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Stability of clavulanic acid in PEG/citrate and liquid–liquid extraction in aqueous two-phase system

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ABSTRACT

β-Lactamases are enzymes responsible for the hydrolysis of β-lactam antibiotics, being produced by several pathogenic bacteria. Clavulanic acid is a commercially and clinically important β-lactamase inhibitor, its extraction being possible by the application of aqueous two-phase system. In this study, clavulanic acid stability was investigated at different molar mass PEG (400, 1 000 and 20 000 g mol^{-1}) and at different citrate concentrations (5 and 20%) PEG/citrate aqueous-two phase systems (ATPS), under different pH values (4.0–8.0). Clavulanic acid extraction was also evaluated. Low citrate concentration and PEG 20 000 (g mol^{-1}) ATPS, at pH 6.0, were shown to be the systems that presented the highest clavulanic acid stability. Based on this, a factorial design 2^2 was used to evaluate CA extraction, being PEG (20, 25 and 30%) and citrate (5, 10 and 15%) concentrations the parameters evaluated. Clavulanic acid was extracted into the PEG-rich phase (133.66 mg L^{-1}), the highest values of the partition coefficient and yield being $K = 5.92$, $Y = 103.53\%$, respectively The ATPS was not only effective on clavulanic acid extraction, but also its degradation was minimal. These results clearly indicate that ATPS can be successfully applied as a first step for the purification of clavulanic acid.

1. Introduction

The use of antibiotics to control infectious diseases is greatly hindered by bacterial resistance. One of the most important resistance mechanisms exhibited by a variety of Gram-positive and Gram-negative bacteria is their ability to produce β-lactamases, as β-lactamases hydrolyse the β-lactam ring inactivating penicillins and cephalosporins [1]. The discovery of clavulanic acid (CA) and its application as a β-lactamase inhibitor in the presence of antibiotics proved to be an effective approach to face antibiotic resistant bacteria [2].

CA is a mechanism-based inhibitor because it is recognized as a substrate by β-lactamase. CA binds covalently to the enzyme producing chemical modifications (rearrangements) that change the enzyme structure in an irreversible way [3]. CA is a potent antibiotic inhibitor of "serine" (or classes A, C, and D) β-lactamases. It is used in conjunction with amoxicillin in a salt (potassium clavulanate) form and prescribed clinically as co-amoxiclav (Augmentin™ in USA, Clavulin™ in Brazil) or with ticarcillin (Timentin™) [4].

The CA molecule is chemically unstable, due to the susceptibility of the carbonyl group linked to the beta-lactam ring, which can, in the presence of water, suffer an acidic (H+)- or alkaline (OH−)-catalyzed attack. This property demands, the development of efficient and cost-effective separation and purification processes that allow for the obtention of CA with the required high quality standards of quality for market approval [5].

Among the several techniques available for the purification of CA, aqueous two-phase systems (ATPS) extraction and purification has been studied [5–7]. Aqueous two-phase systems are formed when two hydrophilic components are mixed with water and they are above a threshold concentration [8]. These solutes may be two polymers or a polymer and a salt. The ATPS are a convenient and appropriate method for the extraction of biological substances due to the high water content (between 70% and 90%) in both phases. This provides a pleasant environment to work with biologically active compounds because it preserves its molecular stability [9].

The use of CA in the pharmaceutical industry is increasing, consequently studies addressing the effect of pH, temperature, types of salts and their concentrations on CA stability are of particular

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Nomenclature

ATPS	aqueous two phase system
CA	clavulanic acid
PEG	polyethylene glycol
k	degradation rate constants (h^{-1})
K	partition coefficient
Y	yield (%)
MB	mass balance (%)
C_T	clavulanic acid concentrations at the top phase ($mg\,L^{-1}$)
C_B	clavulanic acid concentrations at the bottom phase ($mg\,L^{-1}$)
C_TV_T	clavulanic acid mass in top phase (mg)
C_BV_B	clavulanic acid mass in bottom phase (mg)
C_iV_i	initial clavulanic acid mass (mg)
C_{PEG}	PEG concentration
C_{CIT}	citrate concentration

importance [10]. Several research-groups have conducted studies on the stability of the CA from various sources in aqueous solutions under different conditions. Generally, CA stability in aqueous solutions is better at pH between 6 and 7 and at temperatures in the range 20–30 °C [5,7,11,12].

The main aim of this study was the evaluation of the stability of CA in PEG/citrate ATPS at different concentrations and pH values. In addition, the influence of system composition on CA was characterized.

2. Materials and methods

2.1. Materials

Potassium salt of CA used for all the solutions and the Imidazole used in CA determination were provided by Sigma–Aldrich (St. Louis, MO, USA) with 99% purity. Ultra-pure polyethylene glycols (PEG) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All the other reagents were of analytical grade.

2.2. Clavulanic acid stability in PEG and citrate salt solutions

To evaluate CA the effect of PEG molecular weight in CA stability, 25% (w/v) solutions for the several molecular weight PEG considered (400, 10 000 and 20 000 $g\,mol^{-1}$) were prepared in McIlvaine buffer at different volumes for pH values at 4.0–8.0. CA stability in citrate salts was done by preparing solutions of citrate salts at 5 and 25% (w/v) using different concentrations of sodium citrate and citric acid for pH values ranging from 4.0 to 8.0.

CA stability experiments were done by adding a concentrate CA stock solution (300 $mg\,L^{-1}$), reaching an initial CA concentration of 30 $mg\,L^{-1}$, to test tubes containing 50 mL of PEG solution or citrate salts solutions. The solutions were then homogenized and aliquots of the solutions were withdrawn during a 24 h period at time intervals of 15 min in the first hours, and then at three hours intervals. The concentration of non-degraded CA was determined. According to Bersanetti et al. [12], CA degradation in aqueous solutions follows a pseudo-first-order kinetics according to Eq. (1), where k_2 is the second order degradation rate constant and C is CA concentration. After integration (Eq. (2)), the degradation rate constant (k_2), can be estimated by linear regression of the experimental data.

$$\frac{-dC}{dt} = k_2 C \tag{1}$$

Table 1
Levels of independent variables in the 2^2 factorial design for CA extraction in aqueous two-phase system (ATPS) PEG/citrate.

Variables	Levels		
	−1	0	+1
PEG concentration (%)	20.0	25.0	30.0
Citrate concentration (%)	5.0	10.0	15.0

$$-\ln\frac{C}{C_0} = k_2 t \tag{2}$$

2.3. Clavulanic acid extraction in aqueous two-phase system (ATPS) PEG/citrate

A concentrated citrate solution (40% w/w) was prepared by mixing appropriate amounts of sodium citrate ($Na_3C_6H_6O_7{\cdot}2H_2O$) and citric acid ($C_6H_8O_7$) at pH 6.0. The required amount of this solution was mixed with a PEG 20 000 $g\,mol^{-1}$ solution in 15 mL graduated tubes with conical tips to achieve the desired composition system. PEG 20 000 concentrations and citrate salt in each system varied according to the factorial design 2^2 shown in Table 1. The systems (10 g) were loaded with 2 g of the CA stock solution with initial concentration of 500 $mg\,L^{-1}$. After mixing for 1 min in a vortex, the mixture was allowed to settle for 60 min at 20 °C, for phase separation. The phase volumes were measured, and each phase was separately withdrawn using pipettes and used for determining the CA concentration in each phase. The response variables were the partition coefficient (K) and yield (Y) on top and bottom phases. After all experiments, statistical analyzes was conducted using Statistica 8.0 software (Statsoft, Inc., Tulsa-OK, USA) [13].

2.4. Clavulanic acid assay

Bird et al. [14] described the spectrophotometric assay by reaction with imidazole, selected in this study. According to this method, the CA concentration is determined by measuring the amount of [1-(8-hydroxy-6-oxo-4-azooct-2-enol)-imidazole] at 312 nm. This product is released by the reaction between CA and imidazole. To determine the CA concentration, a calibration curve from standard stock solutions of potassium clavulanate was used.

2.5. Calculation of partition coefficient (K), yield (Y) and mass balance (MB)

The CA distribution between phases was expressed in terms of the partition coefficient (K), calculated as follows:

$$K = \frac{C_T}{C_B} \tag{3}$$

where C_T and C_B are CA concentrations (mg/L) at the top and bottom phases, respectively.

To evaluate the efficiency of extraction of CA, the yield (Y) for each phase was calculated according to:

$$Y_x = \left(\frac{C_xV_x}{C_iV_i}\right) \times 100 \quad \text{for } x = T \text{ or } B \tag{4}$$

where C_xV_x is the mass of CA (mg) in the top (T) and bottom (B) phase volume, respectively. The C_iV_i is the initial mass of CA (mg).

The mass balance (MB) for CA is:

$$MB = \left(\frac{C_TV_T + C_BV_B}{C_iV_i}\right) \times 100 \tag{5}$$

where C_TV_T and C_BV_B is the mass of CA in top phase (T) end bottom phase (B), respectively, and the C_iV_i is the initial mass of CA (mg).