

## Extraction of Oxytetracycline Hydrochloride in Aqueous Two-phase System of Acetone and Ammonium Sulfate

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**Summary:** Aqueous two-phase system (ATPS) is an efficient implement for separation of various substrates, and extracted by an aqueous two-phase system has been successfully applied in the downstream processing of various biological compounds. In this research, the extraction of oxytetracycline hydrochloride (OTC·HCl) was carried out in an aqueous two-phase system containing acetone and ammonium sulfate solution, which partitioned the antibiotic to the upper phase. The effects of some parameters on the extraction efficiency of OTC·HCl were studied in detail, including temperature, the volume of acetone, the pH value of ammonium sulfate solution, the concentrations of  $(\text{NH}_4)_2\text{SO}_4$  and OTC·HCl. The results showed that the volume of acetone, the pH value of ammonium sulfate solution and the concentration of OTC·HCl in feed had significant effects on the extraction efficiency of OTC·HCl, but the effects of temperature on the extraction of OTC·HCl was not obvious.

**Keywords:** Oxytetracycline hydrochloride; Acetone; Ammonium sulfate; Aqueous two-phase system; Extraction.

### Introduction

Aqueous two-phase systems (ATPSs) are composed of two aqueous solutions of structurally different components, such as two or more water-soluble polymers [1], a polymer and a salt [2, 3], two surfactants [4, 5], an ionic liquid and a salt [6], or a small molecular water-miscible organic solvent and a salt [7], at a definite concentration. This phenomenon has been firstly reported in the literature by Beijerinck in the 19th century [8]. Albertson discovered [1] that some polymers (polyethylene glycol), electrolytes (potassium phosphate) and water formed a two-phase system above a certain critical concentration. He found that when two water-soluble polymers or a polymer and a strong electrolyte were dissolved in water, a two-phase system was formed, in which one-phase was mainly composed of polymer solution and the other phase was mainly composed of electrolyte solution. Albertson realised the potential use of these systems as an important separation technique in the downstream processing of biomolecules.

Compared with conventional organic solvent extraction or solid phase extraction (SPE), aqueous two-phase extraction (ATPE) has many outstanding advantages, such as low viscosity, simple operation, no need of using traditional volatile solvents, rapid phase separation, high extraction efficiency, and friendly to both environment and operator. And nowadays ATPE has become an important emerging

technique used in separation and purification of biological compounds such as DNA [9], proteins [10], enzymes [11] and antibiotics [12]. Despite these advantages, the applications of the polymer, surfactant or ionic liquid-based ATPSs have not found extensive industrial application. One of the reasons is that it's difficult to recover the phase-forming polymers or ionic liquid from the aqueous phase containing extract. As acetone is volatile, it needn't back extraction for the recovery of acetone. In this research, we try to use water-miscible acetone instead of polymer or ionic liquid to form ATPS with  $(\text{NH}_4)_2\text{SO}_4$  solution and to extract OTC·HCl in aqueous solution.

In this paper, the extraction efficiency of OTC·HCl in the acetone– $(\text{NH}_4)_2\text{SO}_4$  ATPS has been investigated. OTC·HCl ( $\text{C}_{22}\text{H}_{24}\text{N}_2\text{O}_9\cdot\text{HCl}$ ) is a broad-spectrum antibiotic which belongs to the tetracycline family. The molecular structure of OTC·HCl is shown in Fig. 1. It is clinically used to treat the infections caused by Gram-positive and Gram-negative bacteria. As far as we know, there is no report on partitioning of OTC·HCl using acetone– $(\text{NH}_4)_2\text{SO}_4$  ATPS. The effects of such parameters as OTC·HCl concentration, temperature, volume of acetone,  $(\text{NH}_4)_2\text{SO}_4$  concentration and the pH of ammonium sulfate solution on the extraction efficiency were studied in detail.

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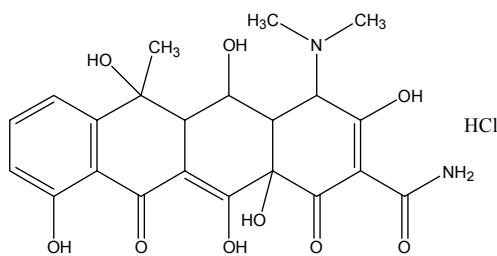


Fig. 1: Structure of OTC·HCl.

## Results and Discussion

### Phase Diagram

The phase diagram of the acetone (1) + ammonium sulfate (2) + water (3) system at 298.15K and 283.15K was shown in Fig. 2. OTC·HCl was easier denatured in alkaline solution, so an acid salt was chose as the phase-separation salt. Ammonium sulfate was an acid salt and could form an ATPS with acetone solution at a definite concentration. In Fig. 2 the region L denote the liquid phase. In the region L+ L, two liquid phases, an upper phase rich in acetone and a lower phase rich in (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, are in equilibrium. The following experiments were carried out in the 2L region. In this region we optimized the extraction efficiency of OTC·HCl by changing the ratio of salt and acetone.

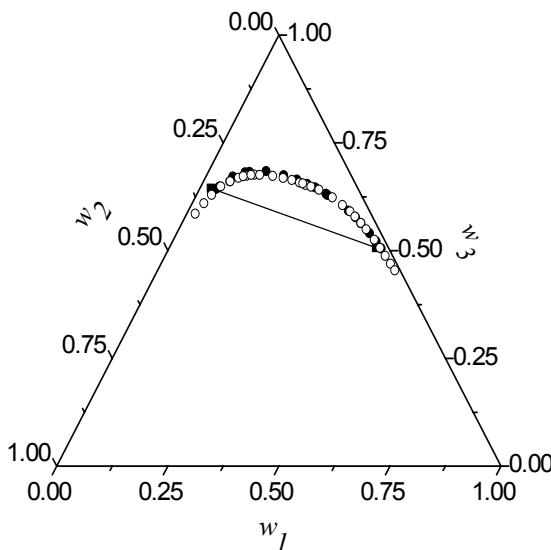
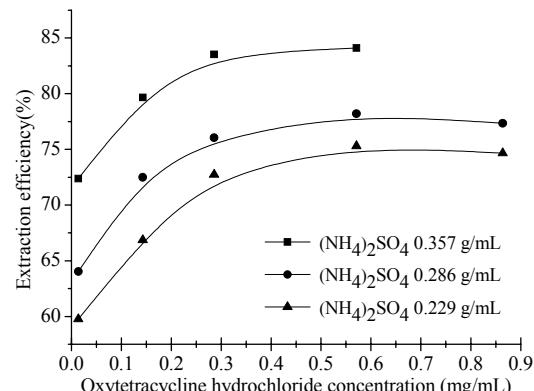


Fig. 2: Binodal curve for the acetone (1) + ammonium sulfate (2) + water (3) system at 298.15K and 83.15K.

### Effect of OTC·HCl Concentration on the Extraction Efficiency

The effect of OTC·HCl concentration on the extraction efficiency in the acetone-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> ATPS at different salt concentration were studied in this part. It was found from Fig. 3 that in the acetone-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> ATPS, when 0.357 g/mL, 0.286 g/mL or 0.229 g/mL (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 3 mL acetone were used, the extraction efficiency of OTC·HCl correspondingly changed with the increase of the OTC·HCl concentration. When the salt concentration of 0.357 g/mL, the extraction efficiency of OTC·HCl increased from 72.4 to 84.1%, with the increase of the concentration of OTC·HCl from 0.014 to 0.571 g/mL. The result also showed that the tendency of the extraction efficiency of OTC·HCl as a function of OTC·HCl concentration was same at the different salt concentration in the acetone-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> ATPS.

Fig. 3: Effect of concentration of OTC·HCl on the extraction efficiency: (■) (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.357 g/mL; (●) (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.286 g/mL; (▲) (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.229 g/mL.

From Fig. 4 it can be seen that the volume of the upper phase was basically unchanged with the different concentration of OTC·HCl added into the ATPS. So the concentration of OTC·HCl had little influence on the compositions of the ATPS. When the components of the ATPS was unchanged, the solubility of OTC·HCl in the upper and lower phases were certain. But the solubility of OTC·HCl in the lower phase became very small in the lower phase with a high salt content. Before the antibiotic reach the saturation solubility in the upper, the extraction efficiency increased with the increase of concentration of OTC·HCl. And when the antibiotic

reach the saturation solubility in the upper and lower phases, OTC·HCl precipitated at the interface. This caused the decrease of proportion of OTC·HCl in upper phase above a definite concentration.

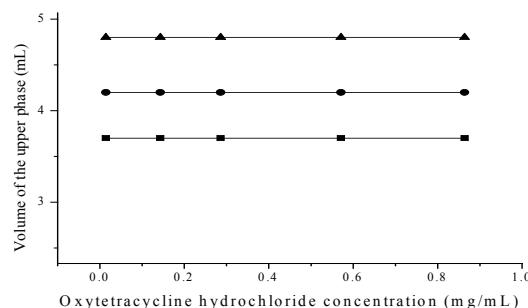


Fig. 4: Effect of the concentration of OTC·HCl on the volume of the upper phase: ■, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.357 g/mL; ●, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.286 g/mL; ▲, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.229 g/mL.

#### Effect of Temperature on the Extraction Efficiency

Fig. 5 illustrated the extraction efficiencies of OTC·HCl in the acetone-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> ATPS at two different salt concentration of 0.457 g/mL and 0.357 g/mL. The extraction was carried out over a temperature range of 283.15~308.15K. As the result shown in the Fig. 5, when 0.457 g/mL and 0.357 g/mL (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.571 mg·mL<sup>-1</sup> OTC·HCl solution and 3 mL acetone were used, the extraction efficiency of OTC·HCl changed a little with the increase of the temperature. It is obvious that when the temperature below the room temperature, the extraction efficiency increases along with the temperature ascension. With the increased of temperature, the binodal curve moved up and the two-phase region below the binodal curve become larger. The change in the extraction efficiency of the OTC·HCl with temperature may be attributed to this variation in the phase compositions. While the temperatures above the room temperature, the extraction efficiency decreased along with the temperature ascension. This probably because of the nature of OTC·HCl, higher temperature could speed up the degradation of OTC·HCl.

The results indicates that temperature had little influence on the distribution behavior of steroids. Thus, this method provides a relatively wide range of temperature for the study on the extraction efficiencies of OTC·HCl. The following studies were carried out at room temperature.

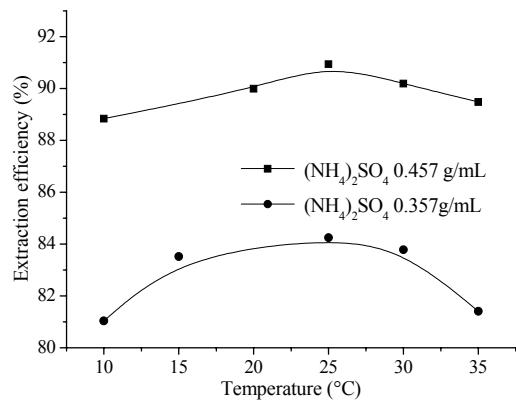


Fig. 5: Effect of temperature on extraction efficiency: (■) (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.457g/mL; (●) (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.357g/mL.

#### Effect of the Volume of Acetone on the Extraction Efficiency

The volume of acetone had an important influence on the extraction efficiency of OTC·HCl. Fig. 6 showed the influence of the amount of acetone on the extraction efficiencies of OTC·HCl. Herein, 5.00 mL 0.357 g·mL<sup>-1</sup>, 0.286 g/mL and 0.229 g/mL (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> solution and 0.571 mg·mL<sup>-1</sup> OTC·HCl solution were used for study. As show in Fig. 6, with the increase of the volume of acetone, the extraction efficiency of OTC·HCl increased obviously. The extraction efficiency of OTC·HCl was urgently increased with the increase of volume of acetone from 1 mL to 2.5 mL, When the volume over 2.5 mL, the effect of volume was inconspicuous. In the acetone-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> ATPS at the salt concentration of 0.357 g/mL, when the increase of volume of acetone from 1 mL to 4 mL, the extraction efficiency of OTC·HCl increased from 36.5 to 88.4%.

In the acetone-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> ATPS, acetone not only was used as the extractant, but also acted as a solvent. When this ATPS was chose to extract OTC·HCl, the chief use of acetone was acted as solvent. Within lower concentration of acetone, the concentration of OTC·HCl dissolved in the upper phase increased with the volume of acetone. While the salting-out ability of salt in a certain critical concentration, there was a maximum of the concentration of OTC·HCl that can be extracted in the upper phase. So when the salting-out ability of salt reach the maximum value, the change of the extraction efficiency trended toward stable.

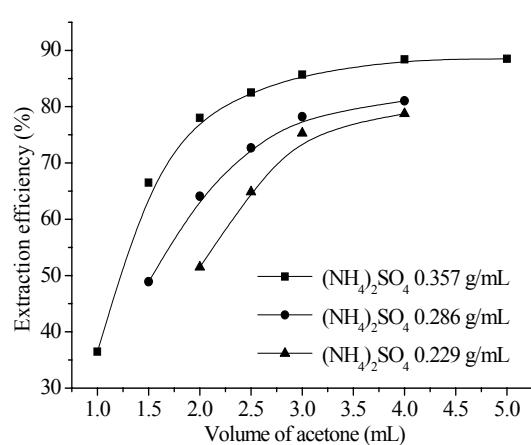


Fig. 6: Effect of the volume of acetone on the extraction efficiency: (■)  $(\text{NH}_4)_2\text{SO}_4$  0.357 g/mL; (●)  $(\text{NH}_4)_2\text{SO}_4$  0.286 g/mL; (▲)  $(\text{NH}_4)_2\text{SO}_4$  0.229 g/mL.

#### Effect of Salt Concentration on the Extraction Efficiency

Fig. 7 showed the effect of salt concentration on the extraction efficiency of OTC-HCl at 283.15K and 298.15K. As shown in Fig. 7 the results indicate that extraction efficiency changed with the concentration change of the salt: when the concentration of salt was not too high, the extraction efficiency significantly increased with the increase of the salt concentration. But when the concentration closes to the saturation of  $(\text{NH}_4)_2\text{SO}_4$ , the extraction efficiency remains the same.

By the hydration theory [13], two-phase formation occurred with the sulfate added which had a marked salting-out effect on non-electrolytes. The formation of the two phases in acetone-salt system is due to the “salting-out” effect of salt, in other words, this is due to the preferential orientation of water molecules around salt ions. So when  $(\text{NH}_4)_2\text{SO}_4$  was added to the aqueous solution of OTC-HCl, the solubility of OTC-HCl in the lower phase was reduced because of the effective removal of water molecules, and the extraction efficiency was increasing rapidly with the concentration of the salt. When the concentration closes to the saturation of  $(\text{NH}_4)_2\text{SO}_4$ , the salt was salted out, and the concentrations of  $(\text{NH}_4)_2\text{SO}_4$  in both upper and lower phases remain the same, and the change of the extraction efficiency trended toward stable.

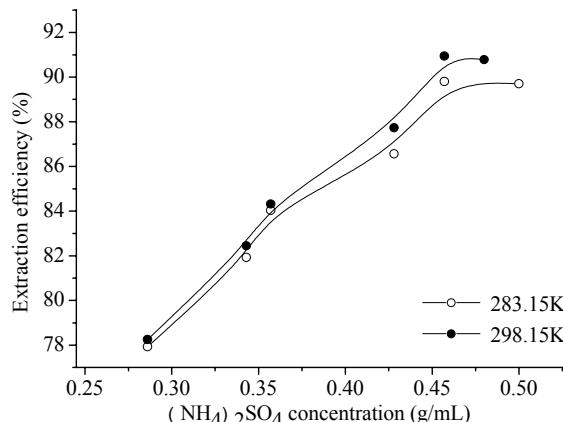


Fig. 7: Effect of  $(\text{NH}_4)_2\text{SO}_4$  concentration on the extraction efficiency: (●) 283.15K; (○) 298.15K.

#### Effect of the pH value on the extraction efficiency

Fig. 8 showed the changes of extraction efficiency of OTC-HCl in the ATPSs include different salt concentration at different pH values. In this part 5.00 mL 0.286 g/mL, 0.357 g·mL<sup>-1</sup> and 0.429 g/mL  $(\text{NH}_4)_2\text{SO}_4$  solution and 0.571 mg·mL<sup>-1</sup> OTC-HCl solution were used, and the influence of the pH value on the extraction efficiencies of OTC-HCl was investigated. The results indicate that the minimum extraction efficiency appears at pH about 5.4, and higher extraction efficiency was observed when pH was greater or less than 5.4. Because of the nature of OTC-HCl, higher pH value of the solution could speed up the degradation of OTC-HCl, especially in alkaline solution. Studies on the extraction of OTC-HCl were carried out in acid solution.

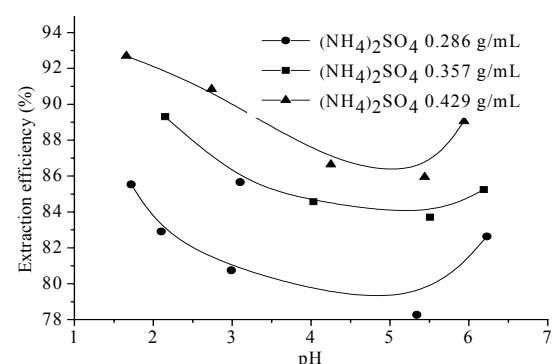


Fig. 8: Effect of pH on the extraction efficiency: (▲)  $(\text{NH}_4)_2\text{SO}_4$  0.429 g/mL; (■)  $(\text{NH}_4)_2\text{SO}_4$  0.357 g/mL; (●)  $(\text{NH}_4)_2\text{SO}_4$  0.286 g/mL.

From Fig. 9 we can see the volume of the upper phase had not an obvious change with the different pH values. So within this range the pH value, pH had little influence on the ATP system. The result in Fig. 9 showed that the effect of the pH value on the extraction efficiency may be mediated by changing the OTC·HCl surface charge and the solubility in the water. The isoelectric point (pI) of four tetracycline antibiotics was about 5.4, and around this pH the solubility of OTC·HCl was lowest. When the pH of the aqueous solution was below the pI, the charge of OTC·HCl became positive, and OTC·HCl mainly existed in its cation form in the smaller pH, while when the pH of the system was above the isoelectric point, the charge of OTC·HCl became negative. The studying result indicated that the change of the charge of OTC·HCl was helpful to extracting OTC·HCl from the lower phase to the upper phase.

## Experimental

### Material

All reagents used were of analytical grade. Oxytetracycline hydrochloride was obtained from the Beijing Meng Yi Mei Co. Ltd. (Beijing, China). Acetone and ammonium sulfate were obtained from the Sinopharm Chemical Reagent Co. Ltd. (Beijing, China).

### Determination of the Phase Diagram of ATPS

The phase diagram for the acetone– $(\text{NH}_4)_2\text{SO}_4$ –water ATPS was determined by the cloud point method at 298.15K. Simply, the acetone was added drop wise into an aqueous solution containing preweighted  $(\text{NH}_4)_2\text{SO}_4$  until the clear solution turned turbid. The concentration of acetone was recorded. The compositions of the mixture were determined by a Sartorius analytical balance (model BS 124S) with a precision of 0.0001 g. The concentration of acetone was recorded. More water was added to get a clear one-phase system and then the mixture was weighed. More acetone was added again to form a two-phase system. The procedure was repeated until a sufficient number of points for the construction of the binodal curve were obtained.

### Extraction of Oxytetracycline Hydrochloride in ATPS

A certain volume of acetone was added into 10 mL aqueous solution containing certain amount of OTC·HCl and  $(\text{NH}_4)_2\text{SO}_4$ . The mixture was stirred well and centrifuged at 1000 rpm for 10 min at room

temperature to facilitate phase separation, and then the mixture was performed at 298.15K in constant water bath for 2 hours. The upper phase was rich in acetone solution, while the lower phase was rich in salt solution. The volumes of the two phases were recorded. The partition behaviors of OTC·HCl were described by the extraction efficiency ( $E$ , %) (defined by  $E = V_u c_u / m_s$ , where  $m_s$  is the amount of the antibiotic added,  $c_u$  is the concentration of the antibiotic in the upper phase).

### Determination of Antibiotic by UV Absorption

The ATPS was prepared as described previously. A certain volume of the upper phase (containing acetone and the antibiotic mainly) was transferred with a syringe into a bottle, and then the contents were diluted to the mark by deionized water.

The determination of OTC·HCl, was carried out on a UV-vis spectrophotometer (SHIMADZU UV-2450). The wavelengths used for quantitative analysis was 354 nm for OTC·HCl, and no clear spectral interference was observed under the optimum wavelength of analytical line when acetone and  $(\text{NH}_4)_2\text{SO}_4$  are present.

The UV spectral characteristics of OTC·HCl were investigated at various concentrations from 0.8 to 100  $\mu\text{g}/\text{mL}$ . Each standard was analyzed twice, and the standard curve was shown in Fig. 10. Excellent correlation coefficient of 0.99997 was achieved which proves the good accuracy of the following equation.

$$A = 0.027C + 0.026 \quad (1)$$

where  $C$  is the concentration of OTC·HCl ( $\mu\text{g}/\text{mL}$ ) and  $A$  is the absorbance

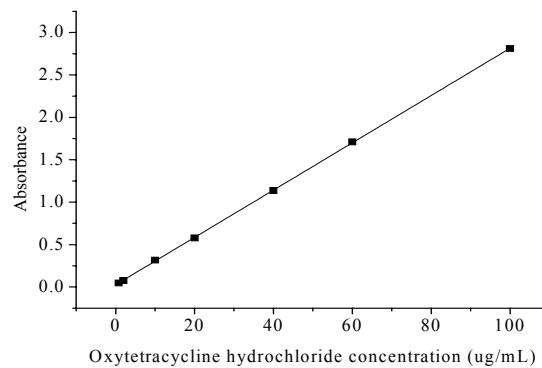


Fig. 10: The standard curve: the UV absorbance of OTC·HCl at the various concentrations at 354 nm

## Conclusion

The proposed extraction technique based on ATPS of acetone and  $(\text{NH}_4)_2\text{SO}_4$  was demonstrated to be an excellent strategy for the extraction of OTC-HCl. Compared with traditional LLE or SPE, the method is simpler, more rapid, higher in extraction efficiencies in one-step extraction, and more environmentally friendly due to no use of toxic organic solvent and low consumption of extraction solvent because the dominating component in both phases was water. In this research, the extraction of OTC-HCl was studied, and the parameters of OTC-HCl concentration, temperature, volume of acetone,  $(\text{NH}_4)_2\text{SO}_4$  concentration and pH on the extraction efficiency were analyzed. The result shows that  $(\text{NH}_4)_2\text{SO}_4$  concentration and pH in feed had moderate effects on the extraction efficiency. The results also indicated that temperature and pH of ammonium sulfate solution had no significant effect on the antibiotic partitioning. The pH value on the extraction efficiency may be mediated by changing the OTC-HCl surface charge. The salt is not only participating in the formation of ATP, but also change the partition of OTC-HCl in the upper and lower phases. It is the dominant driving force of transfer between upper and lower phases.

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