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Determination of betamethasone disodium phosphate in the in vitro media of PLGA microspheres by high-performance liquid chromatography

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Abstract

Betamethasone disodium phosphate is a kind of corticosteroid, which is commonly used in the intralesional treatment of keloids. In this work, polymeric microspheres loaded with betamethasone disodium phosphate were fabricated to design a sustained-release system. A validated HPLC technique for determination of betamethasone disodium phosphate in in vitro media of the polymeric microspheres has been developed. The operation conditions were optimized. The analytical column was ZORBAX® Bonus-RP column with 4.6 × 150 mm ID and a particle size of 5 μm. The mobile phase consisted of acetonitrile–0.01% phosphoric acid water solution (40:60, v/v). The flow rate was 1.0 ml/min and injection volume was 50 μl. The elutes were detected at 240 nm. Linearity, repeatability, inter- and intra-assay precision and accuracy of the method were evaluated. The liner range was obtained in a concentrated range of 20–1000 μg/ml, with a coefficient of correlation $r = 0.999978$. The limit of detection for betamethasone disodium phosphate in the in vitro test samples was 0.25 μg. Recovery of betamethasone disodium phosphate from the in vitro test samples was $99.7 \pm 5.2\%$ (mean ± SD). Stability of betamethasone disodium phosphate at different pH values and temperatures were also investigated.

Keywords: Betamethasone disodium phosphate; PLGA microspheres; High performance liquid chromatography; Stability; Validation

1. Introduction

Keloids are formed after skin injury due to proliferative dermal growths, the scar tissue extends beyond the borders of original wound, which results in cosmetic disfigurement for the patients, and it can also be pruritic, tender and complicated by second infections. There are numerous therapeutic options for the treatment of keloids such as occlusive dressings, compression therapy, intralesional corticosteroid injections, cryosurgery, excision, radiation therapy and laser therapy [1]. Among these methods, corticosteroid injections have been a standard treatment for

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keloids for many years [2]. The disadvantage of this treatment is that 4–6 injections need to be administered at intervals of 4–6 weeks for several months or until the scar is flattened and the pain is experienced by patients during the injections [3]. Excessive injection of corticosteroids may affect the surrounding normal skin, with resultant atrophy, telangiectasia formation or altered pigmentation and occasionally, skin breakdown. To overcome these problems, sustained-release microsphere systems were being developed in our laboratory to deliver betamethasone disodium phosphate by single injection. Betamethasone disodium phosphate is water-soluble. Its chemical structure is shown in Fig. 1. Betamethasone disodium phosphate was encapsulated within poly(DL-latide-*co*-glycolide) (PLGA) microspheres using a water-in-oil-in-water double emulsion process [4]. In order to evaluate in vitro release kinetics of betamethasone disodium phosphate from the microspheres, high-performance liquid chromatography (HPLC) was employed to determine betamethasone disodium phosphate concentration in the in vitro samples. There were several HPLC approaches suggested for determining betamethasone or betamethasone dipropionate in blood or urine samples or pharmaceutical products [5–9]. However, less research work was focused on determination of betamethasone disodium phosphate [10–12]. Mixtures of methanol or acetonitrile and phosphate buffer were used as mobile phase in these methods. The large amount of samples we need for analysis would keep the HPLC system in the buffer for a long period of time, which may be harmful for the system maintenance. The internal standard method they used may not be practical for our routine testing.

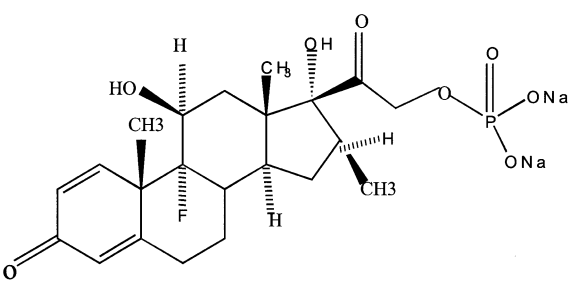

Fig. 1. Structure of betamethasone disodium phosphate.

This present work was undertaken to develop a rapid, reliable and simple HPLC procedure to determine betamethasone disodium phosphate in in vitro media of PLGA microspheres.

2. Experimental

2.1. Instrumentation

The chromatographic system, 2690D Separations Module, was purchased from Waters (Milford, USA). It consisted of an auto-sampler system and a 996 Photodiode Array Detector. The chromatographic data were collected and processed using MILLENNIUM^{32} software (Waters, Milford, USA). The separation was performed by a ZORBAX® Bonus reverse phase analytical column of 4.6 × 150 mm ID and a particle size of 5 μm. This was protected by a ZORBAX® Bonus-RP guard column of 4.6 × 12.5 mm ID (Hewlett–Packard, Santa Clarita, USA).

2.2. Materials

Betamethasone disodium phosphate was purchased from Sigma Chemical Co. (Deisenhofen, Germany). Acetonitrile of HPLC grade was obtained from J.T. Baker, Phillipsburg, USA. Water was obtained from a Milli-Q ultrapure water system and filtered by 0.22 μm Millipak® 40 filter (Millipore, Molsheim, France). Analytical phosphoric acid, sodium hydroxide, sodium phosphate, potassium chloride and sodium azide were from Merck KgaA, 64271 Darmstadt, Germany. Sodium chloride was from BDH Laboratory Supplies, Poole, BH 15 1TD, England.

2.3. Chromatographic conditions

The mobile phase was acetonitrile–0.01% phosphoric acid aqueous solution (40:60, v/v) which was filtered through a Millicup filter (0.45 μm) and degassed by vacuum for 15 min prior to use. The flow rate was 1.0 ml/min and the column temperature was ambient. The injection volume

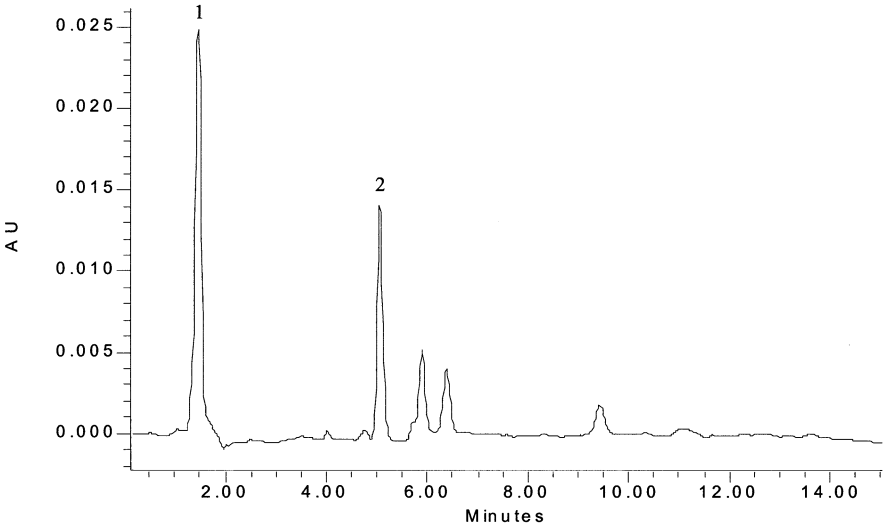

Fig. 2. Typical chromatogram of betamethasone disodium phosphate in the in vitro media of PLGA 65:35 microspheres. Peaks 1 and 2 are indicative of PBS and betamethasone disodium phosphate, respectively. The rest comes from degradation products of PLGA 65:35.

was 50 μl, and the eluate was monitored at 240 nm.

2.4. Sample preparation

Thirty millograms onwards of microspheres containing betamethasone disodium phosphate was accurately weighted in culture tubes and suspended in 1 ml phosphate buffered saline (PBS) with pH 7.4. PBS was prepared by dissolving 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 1 l of distilled water and adjusted to pH 7.4 with HCl. Samples were collected from the test tubes at predetermined intervals after being centrifuged for 6 min at the speed of 10 000 rpm/min. The supernatant was replaced with fresh PBS and the release study was continued after the replacement. The supernatant was filtered with a 0.2 μm syringe filter. Each in vitro release study was performed in triplicate. The samples were kept at 4 °C before being quantified by HPLC.

2.5. Stability of betamethasone disodium phosphate

The stability of betamethasone disodium phosphate incubated in PBS buffer was evaluated under different conditions by analyzing its concentration change as a function of incubation time.

3. Results and discussion

3.1. Optimization of chromatographic conditions

Optimization of chromatographic conditions was carried out with respect to mobile phase composition. Adding phosphoric acid to the mobile phase was to modify retention time of betamethasone disodium phosphate peak and eliminate interference of degradation products of PLGA polymer or other compounds present in the PBS buffer. Reproducibility of betamethasone disodium phosphate retention time was significantly improved due to the presence of phosphoric acid. Good separation of betamethasone disodium phosphate from the polymer degradation products was achieved when acetonitrile–0.01% phosphoric acid (40:60, v/v) was used as the mobile phase. The run-time of one sample was 12 min. Typical chromatogram is shown in Fig. 2.

3.2. Calibration curve and linearity

Betamethasone disodium phosphate was weighed accurately and dissolved in PBS to prepare a 10.0 mg/ml stock solution. The stock solution was diluted with PBS to obtain 20, 40, 60, 80, 100, 200, 400, 600, 800, 1000 μg/ml work standards. Each standard was injected three times. Calibration curve was generated based on plotting peak-height versus betamethasone disodium phosphate concentration using MILLENNIUM[32] software (Waters, Milford, USA). A linear curve was obtained for betamethasone disodium phosphate in a concentration range of 20–1000 μg/ml, with a coefficient of correlation $r = 0.999978$. The least-square plot gave the equation $y = 70.809x - 104.339$ (RSD = 2.6 and 9.8% for slope and intercept, respectively, $n = 3$).

Table 1
Inter-assay ($n = 6$) precision and accuracy of betamethasone disodium phosphate

Added (μg/ml)	Found (μg/ml)	RSD (%)	Relative error (%)
20.3	20.6	4.9	1.7
40.5	40.6	2.7	0.1
60.8	60.9	1.4	0.1
81.0	80.6	1.0	−0.5
101.3	101.6	1.3	0.3
202.6	203.9	0.5	0.7
405.2	399.6	0.3	−1.4
607.8	597.5	0.4	−1.7
803.2	802.7	0.3	−0.1
1013.0	1020.9	0.8	0.8

Table 2
Intra-assay ($n = 3$) precision and accuracy of betamethasone disodium phosphate

Added (μg/ml)	Found (μg/ml)	RSD (%)	Relative error (%)
20.1	20.4	0.8	1.8
40.2	40.5	2.8	0.9
60.2	61.7	2.5	2.5
80.3	80.8	0.8	0.6
100.4	99.6	0.8	−0.8
200.8	197.2	0.8	−0.8
401.6	399.8	1.1	−0.5
602.4	599.1	0.3	−0.5
803.2	799.8	0.7	−0.4
1004.0	1013.0	0.4	0.9

3.3. Precision and accuracy

Inter-day precision ($n = 6$), intra-day precision ($n = 3$) and accuracy of method were evaluated from the data obtained during the 3-day validation. Concentration measured was from 20 to 1000 μg/ml within the concentration range of the calibration curve. Ten different concentration points were investigated for this purpose. For each concentration the relative standard deviation (RSD) of the mean served as measure of the precision. The accuracy was determined by comparing the measured concentrations to the actual concentrations of betamethasone disodium phosphate in the sample and expressed as the mean relative error (RE). Tables 1 and 2 listed the inter- and intra-day reproducibility data. In the intra day precision study, the RSD varied from 0.4 to 2.8% but did not exceed 5.0% on the calibration points for inter-day precision data. It is concluded that the accuracy of this method was quite good, and both intra- and inter-day assay errors were less than 3%.

3.4. Recovery

Recoveries of betamethasone disodium phosphate from in vitro test samples were determined in triplicates by comparing the determined amount with the actual added one in the in vitro test samples. Different levels of betamethasone disodium phosphate (e.g. 20, 100, 200 or 500 μg/ml) were added into the in vitro test samples. Average recovery from the in vitro samples was $99.7 \pm 5.2\%$ for betamethasone disodium phosphate as shown in Table 3.

3.5. Limits of detection (LOD) and limits of quantitation (LOQ)

The limit of detection was identified by repeated injections ($n = 10$) of blank sample into the column. The present validation measurements revealed that a limit of detection (signal-to-noise

Table 3
Recoveries of betamethasone disodium phosphate ($n = 3$)

Amount added (μg)		Amount measured (μg)	Recovery (%)	SD (%)
20.08	1	19.70	98.1	
	2	20.27	101.0	
	3	20.30	101.1	
	Average	20.09	100.1	1.7
100.4	1	101.5	101.1	
	2	101.8	101.4	
	3	102.0	101.6	
	Average	101.8	101.4	0.3
200.8	1	211.1	104.8	
	2	204.1	101.6	
	3	216.7	107.9	
	Average	210.6	104.8	3.2
502.0	1	463.7	92.4	
	2	463.6	92.4	
	3	465.3	92.7	
	Average	464.2	92.5	0.2

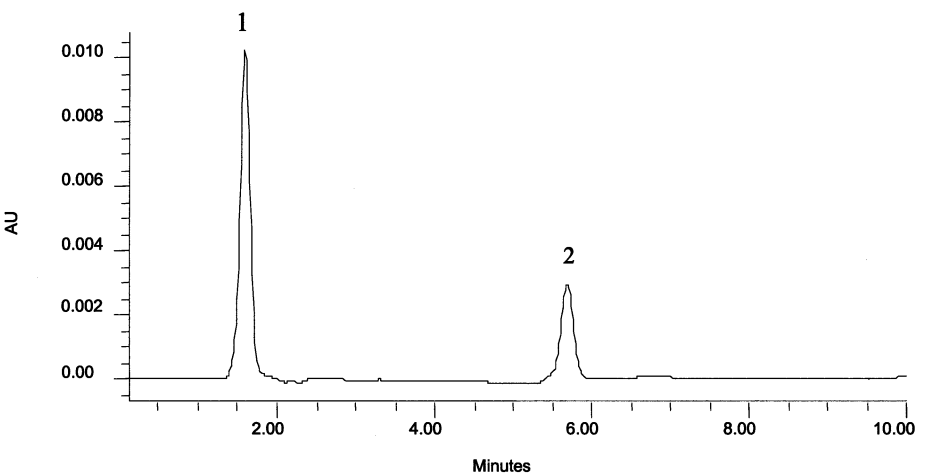

Fig. 3. Chromatogram of blank PLGA 65:35 microspheres in the in vitro media. Peaks 1and 2 are indicative of PBS and PLGA degradation product, respectively.

ratio of 3:1) was 0.25 μg, and limit of quantitation (signal-to-noise ratio of 5:1) was 0.42 μg.

3.6. Specificity

PLGA microspheres without drug loading were put into PBS buffer as described previously in Section 2.4. The supernatant was injected into the HPLC system (Fig. 3). No interference from PLGA degradation product presented.

3.7. Stability of betamethasone disodium phosphate

It was reported that pH within the PLGA microspheres dropped precipitously during degradation and it was down to as low as 1.5 in the center of the microspheres [13]. In this work, the stability of betamethasone disodium phosphate was investigated at various pH values, temperatures and concentrations. Tables 4–6 listed effects of pH, concentration and incubation time on

Table 4
Effects of pH value and incubation temperature on stability of betamethasone disodium phosphate at a concentration of 53 ppm

Day	26 °C			37 °C		
pH	7.4	5	2	7.4	5	2
0	53.0	53.0	53.0	53.0	53.0	53.0
2	52.2	52.6	52.6	52.3	52.5	52.7
4	52.5	53.3	53.4	52.8	53.1	53.3
7	52.9	53.5	53.1	53.1	52.2	52.7
11	49.2	49.9	50.1	52.7	52.7	48.7
15	48.7	49.4	49.8	47.4	47.8	48.6
18	48.6	49.4	49.3	48.2	48.0	48.9
22	47.9	49.3	49.5	47.5	47.4	48.7

Table 5
Effects of pH value and incubation temperature on stability of betamethasone disodium phosphate at a concentration of 101 ppm

Day	26 °C			37 °C		
pH	7.4	5	2	7.4	5	2
0	101.0	101.0	101.0	101.0	101.0	101.0
2	101.3	101.5	101.8	101.0	101.8	101.9
4	101.1	101.4	101.4	101.4	101.6	101.2
7	101.9	101.3	101.7	101.4	101.3	101.2
11	96.1	99.8	99.2	101.1	101.2	96.9
15	95.5	98.1	99.1	91.9	97.7	96.8
18	95.5	99.6	98.3	92.5	95.9	96.4
22	95.7	97.7	98.3	91.2	95.8	95.9

Table 6
Effects of pH value and incubation temperature on stability of betamethasone disodium phosphate at a concentration of 505 ppm

Day	26 °C			37 °C		
pH	7.4	5	2	7.4	5	2
0	505.2	505.2	505.2	505.2	502.2	505.2
2	505.1	502.6	505.9	498.9	507.5	505.4
4	497.1	507.9	505.2	495.1	506.6	505.5
7	500.2	505.5	504.0	496.1	502.5	505.2
11	497.4	494.5	503.4	495.2	503.2	491.7
15	498.3	496.2	498.6	473.6	490.5	489.0
18	485.7	491.0	497.1	467.6	481.0	486.7
22	484.2	485.6	494.2	468.7	473.8	488.6

degradation of betamethasone disodium phosphate. It is observed that betamethasone disodium phosphate was more stable at lower pH values and temperatures. A reduced concentration led to slightly faster degradation of betamethasone disodium phosphate. For instance, when its initial concentration was 101 ppm, 5.1 and 9.7% betamethasone disodium phosphate degraded at pH 2 and 7.4, respectively, after a 22-day incubation at 37 °C but 5.3% degradation at pH 7.4 at 26 °C.

4. Conclusions

A fast, simple and reliable HPLC method has been developed to quantitatively determine betamethasone disodium phosphate in the in vitro media of PLGA microspheres. The samples were injected automatically and directly into the HPLC and the run-time for each sample was 12 min. This method is suitable for routine tests to guide the design of desirable release profiles of betamethasone disodium phosphate from the microspheres. In addition, stability of betamethasone disodium phosphate in different pH was evaluated by using this established method. Betamethasone disodium phosphate was more stable at lower pH values and temperatures as well as higher concentrations.

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