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Article

Fast and simultaneous analysis of two immunosuppressants in whole blood using high-performance liquid chromatography

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Abstract: Cyclosporine A and sirolimus are commonly used immunosuppressants in organ transplantation, known for their complementary and synergistic mechanisms of action. However, due to their narrow therapeutic index and critical dosing requirements, maintaining their concentrations within a specific blood range is essential for optimal efficacy and safety. This study systematically compared the chromatographic behaviors of cyclosporine A and sirolimus using a biological liquid chromatography (BioLC) column and traditional liquid chromatography (TraLC) columns under identical conditions. The results indicated that the BioLC column, specifically ZORBAX 300SB C8 (250 mm × 4.6 mm, 5.0 μm), provided the highest peak heights and the narrowest peak widths for both drugs. The number of theoretical plates for both drugs significantly increased on this column when acetonitrile in the mobile phase exceeded 70%. Retention times on both column types were minimally affected by formic acid and trifluoroacetic acid in the mobile phases. Additionally, the ZORBAX 300SB C8 column exhibited a higher number of theoretical plates and a distinct relationship between retention factor and column temperature compared to the TraLC columns. For analysis, a 50 μL whole blood sample was prepared through protein precipitation with 1 mol/L sodium hydroxide, extracted into 500 μL ether-methanol (95:5, v/v), and then centrifuged. The organic layer was evaporated under nitrogen at 50 ℃, and the residue was reconstituted in 200 μL methanol. Cyclosporine A and sirolimus were then separated via isocratic elution on the ZORBAX 300SB C8 column.

Keywords: high performance liquid chromatography (HPLC); immunosuppressant; cyclosporine A; sirolimus; biological liquid chromatography column

1. Introduction

As immunosuppressants, cyclosporine A and sirolimus (see **Figure 1** for chemical structure) have been successfully used in many organ transplantation operations [1–3]. The therapeutic window of these two immunosuppressants is very narrow, and they only have the expected therapeutic effect within a specific blood concentration range. When the blood concentration is low, there is a risk of organ rejection, while when the blood concentration is high, there may be serious side effects such as nephrotoxicity, cardiotoxicity, and neurotoxicity [4]. On the other hand, when these two immunosuppressants are used together, they will produce synergistic effects, and they are usually used together in clinical practice [5]. The therapeutic range of immunosuppressants is affected by many factors, such as transplant organs, patient age, etc. It is reported that the therapeutic range of cyclosporine A is 50–300 ng/mL [6], and the therapeutic range of sirolimus is 12–30 ng/mL. When combined with cyclosporine A, the therapeutic range is 4–12 ng/mL [5]. Therefore, monitoring the blood concentration of these two immunosuppressants

at the same time plays an important role in the drug delivery program after organ transplantation.

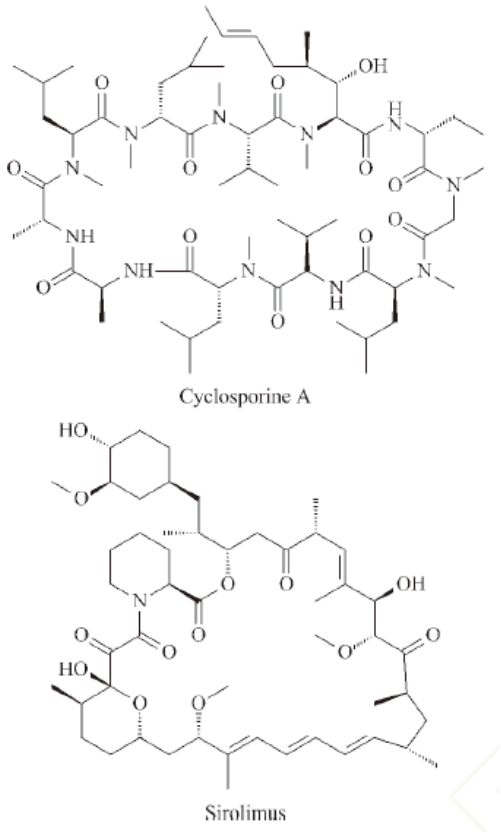

Figure 1. Chemical structures of immunosuppressants.

The methods reported in the literature for quantitative analysis of immunosuppressants such as cyclosporine A, sirolimus and tacrolimus mainly include immunoassay [7], high performance liquid chromatography (HPLC) [8] and high performance liquid chromatography tandem mass spectrometry (HPLC) [9–12]. The operation of immunoassay is simple, but the antibody may cross react with the inactive product of immunosuppressant metabolism, thus overestimating the drug level [13]. HPLC is also commonly used for quantitative analysis of single or multiple immunosuppressants in whole blood, which usually requires a long chromatographic run time to separate multiple targets and reduce matrix interference [14]; recently, a high performance liquid chromatography method based on intelligent chemometrics was reported, which realized rapid quantitative analysis of three immunosuppressants in whole blood by processing the ultraviolet spectrum [15]. HPLC-MS/MS is mainly used to detect the content of immunosuppressive agents in animal viscera, blood, urine and other biological samples with unique selectivity and sensitivity, but this method is also limited by such factors as matrix effect, high instrument cost, and the need for.

Because the conformational isomer of cyclosporine A has a certain resolution (*R*) on the traditional reversed-phase C8 or C18 column, it has an obvious chromatographic peak broadening effect [16].

In order to achieve better separation effect and higher detection sensitivity,

cyano column [17] or phenyl cyclohexyl column [10,18] become a better choice for separating and detecting these immunosuppressants; in addition, the rapid separation and determination of cyclosporin A and sirolimus in whole blood by HPLC are rarely reported. With the development of chromatographic column technology, biological liquid chromatographic columns for proteins, peptides and other macromolecules have been continuously developed. This study investigated the chromatographic behavior of cyclosporine A and sirolimus on biological liquid chromatographic columns and traditional liquid chromatographic columns, and established an HPLC analysis method based on biological liquid chromatographic columns that can quickly separate and detect cyclosporine A and sirolimus in whole blood.

2. Experiment

2.1. Instruments, reagents and materials

Agilent 1260 Infinity II high performance liquid chromatograph (equipped with diode array detector (DAD)); XP105 balance (Shanghai Mettler Toledo Company).

Acetonitrile and methanol (HPLC grade, Merck KGaA, Germany); Formic acid, trifluoroacetic acid, ether and sodium hydroxide (analytically pure, Shanghai MacklinBiochemical Company); Cyclosporine A and sirolimus (purity ≥ 98%, Nanjing Dulai Biotechnology Co., Ltd.). The experimental water is ultrapure water.

2.2. Preparation of standard solution

Standard stock solution: Accurately weigh appropriate amount of cyclosporine A and sirolimus, dissolve them in methanol and make them into 1000 μg/mL and 500 μg/mL standard stock solution, stored at 4 ℃ away from light.

Mixed standard stock solution: take appropriate amount of each standard stock solution, dilute it with methanol to obtain mixed standard stock solution, and prepare it immediately.

Matrix standard working solution: use the blank matrix after sample pretreatment to dilute and mix the standard stock solution step by step to prepare the matrix standard working solution, which is ready for use.

2.3. Sample pretreatment method

Accurately measure 50 μL whole blood sample, add 100 μL sodium hydroxide aqueous solution (1 mol/L) and shake for 30 s, then add 500 μL ether methanol (95:5, v/v) and shake for 30 s, then centrifugate at 14,000 r/min for 10 min, take the organic layer solution and blow it dry with nitrogen at 50 ℃, finally add 200 μL Dissolve in methanol for later use.

2.4. Liquid chromatographic conditions

Chromatographic column: ZORBAX300SBC8 chromatographic column (250 mm × 4.6 mm, 50 μm. Agilent, USA); Column temperature: 60 ℃; Mobile phase: acetonitrile water (70:30, v/v); Flow rate 10 mL/min; Detection wavelength: 205 nm (cyclosporine A) and 278nm (sirolimus); Injection volume 20 μL.

3. Results and discussion

3.1. Effect of chromatographic conditions on chromatographic behavior of immunosuppressants

In the experiment, keeping the same concentration of cyclosporine A and sirolimus solution, under the conditions of flow rate of 1 mL/min, injection volume of 20 μL and detection wavelength of 205 nm and 278 nm, respectively, the composition of chromatographic column and mobile phase were investigated. The effects of mobile phase additives and column temperature on the chromatographic behavior of two immunosuppressants.

Chromatographic column:

Under the condition that the mobile phase is acetonitrile water (80:20, v/v) and the column temperature is 50 ℃, the chromatographic retention behavior of cyclosporine A and sirolimus on six Agilent reverse phase chromatographic columns (see **Table 1** for basic column information) was investigated. The chromatogram and half peak width values are shown in **Figure 2**. The results showed that the retention behaviors of cyclosporine A and sirolimus on different chromatographic columns were significantly different, especially the peak broadening effect of cyclosporine A on different chromatographic columns; Compared with the traditional liquid chromatographic column, the two immunosuppressants have the highest chromatographic peak and the smallest half peak width on ZORBAX300SBC8 biological liquid chromatographic column. In particular, the chromatographic peak height and half peak width of cyclosporine A on this column are 25–75 times and 013–039 times of those on the other five chromatographic columns, respectively. It can be seen that the detection sensitivity of cyclosporine A and sirolimus can be significantly improved by using a biological liquid chromatographic column. This may be related to the pore size of the chromatographic column. ZORBAX 300SB C8 column has the largest pore size, followed by InfinityLab Poroshell 120 EC C8 column, and ZORBAX Eclipse XDB C18 column is the smallest. The change trend of the chromatographic peak height and half peak width of the two immunosuppressants is also basically positively and negatively related to the change of the pore size of the chromatographic column.

Table 1. Basic information on the employed chromatography columns.

No.	Column style	Model	Specification	Maximum operating temperature/*t*	Status
1	Traditional	ZORBAX Eclipse XDB C18	250 mm × 4.6 mm, 5.0 pm, 8 nm	60	Used
2	Traditional	ZORBAX Eclipse Plus C18	250 mm × 4.6 mm, 5.0 pm, 9.5 nm	60	Used
3	Traditional	ZORBAX Eclipse Plus C8	250 mm × 4.6 mm, 5.0 pm, 9.5 nm	60	New
4	Traditional	ZORBAX Eclipse PAH	250 mm × 4.6 mm, 5.0 pm, 9.5 nm	60	New
5	Traditional	InfinityLab Poroshell 120 EC C8	150 mm × 4.6 mm, 2.7 pm, 12 nm	60	New
6	Biological	ZORBAX 300SB C8	250 mm × 4.6 mm, 5.0 pm, 30 nm	80	New

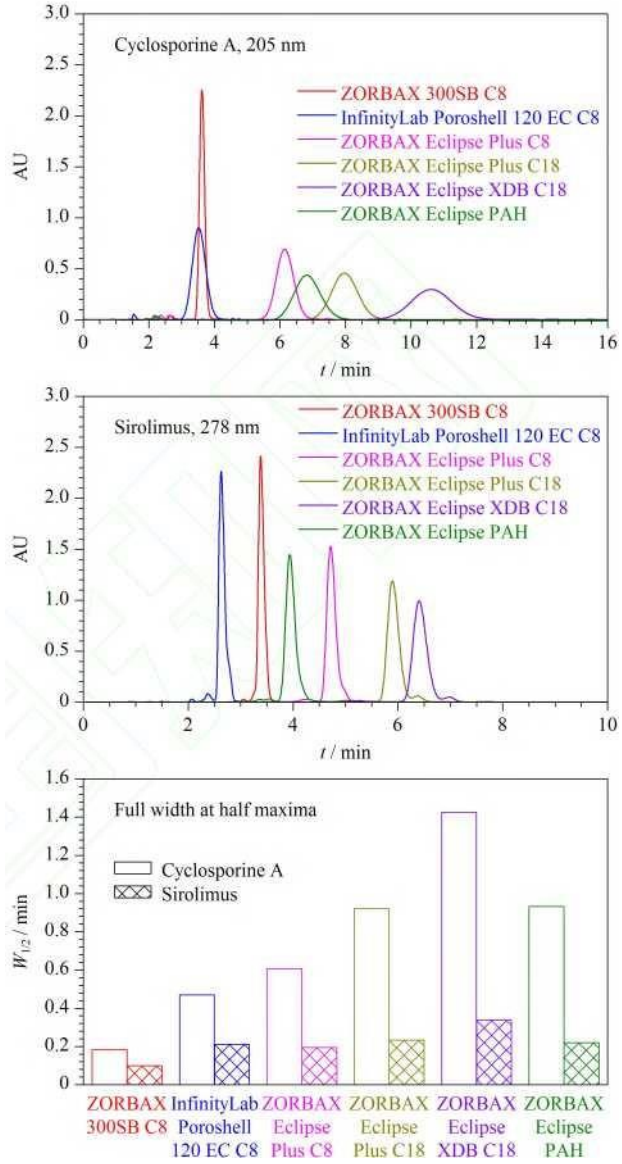

Figure 2. Chromatograms and full width at half maxima of cyclosporine A and sirolimus on different chromatography columns.

Mobile phase composition:

At the column temperature of 50 ℃, the influence of acetonitrile water mobile phase system on the chromatographic behavior of cyclosporine A and sirolimus on three chromatographic columns was investigated. The relationship between mobile phase composition and retention factor (k) and theoretical plate number (N) is shown in **Figure 3**. The results showed that the retention time (t_R) of cyclosporine A and sirolimus increased exponentially with the decrease of acetonitrile volume fraction in mobile phase on the three chromatographic columns; When the volume fraction of acetonitrile in the mobile phase is more than 70%, the theoretical plates of cyclosporine A and sirolimus on ZORBAX 300SB C8 column increase significantly with the increase of the volume fraction of acetonitrile, and are significantly higher than the theoretical plates on the other two chromatographic columns.

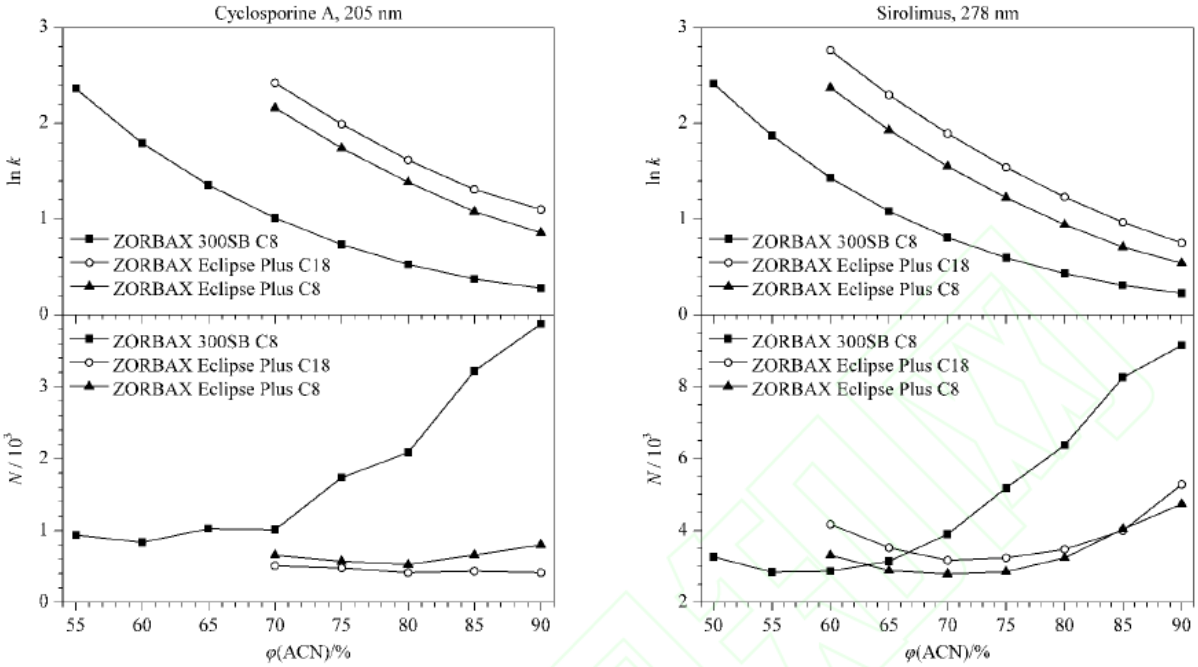

Figure 3. Chromatographic peak parameters of cyclosporine A and sirolimus in ACN-water mobile phases.

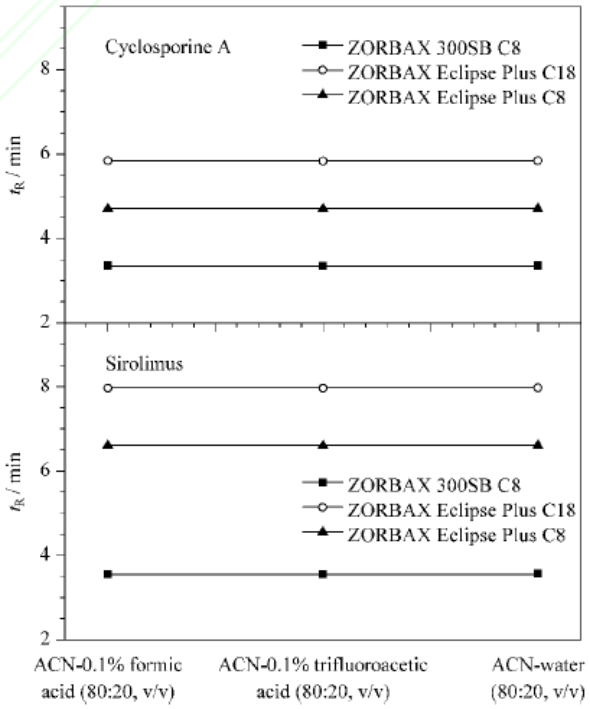

Figure 4. Retention of cyclosporine A and sirolimus in different mobile phases.

Mobile phase additive:

Under the column temperature of 50 ℃, the influence of trifluoroacetic acid and formic acid on the retention time of cyclosporine A and sirolimus was investigated by adding two ion pair reagents to the mobile phase. The results are shown in **Figure 4**. The results showed that two mobile phase additives had little effect on the retention time of cyclosporine A and sirolimus on three chromatographic columns. This is because there are no free amino and carboxyl groups in the chemical structure

of cyclosporine A and sirolimus, so acid ions have no significant effect on the chromatographic retention behavior of the reagents.

Column temperature:

Under the condition that the mobile phase is acetonitrile water (80:20, v/v), the influence of column temperature on the chromatographic behavior.

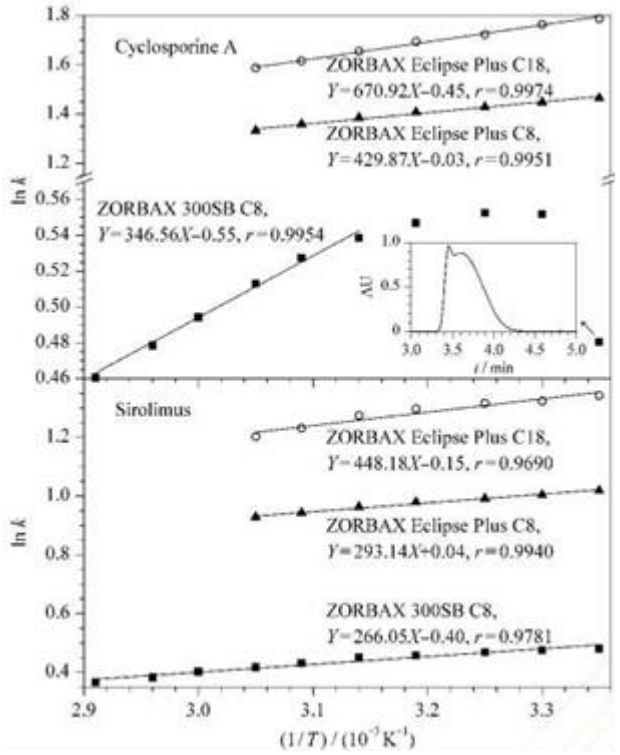

Figure 5. Relationships between retention factor and column temperature of cyclosporine A and sirolimus on different chromatography columns.

of cyclosporine A and sirolimus was investigated using Van't Hoff equation [19–21]. The relationship between column temperature and retention factor (k) and theoretical plate number (*N*) is shown in **Figures 5** and **6**. The results showed that the retention factors of cyclosporine A and sirolimus basically decreased with the increase of column temperature on the three chromatographic columns. In the range of experimental column temperature, the linear relationship between the ln*k* of sirolimus and the reciprocal (1/*T*)of column temperature on the three chromatographic columns is good ($r > 0.96$), and the linear relationship between cyclosporine A and ZORBAX Eclipse Plus C18 and ZORBAX Eclipse Plus C8 columns is good ($r > 0.99$); When the column temperature is 45–70 ℃ ($1/T$: 3.14×10^{-3}–2.91×10^{-3} K^{-1}), the linear relationship of cyclosporine A on ZORBAX 300SB C8 column is good ($r > 0.99$), while the column temperature is 25–40 ℃ (3.35×10^{-3}–3.19×10^{-3} K^{-1}), and the column temperature is 25 ℃ (3.35×10^{-3} K^{-1}); In the range of experimental column temperature, the theoretical plates of cyclosporine A and sirolimus in ZORBAX Eclipse Plus C18 and ZORBAX Eclipse Plus C8 columns are similar, while the theoretical plates of ZORBAX 300SB C8 columns are significantly higher than those of the other two columns. According to the theory [16], increasing the column temperature can reduce the conformational separation

and chromatographic peak width of immunosuppressants, thereby improving the column efficiency, and increasing the column temperature can reduce the viscosity of mobile phase, which is conducive to reducing the mass transfer resistance of mobile phase and further improving the column efficiency; In addition, ZORBAX 300SB C8 column has larger pore diameter, less mass transfer resistance of stationary phase and higher column efficiency. Therefore, at higher column temperature, the column efficiency and detection sensitivity of cyclosporine A and sirolimus on ZORBAX 300SB C8 column will be significantly better than those of the other two columns.

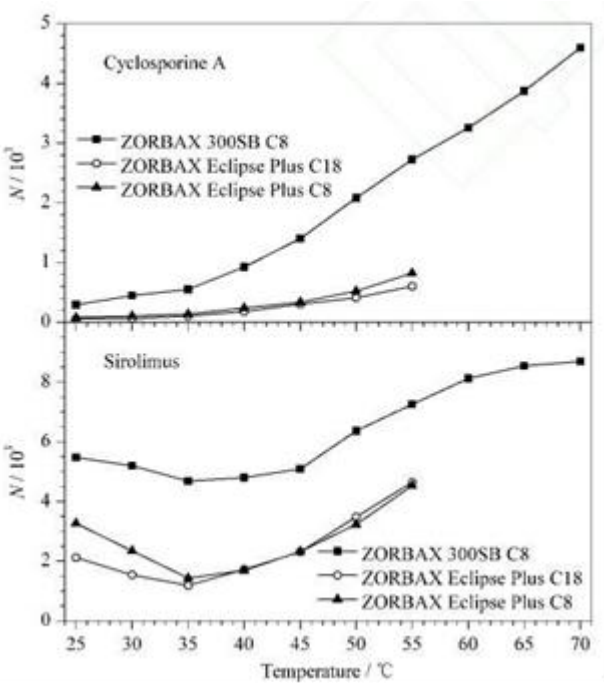

Figure 6. Relationships between the column temperature and chromatographic efficiency of cyclosporine A and sirolimus on different chromatography columns.

3.2. Optimization of liquid chromatography conditions

The influence of biological liquid chromatographic column and traditional liquid chromatographic column on the chromatographic behavior of two immunosuppressants shows that biological liquid chromatographic column has significant advantages in the detection of cyclosporine A and sirolimus. Therefore, the chromatographic conditions for the separation of two immunosuppressants were optimized on ZORBAX 300SB C8 column. The resolution results under different chromatographic conditions are shown in **Figure 7**. The results showed that the resolution increased with the decrease of acetonitrile volume fraction in mobile phase. When the acetonitrile volume fraction was less than 80%, the chromatographic peaks of cyclosporine A and sirolimus could be completely separated ($R \geq 15$); The resolution increased with the increase of column temperature. When the column temperature was higher than 50 ℃, the chromatographic peaks of cyclosporine A and sirolimus could be completely separated. Therefore, the complete separation of the two immunosuppressants can be achieved by reducing the volume fraction of acetonitrile in the mobile phase or/and

raising the column temperature.

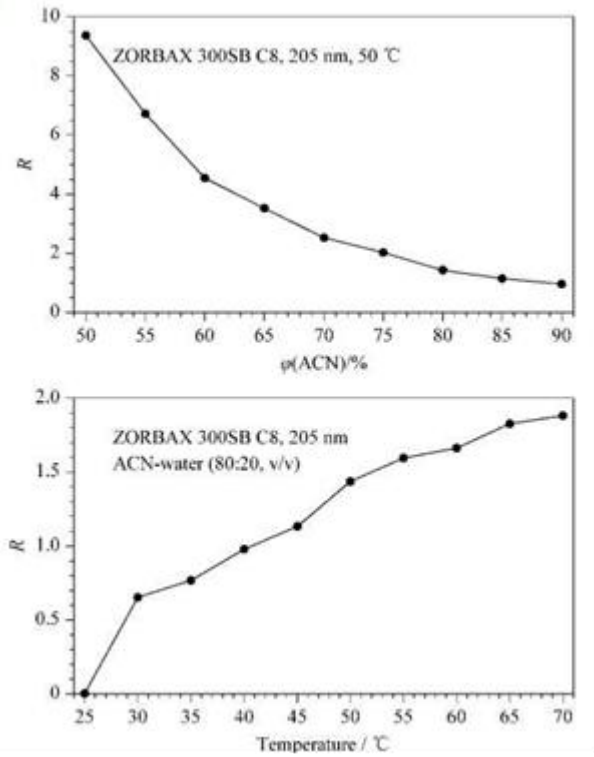

Figure 7. Resolutions of cyclosporine A and sirolimus under different chromatography conditions.

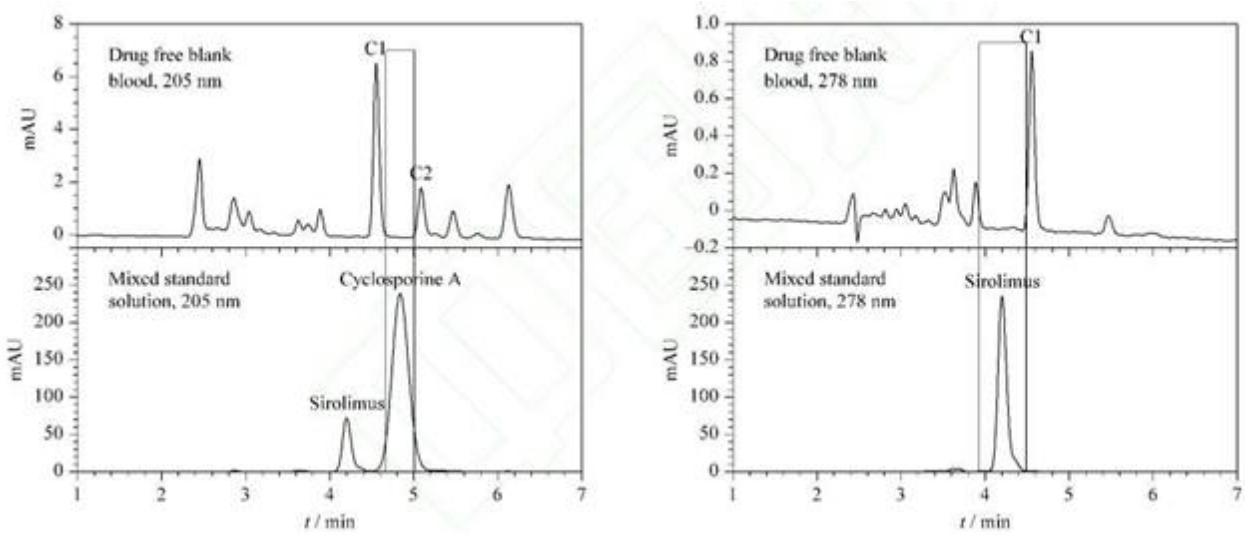

Figure 8. Chromatograms of a blank sample of whole blood and a mixed standard solution of cyclosporine A and sirolimus.

Because the whole blood sample contains a large amount of protein and polypeptide, even after pretreatment, it may still interfere with the detection of these two immunosuppressants. Therefore, the matrix effect of blank whole blood was further investigated. The blank whole blood and mixed standard stock solution after 13 sections of pretreatment were detected using the liquid chromatographic conditions in Section 2.4. See **Figure 8** for the chromatogram. The results showed

that there were still endogenous substances C1 and C2 in the blank whole blood after pretreatment, which might interfere with the detection of these two immunosuppressants; When the mobile phase is acetonitrile water (70:30, v/v) and the column temperature is 60 ℃, C1 and C2 have little interference with cyclosporine A and almost no interference with sirolimus, and cyclosporine A and sirolimus can be completely separated within 6 min (R = 3.7, 205 nm). Therefore, taking into account the separation degree, detection sensitivity and matrix effect of whole blood samples, the HPLC analysis conditions for rapid separation and detection of two immunosuppressants in whole blood established in this paper are detailed in Section 2.4.

3.3. Linear range, detection limit and quantification limit of the method

The matrix standard working solution prepared in Section 12 was determined by using the liquid chromatography conditions in Section 14. The linear regression was performed with the peak area (Y) of the analyte and the corresponding mass concentration (X, ng/mL) to obtain the linear equation and correlation coefficient of cyclosporine A and sirolimus. The detection limit and quantitative limit of the method were determined with the concentration of $S/N \geq 3$ and 10. See **Table 2** for the results. The results showed that cyclosporine A and sirolimus had a good linear relationship within a certain range, the correlation coefficients were greater than 0.997, the detection limits were 10 ng/mL and 1ng/mL, respectively, and the quantification limits were 30 ng/mL and 2 ng/mL, respectively.

Table 2. Linear ranges, linear equations, correlation coefficients (r), LODs, and LOQs of cyclosporine A and sirolimus.

Immunosuppressant drug	Linear range/(ng/mL)	Linear equation	r	LOD/(ng/mL)	LOQ/(ng/mL)
Cyclosporine A	30–2000	$Y = 0.053X + 0.045$	0.9976	10	30
Sirolimus	2–200	$Y = 0.098X + 0.538$	0.9989	1	2

Y: peak area; X: mass concentration, ng/mL.

3.4. Precision, accuracy and recovery rate

In the blank whole blood, three levels of cyclosporine A and sirolimus solution were added respectively, and the precision, accuracy and recovery rate of spiked samples were tested. The spiked samples were determined after being processed according to the methods in Section 2.3. The results are shown in **Table 3**. The results showed that the average recoveries of cyclosporine A and sirolimus were 83.5%–89.7% and 95.8%–97.8%, respectively, and the relative standard deviations were 3.2%–90% and 3.4%–67%, respectively. This method can well meet the requirements for the determination of two immunosuppressants in whole blood samples.

Table 3. Spiked recoveries and RSDs of cyclosporine A and sirolimus in a blank whole blood sample (n = 5).

Immunosuppressant drug	Added/(ng/mL)	Found/(ng/mL)	Recovery/%	RSD/%
Cyclosporine A	30	25.06	83.5	9.0
	60	51.26	85.4	6.0
	300	269.01	89.7	3.2
Sirolimus	2	1.94	97.0	6.7
	4	3.83	95.8	4.2
	20	19.56	97.8	3.4

4. Conclusion

This study investigated the chromatographic behavior of cyclosporine A and sirolimus on biological liquid chromatographic column and traditional liquid chromatographic column, and established a high performance liquid chromatographic method based on biological liquid chromatographic column for rapid and simultaneous determination of the content of these two immunosuppressants in whole blood. This method has the advantages of simple mobile phase, short analysis time, wide linear range and high sensitivity. It can provide technical support for the detection of cyclosporin A and sirolimus in whole blood.

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Conflict of interest: The authors declare no conflict of interest.

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