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Liquid–liquid extraction of clavulanic acid using an aqueous two-phase system of polyethylene glycol and potassium phosphate

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Abstract

An aqueous two-phase system of polyethylene glycol (PEG) and potassium phosphate was developed for *in situ* extraction of clavulanic acid from fermentation broth. Preliminary studies to identify the relevant parameters that determine the partitioning of clavulanic acid in the two-phase system were carried out using potassium clavulanate. The effect of pH, polymer molecular mass and tie-line length were investigated. For all conditions tested potassium clavulanate showed a high affinity to the PEG-rich top phase ($K = 1.5–114$), and high recoveries (90–99%) were obtained.

1. Introduction

Clavulanic acid is a naturally occurring compound isolated from *Streptomyces clavuligerus* [1], and it consists of a β-lactam ring fused to an oxozilidine ring [2]. It shows weak antibacterial activity against most bacteria, but is a potent inhibitor of a wide range of β-lactamase and is able to potentiate the antibacterial activity of penicillins and cephalosporins against many β-lactamase-producing resistant bacteria [3]. It is currently used in combination with amoxicillin for the treatment of infections caused by β-lactamase-producing bacteria.

Clavulanic acid is produced industrially by fermentation and is isolated and purified from the fermentation medium in several steps. The first step involves clarification of the medium by filtration or centrifugation followed by either adsorption or liquid–liquid extraction with an organic solvent, normally butanol. Further purification is achieved by anion-exchange chromatography. Owing to the unstable nature of the free acid, clavulanic acid is isolated as the lithium, potassium or sodium salt.

In this work, an aqueous two-phase system of polyethylene glycol (PEG) and potassium phosphate was developed as an alternative process to be used as the first step in the isolation and purification of clavulanic acid, in order to decrease the number of downstream processing steps. Potassium clavulanate was used as a model system for the partitioning studies in PEG–potassium phosphate two-phase systems. Some relevant parameters, such as pH, polymer molecular mass and polymer and potassium salt concentrations, which affect the partitioning behaviour of potassium clavulanate in PEG–salt phases were investigated.

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2. Experimental

2.1. Chemicals

PEG 400, 1000, 4000 and 6000 were supplied by Sigma. Potassium phosphates (analytical-reagent grade) and imidazole (99% purity) were obtained from Merck (Darmstadt, Germany). Potassium clavulanate (70% purity) was a kind gift from CIPAN (Lisbon, Portugal).

2.2. Preparation of aqueous two-phase systems

Phase systems of 5 g each were prepared in 10-ml graduated centrifuge tubes by weighing the PEG (100%, w/w) and by addition of appropriate amounts of KH_2PO_4–K_2HPO_4 solutions at pH 7.0 (22.9%, w/v) and pH 8.0 (32.7%, w/v). This mixture was vortex mixed for 5–30 min until a two-phase system was obtained. The system was completed by addition of 50 μl of potassium clavulanate stock solution (5 mg/ml) with agitation. The phases were separated by centrifugation at 1000 *g* for 5 min. After phase separation the phase volumes were noted and potassium clavulanate was determined, in both phases, by the imidazole assay method, as described below. The assays were performed in triplicate.

The study of potassium clavulanate partitioning in PEG–KH_2PO_4–K_2HPO_4 systems was carried out at pH 7.0 and 8.0 with PEG 400, 1000, 4000 and 6000 and for different tie-line lengths by varying the PEG and salt concentrations. The tie-line lengths were measured directly from the phase diagram.

2.3. Potassium clavulanate assay

Potassium clavulanate in PEG and phosphate phases was determined spectrophotometrically at 312 nm by analysis of the reaction product with imidazole reagent [4].

Imidazole reagent was prepared by dissolving 8.25 g of imidazole in 100 ml of distilled water, and the pH was adjusted to 6.8 ± 0.05 with 5 *M* hydrochloric acid. A 1-ml volume of top/bottom phase was added to a tube containing 5 ml of imidazole reagent (tube A). This mixture was incubated in a water-bath at 30°C for 12 min and then cooled rapidly to 20°C. To a second tube (tube B), 1 ml of top/bottom phase was mixed with 5 ml of distilled water. The absorbances at 312 nm of the solutions in tubes A and B were measured using as a reference a mixture of 1 ml of top/bottom phase and 5 ml of imidazole reagent heated at 30°C for 12 min. The absorbance difference between solutions A and B was calculated and the clavulanate concentration of the samples was determined from a calibration graph obtained from a sample of known purity reacted under the same conditions for the same time. Interferences from PEG molecular mass and high PEG and salt concentrations in the potassium clavulanate assay method were checked; none were observed.

3. Results and discussion

The effect of PEG molecular mass, tie-line length and pH on potassium clavulanate partitioning in the PEG–phosphate potassium two-phase system is shown in Figs. 1 and 2. *K* is the partition coefficient of potassium clavulanate, defined as the ratio between potassium clavula-

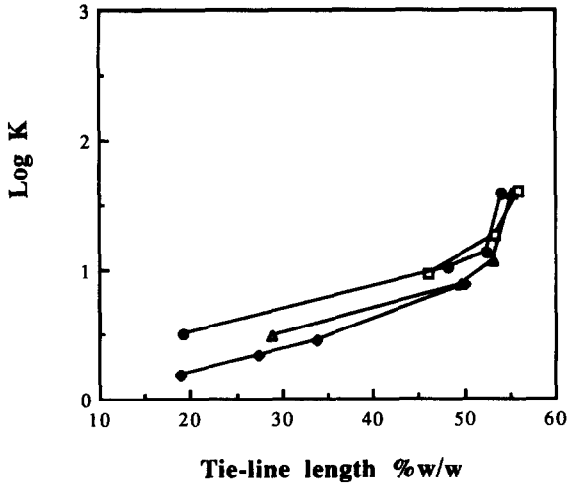

Fig. 1. Variation of the partitioning of potassium clavulanate with tie-line length at pH 7.0 for different PEG molecular masses: ◆ = PEG 6000; △ = PEG 4000; ● = PEG 1000; □ = PEG 400.

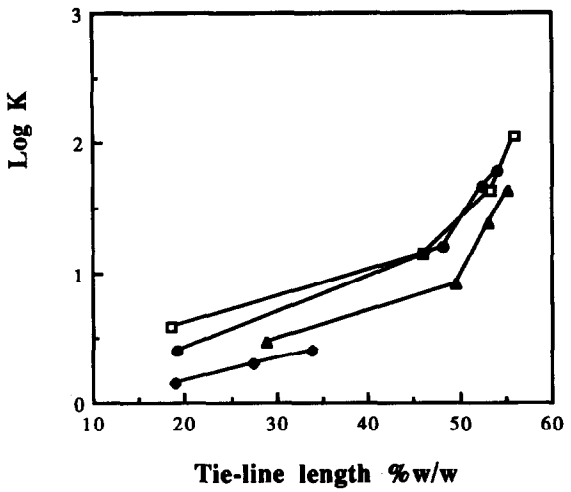

Fig. 2. Variation of the partitioning of potassium clavulanate with tie-line length at pH 8.0 for different PEG molecular masses: ◆ = PEG 6000; △ = PEG 4000; ● = PEG 1000; □ = PEG 400.

nate concentrations in the top and bottom phases. The reported values are the average of three measurements.

The results in Figs. 1 and 2 show that potassium clavulanate has a higher affinity for the PEG phase, as partition coefficients varying from 1.5 to 114 were obtained for the different PEG molecular masses, with short and long tie-lines, and at pH 7.0 and 8.0. High recoveries from 60 to 99% were also observed. The potassium clavulanate recovery, Y, was calculated as the fraction of potassium clavulanate recovered in the PEG phase.

The partition coefficients observed are higher than theoretically expected for small molecules such as clavulanic acid [5]. However, partitioning of penicillin G in PEG–potassium salt systems also showed high partition coefficients (above 10) [6]. Degradation of cholesterol by *Micobacterium* sp. in aqueous two-phase systems has been reported [7] and the substrate partitioned to the upper phase or the interface, while the products had partition coefficients of *ca.* 2. Deacylation of benzylpenicillin (BP) to 6-aminopenicillanic acid (6-APA) using penicillin acylase in a PEG–potassium phosphate system showed partition coefficients of $K_{BP} = 8.3$, for the substrate and $K_{6\text{-APA}} = 1.35$ and $K_{\text{phenylacetic acid}} = 1.7$ for the products [8].

Potassium clavulanate has a great affinity for the PEG-rich phase, leading to high partition coefficients. However, at pH 7.0 and 8.0, clavulanic acid ($pK_a = 2.3$–2.7) is mostly present in the ionic form but is extracted into the more hydrophobic top phase.

The partitioning of potassium clavulanate in the PEG–potassium phosphate system is not greatly influenced by variations in the molecular mass of PEG, for low polymer molecular masses. Similar partition coefficients and recoveries were obtained for PEG 1000 and 400, for a fixed tie-line, at pH 7.0 and 8.0. For higher polymer molecular masses (PEG 6000 and 4000), a decrease in the partition coefficients with increasing polymer molecular mass was observed.

The effect of polymer molecular mass can be attributed to the increasing number of hydrophilic end-groups on shorter PEG chains, which decreases the overall hydrophobicity [9], and to the excluded volume effects which increase with increasing polymer molecular mass [10]. As clavulanic acid is a small molecule it probably is less influenced by the excluded volume effects.

Increasing the tie-line length raised the clavulanic acid salt partition coefficients from 1.5 (lower value) to 114 (highest value) and high recoveries (97–99%) were obtained (Figs. 1 and 2). This was noted for all the PEGs tested. An increase in tie-line length promotes an increase in phosphate concentration in the lower phase whereas in the upper phase it remains relatively constant [11]. This probably leads to salting-out of the potassium clavulanate from the phosphate-rich phase to the PEG-rich phase as its solubility limit is reached. Partitioning of the potassium clavulanate to the top phase increases until its solubility limit in that phase is not exceeded or is molecular excluded.

The pH also influences potassium clavulanate partitioning in the PEG–phosphate potassium two-phase system as higher partition coefficients are obtained with increasing pH for PEG 1000 and 400 (Figs. 1 and 2). For PEG 6000 and 4000 this behaviour was not observed.

The effect of pH on the PEG–salt phase

diagram was studied and it was found that decreasing pH leads to an increase of the polymer and salt concentrations required for phase formation [9]. When the pH is lowered from 8 to 7 the $H_2PO_4^-/HPO_4^{2-}$ ratio increases and owing to the rejection of multivalent anions by PEG [12,13] a higher salt and/or polymer concentration will be needed to obtain a two-phase system. The displacement of the phase diagram towards higher salt concentrations, which can be compared with the effect of shortening the tie-line length, probably accounts for the decrease in K with decrease in pH.

4. Conclusions

Potassium clavulanate showed a high affinity for the PEG-rich phase with partition coefficients ranging from 1.5 to 114 and high recoveries (75–99%).

The partition coefficient of potassium clavulanate is independent of PEG molecular mass for PEG 1000 and 4000, but increases with increasing polymer molecular mass for higher PEG molecular masses.

Increasing the tie-line length and pH raised the potassium clavulanate partition coefficient and 99% of the clavulanate was recovered in the PEG phase.

5. Acknowledgement

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