Measurement of cyclosporine A in aqueous humor of rabbit eyes by HPLC

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ABSTRACT: OBJECTIVE To develop a simple and reproducible high-performance liquid chromatography (HPLC) method for determination of cyclosporine A (CsA, also known as cyclosporin A) in aqueous humor of rabbit eyes using CsD as internal standard. **METHODS** The pretreatment involved one step of liquid-liquid extraction with ether from aqueous humor samples. Chromatography was carried out using an ODS column which was controlled at 75℃ under isocratic elution with the mobile phase of acetonitrile-deionized water (89:11, v/v) at the flow rate of 0.1 mL·min^{-1} and the detector set at 200 nm. **RESULTS** The mean absolute recovery of CsA from aqueous humor was 86% and the mean relative recovery was 100.7%. The linearity was assessed in the range of 25 ~ 500 ng·mL^{-1}, with a correlation coefficient of 0.9986. The limit of quantification and detection of the present method were 25 and 10 ng·mL^{-1}, respectively. **CONCLUSION** This method has been used to analyze large numbers of aqueous humor samples of rabbit eyes for ophthalmic research and proved to be rapid with good accuracy and precision.

KEY WORDS: cyclosporine A; HPLC; aqueous humor of rabbit eyes

反相高效液相色谱法测定兔眼房水环孢素 A

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摘要:目的 以环孢素 D为内标物,建立 RP-HPLC法测定兔眼房水环孢素 A(CsA)药物浓度。方法 兔眼房水标本经乙醚单步提取挥干后,色谱过程使用 ODS柱,乙腈:水(89:11)为流动相,流速:0.1 mL·min^{-1},75℃柱温下进行色谱分析,紫外检测波长为 200nm。结果 方法的绝对回收率平均为 86%,相对回收率平均为 100.7%,CsA兔眼房水浓度在 25 ~500ng·mL^{-1}范围内呈良好的线性关系,r=0.9986,最低定量限与最低检测限分别为 25ng·mL^{-1},10ng·mL^{-1}。结论 眼科药物研究过程中大量房水标本检测实践表明,本方法过程简便、快速、结果准确,精密度符合方法学要求。

关键词:环孢素 A;反相高效液相色谱法;兔眼房水
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1 Introduction

Cyclosporine is a cyclic undecapeptide consisting of 11 amino acids. It is an important immunosuppressive drug used commonly to reduce tissue rejection after organ transplantation. Topical preparation of CsA drug delivery system vehicled by biodegradable PLGA or ophthalmic solution can be used to prevent rejection of penetrating keratoplasty (PKP)[1,2]. Methodological approaches to measuring CsA include high-performance liquid chromatography (HPLC) and a variety of immunoassay-based methods. Although immunoassay-based methods are simple to use, the cross-reactivity of the antibody with the metabolites leads to overestimation of the parent CsA concentrations in samples by these methods. The great different amount of protein in blood and aqueous humor make them not appropriate for determination of CsA in aqueous humor because of the different absorption[3~5].

During the past two decades, many attempts have been made to improve HPLC method for CsA analysis in blood, resulting in numerous published methods most of which involve the use of solid-phase extraction (SPE) procedure or liquid liquid extraction followed by reconstitution with n-hexane using Cyclosporin D (CsD) as internal standard, which is time-consuming[6,7]. What's more, the described chromatographic operation can only offer detection limit of 50 ng·mL^{-1} from 1 to 2mL matrix which can not be followed to determine the small-volumed specimen with low CsA concentration such as aqueous humor with volume of 0.2mL.

The present study aims to modify the reported method for CsA assay in aqueous humor. In this paper, we report a simple and reproducible HPLC method using simple pretreatment proce-

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dure and demonstrate applicability of the method.

2 Materials and method

2.1 Reagents

All chemicals were reagent or HPLC grade. Acetonitrile, methanol, were obtained from Merck, Germany. Both CsA and CsD standard products were provided by National Institute for Control of Pharmaceutical and Biological Products. Deionized water was obtained by using USF-ELGA purification system.

2.2 Instrumentation

Analysis was performed using a Agilent 1100 HPLC system (Agilent, USA) consisting of a on-line degasser, a quaternary pump, a autoinjector, a column oven, a VWD detector and a chemstation. A stablebond C_{18} reversed-phase column (80Å, 5μm, 2.1mm i.d., dpl 50mm ZOBARX, Agilent, USA) was used for chromatographic separation. The detector was set to 200 nm. The mobile phase comprised of acetonitrile-deionized water (89∶11). Analyses were run at flow rate of 0.1mL·min^{-1} at 75℃ for 10min. The samples were quantified using peak height ratio of CsA and CsD.

2.3 Standard solutions

The stock standard solutions of CsA and CsD (1mg·mL^{-1}) were prepared by dissolving the powder in methanol respectively. For constructing calibration curves and for assay validation covering the range, both the working standard solutions of 2.5μg·mL^{-1} for CsA and 25μg·mL^{-1} for CsD were obtained by diluting stock solutions.

2.4 Sample preparation

To a 200μL of aqueous humor in conical test tube, 2μL working standard solution of CsD and 1mL ether were added successively. The tube was vortex-mixed for 60s and centrifuged for 2 min at 3000rpm. The supernatant layer was transferred to another clean tube and evaporated in water bath at 60℃. After reconstitution with 20μL mobile phase, 10μL volume was injected into the chromatograph.

3 Results and conclusion

3.1 Chromatographic behavior

The selectivity of the HPLC method was checked by analyzing different independent blank aqueous humor samples from rabbit eyes respectively. Representative chromatograms showed in Fig 1 indicate that no endogeneous interfering peaks at the retention time of CsA were found.

3.2 Linearity

The linearity was evaluated with six blank aqueous humor spiked with various amounts of working standard solution containing 25, 50, 100, 200, 400, and 500 ng·mL^{-1} of CsA. For each concentration three measurements were performed and

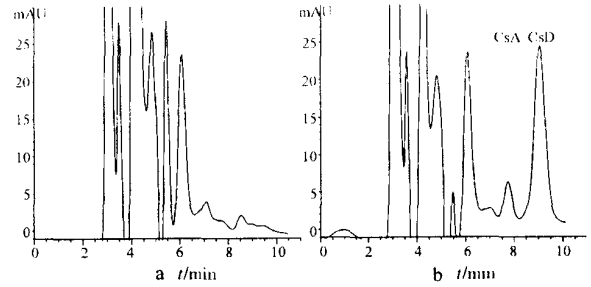

Fig 1 Chromatograms of blank aqueous humor (a) and controlled sample (b) of rabbit eyes

图 1 房水中环孢素 A的 HPLC图

a.空白房水色谱图 b.样品色谱图

calibration curves based on the peak height ratio of CsA and CsD versus nominal concentrations of CsA expressed in ng·mL^{-1} were constructed. The equation was $y = 0.694391x$ with the correlation coefficient of 0.9986 and was calculated by least-square linear regression analysis packed in the chemstation.

3.3 Accuracy and recovery

In the accuracy evaluation, three working standard solutions in aqueous humor covering the calibration range and containing 25, 100 and 500 ng·mL^{-1} of CsA were prepared as described in the pretreatment section previously. Five consecutive measurements were performed for each concentration within the same day. Relative recovery was calculated by comparing the results from the standard curve above and the theoretical concentrations. Extraction recovery was calculated by comparing the heights of CsA from aqueous humor spiked with working standard solution and those resulting from the direct sampling of working standard solutions prepared in mobile phase having the same theoretical concentrations of CsA considering the folding effect and complete recovery of the pretreatment. The individual and the overall mean percent recovery were calculated in Table 1 and a RSD value of maximum 15% was set as the acceptance criteria.

Tab 1 Recovery of the method for analysis of CsA in aqueous humor

表 1 眼前房水 CsA回收率 ($n = 5$)

Concentration	Relative recovery		Absolute recovery	
(ng·mL^{-1})	recovery* (%)	RSD (%)	recovery* (%)	RSD (%)
25	104.4±10.1	9.7	86±7.2	8.4
100	99.4±6.1	6.1	88±6.8	7.7
500	98.46±4.36	4.4	83±7.3	8.8

Note: * calculated from peak height ratio (CsA∶CsD) and expressed as recovery in percent, $n = 5$, $\bar{x} \pm s$.

注: * 以峰高比计算 (CsA∶CsD),以百分数表达, $n = 5$, $\bar{x} \pm s$.

3.4 Reproducibility

The within-day and day-to-day analytical reproducibility were determined by analyzing three different working standard solutions in aqueous humor having 25, 100 and 500 ng·mL^{-1} of

CsA showed in Table 2. Five consecutive measurements were performed for each concentration within the same day and on five different days. The acceptance criteria for within-day and day-to-day R. S. D. values were not more than 15%.

3.5 Sensitivity

The sensitivity in terms of detection and quantification limits were determined by using series of different working standard solutions in aqueous humor varying from 5 to 30 ng·mL^{-1} of CsA, which were prepared as described in the standard solutions and pretreatment sections. The concentration measured with suitable accuracy and precision was accepted as the limit of quantification. The limit of detection at a signal-to-noise ratio of about 3∶1 was regarded as the lowest detectable amount which was 10 ng·mL^{-1}.

Tab 2 Within-day and between-day reproducibility of the method for analysis of CsA in aqueous humor

表 2 精密度测定结果 ($n=5$)

Concentration (ng·mL^{-1})	added	Within-day		Between-day	
		found* (%)	RSD (%)	found* (%)	RSD (%)
25	25	26.1 ±2.52	9.7	26.7 ±3.56	13.5
100	100	100.2 ±6.93	6.9	96.8 ±9.97	10.3
500	500	506.5 ±21.6	4.3	504.7 ±29.1	5.8

Note: * Calculated from peak height ratio (CsA∶CsD) and expressed as found amount in percent, $n=5$, $\bar{x}\pm s$.

注: * 以峰高比计算 (CsA∶CsD),以百分数表达, $n=5$, $\bar{x}\pm s$.

4 Discussion

Most of the HPLC methods[3~7] use extensive sample clean-up procedure following deproteinization that complicates HPLC assay of CsA in the whole blood. This may explain why most of the laboratories prefer to use immunoassay methods. In the present study, it is demonstrated that CsA in aqueous humor could be analyzed by HPLC following only one-step extraction.

Commercially available HPLC columns show great differences in their chromatographic behavior. Columns with different packing materials such as C18, C8, and CN were tested and all were found suitable according to the refference[8]. Columns like CN should be operated at 60℃ with the mobile phase of 50% acetonitrile, while 70℃ and 75% acetonitrile should be used for C18 or C8 columns. To improve the sensitivity, analytical narrow-bore column of C18 controlled at 75℃ was applied in this study with better performance. The detective wave length of 200 nm was used in this study which was different from reference but showed strongest absorption. The reduction of flow rate from usual 1 mL·min^{-1} to 0.1 mL·min^{-1} causes the probability of improving sensitivity greatly which makes it possible to measure CsA in aqueous humor.

The storage condition is usually considered important when validating a method. As is reported that freezing of blood samples increases interferences dramatically and should be avoided. Several studies show that CsA is stable in blood samples for 2 ~ 3 months when stored at 1 ~ 4℃[8]. In this study, even though stored up to more than 3 months at 4℃ or below 0℃, the number and intensity of interfering peaks were not increased and the CsA was still well separated in aqueous humor samples. Representative chromatograms obtained with CsA are shown in Fig. 1. The CsA peak, which had a retention time of 7.8 min, was well resolved and free of interference from endogenous compounds in the aqueous humor. One fact should be noted that polypropylene tubes can not be used for pretreatment to avoid interfering additives which have much similar chromatographic behavior as CsA in them.

In conclusion, the HPLC method described here is simple, sensitive, reproducible and is applicable to pharmacokinetic studies of CsA in aqueous humor of rabbit eyes during topical therapy.

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