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Research Article

A Multi-step High-Performance Liquid Chromatography (HPLC) Technique for the Extraction and Purification of Medical-Grade Cyclosporine A

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Abstract: Cyclosporine A (CsA), a potent immunosuppressive drug, is used to treat autoimmune diseases and prevent organ transplant recipients from rejecting their new organs. Maintaining CsA's uniformity and purity is essential to maintaining its therapeutic efficacy and safety. Impurities and contaminants in CsA formulations can exacerbate side effects and patient toxicity. Purification processes can improve the safety profile of the drug and reduce the likelihood of adverse effects by eliminating impurities. Using High-performance liquid chromatography (HPLC) methods, CsA has been isolated and refined from crude in recent years. However, after the procedure, the CsA purity was not at its best. To solve this issue, this work employs a multi-step HPLC method to increase purity levels to above 95%. When exploring the HPLC detection conditions of CsA, we found that the method from the Chinese pharmacopoeia: acetonitrile-water-tert-butyl methyl ether-phosphoric acid (430 : 520 : 50 : 1) was a better mobile phase solution, our further research, including the substitution of tert-butyl methyl ether with other buffer solvents, showed no significant improvement. At the same time, we established that the key factor affecting its separation was temperature. When the temperature is lower than 70 ℃, the HPLC separation effect worsens, the retention time increases, and the peak width becomes longer. Finally, the utilization of acetonitrile-water-tert-butyl methyl ether-phosphoric acid (430 : 520 : 50 : 1) as the mobile phase with a column temperature of 70 °C, petroleum ether: acetic acid Ethyl ester (70 : 30) as the mobile phase, and filling the φ 40 mm × 500 mm chromatography column using a 40~60 μm silica gel so that a 10 : 1 height-to-diameter ratio of the packed part is obtained, under normal temperature conditions, resulted in an average cyclosporine A yield of 80.1%. The corresponding average purity was 97.2%, of which the first isolation yield was 82.3%, and the purity was 98.1%.

***Keywords*:** cyclosporine A, high-performance liquid chromatography, extraction and purification, medical grade, mobile phase

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1. Introduction

The chemical structure of Cyclosporine (Cs) is ($C_{62}H_{111}N_{11}O_{12}$),[1] which is a non-polar chemical that is soluble in organic solvents such as acetone and ethyl acetate but not in water.[2] Cs is typically a white powder[3] and can bind to the cellular protein cyclophilin, which inhibits the enzyme calcineurin.[4] Cs tend to suppress immunocompetent cells in the G0- or G1-phase of the cell cycle in a particular and reversible manner.[1] T-lymphocytes are suppressed selectively, with T-helper cells being the principal target. Lymphoma formation and release are also inhibited by Cs.[3] In the early 1970s, Brayman and colleagues reported CsA as a good medication for human organ transplantation.[5] The U.S. Food and Drug Administration (FDA) approved it for clinical kidney transplantation in 1983.[5] In the same year, the Fujian Institute of Microbiology discovered and isolated CsA from another cyclosporine-producing fungus, Fusarium Solan, in Chinese soil.[5] In terms of physical and chemical characteristics,[6] as well as spectral detection,[4] the CsA isolated from Fusarium solanacearum and the foreign CsA were identical.[7] In 1991, China released the first CsA preparation that it had created independently.[3]

CsA is an immunosuppressant medicine that reduces the activity of the patient's immune system and hence the risk of organ rejection after an allogeneic organ transplant.[8] It is a cyclic nonribosomal peptide,[9] with eleven amino acids. Immunocompetent lymphocytes are also reversibly inhibited in the G0- and G1-phases of the cell cycle.[3-4] It functions as an antifungal,[10] antirheumatic, dermatologic, immunosuppressive, metabolite, carcinogenic,[11] anti-asthmatic,[10] and phosphoprotein phosphatase inhibitor, among others. The FDA has approved CsA for the treatment and prevention of graft-versus-host disease in bone marrow transplantation, as well as the prevention of kidney, heart, and liver transplant rejection,[12] In the United States, it's also authorized for the treatment of rheumatoid arthritis and psoriasis, as well as persistent nummular keratitis after adenoviral kerato-conjunctivitis and as eye, drops to treat dry eyes caused by Jorgen's syndrome and Meibomian dysfunction.[13]

While upstream methods have gotten a lot of interest in the extraction and purification of crude compounds like that of CsA and monoclonal antibodies (mAb), and have progressed significantly over the years, resulting in extraordinarily high mAb titres in the broth, downstream processing (DSP), on the other hand, has been neglected.[14-15] Despite its flaws, conventional DSP purification strains and chromatography can still be considered the workhorse of practically every industrial-scale biotech product manufacturing.[16-17] The most noticeable is the significant cost, which is related to the need for many pre-purification processes to avoid column obstruction. For two basic reasons, excess physical processes are undesirable: more stages equal less recovery and longer, more expensive process duration.[18]

Integrative new technologies, which decrease the number of unit activities by viewing them as a single unit, are the solution to this particular flaw.[19] For the analysis of Cs, several liquid chromatographic techniques have been reported.[20] To isolate CsA from physiological fluids, many of them require labour-intensive multistep liquid-liquid extractions, while certain solid-phase extraction techniques have low analytical recovery and are prone to interferences from late-eluting peaks.[21] Fermentation processes are used to make Cs antibiotics.[4] The isolation of CsA and B obtained by culturing the fungus strain *cylindrocarpon* lucid booth 5,760 was described for the first time in Swiss patent No. 589716.[22] Different components with chemical and physical properties that are quite similar to each other are extracted from the fermentation liquid.[4] A multistep chromatographic preparative process was used to extract the components from the resulting crude Cs combination. Our group hence employed a high-performance liquid chromatographic method for the separation and purification of CsA from the as-prepared crude Cs combinations. Our techniques employed parameters such as column temperature, flow rate, and other column characteristics to establish a high-performing technique with high recovery, purity, and shorter process duration.

2. Experimental section

2.1 *Materials and reagents*

High-performance liquid chromatography and glass chromatography column (C18 column: 4.6 mm × 250 mm, and the particle size of 3 μm) used were purchased from Beijing Puyuan Jingdian Technology Co., Ltd. Ethyl acetate, petroleum ether, and phosphoric acid were purchased from Sangon Co. Ltd, Shanghai, China. Acetonitrile was obtained from OCEANPAK, China. Crude Cs samples were obtained from Jiangsu Hanbon Sci. and Tech. Ltd (Chinese Academy

of Science), Huai'an, Jiangsu, China. Silica gel and CsA standard were obtained from Beijing Puyuan Jingdian Technology Co., Ltd.

2.2 *Solutions configuration*

2.2.1 *Standard solution configuration*

100 mg of CsA standard was accurately weighed and dissolved in 50 mL of solution (1 chromatography grade acetonitrile : 1 water) into a volumetric flask and made up to volume. 2 mg/mL of the as-prepared standard solution was pipetted into a tube, seal with Parafilm and stored in a refrigerator at 2 ℃ for future use.[23]

2.2.2 *Crude Cs configuration*

100 mg of crude Cs sample was accurately weighed and dissolved in 50 mL of solution (1 chromatography grade acetonitrile : 1 water) into a volumetric flask and made up to volume. 2 mg/mL of the as-prepared standard solution was pipetted into a tube, seal with Parafilm and stored in a refrigerator at 2 ℃ for future use.[24]

2.2.3 *Configuration of loading solution*

2 mL of mobile phase (acetonitrile, ultrapure water, tert-butyl methyl ether, phosphoric acid) was used to dissolve each gram of crude product. The solution was vigorously shaken to ensure a uniform mixture and set aside.[25]

2.2.4 *Mobile phase solution configuration*

High-performance liquid chromatography (HPLC) grade, acetonitrile, tert-butyl methyl ether, and phosphoric acid were filtered using a 0.2 micron organic filter membrane and ultrasonicated for 15 mins.[24]

2.3 *Preparations before the HPLC procedure*

2.3.1 *Packing silica gel column*

Silica gel was weighed according to the volume of the column bed. Petroleum ether twice the volume of dry silica gel was then added and stirred thoroughly with a glass rod until there were no bubbles.[23] Finally, the glass column was properly cleansed and fixed on the retort stand.

2.3.2 *Packing of silica gel into the C18 column*

The mobile phase was poured into the C18 column, accounting for about one-third of the column bed volume, the C18 column piston was opened to let the mobile phase flow out slowly, at the same time, the previously mixed petroleum ether-silica gel homogenate was slowly poured out. The silica gel relies on gravity to settle in column.[26] The mobile phase was slowly added to the glass column to maintain a certain liquid level that facilitates the settling of the silica gel. Cotton wool was finally used to firmly seal the silica gel.[27]

2.4 *Sample preparation and loading*

The crude Cs sample to be separated was dissolved in a small amount of eluent, the same as that used for column packing, and also to prepare a small volume and highly concentrated sample solution which was then added to the silica gel surface. After the sample solution had completely entered the silica gel, it was rinsed by adding a small amount of mobile phase.[20]

2.4.1 *Elution procedure*

The tap/piston was initially opened at the lower end of the column, while concurrently connecting the upper end of the column to the reservoir ball. The flow rate was then adjusted and using a constant flow pump, the mobile phase was continuously added to keep the liquid level in the reservoir ball stable. Ensuring a stable glass column during elution,

and significant shaking was a prerequisite.[21]

2.4.2 *Collection and processing of eluent*

The eluent was collected in aliquots, using a test tube or EP tube (pre-test to ensure that the organic solvent would not dissolve the EP tube).[28] Each tube collected about a quarter of the column volume and was labeled in sequence, to be tested.

2.5 *Detection procedure*

Each collected eluent was injected into a 1 mL sample bottle before being placed on the tray in the order specified on the label. The chromatographic detection conditions are set using chromatography software: acetonitrile : ultrapure water : tert-butyl methyl ether : phosphoric acid (430 : 520 : 50 : 1 respectively).[29] The injection volume was 15 μL, with each sample having a 30 minute detection duration, as well as multiple (thrice check). The process of HPLC separation and purification of CsA is illustrated in Figure 1 below.

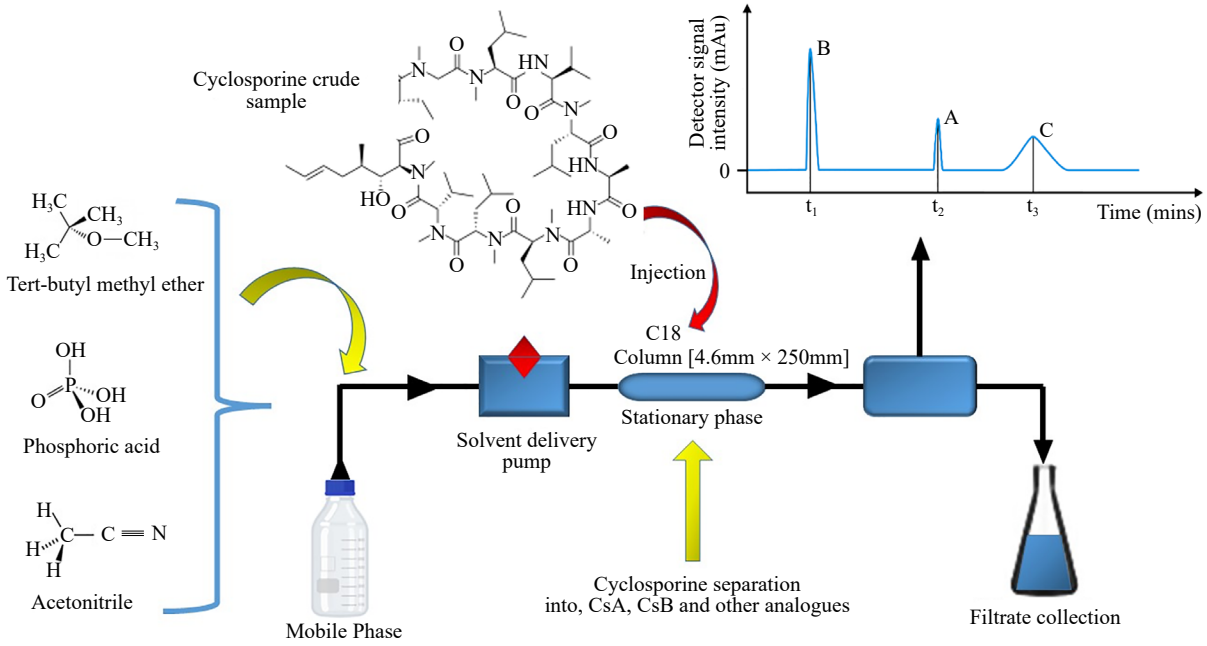

Figure 1. Illustration of the step-by-step HPLC procedure for the separation and purification of crude cyclosporine into medical-grade CsA

Other parameters were based on the HPLC machine's default settings. Prior to testing, we pre-heated the column oven and then released the exhaust through the exhaust valve. After that, we equilibrate the column by running empty samples (no sample injection) for more than 30 minutes. The standard was tested first, followed by the eluted solution. As a result, each peak area of the chromatogram was recorded.[20]

The peak area was positively associated with the concentration, and the peak time of the same chemical was the same. The concentration of CsA in the eluent and the content of other contaminants can be determined via comparative analysis of the peak time and area of the corresponding peak in the crude product and the standard product, as well as the standard product concentration.[27] Finally, the yield and purity are calculated using the eluent that meets the requirements.

2.6 *Calculation and data processing of standard curve*

2.6.1 *Drawing CsA standard curve*

To determine the peak area, the CsA standard solution (2 mg/mL) was diluted 1 time, 2 times, 4 times, 8 times, and 16 times and 15 μL was injected into the liquid chromatography. A standard curve was drawn using the peak area as the ordinate, the sample volume as the abscissa, and the highest concentration of CsA in the eluent as the abscissa (the maximum concentration of CsA in the eluent could not be higher than 2 mg/mL). The average value was calculated after measuring each concentration three times.

The fitting of the standard curve was done using the fitting feature of the liquid chromatography control software to guarantee that the correlation coefficient was more than 0.9999, as shown in Figure 2. The concentration was computed the concentration by plugging the peak area into the standard curve, then the recovery rate and purity.

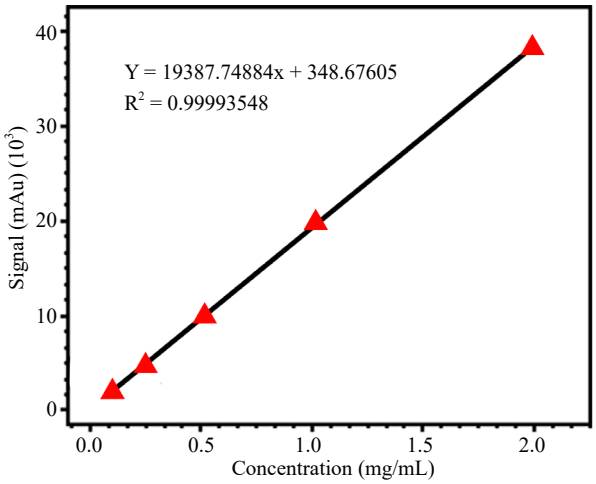

Figure 2. Standard curve produced from the CsA standard solution (2 mg/mL) diluted 1 time, 2 times, 4 times, 8 times, and 16 times. Mobile phase: V (petroleum ether) : V (acetic acid Ethyl ester) = 70 : 30. Volume: 2 mg/mL. Column: C18 particle size of 3 μm. Column temperature: 70 °C

2.6.2 *Chromatograms of standard CsA sample and crude Cs*

2 mg/mL of crude Cs and CsA were independently diluted 1 time, 2 times, 4 times, 8 times, and 16 times and 15 μL was injected into the HPLC and a standard chromatogram for each sample was detected (Figure 3). In the crude Cs, a total of 4 related substances (11, 12, 13, and 14) with high content were detected in the crude product of CsA, of which 14 was CsA, and substances 11, 12, and 13 could be cyclosporine L, Cyclosporine C, Cyclosporine U through literature search.

Firstly, the effect of different proportions of petroleum ether-ethyl acetate as a mobile phase on the separation and purification of CsA. Under other conditions (70 °C column temperature) unchanged, we changed the mobile phase matching ratio (V petroleum ether : V ethyl acetate = 40 : 60; 50 : 50; 60 : 40; 70 : 30; 80 : 20). After the optimal mobile phase ratio was obtained, the flow rate was adjusted by changing the closing degree of the glass column tap/piston. In the optimal ratio V (petroleum ether) : V (ethyl acetate) = 70 : 30, the chromatogram of the crude Cs and the CsA sample were determined using flow rates of 2 mL/min, 3 mL/min, 4 mL/min, and 5ml/min separate effects.

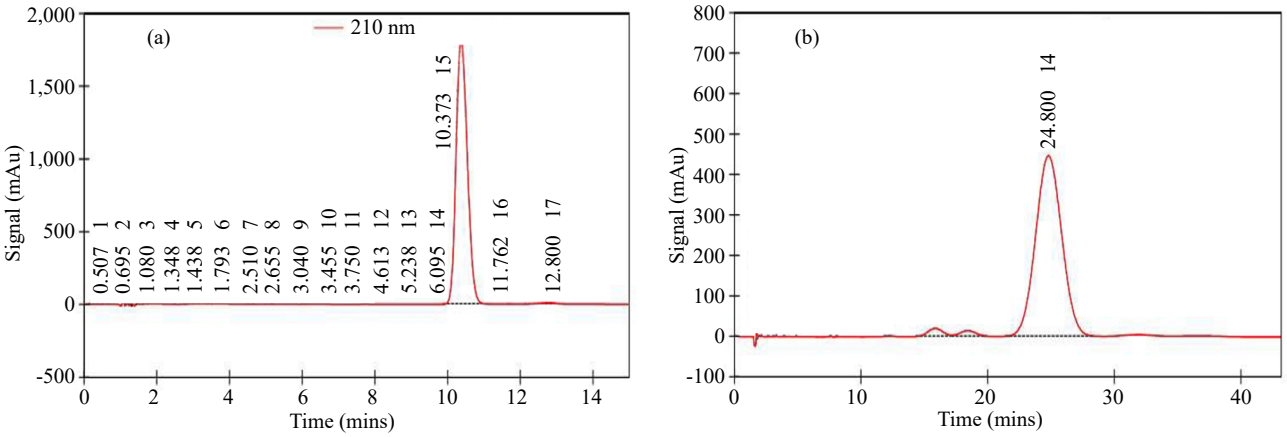

Figure 3. Chromatograms of (a) standard CsA sample and (b) crude Cs sample

3. Results and discussion

3.1 *Varying column temperature*

Five cyclosporine analogues were quickly separated using Supercritical Fluid Chromatography, according to Shao and colleagues.[30] The team found that selectivity remained essentially unchanged as column temperature rose. Peak widths, however, were dramatically lowered, suggesting improved column efficiency. The resolution of the five cyclosporine analogues was dramatically enhanced with higher temperatures since the resolution was dependent on retention, selectivity, and column efficiency. Peaks were quite broad and had poor separation at lower temperatures, such as 0.0 °C to 10.0 °C. Baseline resolution was attained for each of the five peaks at 50.0 °C. Up to 80 °C, the peak shape and resolution were getting better.[30] Furthermore, the impact of column temperature on the plate count and retention periods of the cyclosporine congeners was investigated by Sakai-Kato and Yoshida. They also noted that when the column temperature was raised from 50 to 80 °C, the peak shapes became sharper but the retention times remained relatively unaffected, leading to an increase in the plate count. These trends were similar to those seen with cyclosporine A, as previously described by several other researchers. As a result, they employed 75 °C for the cyclosporine's further analysis.[31]

Scrutiny of the HPLC detection conditions for CsA revealed that the Chinese Pharmacopoeia's approach of acetonitrile-water-tert-butyl methyl ether-phosphoric acid (430 : 520 : 50 : 1) was a superior mobile phase solution. Further studies yielded no significant differential outcome when tert-butyl methyl ether was replaced with alternative buffer solvents. At the same time, it was discovered that temperature was an important parameter in chromatographic separation. Although the denaturing conditions are inherent to detecting substances, one particularly useful process parameter to adjust was the column temperature. Variability in temperature modulates the structure of CsA, which can be utilized to ultimately affect peak shape and even selectivity; presumably, slight changes in conformation affect the interaction thereby affecting the detection of crude cyclosporine A. The effect of temperature was more clearly observed in the HPLC analysis of CsA. An increase in temperature improves peak width at half height when comparing 70 °C and 80 °C (Figure 4(a)), with improvements in peak widths for the light chain. However, peak widths are slightly worse when comparing 80 °C and 90 °C. More interesting are the impurity profiles for the heavy chain, which vary between each method column temperature (Figure 4(b)). Depending on the importance of characterizing each impurity, one profile may be more desirable than another. The chromatographic separation effect deteriorates when the temperature drops below 70 °C, the retention time increases, the peak width widens, and the detection effect deteriorates. Finally, with a column temperature of 70 °C, it was chosen to employ acetonitrile-water-tert-butyl methyl ether-phosphoric acid (430 : 520 : 50 : 1) was employed as the mobile phase.

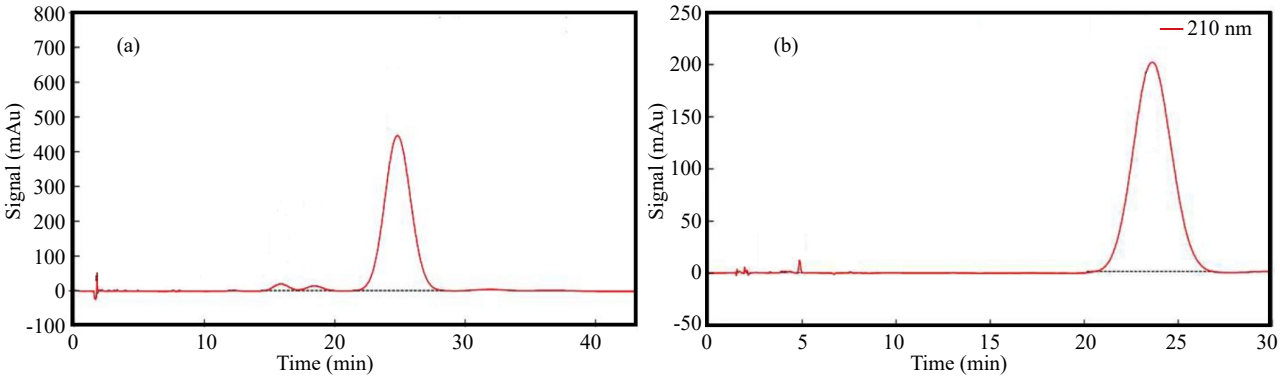

Figure 4. Altering column temperature (a) improves peak width at half height when comparing 70 °C and 80 °C (b) impacts the impurity profiles for the heavy chain, which vary between each method column temperature. Mobile phase: V (petroleum ether) : V (acetic acid Ethyl ester) = 70 : 30. Volume: 2 mg/mL. Column: C18 particle size of 3 μm

3.2 *The flow rate ratio of mobile phase and elution time*

The flow ratio of the mobile phase, the choice of packing, the flow rate of the mobile phase, the volume of the sample, and the ratio of the height and diameter of the chromatography column are all factors that affect CsA separation and purification. Yet, this research only considered the flow rate ratio and sample volume. First, the influence of the different proportions of petroleum ether-ethyl acetate as a mobile phase on CsA separation and purification was studied. Here, only the mobile phase matching ratio (V petroleum ether : V ethyl acetate = 40 : 60; 50 : 50; 60 : 40; 70 : 30; 80 : 20) was altered, while keeping all other conditions the same. Figure 5 below shows how changing the mobile phase matching ratios affected the CsA elution duration from the column and the corresponding signal. Under normal temperature and natural flow rate (about 4.0 mL/min), four different ratios of petroleum ether/ethyl acetate mobile phases were chromatographed using 40~60 m silica gel to fill the 40 mm × 500 mm chromatography column, so that a resultant packed height-to-diameter ratio of the packed part was 10 : 1 (column bed volume 0.5 L), the sample volume was 10.0 g.

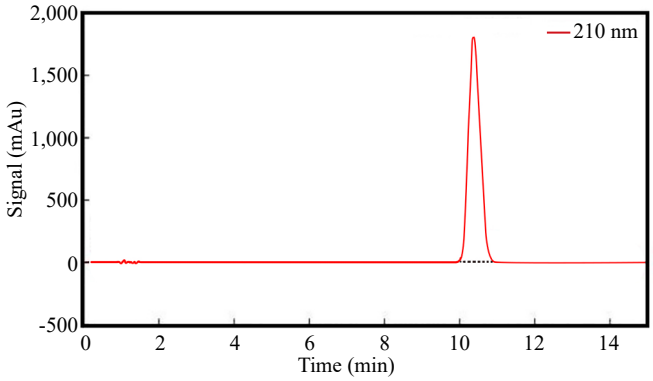

Figure 5. Correlation between variability of mobile phase matching ratios and the duration required for CsA elution from the column. Mobile phase: V (petroleum ether) : V (acetic acid Ethyl ester) = 70 : 30. Volume: 2 mg/mL. Column: C18 particle size of 3 μm

The findings revealed that when the amount of ethyl acetate in the mobile phase drops, the polarity of the mobile phase reduces, the separation effect of cyclosporine rises, and the purity of Cs falls (see Table 1 below). This resulted in a rise in the amount collected, but the amount of mobile phase required increased as well, and the recovery rate reduced; overall, the mobile phase ratio increased. The optimum matching ratio and the highest signal were matched at 10.3 mins. The ideal mobile phase matching ratio was determined. V (petroleum ether) : V (ethyl acetate) to be 70 : 30.

Table 1. Manipulation of the mobile phase matching ratio (V is the amount of mobile phase; l is loading weight; R is the recovery rate of cyclosporine A; P is the purity of CsA)

V (petroleum ether)	V (ethyl acetate)	L/L	R%	P%
40	60	0.64	90.1	71.2
50	50	1.13	88.7	80.6
60	40	1.42	86.9	88.3
70	30	1.64	82.1	95.4
80	20	1.85	77.1	97.8

3.3 *Flow rate ratios and corresponding recovery time*

The findings of the investigation revealed that the flow rate affects the potential tray height and, as a result, the resolution. The flow velocity was too fast, the chemical liquid's contact time with the silica gel as well as the components' equilibrium period in the two-phase (solid-liquid) was too short, and the components' equilibrium period in the solid-liquid two phases was too short. There was no full adsorption or even flow out of the combined components, resulting in waste and time expense; if the flow rate was too sluggish, growth ensued. The substance's diffusion also fails to produce the desired separation effect, resulting in inadequate yield and purity. In gradient elution, the tail of the peak was always under the influence of the stronger mobile phase as compared to the peak front, especially with a smooth gradient form without flat portions. The flow rate was changed (in Table 2 below) by altering the closure degree of the glass column tap/piston once the ideal mobile phase ratio had been determined. V (petroleum ether) : V (ethyl acetate) = 70 : 30 was the ideal ratio.

The flow rate at 2.0 mL/min, 3.0 mL/min, 4.0 mL/min, and 5.0 mL/min separate impacts, as well as the flow rate at 2.0 mL/min, 3.0 ml/min, 4.0 mL/min, and 5.0 mL/min were investigated. Figure 6 shows the experimental findings that summarize that if the flow rate was too high, the components in the solution could not be properly absorbed by the silica gel. The chemicals in the solvent will seep into the silica gel if the flow rate is too slow, resulting in poor results. As a result, 4.0 mL/min was the optimum flow rate upon examination.

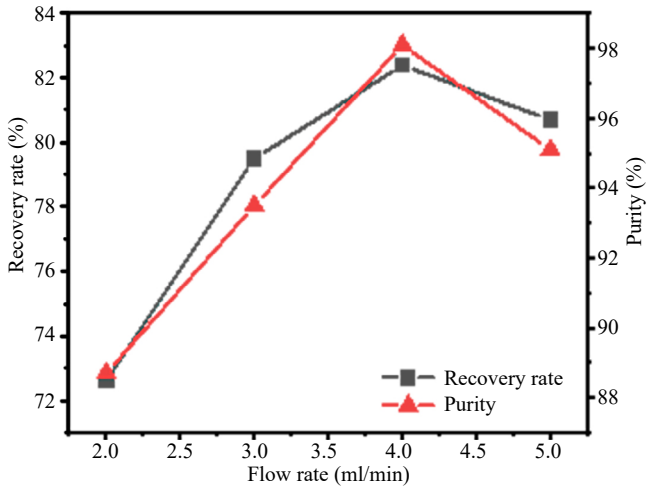

Figure 6. Varying flow rate by altering the closure degree of the glass column tap/piston. Mobile phase: V (petroleum ether) : V (acetic acid Ethyl ester) = 70 : 30. Volume: 2 mg/mL. Column: C18 particle size of 3 µm

Table 2. Altering the closure degree of the glass column tap/piston once the ideal mobile phase ratio has been determined. V (petroleum ether) : V (ethyl acetate) = 70 : 30 is the ideal ratio (V is the amount of mobile phase; R is the recovery rate of CsA; P is the purity of CsA)

Flow rate (mL/min)	Recovery rate, R (%)	Purity, P (%)
2	72.6	88.7
3	79.5	93.5
4	82.4	98.1
5	80.7	95.1

3.4 *Varying sample loading*

The effect of varying sample loading on the final result was examined under the defined circumstances of the flow ratio and flow rate in the above two portions. The sample loads were 9.0 g, 12.0 g, 15.0 g, 18.0 g, and 21.0 g as shown with corresponding results in Table 3 below. The following are the final conditions for optimal separation and purification of CsA, as determined by the aforementioned three tests: Filling the 3 μm chromatography column with 40~60 m silica gel, so the height of the packed part was 10 : 1 (column bed volume 0.5 L), the optimum sample volume was 15.0 g, the flow matching ratio was V (petroleum ether) : V (ethyl acetate) was 70 : 30, and the flow rate was controlled at 4.0 mL/min under normal temperature conditions. Three distinct separate separation tests were carried out under this circumstance to investigate how the separation effect changed over time without modifying the silica gel in the glass column. The results of the investigation showed (in Figure 7) that when the loading volume was too large, the elution peaks of cyclosporine and its homologues overlap, reducing the separation effect and lowering the purity and yield of CsA. The distinct effect was optimal when the loading volume was 15.0 g.

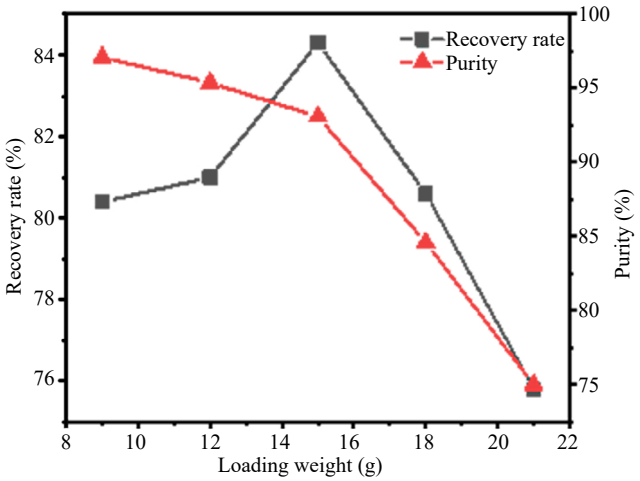

Figure 7. The effect of varying sample loading under the defined constant of the flow ratio and flow rate. Mobile phase: V (petroleum ether) : V (acetic acid Ethyl ester) = 70 : 30. Volume: 2 mg/mL. Column: C18 particle size of 3 μm

Table 3. Varying sample loading (g) and its impact on recovery and purity of CsA

Sample load (g)	Recovery rate R (%)	Purity P (%)
9	80.4	97.1
12	81.0	95.4
15	84.3	93.1
18	80.6	84.6
21	75.8	75.0

3.5 *Elusion times, recovery times, and purity*

Gradient elution is the process of changing the mobile phase composition over time during a chromatographic run, hence could be compared to the temperature programming used in gas chromatography.[32-35] Gradient elution's major goal was to transfer the mixture's strongly retained components quickly while keeping the least retained component stationary. Starting with the organic component's low content in the eluent, the least retained components are separated. Components that are strongly retained will either sit on the adsorbent surface at the top of the column or move extremely slowly.[36-38] Because of the constant increase in competition for adsorption sites, as the amount of organic component in the eluent (acetonitrile) was increased, strongly retained components moved faster and faster. Gradient elution also improves the column's quasi-efficiency. The longer a component is kept in the isocratic elution, the wider its peak becomes.

The detector response acquired from an amount of analyte added to and removed from the biological matrix, as opposed to the detector response obtained for the real concentration of the analyte in a solvent, was the recovery of an analyte in an assay. In Table 4 below, employing gradient elution for the replicate test, the tail of the peak was always influenced by the stronger mobile phase when compared to the peak front, especially with the smooth gradient form without flat sections. Higher elution periods resulted in lower CsA recovery rates and purity, whereas lower recovery times resulted in higher CsA recovery rates and purity as shown in Figure 8 below. A comparison was made between the purity performance of the as-prepared multi-step HPLC approach and previous published studies for the extraction and purification of medical-grade CsA (Table 5). Good operating parameters, such as recovery and elusion times, column temperatures, flow rates, and purity, were demonstrated by this approach.

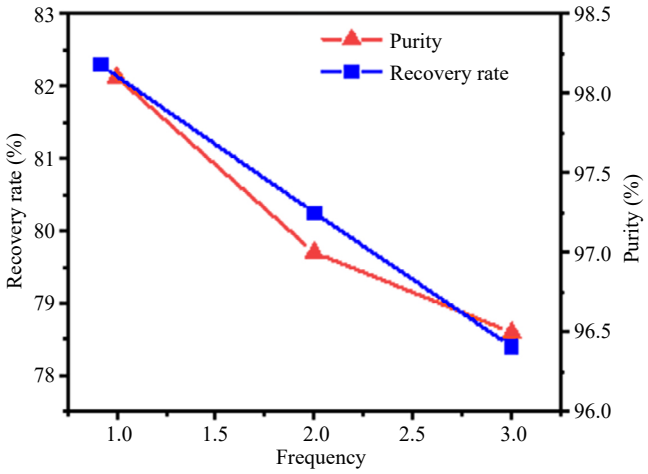

Figure 8. Replicate recovery rate (%), and purity (%) when gradient elution was employed. Mobile phase: V (petroleum ether) : V (acetic acid Ethyl ester) = 70 : 30. Volume: 2 mg/mL. Column: C18 particle size of 3 μm. Column temperature: 70 °C

Table 4. Replicate test results when gradient elution was employed

Elution times	Recovery rate R (%)	Purity P (%)
1	82.3	98.1
2	80.1	97.0
3	77.9	96.5

Table 5. Purity P (%) comparison of multi-step HPLC Technique to other techniques for the purification CsA

Method	Stationary phase	Mobile phase	Column temperature	Purity P (%)	Ref.
Centrifugal partition chromatography	CPC column	Heptane/ethyl acetate/acetone/methanol/ water (1 : 2 : 2 : 1 : 2, v/v)	80 °C	Run1: 67% Run2: 94% Run3: 82%	39
Semi-automated HPLC	C18 column	Acetonitrile, methanol and trifluoroacetic acid	np	75 ± 3%	40
Reverse phase HPLC	C18 column	Acetonitrile in 0.1% trifluoro acetic acid buffer	np	98.08 to 101.55%	41
Reverse phase HPLC	C18 column	Methanol-water: acetonitrile-water	37.5 ± 0.5 °C	< 80%	42
Multi-step HPLC	C18 column	Petroleum ether: acetic acid Ethyl ester 70 : 30	70 °C	98.1%	This work

Np: not provided

3.6 *Rate, frequency, and purity measurement*

As a result, molecules in the chromatographic zone's tail (peak) will move faster. This has the effect of compressing the zone and narrowing the resulting peak. The instrumentation greatly influences the efficiency of gradient elution. Thus, these two points are worth paying attention to during instrumentation: the volume of air between the component mixing point and the column inlet and the system's ability to combine effluent components. This capacity of the former usually corresponds to the pump volume in low-pressure gradient systems, which was around 2-3 mL. In addition, if the system's ability to combine eluent components. If the system does not properly mix the components, it will supply one component at a time, then another, and so on. Such a system will demonstrate poor chromatographic performance, especially for the least retained components.

3.7 *Replicate test*

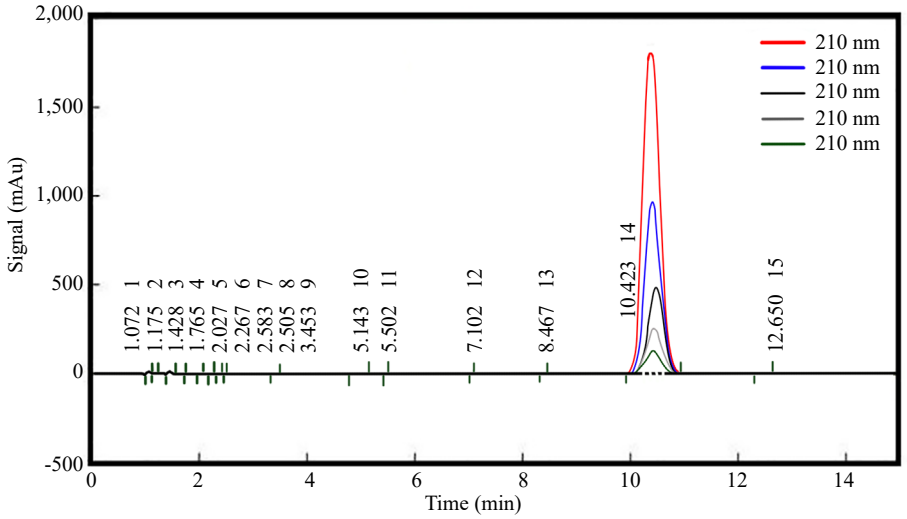

Figure 9. Overlay of multiple chromatograms: establishing repeatability of experimentation. Column temperature chromatogram: Mobile phase matching ratios chromatogram: Flow rate chromatogram, Sample loading chromatogram: and Replicate test chromatogram. Mobile phase: V (petroleum ether) : V (acetic acid Ethyl ester) = 70 : 30. Volume: 2 mg/mL. Column: C18 particle size of 3 µm. Column temperature: 70 °C

In a single experiment, the numerous HPLC parameters under evaluation, such as absorbance, retention duration, sample volume, column temperature, and flow rate, were all altered simultaneously.[39,43] The goal of this modification was to develop a detailed final method for detecting, extracting, and purifying CsA from crude form, based on the established

optimum conditions of the parameters as stated in this study, especially creating a novel method with optimized purity, recovery time, and loading weight. In high-performance liquid chromatography methods for the detection and extraction of CsA, temperature changes lead to gains in peak width and retention time. Furthermore, selectivity was evaluated individually because temperature rises could be detrimental to separation. Partially separated impurities may be visible at the intact level, resulting in overall peak broadening but a more detailed impurity profile. Because changes in impurity profiles may be seen at the sub-unit level, the temperature was included in the experiment design. Multiple samples of the crude Cs sample were analyzed to improve the accuracy of this work. The results are expressed in an overlapping form (Figure 9) through the chromatograms. The use of numerous overlapping chromatograms facilitates easy and effective viewing and comprehension of the data. The repeatability of experimentation was established after test sample results overlapped in this manner.

4. Conclusion

Strong immunosuppressive medications, CsA are used to treat autoimmune illnesses and stop organ transplant recipients from rejecting their new organs. Sustaining the safety and therapeutic efficacy of CsA requires preserving its homogeneity and purity. Contaminants and impurities in CsA formulations can increase patient toxicity and adverse effects. By removing contaminants, purification procedures can raise the drug's safety profile and lower the chance of side effects. In addition, there has been interest in the development of methods to solubilize active pharmaceutical ingredients (APIs) that are poorly soluble in water. Recently, CsA isolation and refinement using HPLC have become more common. To improve the purity, more research is necessary on the process. To raise purity levels, this work uses a multi-step HPLC approach. In this study, the extraction and purity of crude Cs were determined utilizing several HPLC procedures in the C18 column with silica gel as the stationary phase. Gradient elution of the mobile phase yields significant research findings, resulting in overlapping chromatograms for simple analysis and conclusions. Filling the 3 μm chromatography column with 40~60 m silica gel, so that the height-to-diameter ratio of the packed part was 10 : 1 (column bed volume 0.5 L), the sample volume was 15 g, and the mobile phase was V (petroleum ether) : V (acetic acid Ethyl ester) = 70 : 30, and the flow rate was controlled to 4.0 mL/min under normal temperature conditions. CsA had an average yield of 80.1% and a purity of 97.2%, with the first isolation being 82.3% and the purity being 98.1%. In the future, a better route for further optimization of the solvent used for washing silica gel, as well as a better stationary phase, is required to improve recovery and purity.

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Data availability

Data will be made available on request.

Author contributions

Eliasu Issaka: Conceptualization, Methodology, Investigation, Formal analysis, Writing-Original Draft, Writing-Review & Editing, Supervision; Nii Okai Amu-Darko: Material preparation, data curation, Formal analysis, Writing-

Original Draft; Mabruk Adams: Methodology, Investigation, Formal analysis, Writing-Review & Editing; Sylvenus Aguree: Resource, Methodology, Investigation, Writing-Original Draft; Michael Enyan: Methodology, Investigation; Husseini Sulemana: Writing-Original Draft, Resource, Data curation; Princess Firdaus Suleimana: Resource, Data curation.

Conflict of interest

The authors declare no competing financial interest.

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