Bioactive Polymers, XIV
Immobilization of Ampicillin on BIOZAN R

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The ampicillin coupling on BIOZAN R (a polysaccharide of microbial synthesis Xanthomonas campestris) is studied by the activation of carboxylic groups by dicyclohexylcarbodiimide. The following optimum reaction parameters are settled on the basis of an experimental program: time = 7.5 h, temperature = 10 °C, dicyclohexylcarbodiimide concentration = 1.10^{-3} m mole, ampicillin concentration = 2.4 · 10^{-3} m mol. Under these conditions up to 37% of ampicillin with respect to the polymer is coupled.

The microbiological and hydrolysis studies under the conditions of the human physiological pH attest the ampicillin maintains its activity and may be released in oral administration.

Introduction

There are two highly desirable objectives in chemotherapy: to enhance the specificity of a drug and to increase the duration of its action. The first objective is attained by the systematic chemical modification of drugs, strict control of concentration and restriction of the action to one bondy compartment [1—3]. The second is attained by the drug coupling on biologically inactive polymers, natural [4—11] and synthetic [12], bio- and nonbiodegradable. Antibiotics [8—11, 13], insulin [4—6], cytostatic agents [14—17] were thus immobilized.

The support nature determines the amount of the bound antibiotic and the therapeutic applications of the new drug. The BIOZAN R is a polysaccharide of microbial synthesis tolerated by the human body when administrated by both oral and intravenous way.

In the present paper the ampicillin immobilization on BIOZAN R by the activation with dicyclohexylcarbodiimide is reported.

Experimental

Immobilization reaction

The ampicillin and 0.5 g of BIOZAN R are added in 25 ml distilled water under stirring. The mixture is maintained at 10 °C and dicyclohexylcarbodiimide (DCI) solved in 2 ml THF added. After the reaction took place the product is precipitated in acetone, washed on the filter with THF and on acetone-water mixture (70%—30% by vol.) in which the unreacted ampicillin is soluble and dried finally under vacuum.

Determination of the immobilized ampicillin

0.08 g product is solved in 50 ml of phosphate buffer at pH = 6.1 1N NaOH solution is added to 5 ml solution and then allowed to stay for 20 min. 1 ml 1 N HCl, 5 ml acetate buffer of pH = 4.6 and 10 ml 0.01 N iodine solution are added and the mixture is kept in the dark for 20 min. After adding 1 ml starch 1% the solution is titrated with 0.01 N sodium thiosulphate. To 5 ml of the initial solution 5 ml of acetate buffer of pH = 4.6 and 10 ml 0.01 N iodine are added; the solution is kept in the dark for 20 min, then 1 ml starch solution added and titrated with 0.01 N sodium thiosulphate. The difference between the two titrations represents the iodine consumption by the ampicillin in 5 ml of the sample solution. 1 ml 0.01 N iodine solution corresponds to 0.000437 g C_{16}H_{18}N_{3}O_{8}S.

Kinetical study of ampicillin releasing

a. Basic hydrolysis: 64.10^{-5} mol sodium hydroxide in 100 ml distilled water at 30 °C (pH 11.36). Polymer containing 25.5% by wt. immobilized ampicillin (corresponding to 16.10^{-5} mol/l coupled ampicillin) were introduced. The pH variation in time was followed.

b. Acid hydrolysis: The polymer is introduced into 70 ml of HCl solution of pH = 4.29 under the same conditions as above and the pH variation in time at 30 °C is followed.

Results and Discussion

The BIOZAN R contains a main chain consisting of β-anhydroglucosidic units 1—4 bound on which
short chains of acetylated mannose, glucuronic acid and partially piruvilated mannose are grafted. The polymer is soluble in water, alkali and acid solutions, low alcohols, nontoxic when administrated orally [18, 20].

In order to find the optimum parameters for the condensation of BIOZAN R with ampicillin a series of experiments according to a central composite rotatable (second order degree) design plan limiting to minimum the number of attempts [21].

The values of the parameters (independent variable) were coded as in Table I.

Tab. I. Coded variable for experimental plan.

<table>
<thead>
<tr>
<th>Variable code</th>
<th>-1.682</th>
<th>-1</th>
<th>0</th>
<th>1</th>
<th>1.682</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin amount (g)</td>
<td>0.3136</td>
<td>0.45</td>
<td>0.65</td>
<td>0.85</td>
<td>0.9864</td>
</tr>
<tr>
<td>DCI amount (g)</td>
<td>0.0318</td>
<td>0.10</td>
<td>0.20</td>
<td>0.30</td>
<td>0.3682</td>
</tr>
<tr>
<td>Reaction time (h)</td>
<td>0.636</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>7.364</td>
</tr>
</tbody>
</table>

Bio-COOH + C₆H₅-N=CH₂-N₃ = Bio-COOH + NH₃⁺ + C₆H₅-NH₂

By means of the proposed mathematical model (eq. (1)):

\[
Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j
\]

the regression coefficients are determined and this function estimated. \(Y\) denotes the ampicillin amount bound on BIOZAN R (Table II).

By applying the least squares method the regression coefficients in eq. (1) are calculated based on the following relation ships:

\[
\beta_0 = 0.166388 \sum Y - 0.056791 \sum x_i^2 Y
\]

\[
\beta_i = 0.073224 \sum x_i Y
\]

\[
\beta_{ij} = 0.0625 \sum x_i x_j Y + 0.006889 \sum x_i^2 Y - 0.056791 \sum Y
\]

\[
\beta_{ij} = 0.125 \sum x_i x_j Y
\]

The unsignificant regression coefficients were removed by using the test \(t\) which gives the coefficients \(\beta \geq t \cdot s_{\beta}\) (6) as significant, where \(\beta\) represents the regression coefficients and \(s\) is determined by means of the relations:

\[
s_{\beta} = s \sqrt{0.073224}\] for coefficients \(\beta_i\) (7)

\[
s_{\beta} = s \sqrt{0.0625 + 0.006889}\] for coefficients \(\beta_{ii}\) (8)

\[
s_{\beta} = s \sqrt{0.125}\] for coefficients \(\beta_{ij}\) (9)

In the relation (7), (8) and (9) the average square deviation \(s\) of \(Y\) for the values in the center of the experimental region \((n = 6)\) was calculated by means of the relation:

\[
s_{\beta} = \sqrt{\frac{(Y - \bar{Y})^2}{n - 1}}\]

The following equation (11) gives the correlation between the experimental and theoretical data (Table II) and expresses the influence of the reaction conditions on the amount of the bound ampicillin.

\[
Y = 25.388 + 5.3817x_1 + 2.8915x_2 + 3.788x_3 - 1.78x_1^2 - 1.96x_2^2 + 1.99x_1x_3 - 0.667x_2x_3
\] (11)

In order to reduce the regression space dimensions \(x_1 = 0\) is taken and the equation (11) becomes:
\[ Y = 25.388 + 2.8915x_1 + 3.788x_2 - 1.78x_2^2 - 1.96x_3^2 - 0.667x_2x_3 \]  

(12)

By means of the equation (12) the coordinates of the stationary point and the coefficients of the canonical form are settled

\[ \frac{\partial Y}{\partial x_1} = 2.8915 - 3.562x_2 - 0.667x_3 = 0 \]  

(13)

\[ \frac{\partial Y}{\partial x_3} = 3.788 - 3.926x_3 - 0.667x_2 = 0 \]  

(14)

with the solutions \( x_{s1} = 0.652; x_{s3} = 0.854 \) and the determinant

\[
\begin{vmatrix}
-3.562 - \lambda & -0.667 \\
-0.667 & -3.926 - \lambda
\end{vmatrix} = 0
\]

(15)

which gives the equation (16)

\[ \lambda^2 + 7.488\lambda + 13.667 = 0 \]  

(16)

with the solutions: \( \lambda_1 = -3.152; \lambda_2 = -10.67 \)

The negative values obtained for \( \lambda_1 \) and \( \lambda_2 \) attest that the regression space (eq. (17))

\[ Y - 27.94 = -10.67x_2^2 - 3.152x_3^2 \]  

(17)

has a single maximum in the stationary point, \( S (Y_s = 27.94) \).

The defining of the regression space gives the possibility of drawing the level lines (Fig. 1). The optimum values DCI = 1 \( \times 10^{-3} \) mol and time = 7.5 h are thus found.

The amount of bound ampicillin was found to increase linearly with increasing concentration in the reaction medium (Fig. 2). A linear variation was also noticed for different reaction times (Fig. 3).

![Fig. 1. Variation of the amount ampicillin coupled on BIOZAN R as a function of time and DCI amount. Ampicillin = 1.6 \( \times 10^{-3} \) mol, time = 5.1 h.](image)

![Fig. 2. Variation of the amount of ampicillin bound on BIOZAN R as a function of the ampicillin concentration in the reaction medium. DCI = 1 \( \times 10^{-3} \) mol, time = 6 h, temperature = 10 °C.](image)

![Fig. 3. Variation of the ampicillin percentage content of the reaction product as a function of the ampicillin amount for different reaction durations. DCI = 1 \( \times 10^{-3} \) mol, temperature = 10 °C.](image)
The optimum reaction time is noticed to increase with increasing ampicillin concentration. The conclusion may be drawn that the amount of the ampicillin decomposing in the unit time is constant regardless its concentration in the system (Fig. 4).

The carbodiimide acts as a reaction catalyst at stoichiometrical or somewhat higher concentration with respect to ampicillin (Fig. 5). Above these values the bound antibiotic suffers deactivations by the catalysis of the β-lactonic ring hydrolysis.

When the compound is a drug with retard action the study on the release of bound ampicillin is impor-
In this connection we studied the ampicillin bound on polymer in both acid and basic medium. The kinetical study performed by following the variation of the pH solution indicates the splitting of the esteric bond.

In acid medium the rate of HCl consumption is higher for the sample of low ampicillin content since this will substitute the remanent Na and K at a higher rate instead of to participate to the hydrolysis reaction. Inversely, for higher ampicillin content HCl will act preferentially on the amidic bond and its consumption rate will consequently lower.

In alkali medium the NaOH consumption depends on the sample ampicillin content since it acts on the peptide bond only.

Hence, the drug may be released in the physiological medium of intermediate pH value (blood plasma: pH = 7.4; pancreatic digestion pH = 7.5–8; intestinal digestion pH = 6.9–7.7; stomacal digestion pH = 1.0).

Tab. III. Antimicrobial activities of ampicillin coupled on BIOZAN R.

<table>
<thead>
<tr>
<th>Sample nr</th>
<th>Coupled ampicillin activity, calculated&lt;sup&gt;a&lt;/sup&gt; (UI/mg)</th>
<th>Antimicrobial activity found&lt;sup&gt;b&lt;/sup&gt; (UI/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18.02 162.2</td>
<td>226</td>
</tr>
<tr>
<td>2</td>
<td>35.68 321.1</td>
<td>428</td>
</tr>
<tr>
<td>3</td>
<td>19.50 175.5</td>
<td>196</td>
</tr>
</tbody>
</table>

<sup>a</sup> Calculated with respect to the bound ampicillin (initial ampicillin = 900 UI/mg);

<sup>b</sup> determined on agar gel Bacillus subtilis 2589.

The thin-layer chromatography performed with samples of different ampicillin contents (samples 1–3, Table III) indicate the absence of products of chemical degradation in the immobilization product.

The antimicrobial tests of the samples with coupled ampicillin indicate a higher activity (Table III) than the theoretical values due to the gel effects and to the chemical protection by the carboxyl groups. The formation of a microsystem of acid pH assures the ampicillin protection toward the hydrolytic attack of penicillinases [24] while the gel character determines a cage effect [25] causing an increase activity of the ampicillin.

Conclusions

The immobilization of ampicillin on BIOZAN R under the catalytic action of DCI gives a new antibiotic with retard action.

The coupling depends on the time, ampicillin and DCI concentrations, the optimum values being of time = 7.5 h, ampicilline $2.4 \cdot 10^{-3}$ mol, DCI = $1 \cdot 10^{-3}$ mol.

The relatively great amount of the coupled ampicillin (up to 40%) may assure the attack dose necessary in the urgent and long duration treatments.

The studies on the antibiotic release from the support under the pH conditions and ionic concentration characteristic of the physiological medium within which the product is aimed to act made evident the possibility of ampicillin separation and its retard character.