very poor absorption and enzymatic degradation. Intravenous administration has been used effectively, but the drugs suffer from a very short plasma half-life [1] and frequent administration is necessary. For efficacious use of some proteins, targeted or local delivery is required. Many systems have been developed to localize growth factors [2-16]. Several controlled-release formulations have been approved (eg, Leutinizing hormone releasing hormone) agonists, tetanus toxoid, human growth hormone [1-3]). Most often, the approach to controlled delivery uses biodegradable or nonbiodegradable polymers as encapsulation agents, either as microspheres or depots. Recombinant human bone morphogenetic protein-2 (rhBMP-2) is a 32-kd homodimeric protein presumed to promote commitment of multipotential stem cells or progenitor cells to osteoblast lineage [4]. Availability via recombinant DNA technology, cloning, protein expression, and purification science [5-11] has allowed intensive research efforts toward the use of rhBMP-2 in bone restoration and repair [12-21]. The protein's osteoinductive property of causing mesenchymal differentiation into chondrocytes, with subsequent calcification of

A literature survey revealed that HPLC1, HPTLC2, LC-MS3 methods for the estimation of Metoprolol individually and in combination with other drugs. Ramipril has been reported for the estimation as an individual or in combination with other drugs in various analytical methods such as HPLC4,5,6,7,8,9,13, HPTLC10,14, LC-MS-MS11, LC-MS12, and spectrophotometric methods15 in bulk drug as well as in plasma, etc. Literature has revealed the analytical methods for the simultaneous estimation of Metoprolol and Ramipril by RP-HPLC16 and spectrophotometry17. However, there is no analytical method reported to date for the simultaneous estimation of Metoprolol Succinate and Ramipril in a combined dosage formulation by HPTLC method. So this work was taken up for the development and validation by a densitometric method which is advantageous over the existing methods in terms of sensitivity.

EXPERIMENTAL

Materials

Working standards of pharmaceutical grade Metoprolol(Batch no. 2148/009) and Ramipril was (Batch no. 16043/01) obtained from Lupin Limited, Pune, India on a dried basis as a gift sample. It was used without further purification. Commercial tablets of Metoprolol and Ramipril in a combined dosage form were purchased from the local market, brand name Starpress R XL-25 (Lupin). All chemicals and reagents (methanol, toluene, ethyl acetate, ammonia) used were of analytical grade and were purchased from Merck Chemicals, India.

Instrumentation

The samples were spotted in the form of bands of width 6 mm with a Camag 100 microlitre sample (Hamilton, Bonaduz, Switzerland) syringe on precoated silica gel aluminum plate 60 F – 254, (20 × 10 cm) with 250 µm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai) using a Camag Linomat IV applicator (Switzerland). The plates were prewashed by methanol and activated at 110oC for 5 min prior to chromatography. Then the chromatoplate was saturated with ammonia vapors for 30 min. A constant application rate of 0.1 µl/s were employed and space between two bands was kept at 6 mm. The slit dimension was kept at 5 × 0.45 mm and 10 mm/s scanning speed was employed. The monochromator bandwidth was set at 20 nm with K 320 cut off filter, each track was scanned thrice and baseline correction was used. The mobile phase consisted of methanol: toluene: ethyl acetate: 30% ammonia (2.5: 3.0: 5.0, 0.7 v/v/v/v). 11.2 ml of mobile phase was used per chromatography. Linear ascending development was carried out in 20 x 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland). Dimensions: length x width x height= 12x4.7x12.5 cm. It was saturated (lined on the two bigger sides with filter paper that had been soaked thoroughly with the mobile phase) and the chromatoplate development was carried out in dark with the mobile phase. The optimized chamber saturation time for the mobile phase was 30 min at room temperature (25oC ± 2) at a relative humidity of 60 % ± 5. The length of the chromatogram run was 8 cm and approximately 20 min. Subsequent to the development, TLC plates were dried in a current of air with the help of an air dryer in a wooden chamber with adequate ventilation. The flow of air in the laboratory was maintained unidirectional (laminar flow, towards exhaust). Densitometric scanning was performed on Camag TLC scanner III in the reflectance-absorbance mode at 209 nm for all measurements and operated by CATS software (V4.06, Camag). The source of radiation utilized was a deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. The evaluation was via peak areas with linear regression.

Preparation of Standard Stock Solutions

20 mg of Metoprolol and 2 mg of Ramipril were accurately weighed and transferred to the 10ml volumetric flask. Metoprolol and Ramipril were dissolved in 10ml of methanol to get Standard solutions of a concentration of 2 mg/ml of Metoprolol and 0.2 mg/ml of Ramipril. The standard solution was stored at 2- 80C, protected from light.

Optimization of the HPTLC method

The TLC procedure was optimized with a view to developing a simultaneous assay method for Metoprolol and Ramipril respectively. Various solvent systems like toluene: ethyl acetate: methanol, chloroform: methanol: ethyl acetate, toluene: ethyl acetate: methanol: ammonia were tried in different concentrations to separate and resolve spots of Metoprolol and Ramipril from their impurities and other excipients of formulations. Methanol: toluene: ethyl acetate: ammonia (2.5: 3.0: 5.0: 0.7 v/v/v/v) was found to result in the compact spot and best peak shape of Metoprolol and Ramipril. Metoprolol and Ramipril were satisfactorily resolved with Rf 0.67±0.05 and 0.37±0.02 respectively with acceptable resolution and peak shape (figure 3) at a wavelength of 209 nm. In order to reduce the neckless effect, TLC chamber was saturated for 20 min using saturation pads. The mobile phase was run up to a distance of 8 cm; which takes approximately 20 min for the complete development of the TLC plate.

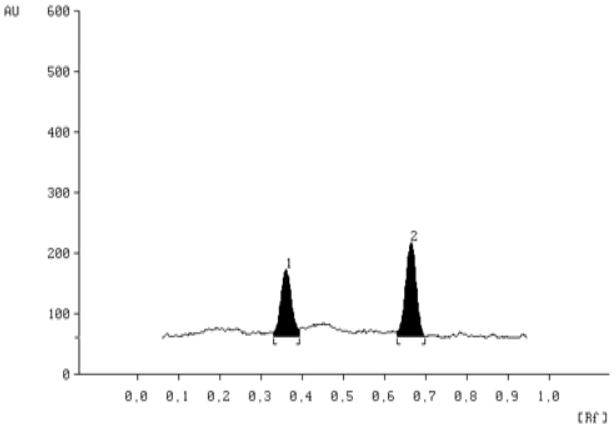

Figure 3: HPTLC Densitogram of standard Peak 1 (200 ng spot-1) of Ramipril (Rf 0.0.37± 0.02), Peak 2 (2000 ng spot-1) of Metoprolol(Rf, 0.67 ± 0.05),

Validation of The Method

Validation of the optimized TLC method was carried out with respect to the following parameters.

Linearity and Range

From the mixed standard stock solution 2 mg/mL of Metoprolol and 0.2 mg/mL of Ramipril, 1 to 6 µL solution spotted on TLC plate to obtain final concentration 2000-12000 ng/spot for Metoprolol and200-1200 ng/spot for Ramipril. The linearity of the method was studied by injecting six concentrations of the drug each concentration was applied three times to the TLC plates. The plate was then developed using the previously described mobile phase and the peak areas were plotted against the corresponding concentrations to obtain the calibration curves.

Precision

The precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analysis of three different concentrations (2000, 6000, 10000 ng /spot for Metoprolol and 200, 600, 1000 ng/spot for Ramipril) of the drug six times on the same day. The intermediate precision of the method was checked by repeating studies on three different days.

Limit of detection and limit of quantitation

Limit of detection (LOD) and quantification (LOQ) represent the concentration of the analyte that would yield signal-to-noise ratios of 3 for LOD and 10 for LOQ, respectively. LOD and LOQ were determined by measuring the magnitude of the analytical background by spotting a blank and calculating the signal-to-noise ratio for Metoprolol and Ramipril by spotting a series of solutions until the S/N ratio 3 for LOD and 10 for LOQ. To determine the LOD and LOQ, serial dilutions of a mixed standard solution of Metoprolol and Ramipril were made from the standard stock solution in the range of 10–200 ng/spot. The samples were applied to the TLC plate and the chromatograms were run and the measured signal from the samples was compared with those of blank samples.

Robustness of The Method

Following the introduction of small changes in the mobile phase composition (± 0.1 mL for each component), the effects on the results were examined. Mobile phases having different compositions, e.g. methanol: toluene: ethyl acetate: ammonia (2.6: 3: 5: 0.7 v/v/v/v), (2.5: 3.1: 5: 0.7 v/v/v/v), (2.5: 3: 5.1: 0.7v/v/v/v), (2.5: 3: 5: 0.8 v/v/v/v), were tried and chromatograms were run. The amount of mobile phase was varied over the range of ± 5 %. The plates were prewashed with methanol and activated at 60°C for 2, 5, and 7 min respectively prior to chromatography. The time from spotting to chromatography and from chromatography to scanning was varied from +10 min. The robustness of the method was determined at three different concentration levels 4000, 8000, 12000 ng/spot for Metoprolol and 400, 800, 1200 ng/spot for Ramipril.

Specificity

The specificity of the method was determined by analyzing standard drug and test samples. The spot for Metoprolol and Ramipril in the samples was confirmed by comparing the RF and spectrum of the spot with that of a standard. The peak purity of Metoprolol and Ramipril was determined by comparing the spectrum at three different regions of the spot i.e. peak start (S), peak apex (M) and peak end (E).

Accuracy

Accuracy of the method was carried out by applying the method to drug sample (Metoprolol and Ramipril combination tablet) to which no amount of Metoprolol and Ramipril standard powder corresponding to 80, 100 and 120% of label claim had been added (standard addition), mixed and the powder was extracted and analyzed by running chromatogram in optimized mobile phase.

Analysis of a marketed formulation

To determine the content of Metoprolol and Ramipril in conventional tablet (Brand name: Starpress R XL25 Label claim: 25 mg Metoprolol and 2.5 mg Ramipril per tablet), ten tablets were weighed, their mean weight determined and finely powdered. The weight of the tablet triturate equivalent to 25 mg Metoprolol and 2.5 mg Ramipril was transferred into a 25 mL volumetric flask containing 10-15 mL methanol, sonicated for 30 min and diluted to 25 mL

with methanol. The resulting solution was centrifuged at 3000 rpm for 5 min and the drug content of the supernatant was determined (1000 and 100 µg/mL for Metoprolol and Ramipril respectively). 2µL of this solution (2000 and 200ng/spot for Metoprolol and Ramipril respectively) was applied to a TLC plate which was developed in an optimized mobile phase. The analysis was repeated in triplicate. The possibility of excipient interference with the analysis was examined.

RESULTS AND DISCUSSION

The results of validation studies on the simultaneous estimation method developed for Metoprolol and Ramipril in the current study involving Methanol: toluene: ethyl acetate: ammonia (2.5: 3.0: 5.0: 0.7 v/v/v/v) as the mobile phase for TLC is given below.

Linearity

The drug response was linear (r2 = 0.997 for Metoprolol and 0.999 for Ramipril) over the concentration range between 2000-12000 ng/spot for Metoprolol and 200-1200 ng/spot for Ramipril. The slope and intercept for Metoprolol and Ramipril were 1.284 (± 0.982), 1979(± 1.25) and 2.947 (± 0.862) and 658 (± 1.06), respectively.

Precision

The results of the repeatability and intermediate precision experiments are shown in Table 1. The developed method was found to be precise as the RSD values for repeatability and intermediate precision studies were < 2 %, respectively as recommended by ICH guidelines.

Table 1: Precision study for Metoprolol and Ramipril

Drug	Concentration ng per band	Intra-day(n=3)		Inter-day(n=3)	
		SD	RSD%	SD	RSD%
Metoprolol	60	14.28	1.040	18.15	1.326
	120	6.96	0.317	5.91	0.269
	180	24.83	0.865	32.73	1.141
Ramipril	60	56.65	1.904	49.86	1.708
	120	33.97	0.671	31.33	0.618
	180	40.51	0.627	41.03	0.635

LOD and LOQ

Signal-to-noise ratios of 3: 1 and 10: 1 were obtained for the LOD and LOQ respectively. The LOD and LOQ were found to be 50 ng/spot and 100 ng/spot for Metoprolol and 50 ng/spot and 150 ng/spot for Ramipril, respectively. The standard deviation of peak areas was calculated for each parameter and the % RSD was found to be less than 2 %. The low values of the % RSD, as shown in Table 2 indicated the robustness of the method.

Table 2: Robustness Testing of Metoprol and Ramipril

Parameters	Metoprolol		Ramipril	
	SD	%RSD*	SD	%RSD*
Mobile phase composition (± 0.1 ml)	10.42	1.235	10.42	1.235
Amount of mobile phase (± 0.5 %)	20.14	1.018	20.14	1.018
Time from spotting to chromatography (± 20 min)	15.36	0.942	15.36	0.942
Time from chromatography to scanning (± 20 min)	20.10	1.085	20.10	1.085

Specificity

The peak purity of Metoprolol and Ramipril was assessed by comparing their respective spectra at the peak start, apex, and peak end positions of the spot,i.e., r (S, M)=0.998 and r (M, E)=0.999. A good correlation(r=0.9997) was also obtained between the standard and sample spectra of Metoprolol and Ramipril, respectively. Also, excipients from formulation were not interfering with the assay.

Recovery Studies

As shown from the data in Table 3 good recoveries of the Thiocolchicoside and Aceclofenac in the range from 98.32 to 99.45 % were obtained at various added concentrations. The average recovery of three levels (nine determinations) for Metoprolol and Ramipril was 98.95 % and 98.98 % respectively.

Table 3: Recovery studies of Metoprolol and Ramipril

Label claim (mg/tablet)	Amount Added (%)	Total amount (mg)	Amount* recovered (mg ± % RSD)	Recovery (%)
Metoprolol 25	80 (20mg)	45	44.65 ± 0.222	99.22
	100 (25mg)	50	49.16 ± 0.154	98.32
	120 (30mg)	55	54.63 ± 0.130	99.32
Ramipril 2.5	80 (2mg)	4.5	4.46 ± 0.526	99.11
	100 (2.5mg)	5.0	4.92 ± 0.360	98.4
	120 (3mg)	5.5	5.47 ± 0.344	99.45

Analysis of a formulation

Experimental results of the amount of Metopr-

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