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Selective removal of Gentamicin C_1 from biosynthetic Gentamicins by facilitated pertraction for increasing antibiotic activity

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ABSTRACT

The less active Gentamicin C_1 have been selectively removed from the biosynthetic mixture containing Gentamicins C_1, C_{1a}, C_2 and C_{2a} by facilitated pertraction with D2EHPA dissolved in dichloromethane as the liquid membrane U-shaped pertraction cell. The pertraction has been analyzed by means of initial and final mass flows, permeability and selectivity factors. The main factors, which control the pertraction selectivity, were identified to be the pH-gradient between the feed and stripping phase, mixing intensity of the aqueous phases and carrier concentration in the membrane phase. The maximum selectivity factor has been recorded for pH 8 of feed phase, pH 3 of stripping phase, rotation speed lower than 100 rpm, and carrier concentration of 10 g/l.

1. Introduction

Gentamicin is an aminoglycoside antibiotic, isolated in 1963 by Weinstein from the *Micromonospora purpurea* cultures. It was introduced in therapeutic practice in 1969 in USA [1]. Gentamicin has a broad spectrum against the aerobic Gram positive and Gram negative bacteria, including the strains resistant to tetracycline, chloramphenicol, kanamycin, and colistin, namely *Pseudomonas*, *Proteus*, *Staphylococcus*, *Streptococcus*, *Klebsiella*, *Haemophilus*, *Aerobacter*, *Moraxella* and *Neisseria*. It was the first antibiotic efficient against *Pseudomonas*, being one of the most important members of the aminoglycoside antibiotics family [1,2].

Gentamicin inhibits the synthesis of the bacterial proteins, by blocking the bacterial ribosomes, thus inducing the misreading of the genetic code. The mechanism of action is based on the electrons transport, which needs the ATP and oxygen to become effective. Therefore, Gentamicin is efficient especially against the aerobic bacteria [2].

This antibiotic is industrially obtained by biosynthesis by *Micromonospora purpurea* or *echinospora*, the product being a complex mixture of some components of very similar structures. Among them, three are the most important: Gentamicins C_1, C_{1a} and C_2 (Gentamicin C_{2a} is considered also to be Gentamicin C_2, because it is its stereoisomer) [2,3]. The biosynthetic complex contains also the active Gentamicin C_{2b}, but its concentration is very low [2,3]. The chemical structures of the major Gentamicins are indicated in Fig. 1 [1,2,4,5].

The ratio of these components in the mixture varies from one biosynthetic product to another, the average values of their concentrations being: Gentamicin C_1 35%, Gentamicin C_{1a} 25%, Gentamicin C_2 (including Gentamicin C_{2a}) 40% [6].

The antibacterial activity of the Gentamicins, respectively their affinity for the bacterial ribosomes, is different. Thus, the most efficient is Gentamicin C_{1a}, its activity being slightly higher than that of Gentamicin C_2. Gentamicin C_1 binds the ribosomal subunits with the lowest efficiency compared with the other two Gentamicins (there are no reports concerning the specific affinity of Gentamicin C_{2a}, probably due to its assimilation with Gentamicin C_2) [7].

The separation of Gentamicin from the fermentation broths at industrial scale is achieved by sorption by cation-exchangers, followed by its desorption with a solution of 4–5% sulfuric acid. After the neutralization, the solution is purified and concentrated under vacuum, the antibiotic being precipitated as sulfate salt by acetone addition [3,8]. But, this technique does not allow the fractionation of the complex mixture of Gentamicins, the use only of Gentamicins C_{1a} and C_2 increasing the specific biological activity per weight unit

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Nomenclature

n	mass flow (moles/m^2 h)
n_f	final (overall) mass flow (moles/m^2 h)
n_i	initial mass flow (moles/m^2 h)
P	permeability factor
S	selectivity factor

Subscript

aq	aqueous phase
o	organic phase

of antibiotic.

Our previous studies on the reactive extraction with di-(2-ethylhexyl) phosphoric acid (D2EHPA) and re-extraction with sulfuric acid of Gentamicins C_1, C_{1a}, C_2 and C_{2a} indicated the possibility to selectively separate the Gentamicin C_1 from the mixture obtained by biosynthesis [9]. Because the Gentamicin C_1 has the lowest activity against the infections, its removal increases the therapeutic activity of the biosynthetic product. In this manner, the therapeutic dose of antibiotic could be reduced and, consequently, its secondary effects diminished.

The separation by reactive extraction method can be improved by Gentamicins extracting and transporting through liquid membranes, technique called *pertraction* or *permeation through liquid membranes*. The principle of this separation method consists in the transfer of a solute between two aqueous phases of different pH-values, phases that are separated by a solvent layer of various sizes. Commonly, liquid membranes can be obtained either by emulsification, when their stability is poor, or by including the solvent in a hydrophobic porous polymer matrix [10,11]. Moreover, the liquid membranes could be obtained using pertraction equipments of special construction, which allow to separate and easily maintain the three phases without adding surfactants (*free liquid membranes*) [12,13].

The pertraction efficiency and selectivity could be significantly enhanced by adding a carrier in liquid membrane, such as organophosphoric compounds, long chain amines or crown-ethers, etc., the separation process being called *facilitated pertraction* [10–15].

Compared with the physical or reactive liquid–liquid extraction, the use of pertraction reduces the loss of solvent during the separation cycle, needs small quantity of solvent and carrier, owing to their continuous regeneration, and offers the possibility of solute transport against its concentration gradient, as long as the pH-gradient between the two aqueous phases is maintained [10–13].

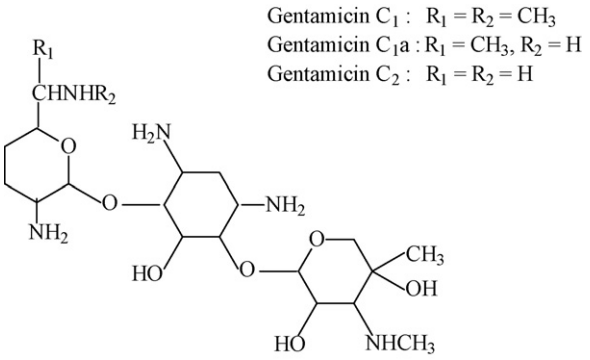

Fig. 1. Chemical structure of biosynthetic Gentamicins.

On the basis of the previous investigations on the mechanism of Gentamicins reactive extraction and on the factors, which influence the efficiency and selectivity of separation, the aim of this paper is to study the possibility to selectively separate the Gentamicins from the biosynthetic mixture by facilitated pertraction with D2EHPA. In this purpose, the influences of the pH-gradient between the aqueous phases, carrier concentration in the liquid membrane and mixing intensity on the efficiency and selectivity of pertraction will be analyzed.

2. Materials and method

The experiments have been carried out using a pertraction equipment that allows obtaining and easily maintaining the free liquid membrane. The pertraction cell has been described in the previous papers and consists on a U-shaped glass pipe having an inner diameter of 45 mm and a total volume of 400 ml, the volume of each compartment being equal [13,16].

The aqueous solutions have been independently mixed by means of double blade stirrers with 6 mm diameter and 3 mm height, having a rotation speed between 0 and 700 rpm. In order to reach high diffusional rates through the solvent layer, the organic phase has been mixed with a stirrer of the same design, at a constant rotation speed of 500 rpm. The area of mass transfer surface, both for extraction and for re-extraction, was of 1.59×10^{-3} m^2. The interfaces between the phases remained flat, and hence the interfacial area constant, for entire rotation speed domain used.

The experiments have been carried out in a continuous system, at the steady state conditions related to the aqueous phases. The aqueous solutions have been separately fed with a volumetric flow of 2.5 l/h.

The liquid membrane phase consisted of a solution of 0–60 g/l D2EHPA (carrier) dissolved in dichloromethane.

The feed phase was an aqueous solution containing the Gentamicins mixture obtained by biosynthesis, the overall concentration of the antibiotic being of 50 g/l. The ratio of the Gentamicins in the initial mixture was as follows: Gentamicin C_1 31.92%, Gentamicin C_{1a} 24.20%, Gentamicin C_2 20.43%, Gentamicin C_{2a} 23.45%. The pH-value of the feed phase was varied in the domain 2–8. The pH adjustment has been made with a solution of 3% sulfuric acid or 3% sodium hydroxide, function on the prescribed pH-value.

The stripping phase was of sulfuric acid solutions with pH 1–3.

The pH-values of both aqueous phases have been determined using a digital pH-meter of Consort C836 type and have been recorded throughout each experiment. Any pH change was noted during the pertraction experiments.

The pertraction has been analyzed by means of the Gentamicins initial and final mass flows, permeability and selectivity factors. The initial mass flow represents the antibiotic mass flow from the feed phase to the liquid membrane, while the final (overall) mass flow the antibiotic mass flow from the liquid membrane to the stripping phase. The permeability factor conveys the capacity of a solute transfer through liquid membrane, and has been defined as the ratio between the final mass flow and the initial mass flow of solute [16]. The selectivity factor has been defined as the ratio between the permeability factor of all Gentamicins and that of Gentamicin C_1.

These parameters have been calculated by determining the Gentamicins concentrations in the feed and stripping phases and by using the antibiotic mass balance for the pertraction system. The Gentamicins concentrations have been measured by high performance liquid chromatography technique (HPLC) with a Phenomenex RP-18 column (4.5 mm diameter, 150 mm length, silica), provided with UV detector at 330 nm [9]. The mobile