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Short communication

HPLC determination of pirenzepine dihydrochloride in rabbit aqueous humor

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Abstract

Pirenzepine was considered as a pharmacologic agent of preventing form-deprivation myopia. To assess the ocular bioavailability of pirenzepine, a HPLC method for determination of pirenzepine in rabbit aqueous humor was developed. An HPLC system was used in the reverse phase mode for the determination of pirenzepine. A Luna RP18 5 μm 4.6 mm × 150 mm column was employed at 35 °C. The mobile phase was methanol/0.02 M KH_2PO_4/sodium 1-pentanesulfonate (350/650/1, v/v/m, pH was adjusted to 8.0 by dropping 1 M NaOH). The flow rate was 1 ml/min. Pirenzepine was monitored at 280 nm. Sample treatment procedure consists of deproteinisation with methanol. Calibration curves fitted by plotting the peak area versus concentration were linear in the range 20–400 ng/ml. The limit of quantification (LOQ) of present method was 20 ng/ml. Within-day and inter-day coefficient of variation was lower than 10%. Analytical recoveries were determined as 92.4, 95.4 and 101.4% at concentrations of 40, 200 and 400 ng/ml. In conclusion, this HPLC method using a simple sample treatment procedure appears suitable for monitoring ocular concentration of pirenzepine.

Keywords: HPLC; Pirenzepine; Concentration in aqueous humor

1. Introduction

Pirenzepine was proved to be able to reduce the axial elongation and myopia in visually impaired chick eye [1]. Currently, pirenzepine ophthalmic gel is being under investigation for the treatment of myopia [2]. Chemically, pirenzepine dihydrochloride is a hydrophilic compound. The corneal permeation is low [3]. Ostrin et al. [4] in University of Houston reported that subconjunctival injections of 0.02% or greater pirenzepine resulted in a significant decrease in accommodation, while subconjunctival injections of 0.002% or less pirenzepine did not decrease accommodation. The results indicated that ocular bioavailability of pirenzepine is important to prevent form-deprivation myopia. Therefore, a sensitive analytic method for pirenzepine in aqueous humor could be useful for ocular pharmacokinetic studies. All published analytical methods refer to the determination of pirenzepine in dosage form or plasma [5,6]. The subject of this paper is to develop a highly efficient, sensitive, and specific HPLC analytical method for determination of pirenzepine in rabbit aqueous humor with a limit of quantification (LOQ) of 20 ng/ml.

2. Experimental

2.1. Materials

Pirenzepine dihydrochloride (purity >99.5%) was purchased from Qiaoguang Pharmaceutical Co. (Guangzhou, China). Methanol of HPLC grade was obtained from Huaiyin Hangbang Sci-Tech Co. Ltd, China. Sodium 1-pentanesulfonate (HPLC grade) was obtained from Tokyo Kasei Kogyo Co. Ltd, Japan. All other reagents were of analytical grade.

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2.2. Equipment

The HPLC system was consisted of a Waters 515 HPLC Pump Waters 2487 HPLC detector (Waters, USA) equipped with Sanrui Chromatography Workstation (Sanrui Sci-Tech Co., Shanghai, China).

2.3. Chromatographic conditions

A Luna RP18 5 μm 4.6 mm × 150 mm column (Phenomenex Sci-Tech Co. Ltd, USA) and a guard column (Huaiyin Hangbang Sci-Tech Co. Ltd, China.) were employed. The column temperature was kept at 35 °C.

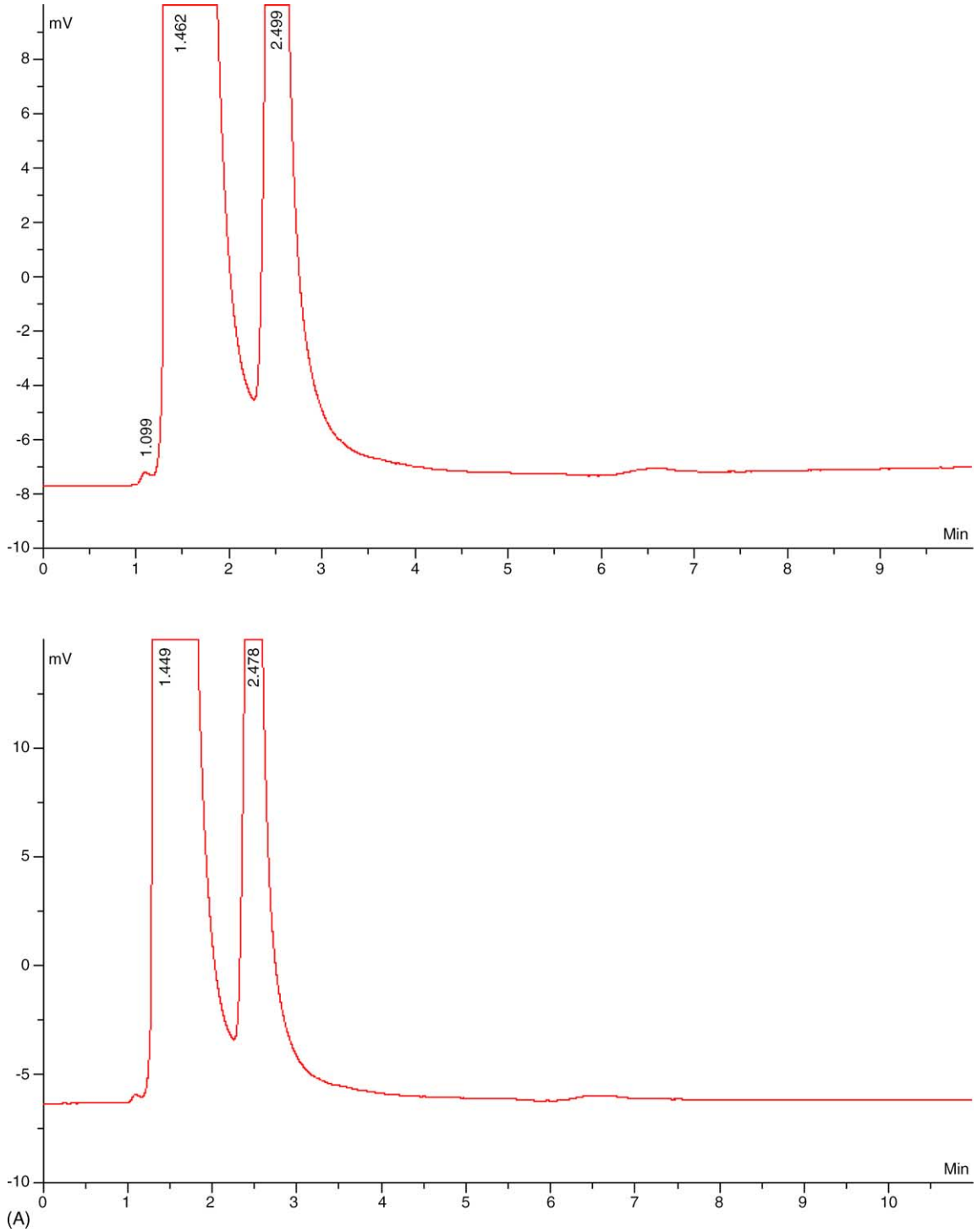

Fig. 1. Representative chromatograms of (A) blank aqueous humor; (B) rabbit aqueous humor spiked with pirenzepine dihydrochloride (20 ng/ml); (C) rabbit aqueous humor sample taken 2 h after dropping 2% pirenzepine ophthalmic gel (about 350 ng/ml).

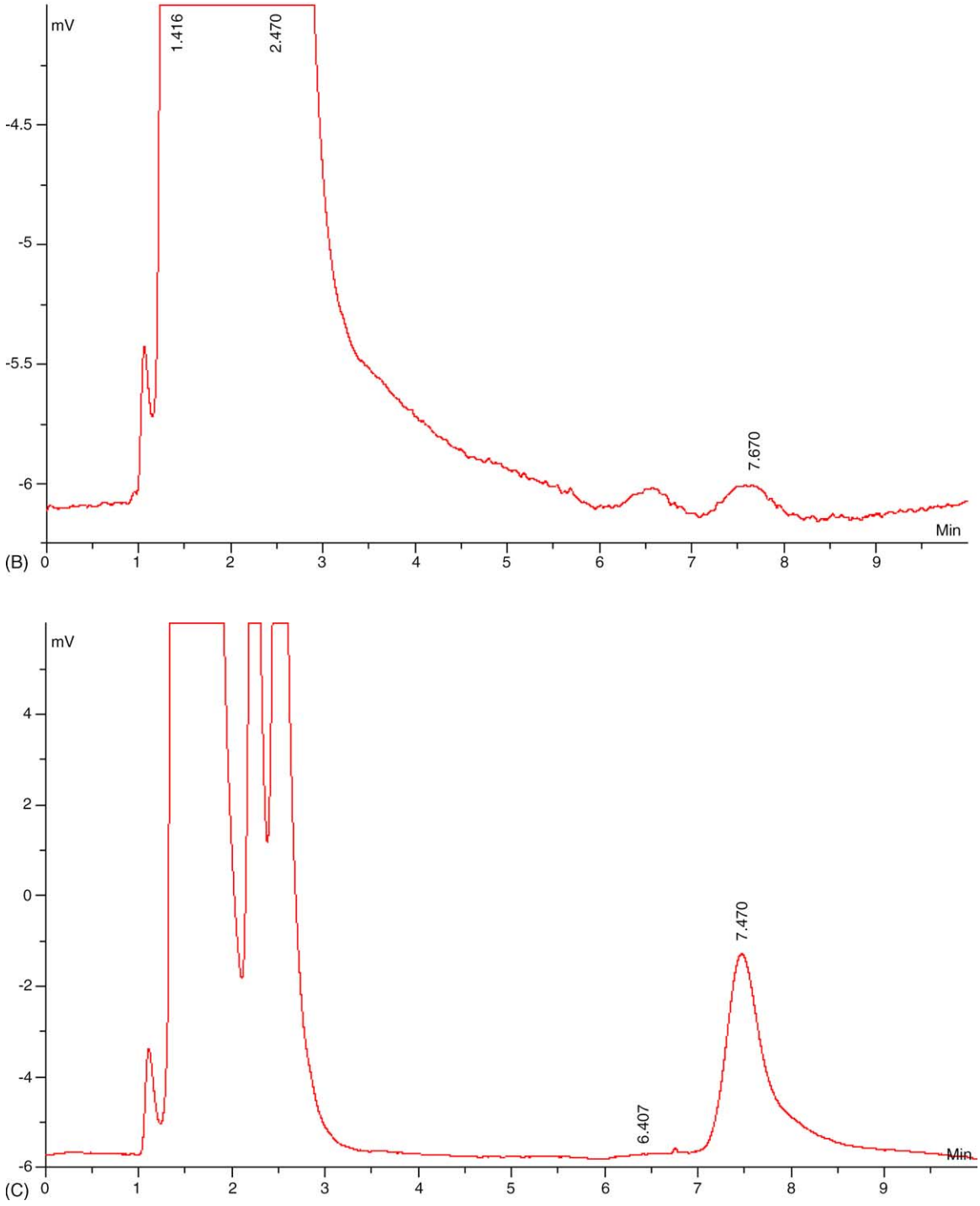

Fig. 1. (*Continued*).

The mobile phase was methanol/0.02 M KH_2PO_4/sodium 1-pentanesulfonate (350/650/1, v/v/m, pH was adjusted to 8.0 by adding 1 M NaOH). The flow rate was 1 ml/min and the UV detector was set at 280 nm. The injected volume was 20 μl.

2.4. Sample preparation

A 100 μl aliquot of rabbit aqueous humor was pipetted into a 1.0 ml Eppendorf tube. A 100 μl aliquot of methanol was added to deproteinization. The sample was mixed vigorously

Table 1
Recovery and accuracy for pirenzepine dihydrochloride ($n = 5$)

Spiked value (ng/ml)	Measured value (ng/ml) (mean ± SD)	Recovery (%)	Relative error (%)
39.95	37.69 ± 0.75	94.4	−5.66
199.75	189.16 ± 2.89	94.7	−5.80
399.5	410.81 ± 14.01	102.8	2.83

Table 2
Precision of pirenzepine in aqueous humor

Spiked value (ng/ml)	Within-day ($n = 5$)		Inter-day ($n = 5$)	
	Measured value (mean ± SD)	RSD (%)	Measured value (mean ± SD)	RSD (%)
20	20.53 ± 1.05	5.20	20.64 ± 0.74	3.58
40	38.89 ± 0.57	1.65	38.47 ± 0.85	2.50
200	201.84 ± 2.89	1.46	195.76 ± 5.65	2.95
400	397.71 ± 3.46	0.93	398.84 ± 2.49	0.63

for 5 min and centrifuged at 5000 × g for 15 min. Twenty microliter of the supernatant were injected into the HPLC system.

2.5. Standard solutions and calibration curve

Aliquots of stock solution of pirenzepine dihydrochloride (800 ng/ml) were transferred into a 1.0 ml ppendorf tube and evaporated to dryness under nitrogen stream. Drug-free rabbit aqueous humor (100 μl) was added and mixed for 5 min. The final concentration of pirenzepine dihydrochloride was 20, 80, 160, 240, 320 and 400 ng/ml. Spiked aqueous humor samples were treated through the assay procedure as stated in Section 2.4 and calibration graphs were obtained by plotting pirenzepine dihydrochloride peak area versus its concentration. Calibration curve was obtained by linear regression analysis.

2.6. Recovery, within-day and inter-day precision

To obtain the recovery and accuracy of pirenzepine dihydrochloride from rabbit aqueous humor, comparison of the spiked value (ng/ml) with the measured value at 40, 200, 400 ng/ml ($n = 5$ for each concentration of pirenzepine used) (Table 1) was conducted.

The within-day variability and the inter-day variability of the developed HPLC method were measured at four different concentrations of pirenzepine (20, 40, 200, 400 ng/ml). Relative standard deviation (RSD) was taken as a measure of precision (Table 2).

3. Results and discussions

Chromatograms of a blank aqueous humor sample, an aqueous humor sample spiked with pirenzepine dihydrochloride (20 ng/ml) and a rabbit aqueous humor sample taken at 2 h after dropping 2% pirenzepine ophthalmic gel are shown in Fig. 1. A sharp peak of pirenzepine was observed under the chromatographic conditions described in this paper. The retention time of pirenzepine was determined as 7.5 min. No interfering peaks were observed in the blank aqueous humor chromatogram, indicating the deproteinisation method is effective. The limit of quantification was determined as 20 ng/ml.

Good linearity between concentration of pirenzepine dihydrochloride (C, ng/ml) in aqueous humor and peak area (A) was obtained for pirenzepine dihydrochloride ($C = 0.0064A + 5.5767$, $r = 0.9991$, $n = 7$) in the range from 20.0 to 400.0 ng/ml.

Table 1 listed the recovery and accuracy data of pirenzepine under present HPLC condition. High recovery data were achieved at 40, 200, 400 ng/ml.

Table 2 showed both the within-day precision, with RSD ranged from 0.93 to 5.20%, and the inter-day precision, with RSD ranged from 0.63 to 3.58%, indicating the very good reproducibility of this method. Both within-day and intra-day RSDs at the concentration of 20 ng/ml were lower than 10%. As shown in Fig. 1C, pirenzepine concentration in rabbit aqueous humor at 2 h after dropping 2% pirenzepine ophthalmic gel was determined as 350 ng/ml. The results indicated that the HPLC method developed in this paper might be sensitive enough for determination of pirenzepine in aqueous humor.

4. Conclusions

As a conclusion, the HPLC method developed in this paper is rapid, sensitive, reproducible and well suited to monitor the concentration of pirenzepine in aqueous humor during ocular pharmacokinetic studies of pirenzepine.

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