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Determination of doxorubicin in rabbit ocular tissues and pharmacokinetics after intravitreal injection of a single dose of doxorubicin-loaded poly-β-hydroxybutyrate microspheres

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Abstract

A validated HPLC method was developed for the quantification of doxorubicin in rabbit ocular tissues using solid phase extraction and ultraviolet detection. Chromatographic separation of doxorubicin in various ocular tissues was performed on a C18 column. The mobile phase was composed of 0.2 M KH_2PO_4 buffer solution, acetonitrile and triethylamine in volumetric ratio of 70/30/0.2, adjusted to pH 4.0 with orthophosphoric acid. The calibration curve was linear over the range of 0.03–10, 0.03–10, 0.05–10 and 0.05–10 μg/ml in vitreous body, iris, retina/choroids and sclera, respectively. The intra-day and inter-day precisions in all ocular tissues were smaller than 4.95% and 5.73%, and the accuracies were about 100%. The extraction recoveries of doxorubicin in all of the ocular tissues were between 83.47% and 96.33%. After intravitreal administration of doxorubicin-loaded poly-β-hydroxybutyrate microspheres, doxorubicin level in ocular tissues was much lower than that for administration of free doxorubicin, which was helpful to reduce the associated toxicity to surrounding tissues. Doxorubicin was detectable even after tens of days in the studied ocular tissues.

Keywords: Doxorubicin; Microspheres; RP-HPLC; Pharmacokinetics; Ocular

1. Introduction

Diseases at the site of posterior segment of the eye remain a challenge clinically till now, because it is difficult for drugs, especially those without suitable balance between hydrophilicity and hydrophobicity, to penetrate the ocular barriers to the posterior chamber by a routine topical administration of eye drops [1]. Intravitreal injection of active agents was effective for diseases of the posterior segment, like proliferative vitreoretinopathy (PVR), uveitis, cytomegalovirus retinitis, etc. [1–3]. Sustained drug delivery systems are preferred not only to prevent the trauma caused by frequent intravitreal injection but also to achieve prolonged drug level [4–7]. The sustained local drug level should be therapeutically beneficial for enhanced efficiency, and reduced dosage may be desired for less systemic toxicity [8,9]. Microparticle systems (microspheres, microcapsules, etc.) prepared with biodegradable polymers like poly(lactic acid) or poly(lactic acid-*co*-glycolic acid) have been investigated as such sustained release drug delivery systems [4–7,10,11].

Doxorubicin (Fig. 1), an anthracycline antibiotic, is widely used in clinical practice and has significant antitumor activity against several human malignancies, including leukemia and breast cancer [12]. Its clinical use is hampered by undesired cytotoxic properties mediated by reactive oxygen species produced by the redox cycling of its semiquinone metabolites [13]. The oxygen radicals produced by doxorubicin metabolism induce production of lipid peroxides that are transported systemically and will damage a variety of cells [14]. Intravitreal injection of doxorubicin was effective for the treatment of experimental PVR [15,16]. However, high local level of doxorubicin is suspected for toxicity at the injection site. Development of intravitreally injectable doxorubicin sustained delivery system will reduce the cytotoxicity and maintain a therapeutic level for prolonged time duration. In our previous study, doxorubicin-loaded microspheres were prepared by emulsification/solvent evaporation method [17] using a biocompatible and biodegradable poly-

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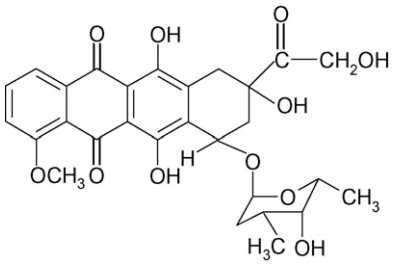

Fig. 1. Chemical structure of doxorubicin.

mer, poly-β-hydroxybutyrate (PHB) [18,19]. To understand the effectiveness of sustained and decreased doxorubicin level in ocular tissues after intravitreal injection of doxorubicin-loaded microspheres, it is mandated to monitor the intraocular doxorubicin level and study its pharmacokinetics after intravitreal administration.

Several methods have been reported for the determination of doxorubicin, including radiolabelled assay [20], fluorescence assay [21,22] and favorably HPLC [23–29]. The HPLC method of high specificity and sensitivity has been employed to determine doxorubicin in various biological samples either with fluorescent or ultraviolet detection. However, methods for the determination of doxorubicin in ocular tissues have not been reported. In the present study, an RP-HPLC method was developed to determine doxorubicin in various ocular tissues of rabbits and applied for the pharmacokinetics study after intravitreal injection of a single dose of doxorubicin-loaded PHB microspheres.

2. Materials and methods

2.1. Chemicals and reagents

Doxorubicin hydrochloride (>99.0%) was kindly gifted from Haizheng Pharma Co. Ltd. (Zhejiang, China). Poly-β-hydroxybutyrate (PHB) with an average molecular weight of 200,000 was kindly gifted by Prof. Guoqiang Chen of Tinghua University (Beijing, China). Polyvinyl alcohol (PVA, 87–89% hydrolyzed) with an average molecular weight of 20,000 was purchased from Aldrich Chemical Co. (Milwaukee, USA). HPLC grade acetonitrile was a Merck product (Darmstadt, Germany). All other reagents were of analytical grade and purchased from Shanghai Chemical Co. Ltd. (Shanghai, China). Daunomycin, the internal standard, was provided by the National Institute for the Control of Pharmaceutical and Biological Products (>99.0%, Beijing, China).

2.2. Apparatus and chromatographic conditions

The RP-HPLC measurements were carried out using an Agillent 1100 series system (Agillent, USA) consisted of a quaternary pump, an on-line degasser, an auto sampler, a column-heating assembly and a UV detector. The separation was performed on a Diamonsil® C18 HPLC column (250 mm × 4.6 mm, particle size 5 μm, Dikma Technologies, Beijing, China). The mobile phase was composed of 0.2 M KH_2PO_4 buffer solution, acetonitrile and triethylamine in volumetric ratio of 70/30/0.2, adjusted to pH 4.0 with orthophosphoric acid. The flow rate was set to 1.0 ml/min, while the temperature to 40 °C and wavelength to 234 nm.

2.3. Preparation of standard samples

A stock solution of doxorubicin was prepared in methanol (1.0 mg/ml) and stored at 4 °C. The stock solution was diluted with methanol to prepare working solutions at a variety of final concentrations. Internal standard stock solution (1.0 mg/ml) was also prepared in methanol and diluted with methanol for a working solution of 5 μg/ml. Working calibration curve for each ocular tissue was developed by adding a stock solution of doxorubicin to each of the blank matrix of vitreous body or homogenates of iris, sclera or retina/choroids.

2.4. Sample preparation

To 0.1 ml of vitreous body, add 0.1 ml of internal standard solution and mix thoroughly through vortexing before subjecting to solid-phase extraction. Samples of iris and retina/choroids were homogenized with 0.8 ml of hydrochloric acid (pH 1.0) using a homogenizer. After addition of 0.1 ml of internal standard and phosphate buffered saline (pH 7.4) to make a final volume of 2 ml, the mixture was centrifuged at 5000 × *g* for 10 min. The supernatant was withdrawn and subjected to solid-phase extraction. Samples of sclera was firstly minced, and then homogenized with 1.9 ml of water. After addition of 0.1 ml of internal standard, the mixture was then centrifuged at 5000 × *g* for 10 min. The supernatant was also withdrawn and subjected to solid-phase extraction.

The supernatant of ocular tissue homogenates was applied to a C18 solid phase extraction cartridges (Supelco LC-8, 100 mg, USA) preconditioned with 2 ml of methanol and 2 ml of deionized water consecutively. Some of the impurities in the tissue extracts were removed by washing the columns with a further 2.0 ml of ethyl acetate. Recovery of doxorubicin was performed by eluting the column with 1.0 ml of methanol. The elute was dried under a gentle stream of nitrogen at 50 °C. The residue was dissolved by 0.1 ml of mobile phase, and 20 μl aliquots were injected into the HPLC system.

2.5. Preparation of doxorubicin-loaded PHB microspheres

Microspheres were prepared by an emulsification/solvent evaporation method as described previously [17]. Briefly, 100 mg of PHB was dissolved in 2 ml of chloroform. To this solution, 6 mg of micronized doxorubicin was added and dispersed by ultrasonication. This organic phase was then slowly injected into 100 ml of 6.5% (w/v) PVA solution and stirred with a mechanical stirrer (Ika, Germany) at 800 rpm at 0 °C for 3 h. The microspheres were filtered and washed with deionized water and dried at room temperature in a desiccator. The final products were stored under reduced light at 4 °C. Ten batches of microspheres were pooled and characterized for *in vitro* properties thereafter. Drug loading of doxorubicin, which was tunable through adjusting preparative variables, was determined by

HPLC under conditions similar to that described in this study. As high drug loading is always associated with faster drug release and more significant burst release, especially for water-soluble drugs like doxorubicin hydrochloride, it was tuned to a much lower level of 1.01% ± 0.03% ($n = 3$) in favor of prolonged release in this study. Although lower drug loading may demand larger volume of the final dosage, this was not a problem here because the therapeutic dosage of doxorubicin was as small as a few micron grams. Diameter of the microspheres was also an important factor influencing their *in vitro* and *in vivo* behaviors. Geometric mean diameter of the microspheres was calculated as the mean of the projection diameters of microspheres in scanning electron micrographs. At least 100 microspheres were counted and the diameter was presented as 43.13 ± 19.86 μm.

2.6. Pharmacokinetics study

New Zealand rabbits, weighing about 2–2.5 kg, were provided by Shanghai Shenwang Experimental Animal Center, and the experiments were carried out in accordance with the guidelines issued by the Ethical Committee of Fudan University.

Forty-five female rabbits were divided into three groups of 11, 17 and 17 animals each, which was injected to posterior segment of the eyes either with free doxorubicin solution or doxorubicin-loaded microspheres. Suspending vehicle was 0.9% (w/v) sodium chloride solution containing 1% (w/v) hydroxypropylmethylcellulose (15 Pa s, Dow Chemicals, USA). After anesthetization by intramuscular injection of ketamine hydrochloride, rabbits in the control group ($n = 11$) were injected with 0.1 ml of free doxorubicin solution into the posterior vitreous cavity at the site of pars plana in both eyes at a dose of 5 μg of free doxorubicin base per eye. At time intervals of 0.167, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0 and 12.0 h after injection, one rabbit was sacrificed with both of its eyes enucleated. Rabbits in the treatment group ($n = 17$) were injected with 0.1 ml of doxorubicin-loaded microspheres suspension into the posterior vitreous cavity at the site of pars plana in both eyes at dose levels of 5 and 9 μg of free doxorubicin base equivalent, respectively. A gauge 7 needle was used for injection of both doxorubicin solution and microspheres suspension. At time intervals of 0.5, 1.0, 1.5, 3.0, 6.0, 12.0 h, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 15.0, 30.0, 45.0 and 60.0 d after injection, one rabbit was sacrificed with eyes enucleated. Conjunctive tissue was carefully removed from the surface of the eye spheres. After rinsing with physiological saline, the eyes were dissected and samples of vitreous fluid, iris, retina/choroids and sclera were collected. All the samples were put on filter paper to remove excessive water and weighed afterwards. Store the samples at −20 °C before HPLC analysis.

3. Results and discussion

3.1. Sample preparation

HPLC method for determination of doxorubicin in a variety of biological samples, especially plasma, has been well studied [23–29]. However, regarding the samples of the ocular tissues, there is little experience. During the initial stage of method development, we met difficulties in sample preparation of the ocular tissues. The vitreous body is a viscous fluid composed of 99% water and a small amount of collagen and hyaluronic acid. A liquid–liquid extraction initially applied for plasma was firstly tested to recover doxorubicin from vitreous body. After vortex mixing of the vitreous fluid and several organic solvent like ethyl acetate, ethyl ether and dichloromethane, emulsification occurred and the viscosity of the mixture increased abruptly as a result of gel-forming, after which further extraction and separation was very difficult. In this study, pre-treatment of the vitreous body with methanol was helpful to extract doxorubicin, precipitate proteins and decrease the viscosity of the system. Coupled with solid phase extraction, the samples of vitreous fluid were highly purified with an absolute recovery of doxorubicin of over 80%. When dealing with sclera, retina/choroids and iris, gels also formed during the homogenization process. The addition of methanol in this samples were helpful to extract doxorubicin and prevent gelling of the homogenates. The samples treated with methanol were further purified and concentrate with solid phase extraction with an absolute recovery of doxorubicin of over 80%.

3.2. Chromatographic separation and selectivity

The chromatograms of different tissues including vitreous body, iris, sclera, retina/choroids were shown in Fig. 2. The retention time for doxorubicin under the current conditions was about 6 min, while for internal standard it was about 11 min. Pooled blank samples were also analyzed and confirmed that there were no interfering peaks in the chromatograms. The selectivity of the method was demonstrated by the absence of substantial endogenous interference on the chromatograms of ocular tissue extracts verified on pooled and untreated tissues from at least three rabbits.

3.3. Calibration linearity

Calibration curves for assay in all of the ocular tissues developed with peak area ratio of doxorubicin to internal standard versus drug concentration were found to be linear over a specific range for each ocular tissue using weighted least square method, and the weight was $1/C^2$. The linear regression details were shown in Table 1.

3.4. Intra-day and inter-day precision

Intra-day and inter-day precision of the method was evaluated by analyzing ocular tissue samples spiked with doxorubicin at three different concentration levels of 0.03, 0.3 and 0.9 μg/ml. For intra-day precision, three replicate quality control samples at each concentration were assayed on the same day. The inter-day precision was evaluated on three different days. Results are summarized in Table 2. The intra-day and inter-day precision was all smaller than 4.95% and 5.73%, respectively, which indicated that the method was reproducible.

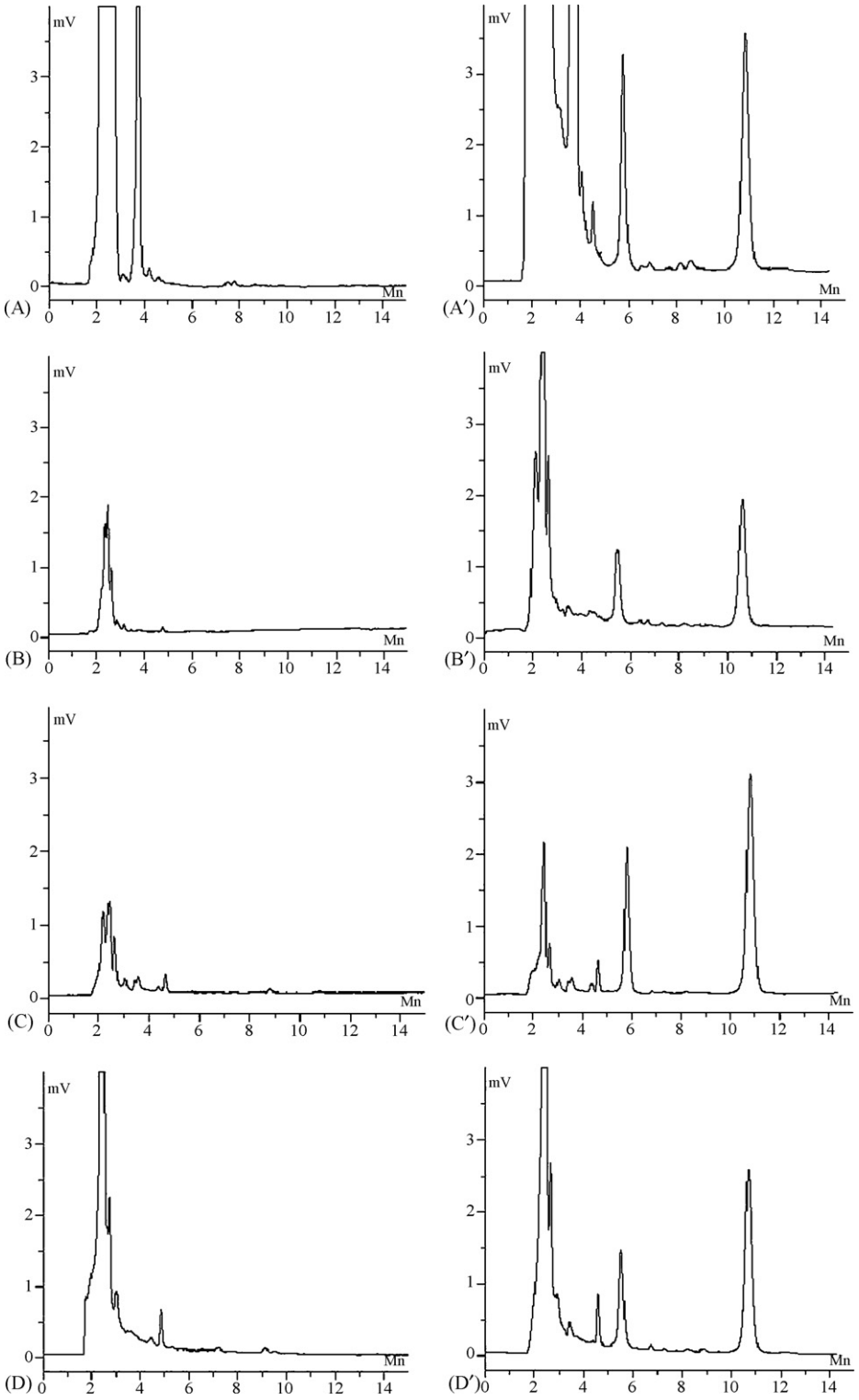

Fig. 2. Chromatograms of ocular tissue extracts: (A) blank vitreous body; (A′) blank vitreous body spiked with doxorubicin and I.S.; (B) blank iris; (B′) blank iris spiked with doxorubicin and I.S.; (C) blank retina/choroids; (C′) blank retina/choroids spiked with doxorubicin and I.S.; (D) blank sclera; (D′) blank sclera spiked with doxorubicin and I.S. (doxorubicin, t_R = 5.8 min; I.S., t_R = 10.7 min).

3.5. Accuracy and extraction recovery

Accuracy of the method was also evaluated by analyzing ocular tissue samples spiked with doxorubicin at three different concentration levels of 0.03, 0.3 and 0.9 μg/ml. Results are shown in Table 3. It was suggested that the method was in good accuracy and complied with criteria for biological sample assay. The extraction recoveries of doxorubicin in all of the ocular tissues were between 83.47% and 96.33%.

Table 1
Calibration linearity for doxorubicin assay in various ocular tissues

Ocular tissues	Linear range (μg/ml)	Intercept ($n=5$)	Slope ($n=5$)	Regression coefficient (r)
Vitreous body	0.03–10	−0.0016 ± 0.0014	0.1168 ± 0.0024	0.9999
Iris	0.03–10	−0.0237 ± 0.0098	0.3954 ± 0.0079	0.9997
Retina/choroids	0.05–10	−0.0085 ± 0.0044	0.4155 ± 0.0110	0.9995
Sclera	0.05–10	−0.0383 ± 0.0132	0.4112 ± 0.0122	0.9998

Table 2
Intra-day and inter-day precision for doxorubicin assay in various ocular tissues ($n=3$)

Tissue	C (μg/ml)	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)
Vitreous body	0.03	4.91	5.73
	0.3	1.77	1.84
	0.9	2.80	1.73
Iris	0.03	4.95	5.63
	0.3	3.24	2.29
	0.9	1.90	2.57
Retina/choroids	0.05	3.18	4.99
	0.3	2.82	1.17
	0.9	2.52	2.97
Sclera	0.05	3.92	3.88
	0.3	1.78	2.52
	0.9	3.25	1.34

3.6. Limits of quantification and detection

The limits of quantification of doxorubicin in vitreous body, iris, retina/choroids and sclera were 0.03, 0.03, 0.05 and 0.05 μg/ml, respectively. The limits of detection of doxorubicin in vitreous body, iris, retina/choroids and sclera were 0.005, 0.01, 0.03 and 0.03 μg/ml, respectively.

3.7. Stability study

The stability experiments of doxorubicin was carried out under three conditions: after storing at room temperature for 24 h, at 4 °C for 7 d and at −20 °C for 30 d. The stability of doxorubicin was investigated in triplicate at the concentrations of 0.03 (for vitreous body and iris), 0.05 (for retina/choroids and sclera) 0.3 and 0.9 μg/ml for doxorubicin, respectively. In all the ocular tissues, doxorubicin was stable for at least up to 7 and 30 d when the samples were stored at 4 and −20 °C, respectively. Doxorubicin was also stable after three cycles of freeze and thaw. At room temperature (25 °C), doxorubicin was unstable and declined to about 67% of initial concentration after 24 h. It was suggested that ocular samples should be stored under cold conditions as soon as possible after collection.

Table 3
Accuracy and extraction recovery of doxorubicin in various ocular tissues ($n=3$)

Tissue	C (μg/ml)	Accuracy (%)	Extraction recovery (%)
Vitreous body	0.03	95.67 ± 4.68	85.71 ± 4.90
	0.3	99.29 ± 1.72	90.31 ± 5.24
	0.9	95.10 ± 2.64	88.89 ± 6.37
Iris	0.03	98.77 ± 3.59	83.96 ± 4.75
	0.3	101.94 ± 2.68	85.63 ± 5.14
	0.9	100.75 ± 1.75	89.77 ± 6.28
Retina/choroids	0.05	100.78 ± 2.76	87.99 ± 4.11
	0.3	100.33 ± 2.95	92.78 ± 5.33
	0.9	104.61 ± 2.68	96.33 ± 2.67
Sclera	0.05	95.73 ± 4.11	83.47 ± 7.25
	0.3	96.55 ± 2.68	88.39 ± 2.36
	0.9	102.27 ± 2.67	91.34 ± 5.24

3.8. Ocular pharmacokinetics

Doxorubicin concentration versus time profiles in various ocular tissues after intravitreal injection of doxorubicin-loaded PHB microspheres at doses of 5 and 9 μg, compared with doxorubicin solution, were shown in Fig. 3. Pharmacokinetic parameters were listed in Table 4.

As for doxorubicin solution, distribution from the vitreous body to other ocular tissues was fast. Doxorubicin concentration versus time profile in retina/choroids was similar to that in vitreous body because the two were adjacent and there was little obstacle preventing drug diffusion. It took time for doxorubicin to diffuse from the injection site to iris and sclera. So, doxorubicin level peaked until 4 h after injection. Elimination was fast and doxorubicin could not be detected in all ocular tissues after 12 h. Even at time point of 8 h, doxorubicin was not detectable in sclera, retina and choroids, while it could not be detected in iris after 10 h.

As for doxorubicin-loaded PHB microspheres, doxorubicin could be detected in all ocular tissues in sustained level for several tens of days, which was in parallel with *in vitro* sustained release characteristics [17]. C_{max} of doxorubin-loaded microspheres was significantly smaller than its solution counterpart. Since the microspheres showed a burst release at the first day of release, t_{max} in these ocular tissues was relatively small, about 24 h or less.

There was a significant increase in relative bioavailability after incorporation of doxorubicin in microspheres. The relative bioavailability calculated on basis of AUC was about 40 times that of doxorubicin solution both in vitreous body and sclera, and about five times in iris and retina/choroids. For the two dosages of microspheres compared, doxorubicin concentration versus time profiles were similar. There was no significant difference in relative bioavailability and t_{max}.

In pharmacodynamic study, we have evaluated the suppression of PVR by intravitreal injection of doxorubicin-loaded PHB

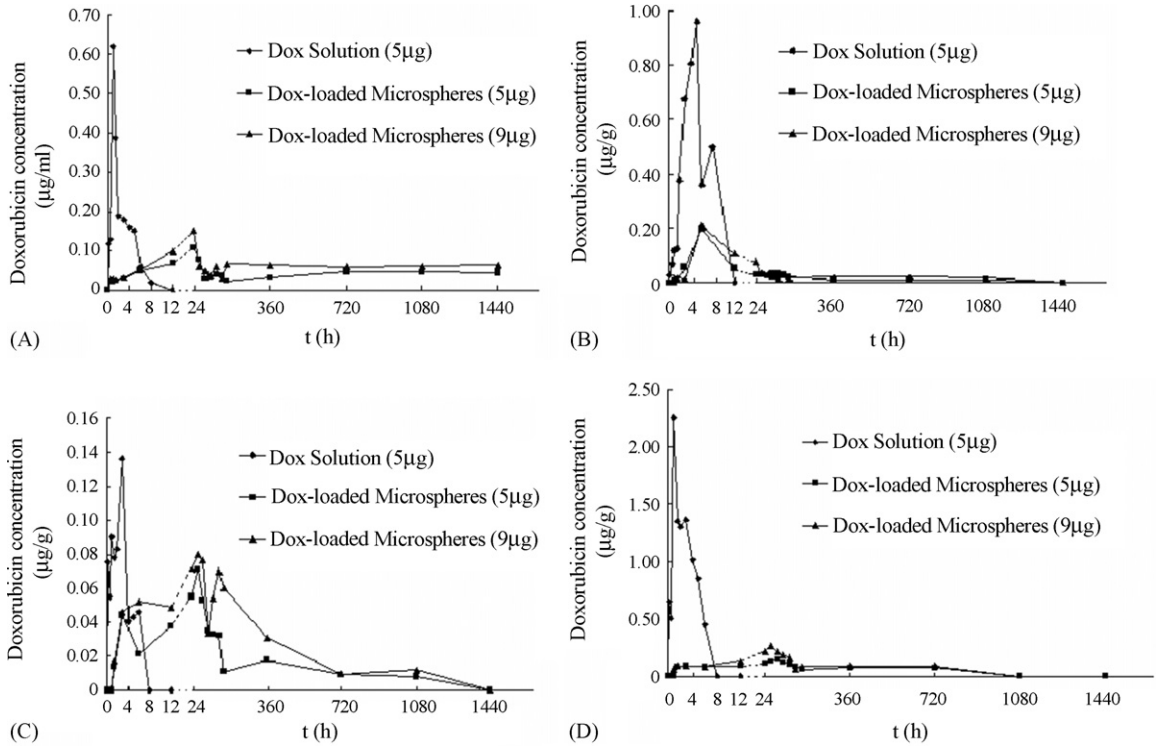

Fig. 3. Doxorubicin concentration vs. time profiles in ocular tissues: (A) vitreous body; (B) iris; (C) sclera; (D) retina/choroids.

microspheres. The result was very encouraging because the Ryan staging of PVR was significantly lower in eyes treated with doxorubicin-loaded microspheres than that treated with solution at the end of 2-month observation (data not shown). However, higher doses of intraocular doxorubicin concentration were associated with local toxicity. So it is important to make sure that the doxorubicin concentrations in ophthalmic tissues is under toxic concentrations. Rong et al. [30] have reported that direct intravitreal injection of doxorubicin at a dose of about 5 μg could result in toxic effect on retina. In our previous study, abnormality in inner layer of retina, especially retinal ganglion cells, was observed at a dose of 5 μg of free doxorubicin. Therefore, in the present study, we applied a dose of 5 μg of free doxorubicin to approximate its toxic levels, and applied the same dose of doxorubicin-loaded microspheres (5 mg/ml, equal to 5 μg of free doxorubicin). A higher dose of 9 mg/ml of microspheres (equal to 9 μg of free doxorubicin) was also applied to see whether the drug concentrations could exceed the therapeutic window when the dose increased. Results showed that intraocular doxorubicin level of sustained release doxorubicin-loaded PHB microspheres

Table 4
Pharmacokinetic parameters in ocular tissues after intravitreal injection of free doxorubicin solution or doxorubicin-loaded poly-β-hydroxybutyrate microspheres

Ocular tissue	C_{max}	t_{max}	AUC_{0-t}	Relative bioavailability[a]
Free doxorubicin solution (5 μg)				
Vitreous body	0.62 μg/ml	1 h	1.24 μg h/ml	
Iris	0.96 μg/g	5 h	4.18 μg h/g	
Retina/choroids	2.26 μg/g	1 h	6.53 μg h/g	
Sclera	0.14 μg/g	3 h	0.43 μg h/g	
Doxorubicin microspheres (5 μg)				
Vitreous body	0.09 μg/ml	1 d	49.18 μg h/ml	39.67
Iris	0.19 μg/g	6 h	16.68 μg h/g	4.00
Retina/choroids	0.15 μg/g	3 d	33.54 μg h/g	5.14
Sclera	0.07 μg/g	2 d	16.55 μg h/g	38.42
Doxorubicin microspheres (9 μg)				
Vitreous body	0.13 μg/ml	1 d	78.67 μg h/ml	35.26
Iris	0.20 μg/g	6 h	28.91 μg h/g	3.85
Retina/choroids	0.33 μg/g	2 d	74.60 μg h/g	6.35
Sclera	0.09 μg/g	2 d	30.47 μg h/g	39.30

[a] Relative bioavailability = $(AUC_{microspheres} \times dose_{solution})/(AUC_{solution} \times dose_{microspheres})$.

was much lower than that of free doxorubicin. It is suggested that intravitreal injection of doxorubicin-loaded microspheres seems to be associated with less toxicity to surrounding ocular tissues like retina, choroids, etc.

4. Conclusion

In the present study, an RP-HPLC method was developed to determine the concentrations of doxorubicin in ocular tissues of rabbits. The method was applied to evaluate the pharmacokinetics and tissue distribution of doxorubicin after intravitreal injection of doxorubicin-loaded PHB microspheres and free doxorubicin solution to rabbits. The result demonstrated that doxorubicin-loaded microspheres could confer a much more longer period of drug release than free drug solution. The study provided sufficient evidence to document the feasibility and safety of doxorubicin-loaded poly-β-hydroxybutyrate microspheres and showed its potential use for intraocular treatment of proliferative vitreoretinopathy.

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