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Solubility-Based Purification of Enzymatic Synthesis Cephradine

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Abstract

Given that processes of chemical synthesis cephradine are cumbersome, and enzymatic synthesis are simple in process, mild in reaction conditions, short in production cycle but the lack of any practical purification method for enzymatic synthesis cephradine. We established an eco-friendly purification and low energy consumption recovery method with industrial significance to make enzymatic synthesis method has potential to be exploited more extensively. Crude product from a new enzymatic synthesis method of cephradine is composed with 96% to 98% of cephradine and 2% to 4% of dihydrophenylglycine which presents as an impurity. Herein, purification process based on solubility and recovery technology through osmatic distillation were presented. A great difference of solubility between cephradine and dihydrophenylglycine in distilled water, as a function of pH, at a temperature of 25°C was observed. By adjusting pH and volume of solvent, two species can be separated and purified successfully, and purity reach to >99.99% in multi-stage of purification. In addition, the purification process is also feasible to purify other compositions of cephradine products with dihydrophenylglycine. Due to heat-sensitivity of cephradine, osmatic distillation was applied to concentrate the solution formed with dissolving cephradine during purification and achieve a higher recovery ratio. The experiments were carried out with a laboratory- scale direct contact membrane distillation by the hollow fiber membrane of polyethylene chlorotrifluoroethylene which takes advantages of its excellent hydrophobicity. The maximum flux is 0.258Kgh^-1m^-2 at a temperature of 25°C with 5.95 M sodium chloride as stripping solution. The quality of cephradine detected by HPLC was well preserved after osmatic distillation process.

Keywords: Cephradine, Direct contact membrane distillation, Ethylene chlorotrifluoroethylene membrane, Purification, Solubility

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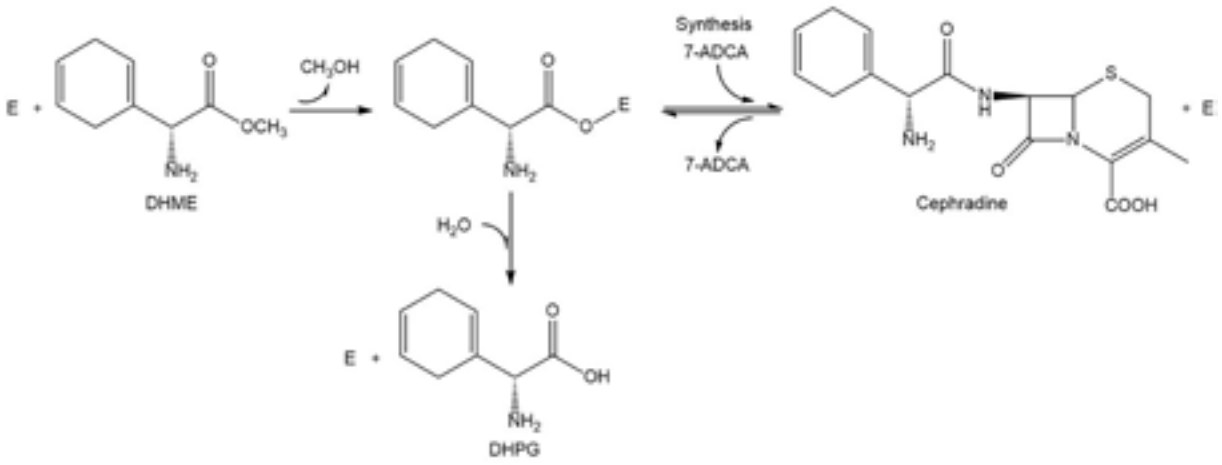
Introduction

Cephradine (CAS Registry No. 38821-53-3) is the first generation of semisynthetic cephalosporin antibiotic developed and produced by Squibb Institute. It is comprehensively used in clinical applications as a general medicine, and can be taken orally or by injection with few adverse reactions and low incidence as well. Its rapid and reliable bactericidal effect that inhibits a broad spectrum of bacterial activity by interfering with the later stages of bacterial cell wall synthesis through inactivation is resistant to β-lactamase, Gram-positive microorganisms and Gram-negative microorganisms. It is used to treat extensive variety of infections caused by sensitive bacteria, including upper respiratory, ear, skin and urinary tract infections[1]. The synthesis methods of cephradine are chemical methods and enzymatic methods, while chemical methods are the main way for industrial production of cefradine. However, chemical synthesis processes are cumbersome, and the reaction conditions are harsh with a large amount of three wastes[2]. Enzymatic methods are simple in process, mild in reaction conditions, short in production cycle, and environmentally friendly[3, 4]. It has the potential to be applied more extensively. A new synthesis of cephradine utilizes modified biological enzyme to catalyze the synthesis of cefradine[5]. He, J. W et al[6]. developed a novel kinetically controlled synthesis route of enzymatic synthesis cephradine, and demonstrated the application of computational active site redesign for engineering enzyme, penicillin G acylase (PGA, EC 3.5.1.11). 7-Aminodesacetoxycephalosporanic acid (7-ADCA) and 2,5- dihydrophenylglycine methyl ester (DHME) are synthetic raw materials. The condensation reaction that was catalyzed by modified enzyme between two reactants takes place in water at ambient temperature and neutral pH, without the requirement for extensive functional group activation and protection; moreover, neither toxic nor harmful reagents are needed. The synthesis reaction route is shown in Scheme. 1.



Scheme. 1 Reaction route for enzymatic synthesis of cephradine

In the beginning, enzyme reacts with DHME to remove a methanol molecule and form an intermediate product. The intermediate product has two reaction routes. The main reaction is a reversible reaction with 7-ADCA to produce the target product, cephradine. The side reaction is hydrolysis of the intermediate product to form dihydrophenylglycine (DHPG) which presents as an impurity in crude product. The crude product from enzymatic synthesis method contains two components which are around 96% to 98% of cephradine and 2% to 4% of DHPG in solid phase. In this study, we focus on purification of crude product to remove DHPG as much as possible and obtain cephradine products with higher purity. As an amino acid drug, there are certain similar characteristics between cephradine and amino acids ; for example, they are both ampholytes and having low solubility in organic solvents. Purification and separation of amino acid in specific solvent wer reported in some studies. The free amino acids in tea and flower of tea can be extracted by a strong polar solvent; that is, distilled water is an ideal solvent and widely used. Wang, L. et al[7] utilized HPLC-DAD with Zorbax Eclipse XDB-C18 column to improve the effect of separation in differently free amino acids extracted by DI water. Strieglerova, L. et al[8] reported another method to separate amino acid by electromembrane extraction. Amino acids present in cationic form in the donor solutions and pass through a supporting liquid membrane constituted of 1-ethyl-2- nitrobenzene / bis- (2-ethylhexyl) phosphonic acid (85:15 (v / v)) then migrates to the tube side of porous polypropylene hollow fiber in an electric field. By discovering and exploiting a great difference of solubility between cephradine and DHPG in DI water, two species can be separated and purified from crude product successfully. This presented method is easy to carry out, with a higher purity and recovery rate. Herein, for the saturated liquid after purification, Osmotic distillation (OD) with poly ethylene chlorotrifluoroethylene (ECTFE) hollow fiber membrane (HFM) was applied to recover available cephradine. With non-thermal membrane separation, OD can still achieve higher concentration with atmospheric pressure and ambient temperature[9], therefore, this technology is particularly suitable for heat-sensitive aqueous solution[10, 11]. A hydrophobic microporous membrane is used for separation, and only water vapor can be transported through the membrane. Driven by the vapor pressure gradient, the water of feed evaporates from feed side, then vapor penetrates through pores of membrane, and eventually condenses on the other side of membrane[12]. OD can effectively remove water from the saturated cephradine solution with the advantages of high selectivity and low operating temperature. ECTFE is a novel polymeric material composed of ethylene and trifluorochloroethylene and it is able to apply under harsh chemical situations and in a broad of temperatures[13], in addition, its excellent hydrophobicity is conducive to concentrate aqueous without being wetted during membrane distillation[14, 15].

Materials and Methods

Materials

Cephradine was purchased from Nanjing Sunsure Chemical Technology Co., Ltd. DHPG was purchased from Tianjin HEOWNS Biochemical of Technology Co., Ltd. An ECTFE HFM mini module was obtained from Tsinghua University, which has 500 fibers with 0.414 μm pore diameter, 0.28 m fiber length, 59 % porosity, 0.21 mm membrane thickness, 0.38 m effective area, 1.27 mm fiber internal diameter, was used in this study.

Crude product

According to the purity of cephradine ranging from 96% to 98% in crude product processed by enzymatic synthesis method, purity of 96.25% was selected to do the following experiments. The simulated crude product solid was prepared in the laboratory containing 96.25% of cephradine and 3.75% of DHPG, by weighting a small and accurate amount (total weight is less than 3 g each time) of two pure compositions in proportion and completely blending.

Analytical measurements

Both solution and remaining solid which did not dissolve in solution were analyzed by the HPLC technique[16, 17] to measure the concentration of each composition after purification process, according to standard line of each substances. An HPLC system was applied in the analysis. A Hypersil ODS 5 μm column (width 4.6 mm, length 250 mm) was used for separation at 25°C. A 75:25 (v/v) 0.03M Potassium dihydrogen phosphate–methanol mixed solvent was used for the mobile phase. The flow rate was kept constant at 0.8 mL/min throughout the analysis. Detection was achieved with the absorbance at 230 nm (A 230). Table. 1 lists the other equipment used in this experiment.

Equipment	Equipment type	Source
pH meter	FE28- Standard	Mettler-Toledo Instruments (Shanghai) Co. Ltd.
Electronic analytical balance	PL203	Mettler-Toledo Instruments (Shanghai) Co. Ltd.
Liquid Chromatography	1200 Series	Agilent Technologies
Magnetic stirrer	CJJ79-1	HUALU Instruments Co. Ltd.
Super thermostat water bath	HH-ZK4	BEIJING XINGDE Instruments Co. Ltd.
Peristaltic pumps	YZ15	CHANGZHOU VCL Fluid Technology Co. Ltd.
Water Circulating Vacuum Pump	SHZ-DIII	BEIJING XINGDE Instruments Co. Ltd.

Table 1. Detailed Information about All of the Equipment Used in This Experiment

Experimental

Solubility of cephradine and DHPG

To determine the solubility of cephradine and DHPG in water at temperature of 25°C as a function of pH, we dissolved the substances in a glass vessel with a valid volume of 100 ml, and all experiments were repeated twice. A fixed amount of each substances was placed in the containers and well mixed with 40 ml distilled (DI) water for 1 h; besides, 0.2 g of sodium bisulfite was additionally added to container for preventing cephradine being oxidized when determining solubility of cephradine[18]. Glass burets were used to add dilute ammonia and hydrochloric acid to maintain and adjust the pH value of solution. If the total solute was dissolved after 1 h of mixing, the amount of 0.05 g solute was added to container, and mixed thoroughly for another 30 min until particles were suspended in the solution. On the contrary, if excess substance still presented in solution as precipitate, added 1 ml DI water into container and stirred for 30 minutes each time, until all the precipitate was dissolved.

Purification experiment

Simulated crude product with purity of 96.25% was prepared, and according to previous solubility data an excess solute (2.696 g of pure cephradine and 0.105 g pure DHPG) was added into the solvent with various pH value or volume of solvent to form two phases of saturated solution and remaining solid. In those experiments that changed pH value, the volume of solution was fixed at 25 ml, while for those experiments that changed volume of solution, the pH value of solution was fixed in the range of pH4.30 to pH4.60. The pH of solution was adjusted by adding diluted hydrochloric acid or diluted ammonia. As the excessive crude product well mixed with 25 ml of solvent at room temperature for 90 minutes to ensure forming a saturated solution, it was dissolved in the solvent, while a part of remaining solid can no longer to be dissolved; therefore, solid phase and liquid phase were formed and the composition of origin crude product was altered. As a result, purifying of one phase could be achieved. Dissolve the remaining solid by a known volume of diluted hydrochloric acid. HPLC was applied to measure the concentration of cephradine and DHPG in each phase.

Osmotic distillation

During purification process, saturated liquid phase is able to be concentrated and crystallized by osmotic distillation with ECTFE HFM. Because cephradine is sensitive to heat, OD process was operated at room temperature. The flow rate of feed and stripper were fixed at 0.108m^3h^-1 and 0.048m^3h^-1 respectively in counter-current flow with peristaltic pumps (YZ15, CHANGZHOU VCL Fluid Technology Co. Ltd.). The feed solution was filtered by a 0.45 μm microfiltration membrane before entering membrane contactor; in addition, the concentration of solution was measured before and after OD process by HPLC. Due to the colligative properties, saturated vapor pressure is changed compared to pure solvent[19]. Shell side was fed with various concentration of sodium chloride solution which caused different vapor pressure gradient between permeate and stripping side; beside, when vapor passed through membrane, the concentration of stripping solution decreased, and resulting an increase in water activity and corresponding vapor pressure according to the following equation[20]. Vapor pressure of saturated cephradine solution was considered as constant because the concentration remained almost the same during OD process.

ECTFE membrane module was washed by feed solution before starting every experiment. Two independent circuits were applied for feed solution and stripping solution. Every stream was looped back to its container with peristaltic pumps. Saturated cephradine solution was introduced into tube side while permeate side was fed with particular concentration of sodium chloride solution at room temperature. Two streams maintained a total recycle mode. Temperatures of feed and stripping solutions were measured by thermometers. Digital balance was used to measure the change in weight of each solution. Fig. 1 is the outline of the OD unit. The initial volume was 200 ml and 500 ml

$$P_w^S = a_S P_w^0$$

Where P_w^S s water vapor pressure of stripping solution, a_S is water activity in stripping solution, and P_w^0 is vapor pressure of pure water.

of feed solution and stripping solution respectively. Every 30 minutes, the decrease in weight of feed solution and increase in weight of stripping solution were recorded. Each experiment was repeated three times with cleaned membrane.

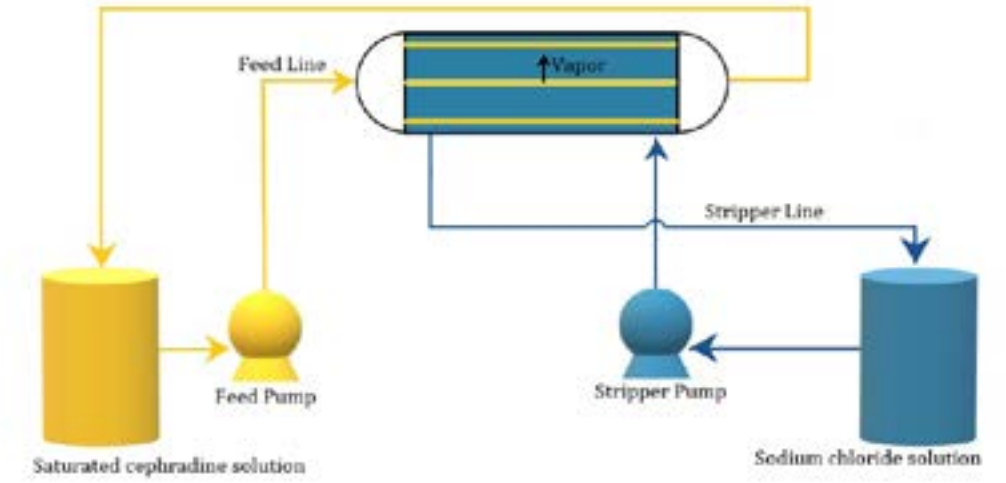


Fig. 1: Flow outline of OD unit with HFM for cephradine solution concentration

Results and Discussion

Solubility of Cephradine and DHPG

To achieve purification of Cephradine product, we measured the solubility of two components in DI water as a function of pH. The data indicates that the pH value has a significant effect on solubility. Fig. 2 shows that cephradine is much more soluble than DHPG. When pH value in the range of pH2.5 to pH7.5 and pH1.2 to pH10.0, the solubilities of cephradine and DHPG maintain similar values respectively, and they increase in more acidic and alkaline conditions. Due to a great difference of solubility between cephradine and DHPG, especially in specific pH value, it is a viable way to separate two components by adjusting pH of solution.

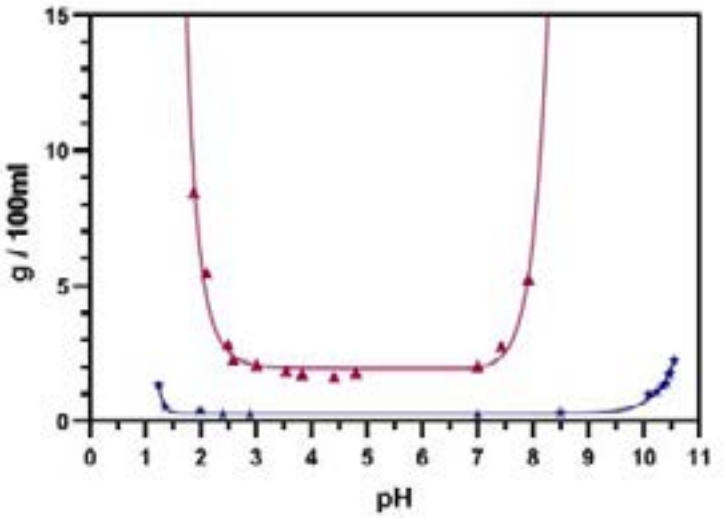


Fig. 2 Solubility of cephradine in 0.5% sodium bisulfate (W/V) solution and DHPG in water as a function of pH at 25 °C. ▲ solubility data of cephradine ★ solubility data of DHPG

Purification via adjusting pH

As Fig. 2 shows, there is a great difference between the solubility of two components below pH2.5 and above pH7.5. Referring to the solubility data, we added excessive crude product (96.25% cephradine, 3.75% DHPG) into the solution, maintained the objective pH value during 90 minutes of experiment. It can be observed in Fig. 3 that the concentraiton of cephradine in solid phase is higher than that in liquid phase in the range of pH2.21 to pH8.15, while it decreases along with more acidic and alkaline conditions. The following result is corresponding to Fig. 2; likewise, solubility of cephradine slightly changes in the section of pH2.5 to pH7.5, and thus the composition also changes slightly in liquid phase and solid phase. However, a phenomenon is contrary to one of the predictions from previous data. According to the result from Fig. 2, we predicted that cephradine might be purified in liquid phase due to its greater solubility compared to DHPG, but the prediction merely takes place under pH2.21 and above pH8.15 in Fig. 3. At other pH values, concentration of cephradine in solid phase is greater than in liquid phase and the original sample, indicating that in this case cephradine more tends to exist as solid form than DHPG, and cephradine was purified a little in solid phase but not in liquid phase during dissolution process. In addition, Fig. 2 demonstrates that pH2.21 and pH8.15 are the intersections which the composition in each phase remains the same as original concentration, and purification cannot be achieved under these conditions. Below and above the intersections, the concentration of cephradine is greatly increased and cephradine is purified in liquid phase. When pH value of solution is equal to pH2.01 or pH8.49, cephradine with a purity of 97.5% can be obtained in liquid phase, which indicted the mass fraction purity was 1.3% higher than original purity. However, after a period of mixing under alkaline conditions, miscellaneous peaks were detected by HPLC, which shows low stability of cephradine due to hydrolyzation of cephradine, and thus cephradine is more appropriate to be purified under acidic condition. Due to the reason, pH2.0 was chosen as the purification condition for further experiments. Significantly, increasing solubility of cephradine starts form pH 2.5 and pH 7.5 in Fig. 2, therefore, an apparent change in concentration of cephradine may be expected at these pH values, however, not only there is no obvious change of concentration in both phases at pH 2.5 and pH 7.5 but the inflected points are at pH2.21 and pH8.15 in Fig. 3. A possible explanation is that the solubility of two components might be affected by each other and causes a small difference between the two results.

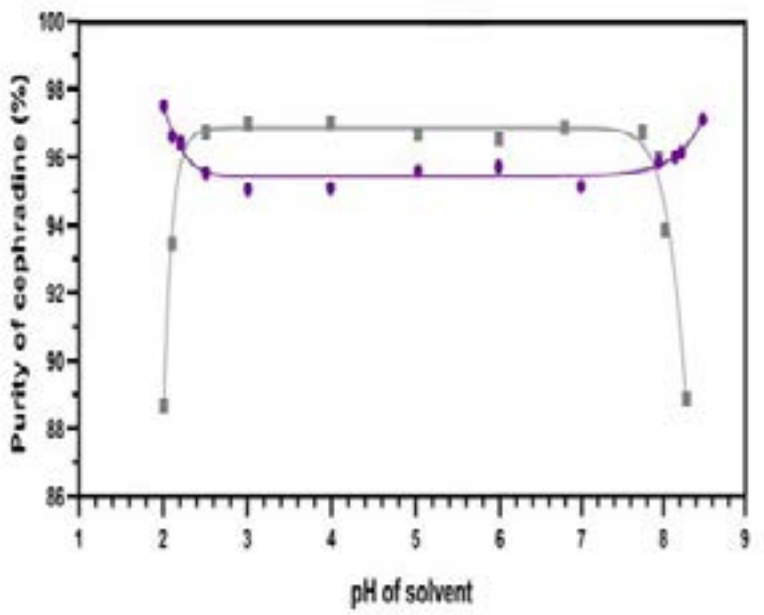


Fig. 3 Effect of pH on the purity of cefradine during purification process based on solubility

● Cephradine in liquid phase ■ Cephradine in solid phase

Purification via adjusting solvent volume

In addition, as shown in Fig. 2, since there is a large difference between the solubility of cefradine and DHPG even in the range of pH4.30 to pH4.60 which is original pH value of solution. The data in Fig. 3 further corroborate that the purification is also achieved without adding diluted hydrochloric acid or diluted ammonia. Therefore, the idea of purification by adjusting the amount of solvent instead of adjusting the pH value was proposed. Excess crude product (96.25% cephradine, 3.75% DHPG) dissolved in various volume of solvent for 120 minutes. Some of crude product solid dissolved in solution while the other remained in solid phase. As depicts in Fig. 4, with the solvent volume between 10ml to 100ml, the purification in solid phase was accomplished. The consequence is surprising, specially when dissolved in 10ml of solvent, with 88.15% and 96.74% of purity of cephradine in liquid phase and solid phase respectively; furthermore, by calculating two concentration in each phase relatively, the maximum separation coefficient (1.15) can be obtained in purification with 10ml of solvent. This data indicates that DHPG more tends to

present in liquid phase than cephradine in this situation, but it seems to be different from solubility data which shows that cephradine is much more soluble than DHPG. Herein, we put forward a reasonable interpretation to describe the discrepancy. According to the solubility data in Fig. 2 without adjusting pH value, the solubility of cephradine and DHPG are 0.180 g and 0.024 g in 10 ml solvent respectively, and the original solubility between two components are in the ratio of 7.5 in DI water. However, an even higher ratio of cephradine to DHPG of 25.7:1 was present in the original mixture. If cephradine and DHPG dissolves into water according to their solubility ratio of 7.5, cephradine will remain in solid phase due to its excessive content. When the volume of solvent as much as to 100ml, most of DHPG is soluble in water, and thus a higher purity of product with purity 98.90% of cephradine can be obtained in solid phase. Based on the data of purification by adjusting the volume of solvent, 100 ml of solvent was chosen for further experiments. Despite a slight increase in purity by dissolving in 10 ml of solvent, the maximum separation coefficient was obtained; besides, more crude product dissolves in liquid phase along with increasing volume of solvent, while solid product can be obtained directly without being concentrated and crystallized in purification with less solvent, and thus 10 ml solvent was chosen for further experiments as well.

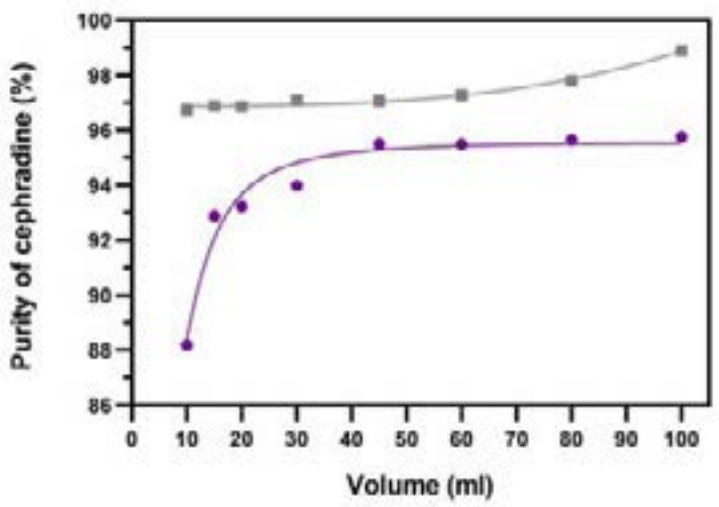


Fig. 4 Effect of volume on the purity of cefradine during purification process based on solubility

Cephradine in liquid phase Cephradine in solid phase

Purification of cephradine with various purity

Herein, cephradine with purity of 96.25% was selected in previous experiments, but cephradine with lower or higher purity might be produced or recycled in industry. Considering other concentrations of cephradine, we further attempted to purify various purity of cephradine based on the previous data. Due to higher amount of cephradine in solid phase, purification with 10 ml of solvent was applied to purify other compositions of crude cephradine products. The simulation crude product which containing cephradine and DHPG was prepared in the laboratory by completely blending two pure compositions in proportion, purity values in the range of 1% to 97% were tested in this study. As Fig. 5 shows, when the initial purity is less than 20%, all of cefradine dissolving in the liquid phase, and thus the purity of DHPG can reach to >99.99% in the solid phase. The concentration of cephradine in solid phase grows linearly along with the increase of initial concentration. As the initial purity reached to 93%, there is an intersection which the concentration of cephradine was the same in each phase, the method was not able to separate the two components under this condition. It is a demarcation point, when the concentration of cephradine is above 93% and the concentration of DHPG is below 7%, DHPG more tends to dissolve in liquid phase, leading a decreased curve of cefradine concentration in the liquid phase. The result indicated that this method can achieve purification of cefradine with various purity of cephradine product when impurity is DHPG, except when the initial purity is 93%. In addition, according to Fig. 5, pure cephradine and DHPG can be obtained in the corresponding phases by performing one or multi-stages of purification process.

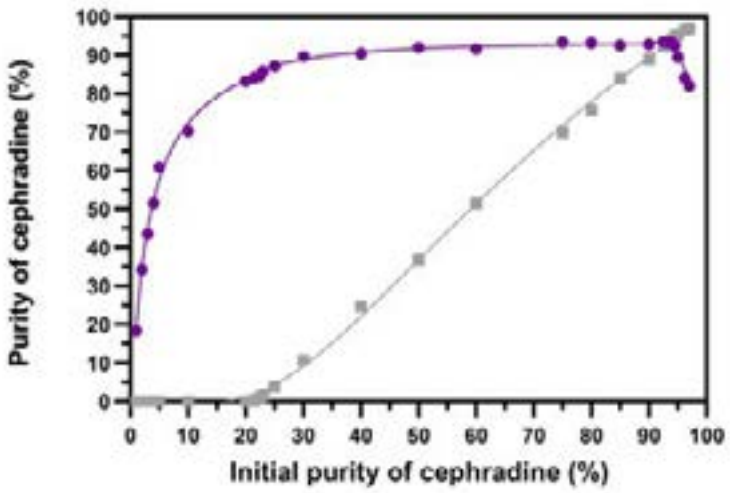


Fig. 5 Correlation results of various initial purity changed in purification process with 10 ml solvent ● Cephradine in liquid phase ■ Cephradine in solid phase

Purification of multi-stage

In order to obtain higher purity products, according to the above purification methods, three conditions were selected for the second stage purification which are same as their first stage. One is to adjust the pH value of solution to pH2.0, and the others are dissolved in the volume of 10ml and 100ml solvent. As Table. 2 shows that higher purity of cephradine products were gained through every chosen method. Corresponding to Fig. 5, as the purity above 93%, higher purity can be obtained indeed in solid phase during purification in the first and second stage, and compared with other methods, obtaining more residual cephradine in solid phase is the advantage of using 10ml of solvent. Purification with 100 ml of solvent, due to a rare amount of DHPG in crude product, 100ml solvent is sufficient to dissolve most of DHPG based on its solubility, and thus the purity of cephradine can reach >99.99% in the solid phase during second stage. Similar to above result, applying pH2.0 solution to purify in second stage purification can get purity 98.07% of cephradine in liquid phase with a higher separation coefficient than the its first stage. Based on the data, multistage of purification process is feasible to reach a higher purity of cephradine. The target purity can be reached along with more stage of purification process. By the repetition of the processes, a high purity cephradine could be prepared successfully.

Condition	Stage	Purity of cephradine	
		Liquid phase	Solid phase
10ml	1	84.18	96.74
	2	94.60	97.02
100ml	1	95.76	98.90
	2	98.62	>99.99
pH2.01	1	97.50	88.68
	2	98.07	83.33

Table 2. Purity of cephadine in first and second stage purification process

Osmatic distillation recovery

With regard to cephradine which was dissolved in liquid phase during multi-stage purification process and cannot be obtained and used directly, OD process with equal temperature on both sides was applied to concentrate and recover available cephradine in the solution. Furthermore, it is able to get solid particle during a longer concentration process. In Fig. 6, either feed solution is DI water or cephradine solution, the higher the concentration of sodium chloride solution on permeate side, the larger reduced of saturated vapor pressure on permeate side will be, hence the flux of OD gradually increased with the concentration on permeate side. Besides, it is indicated that when concentration of permeate side kept the same, with DI water as feed solution, the flux is greater than feeding cephradine solution. The solute decreases the saturated vapor pressure of the cephradine solution, and thus reduce the pressure gradient and driving force between two sides.

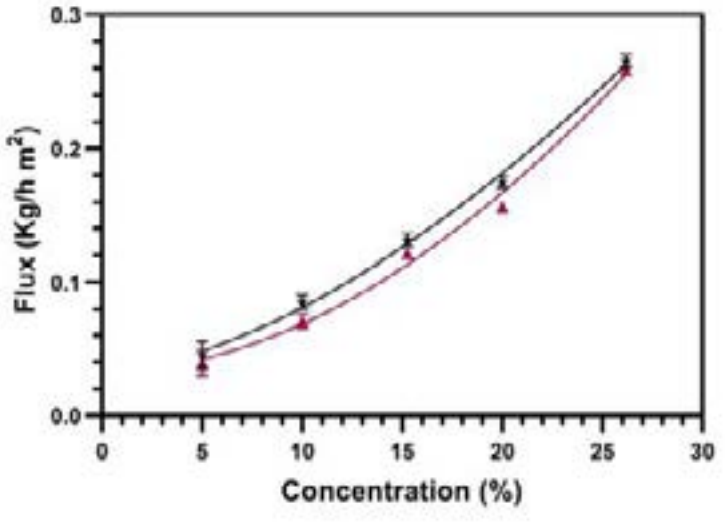


Fig. 6 Effect of stripping solution concentration on flux during osmotic distillation (Ambient temperature, feed flow rate: 0.108m^3h^-1 and 0.048m^3h^-1)

▲ Saturated cephradine solution in tube side ★ DI water in tube side

Conclusion

Within this study, a purification process of enzymatic synthesis cephradine was presented, which takes advantage of a great difference in solubility between cephradine and DHPG. Purification can be achieved in both DI water and acidic solvent. The purity of cephradine increases from origin (96.25%) to 97.02% and >99.99% via second-stage purification with 10 ml and 100 ml DI water respectively: moreover, it reaches to 98.07% through second-stage purification with acidic solvent at pH2.01. To recover cephradine dissolved in solvent during purification, OD process was applied for concentrating saturated solution by ECTFE HFM. The highest flux of 0.258Kgh^-1m^-2 was accomplished under ambient temperature, with feed and stripping solution at flow rate of 0.108m^3h^-1 and 0.048m^3h^-1 respectively and stripping solution of 5.95 M. Recognizing feasibility of the purification is one of the core values, purification can be achieved when purity of cephradine is higher than 93%; while purity is below 93%, the coupling of purification and OD process can recovery available cephradine. Herein, we established an eco-friendly purification and low energy consumption recovery method to make enzymatic synthesis method has potential to be exploited more extensively. Further endeavors are focused on scaling up the process and optimizing crystals collection of cephradine to have more potential in industrial applications.

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Conflict of interest statement

There are no conflicts of interest to declare.

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