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Precipitation of clavulanic acid from fermentation broth with potassium 2-ethyl hexanoate salt

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ABSTRACT

This paper describes a direct precipitation reaction of clavulanic acid (CA) using potassium 2-ethyl hexanoate salt. Clavulanic acid is an important molecule produced by the pharmaceutical industry to overcome problems relating to bacterial resistance to antibiotics. However, precipitation of this organic compound has been little studied and, unlike inorganic compounds, its isolation and precipitation usually involve a complex and meticulous process. The purpose of this work was to improve the purification process and increase the yield of CA from fermented broth by examining the influence of the combined concentrations of clavulanic acid (in organic solvent) and potassium 2-ethyl hexanoate on the potassium clavulanate precipitation reaction. Clavulanic acid was extracted at temperatures below 20 °C and preferably close to 15 °C in the water-immiscible organic solvent ethyl acetate. The drying step was performed with a suitable desiccant to produce an insoluble salt of potassium clavulanate. The resulting precipitate was crystalline and stable, a finding that was confirmed by an NMR ^{1}H analysis.

1. Introduction

Clavulanic acid (CA) from *S. clavuligerus*, which was first reported in 1976 by Brown et al., has a beta-lactam ring fused to an oxazolidine ring. It shows weak antibacterial activity, but it is a potent inhibitor of beta-lactamase enzymes produced by penicillin and cephalosporin-resistant bacteria. However, CA is an unstable hygroscopic oil and is not used in this form for the preparation of pharmaceutical compounds. Potassium clavulanate is more stable than the free acid or other salts such as sodium, calcium and magnesium clavulanate, and is therefore most frequently used in commercial preparations.

In the presence of low CA concentrations, many beta-lactamase producing bacteria become sensitive to commercially available penicillins and cephalosporins [1]. When CA is used together with these antibiotics, an irreversible bond is formed between the beta-lactamase serine hydroxyl group and clavulanic acid, producing a stable acylated intermediate that inactivates the enzyme and allows other antibiotics to act against the infection [2]. The combination of CA with amoxicillin is the most successful example of the use of traditional beta-lactam antibiotics sensitive to beta-lactamases together with an inhibitor of these enzymes [3].

CA is produced by fermentation and purified from the fermentation medium in several steps. These steps may involve a variety of standard techniques such as filtration and centrifugation for cell separation, followed by extraction and/or adsorption techniques for subsequent antibiotic purification. One of these methods is direct extraction with an organic solvent, whereby CA is transferred to the organic phase and subsequently purified. Chromatographic adsorption techniques can also be used with ionic or nonionic adsorbers [3–8].

These methods are the best choice for low molecular weight compounds present in diluted solutions, as is the case of antibiotics produced by fermentation. However, these conditions are not entirely satisfactory for CA because the yields obtained by ion exchange, adsorption and liquid–liquid extraction are very low, i.e., about 20% for the whole process [3,9]. This is because of the great instability of CA allied to its high solubility in water, which hinder the formation of its solid and dry forms that are required for its use in medicinal preparations. To overcome this problem, a promising alternative appears to be CA precipitation as a potassium clavulanate salt in organic solvent without the presence of water.

Little is known about the isolation and purification of CA, but the process apparently begins with classic techniques such as filtration and centrifugation followed by adsorption and ion-exchange techniques.

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Table 1
Conditions for precipitation experiments using CA from Clavulin®.

Experiments	Initial mass of CA (mg)	Initial volume of aqueous solution (mL)	Volume of combined organic phases (mL)	CA conc. in organic solvent (mg/mL)	Potassium 2-ethyl hexanoate conc. (M)	Volume of added potassium 2-ethyl hexanoate (mL)
First combination group						
1A	250	40	120	5	0.5	1.15
2A	250	40	120	5	2.0	0.30
3A	625	100	300	25	0.5	2.85
4A	625	100	300	25	2.0	0.75
Second combination group						
5A	375	60	180	15	0.3	2.85
6A	375	60	180	15	0.7	1.25
7A	875	140	420	35	0.3	6.60
8A	875	140	420	35	0.7	2.85

Several patents describe classic techniques, such as organic solvent extraction and ionic exchange chromatography, to extract clavulanic acid from fermentation broth. These methods often result in a product of low purity, thus requiring more than one purification step for the potassium clavulanate to reach the desired purity. Such assays require longer CA processing and their final results are diluted aqueous solutions, so clavulanate salt must be obtained by freeze-drying or by solvent evaporation. This, in turn, represents high industrial costs and causes greater degradation of the antibiotic due to its labile nature.

The precipitation of potassium 2-ethyl hexanoate salt results in clavulanic acid derivative salt, as mentioned in a patent (Cardoso [10]). This antibiotic production process is very attractive because it does not cause degradation such as that resulting from the solvents drying process or even water drying, so energy requirements are lower [11–14]. However, the conditions used in patented products are not specifically described in detail and no information has been reported about CA concentrations in organic solvent and potassium 2-ethyl hexanoate concentrations or their influence on this precipitation reaction. So far, there are no academic articles in the literature on this subject.

The work reported here consisted of a study of the influence of two combined variables, i.e., CA concentration (in organic solvent) and potassium 2-ethyl hexanoate concentration, on the direct precipitation reaction of potassium clavulanate. The two best combinations were then applied in the precipitation of CA from fermented broth. The precipitation reaction yields were evaluated and the potassium clavulanate precipitate was characterized by ^{1}H NMR.

2. Materials and methods

2.1. Materials

The experiments were carried out with three distinct sources of CA:

- Potassium clavulanate from the pharmaceutical product Clavulin® produced by SmithKline Beecham Laboratory (consisting of 625 mg of amoxicillin and 125 mg of potassium clavulanate);
- CA/cellulose with 37% weight of CA, supplied by Gist Brocades, now DSM-Anti Infective, Delft, The Netherlands; and
- CA from broths fermented with *Streptomyces clavuligerus* ATCC 27064. The culture medium was composed essentially of glycerol solution and soy protein isolate Supro 783 (The Solae Company), supplemented with ornithine, mineral salts, $(NH4)_2SO_4$ and KH_2PO_4 [15]. In some instances, a similar culture medium was used containing soy oil in place of ornithine and Prosan soy flour (The Solae Company) instead of Supro 783 [16].

At the end of the fermentation, the pH of the broth was adjusted to 6.2 with 18 N phosphoric acid, cooled to either 11 or 20 °C, and filtered through a Polysulfone tubular microfiltration membrane with 0.2 μm diameter pores supplied by Amersham Biosciences (CFP-2-E-8A). The permeates from this step were used in the ultrafiltration (polysulfone membranes with pore sizes of 3 kDA (UFP-3-E-3MA) and 50 kDa (UFP-50-E-3MA), supplied by Amersham Biosciences), which was previously filtered by microfiltration. A sample of fermented broth was dried in a SpeedVac and then dissolved in deuterium oxide for the NMR analysis.

The reagent used for the potassium clavulanate precipitation was potassium 2-ethyl hexanoate salt prepared from 2-ethyl hexanoic acid. Potassium hydroxide, calcium chloride and sulfuric acid were also used as reagents.

For the chromatographic separations, 5 g diol-bonded Sep-Pak columns (waters) were used.

2.2. Analytical methods

The concentration of CA from fermentation broth was determined by high performance liquid chromatography (HPLC), as described by Foulstone and Reading [17], by imidazole reaction. The samples were analyzed using a HPLC system equipped with a photodiode array detector (Waters 996 PDA) and a 3.9 × 300 mm C_{18}-μ Bondapak analytical column. The HPLC device was operated at 28 °C and a flow rate of 2.5 mL/min, and standard solutions were prepared from the pharmaceutical product Clavulin®.

Thin layer chromatography (TLC) analyses were performed with plastic-backed Si gel TLC sheets, eluting with different mixtures of solvents. Plates were visualized by ultraviolet light at 254 nm and by spraying phosphomolybdic acid. This chromatographic technique was used to evaluate the nature of the compounds that had to be separated and to select the most suitable chromatographic technique to separate the substances of interest.

The NMR data were recorded on a Bruker ARX400 9.4 T spectrometer operating at 400.35 MHz for ^{1}H and 100.10 MHz for ^{13}C channels, respectively. All the NMR data were obtained at 25 °C, using TMS as internal reference and deuterium oxide as solvent.

2.3. Experimental procedure

The experiments with Clavulin® were carried out in two different groups aiming to identify the optimal combined effects of CA and potassium 2-ethyl hexanoate concentrations.

The tablets were dissolved in distilled water and the mixture was filtered through a 0.45 μm mesh membrane.

This aqueous solution of clavulanic acid was acidified with H_2SO_4 to pH 2.0 (at this pH clavulanic acid is in its molecular form) and shaken with ethyl acetate, which was chosen because water is much less soluble in this solvent than in n-butanol. The ethyl