Inhibitory effects in the side reactions occurring during the enzymic synthesis of amoxicillin: *p*-hydroxyphenylglycine methyl ester and amoxicillin hydrolysis

Luciana R. B. Gonçalves*[1], Roberto Fernandez-Lafuente†, Jose M. Guisan†, Raquel L. C. Giordano‡ and Roberto C. Giordano‡

**Departamento de Engenharia Química, Universidade Federal do Ceará, Campus do Pici s/n, Bloco 709, 60455-760, Fortaleza, Ceará, Brazil, †Instituto de Catálisis y Petoleoquímica, CSIC, Madrid, Spain, and ‡Departamento de Engenharia Química, Universidade Federal de São Carlos, Rod. Washington Luiz, km 235, C.P. 676 CEP, São Carlos, SP, Brazil*

Penicillin G acylase immobilized on glyoxyl-agarose is used to catalyse the reaction between *p*-hydroxyphenylglycine methyl ester (POHPGME) and 6-aminopenicillanic acid (6-APA). Inhibitory effects affecting the side reactions that occur during the synthesis of amoxicillin have been reported and need to be considered when proposing a kinetic model for the enzymic synthesis. In this work, we present a semi-empirical kinetic model that successively includes different inhibitory effects in the rate equations. The model performance was always compared with experimental data on amoxicillin synthesis. Enzyme load and stirring rate were chosen to prevent diffusional effects. Our results indicate that POHPGME and amoxicillin were competitive inhibitors of the hydrolysis of amoxicillin and POHPGME, respectively. 6-APA was a competitive inhibitor of the hydrolysis of amoxicillin. POHPG was a competitive inhibitor and methanol a non-competitive inhibitor of the hydrolysis of both ester and antibiotic, but the action of methanol was only noticeable at very high concentrations. Adding inhibitory effects to the kinetic model led to a significant increase in the accuracy of the simulations of the overall process of synthesis.

Introduction

Amoxicillin is one of the major β-lactam antibiotics, with sales of US $2200 million as a bulk formulated drug in 1994 [1]. Furthermore, as a broad-spectrum antibiotic, this semi-synthetic penicillin is applicable against a wide variety of bacterial infections [2].

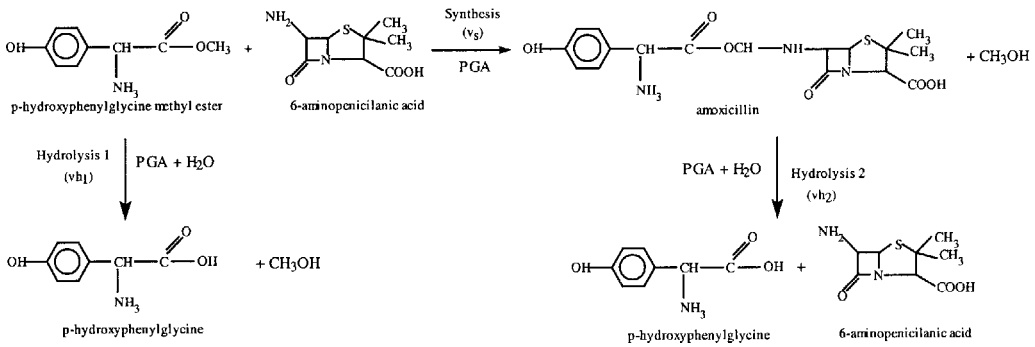
The industrial enzymic synthesis of semi-synthetic penicillins and cephalosporins is only taking its first steps [3]. Nowadays, these antibiotics are still manufactured through chemical routes in almost all pharmaceutical plants. For example, an amino β-lactam such as 6-aminopenicillanic acid (6-APA), usually having its carboxyl group protected, reacts with an activated side-chain derivative, followed by the removal of the protecting group by hydrolysis. These reactions typically involve costly steps such as low temperatures (approx. $-30\ ^{\circ}C$) and toxic organic solvents, such as methylene chloride and silylation reagents [4]. Therefore, enzymic synthesis of semi-synthetic antibiotics is becoming increasingly interesting as an industrial process. It reduces the number of reaction steps and decreases the amount and toxicity of waste products per kg of antibiotic [5]. The use of enzymes such as penicillin G acylase (PGA) is of great interest due to their high selectivity, specificity and activity in mild reaction conditions (aqueous medium, neutral pH and moderate temperatures). These characteristics preclude the use of organic solvents as well as the need for the sequence of protection/unprotection of reactive groups, low temperatures and chemical acylation. Semi-synthetic antibiotics can be produced by two approaches: thermodynamic controlled synthesis [6], which is the reverse reaction of antibiotic hydrolysis, or kinetically controlled synthesis (KCS), which promotes substrate activation. In the first case, yields are only determined by the thermodynamic equilibrium constant of the process. For a kinetically controlled strategy, however, the balance between three different catalytic activities of the same enzyme determines the yields, namely: (i) synthesis of the product (synthetase activity), (ii) hydrolysis of the activated acyl donor (esterase activity) and (iii) hydrolysis of the product synthesized previously (amidase activity) [7,8].

From the reaction scheme and the data in the literature [9–13] one can see that it is necessary to reduce the two hydrolytic activities to achieve a good KCS yield;

Key words: antibiotics, penicillin G acylase, kinetic model.

Abbreviation used: 6-APA, 6-aminopenicillanic acid; KCS, kinetically controlled synthesis; PGA, penicillin G acylase; POHPG, *p*-hydroxyphenylglycine; POHPGME, *p*-hydroxyphenylglycine methyl ester.

[1] To whom correspondence should be addressed (e-mail lrg@ufc.br).



Scheme 1 Enzymic synthesis of amoxicillin, with side reactions

v_S, rate of amoxicillin synthesis (mM/min); v_{h1}, rate of *p*-hydroxyphenylglycine methyl ester hydrolysis (mM/min); v_{h2}, rate of amoxicillin hydrolysis (mM/min).

that is to say, a high conversion of antibiotic nucleus into antibiotic. In fact, it seems evident from Scheme 1, and has been confirmed in the literature, that the hydrolysis of the antibiotic plays a key role in the final yield. When the ester hydrolysis is high (e.g. similar to the synthesis), the maximum 6-APA yield may be improved by increasing the ratio of ester to antibiotic nucleus. However, when the problem is antibiotic hydrolysis, improving this yield implies a reduction of the ester/nucleus ratio. Thus selection of enzymes with reduced specificity against the antibiotic and the use of extraction systems integrated in the reactor, which decrease the risk of hydrolysis of the antibiotic, have been proposed as a good solution [14–17].

Ester and antibiotic hydrolyses may be influenced by different agents. Possible inhibitory effects caused by substrates, products or additives are among the most significant. These inhibitions may also have a great impact on the accuracy of mathematical models of these reactions.

Simulation of this process can certainly facilitate the understanding of the demands that it places on time and equipment. Simulation studies, however, need a reliable kinetic model to represent the synthetic reaction. The accurate prevision of yields for the kinetically controlled approach implies the assessment of the two side reactions that occur simultaneously in this system, not only of the synthetic desired one. The interest in modelling the KCS is evident from the literature [10,12,13,18–20]. However, because of the complexity of this system, many of these papers utilize conditions where the number of kinetic parameters needed for an adequate representation of the system decreases.

Due to the complexity of the system and bearing in mind that different derivatives of PGA seem to present different behaviour in KCS [4,13,18,19,21], in this article we have studied the inhibitory effects that might influence the hydrolysis reactions, catalysed by a derivative that presents very good behaviour in KCS (glyoxyl-agarose) [22].

Therefore, the two main objectives of this paper are (i) to understand the possibilities of controlling the undesired hydrolyses just by using inhibitory effects of the different substrates and products on the undesirable reactions, and (ii) to improve a semi-empirical mathematical model to simulate the reaction courses. Therefore, inhibition models were identified and kinetic constants for those reactions were estimated. While considering the different inhibition effects, the results obtained using these models were compared with experimental data on amoxicillin synthesis. In this way, the importance of these effects in building a global mathematical model of amoxicillin synthesis was evaluated.

Kinetic model

In previous work [20], a simple kinetic model was proposed to describe amoxicillin synthesis. *p*-Hydroxyphenylglycine methyl ester (POHPGME) reacts with 6-APA to yield the antibiotic (amoxicillin) and methanol. Moreover, *p*-hydroxyphenylglycine (POHPG) can be formed by the hydrolysis of either POHPGME or amoxicillin. Scheme 1 shows the whole reaction network.

The main hypotheses of the semi-empirical model were as follows. (i) Antibiotic synthesis only occurs when 6-APA has bound previously to the acyl-enzyme complex. This is not a consensual assumption in the literature. For instance, Schroën et al. [21], treating the synthesis of cephalexin from phenylglycine amide by PGA, assumed that all the antibiotic comes from the nucleophile attack of the acyl-enzyme complex by 7-aminodeacetoxycephalosporanic acid, which is exactly the opposite assumption to ours. These authors took into account the inhibitory action of traces

Table 1 Kinetic models for amoxicillin synthesis

Model 1, without inhibition; model 2, with the inhibitors ester (POHPGME; shown here as AB) and amoxicillin; model 3, inhibitors ester, amoxicillin (AN) and methanol (MeOH); model 4, inhibitors ester, amoxicillin and POHPG (AOH); model 5, inhibitors ester, amoxicillin, POHPG and 6-APA (NH). C_i indicates the concentration of compound i; EZ, enzyme; k_{AB}, inhibition constant (POHPGME); k_{AN}, inhibition constant (amoxicillin); k_{AOH}, inhibition constant (POHPG); k_{MeOH}, inhibition constant (methanol); k_{NH}, inhibition constant (6-APA); K_{EN}, 6-APA adsorption constant; T_{max}, maximum conversion ratio of the complex acyl-enzyme-nucleus into product; v_{AB}, rate of POHPGME consumption (mM/min); v_{AN}, rate of amoxicillin production (mM/min); v_S, rate of amoxicillin synthesis (mM/min); X, fraction of enzyme saturated with 6-APA.

	Ester consumption rate (v_{AB}):	Antibiotic hydrolysis rate (v_{AN}):	Synthesis rate (v_S):
Model 1	$v_{AB} = \frac{k_{cat1} C_{AB} C_{EZ}}{K_{m1} + C_{AB}}$	$v_{AN} = \frac{k_{cat2} C_{AN} C_{EZ}}{K_{m2} + C_{AN}}$	$v_S = v_{AB}\, T_{max}\, X$ where: $X = \frac{C_{NH}}{K_{EN} + C_{NH}}$
Model 2	$v_{AB} = \frac{k_{cat1} C_{AB} C_{EZ}}{K_{m1}\left(1 + \frac{C_{AN}}{k_{AN}}\right) + C_{AB}}$	$v_{AN} = \frac{k_{cat2} C_{AN} C_{EZ}}{K_{m2}\left(1 + \frac{C_{AB}}{k_{AB}}\right) + C_{AN}}$	
Model 3	$v_{AB} = \frac{k_{cat1} C_{AB} C_{EZ}}{K_{m1}\left(1 + \frac{C_{AN}}{k_{AN}} + \frac{C_{AOH}}{k_{AOH}}\right) + C_{AB}}$	$v_{AN} = \frac{k_{cat2} C_{AN} C_{EZ}}{K_{m2}\left(1 + \frac{C_{AN}}{k_{AN}} + \frac{C_{AOH}}{k_{AOH}}\right) + C_{AN}}$	
Model 4	$v_{AB} = \frac{k_{cat1} C_{AB} C_{EZ}}{K_{m1}\left(1 + \frac{C_{AN}}{k_{AN}} + \frac{C_{AOH}}{k_{AOH}}\right) + C_{AB}}$	$v_{AN} = \frac{k_{cat2} C_{AN} C_{EZ}}{K_{m2}\left(1 + \frac{C_{AB}}{k_{AB}} + \frac{C_{NH}}{k_{NH}} + \frac{C_{AOH}}{k_{AOH}}\right) + C_{AN}}$	
Model 5	$v_{AB} = \frac{k_{cat1} C_{AB} C_{EZ}}{K_{m1}\left(1 + \frac{C_{MeOH}}{k_{MeOH}} + \frac{C_{AN}}{k_{AN}} + \frac{C_{AOH}}{k_{AOH}}\right) + C_{AB}\left(1 + \frac{C_{MeOH}}{k_{MeOH}}\right)}$	$v_{AN} = \frac{k_{cat2} C_{AN} C_{EZ}}{K_{m2}\left(1 + \frac{C_{MeOH}}{k_{MeOH}} + \frac{C_{AN}}{k_{AN}} + \frac{C_{AOH}}{k_{AOH}}\right) + C_{AN}\left(1 + \frac{C_{MeOH}}{k_{MeOH}}\right)}$	

of phenylacetic acid that are present in the process and proposed a five-parameter, simplified model. The same hypothesis, that the acyl-enzyme complex is built-up previously to the binding of the β-lactam nucleus, was assumed for simulating the synthesis of ampicillin [23]. (ii) The rate of formation of this complex is not influenced by the presence of 6-APA. Therefore, the kinetic parameters estimated from ester hydrolysis reactions (in the absence of 6-APA) can be used directly in the rate equation of ester consumption in the presence of 6-APA, when the antibiotic is formed.

This model already took into account the following inhibitory effects: the effect of POHPGME and 6-APA on the rate of amoxicillin hydrolysis and the effect of amoxicillin on the rate of POHPGME hydrolysis. In this work, this model is improved and validated. Inhibition effects of other products on the side reactions were investigated and the inhibitory effect of 6-APA on amoxicillin hydrolysis, assumed to be of a non-competitive type in the previous work, has now been checked. Finally, the inhibition constants are re-estimated for a newly prepared enzyme derivative. While adding various inhibitory effects, different kinetic models emerged (see Table 1).

The parameters K_m and k_{cat} of the Michaelis–Menten equation (model 1) were determined after a non-linear fitting of model 1 to experimental data of the initial velocities of hydrolysis of pure POHPGME and pure amoxicillin; in other words, in the absence of inhibitors. The temperature was 25 °C and pH values were 6.5 and 7.5. To estimate T_{max} (the maximum conversion ratio of the complex acyl-enzyme-nucleus into product) and K_{EN} (the 6-APA adsorption constant), initial rates of ester consumption and amoxicillin production were taken during amoxicillin synthesis experiments, at different initial concentrations of POHPGME and 6-APA. The values of these parameters were obtained by fitting the ratio of formation of amoxicillin,

$$f = \frac{v_S}{v_S + v_{hl}}$$

to the experimental data, at pH 6.5 and 25 °C, where v_S is the rate of amoxicillin synthesis and v_{hl} is the rate

of *p*-hydroxyphenylglycine methyl ester hydrolysis (both in mM/min).

Materials and methods

Materials

POHPGME was produced in Universidad de Salamanca, Salamanca, Spain. Amoxicillin was obtained from a commercial source (Clamoxyl, Madrid, Spain). PGA from *Escherichia coli* (EC 3.5.1.11) and POHPG were donated by Antibiotics S. A., León, Spain. Agarose 6BCL was donated by Hispanagar S. A., Burgos, Spain. All other chemicals were of laboratory grade from commercial suppliers.

Preparation of PGA derivatives [15]

Activation of agarose gel was achieved by etherification with glycidol and oxidation with sodium periodate. Further PGA (amine)–agarose (aldehyde) multiple-point attachment was performed by reaction at pH 10 (bicarbonate buffer), in the presence of 100 mM phenylacetic acid. Final reduction of the Schiff bases was obtained using sodium borohydride (1 mg/ml solution).

Enzyme activity

Enzyme activity was evaluated by colorimetric analysis of *p*-nitro-anilide benzoic acid, released during hydrolysis of 6-nitro-3-phenylacetamide benzoic acid (33 mg/l in 50 mM phosphate buffer, pH 7.5). The difference between enzymic activities of the supernatant before and after immobilization was used to assess the enzymic load of the gel. The enzymic activity of the immobilized preparation was also measured directly, using 6-nitro-3-phenylacetamide benzoic acid as a substrate, to ensure no diffusional limitations existed. 1 i.u. of enzyme activity is the amount of enzyme that hydrolyses 1 μmol of penicillin G (5% w/v) per min at pH 8.0 and 38 °C.

Analysis

Concentrations of POHPGME, amoxicillin, 6-APA and POHPG were determined using HPLC. A 20 μl sample was analysed using a C_{18} column (Waters Nova-Pak C_{18}, 60 Å, 4 μm, 3.9 mm $\times$ 150 mm) and the mobile phase composed of 35% acetonitrile, 2‰ SDS, 10 mM H_3PO_4 and 5 mM $K_2H_2PO_4$ at 25 °C, with a flow rate of 1.5 ml/min and $\lambda = 215$ nm. The four components were eluted in the following order: POHPG (1.5 min), 6-APA (2.9 min), amoxicillin (4.1 min) and POHPGME (5.9 min).

Kinetic experiments

Kinetic experimental data were obtained using the initial-rates approach. Initial velocities, for different concentrations of substrate, were determined from the slopes of the curves in the beginning of the reaction (less than 10% of substrate conversion). During inhibition experiments, a known amount of the inhibitor was added to the reaction medium. A 50-ml jacketed batch reactor with mechanical stirring was used in all the experiments. pH and temperature of the solutions during the enzymic hydrolysis reactions were kept constant by an automatic Metrohm pHstat (model Titrino) and a thermostatic bath, respectively. Enzymic reactions were carried out in 30 ml of 25 mM phosphate buffer solution, pH 7.5 or 6.5, at 25 °C. The same amount of biocatalyst was used in all assays: 1 g of glyoxyl-agarose with 30 i.u./ml immobilized PGA. The enzyme load and the stirring rate used in this work ensured the absence of external and intra-particle diffusional effects. These conditions were checked in a previous study [20]: initial-rate assays were carried on, measuring the velocity of synthesis for increasing loads of enzyme, keeping constant the concentration of reactants and the gel diameter. Stirring rate was 800 rev./min (high enough to avoid extra-particle film resistances). The enzymic load ranged from 10 to 190 i.u./g_{gel} (g of glyoxyl-agarose gel). A linear variation of the rate of synthesis with respect to the load was observed up to 90 i.u./g_{gel}, indicating that in this region the reaction was the rate-controlling step.

Amoxicillin synthesis experiments

Batch assays were performed at 25 °C and pH 6.5, using the same equipment described above.

Inhibition studies in enzymic reactions

Dixon graphical analysis was used to identify the inhibition pattern; Giordano and Schmidell [24] concluded that the Dixon plot was the best method for this purpose. The kinetic parameters of the rate equations were fitted from data of initial rates, obtained in the presence and absence of inhibitors [25]. Marquardt's algorithm [26] was used for that purpose.

Results and discussion

Performance of a simple semi-empirical model (model 1)

Table 2 shows the values of K_m and k_{cat} for the glyoxyl-agarose derivative. The K_m values for ester and amoxicillin are similar at pH 7.5, whereas at pH 6.5 K_m decreases for the ester while it slightly increases for the antibiotic. By contrast, k_{cat} for the antibiotic is around 50% higher than for the ester at pH 7.5, and almost double at pH 6.5. This means that the specificity $[(k_{cat2}/K_{m2})/(k_{cat1}/K_{m1})]$ of glyoxyl-PGA is 1.67 at pH 7.5 and 1.1 at pH 6.5, favouring hydrolysis of the antibiotic.

Table 2 Kinetic parameters for all enzymic reactions at pH 7.5 or 6.5 and 25 °C, agarose-glyoxyl-PGA (30 i.u./ml_{gel})

$k_{POHPGME}$, inhibition constant (POHPGME); k_{POHPG}, inhibition constant (POHPG); $k_{methanol}$, inhibition constant (methanol); $k_{amoxicillin}$, inhibition constant (amoxicillin); $K_{6\text{-}APA}$, 6-APA adsorption constant; T_{max}, maximum conversion ratio of the complex acyl-enzyme-nucleus into product.

Parameter	pH 7.5	pH 6.5
k_{cat1} (μmol/i.u. per min)	0.32	0.18
k_{cat2} (μmol/i.u. per min)	0.47	0.33
K_{m1} (mM)	12.4	7.91
K_{m2} (mM)	11.0	12.5
T_{max}	0.99	0.61
$K_{6\text{-}APA}$ (mM)	12.04	14.4
$k_{POHPGME}$ (mM)	8.71	3.78
$k_{amoxicillin}$ (mM)	–	9.17
k_{POHPG} (mM), POHPGME hydrolysis	17.7	–
k_{POHPG} (mM), amoxicillin hydrolysis	14.4	10.9
$k_{methanol}$ (mM), amoxicillin hydrolysis	1563	1691
$k_{methanol}$ (mM), POHPGME hydrolysis	1170	–

Table 3 Maximum yields of conversion of 6-APA for the enzymic synthesis of amoxicillin at pH 7.5 or 6.5 and 25 °C, agarose-glyoxyl-PGA (30 i.u./ml_{gel})

All species were in soluble form.

Initial concentrations (mM; POHPGME/6-APA)	Maximum yield (%)	
	pH 7.5	pH 6.5
50/50	12.7	30.4
30/30	17.2	19.9
30/5	10.9	20.0
112/37	28.0	51.4
5/5	7.3	–
10/5	18.5	–
30/66	6.8	–
80/5	–	53.3
80/50	–	38.6
80/100	–	16.0

The lower specificity of glyoxyl-PGA at pH 6.5, promoted mainly by the K_m value and very likely related to the pK of the substrates, may be one of the reasons why KCS of amoxicillin proceeds better at pH 6.5 than at pH 7.5.

Table 3 summarizes the maximum yields of conversion of 6-APA for the enzymic synthesis of amoxicillin. The experimental data were obtained at pH 6.5 or 7.5, 25 °C and different values of initial substrate concentrations. It should be stressed that higher yields with respect to 6-APA could certainly be achieved, if precipitation of the antibiotic was promoted (for instance, using a fed-batch reactor). However, the focus of this study was the kinetics of the system rather than the optimization of the reactor operation.

Figure 1 compares the predictions of model 1 (which disregards inhibition) with data from an amoxicillin synthesis experiment. It can be seen that this model gives a poor representation of the concentration profiles of amoxicillin and POHPG. Therefore, a more complex model must be used.

Inhibitory effects of POHPGME on the hydrolysis of amoxicillin and of amoxicillin in the hydrolysis of POHPGME

In this work, the ester (POHPGME) was taken as a competitive inhibitor of amoxicillin hydrolysis and the antibiotic was considered a competitive inhibitor of POHPGME hydrolysis. The inhibition constants of amoxicillin and POHPG in the hydrolysis of POHPGME and amoxicillin, respectively, catalysed by glyoxyl-PGA are given in Table 2. The results are congruent with the K_m data. POHPGME seems to be a powerful inhibitor in the hydrolysis of the antibiotic mainly at pH 6.5. Note that the inhibition constant of the antibiotic in the hydrolysis of the ester was much smaller.

These results explain some increments in the yields described in the literature, obtained by increasing the ester concentration, mainly an acidic pH value: not only the possibilities of increasing antibiotic formation (due to the fact that more acyl-enzymes are formed), but the ester inhibition in the hydrolysis of the antibiotic plays an important role in the improvement of the results. In addition, the results suggest that these inhibition constants must be considered in the development of a model to explain the complex process of KCS catalysed by this enzyme.

The competitive inhibitory effect of amoxicillin on the rate of POHPGME consumption and the competitive inhibitory effect of POHPGME on the amoxicillin hydrolysis were considered by model 2. Figure 1 shows that the agreement of the model with the actual experiments improved considerably, especially with respect to the amoxicillin concentration profile. However, it is not accurate for POHPG, indicating that some improvement is still needed. Therefore, inhibitory effects of POHPG on the hydrolysis of POHPGME and amoxicillin were investigated.

Inhibitory effects of POHPG on the hydrolysis of POHPGME and amoxicillin

Results indicate that POHPG acts as a competitive inhibitor in the hydrolysis of POHPGME and amoxicillin. Table 2 shows the POHPG competitive inhibition constants in both hydrolyses. The inhibition constant was only marginally lower for amoxicillin hydrolysis, and again was lower at pH 6.5 than at pH 7.5 (another likely reason for improvement of the yield at this pH value).

The competitive inhibition by POHPG was now included in the rate equations for POHPGME consumption and amoxicillin hydrolysis (model 3). Figure 2 shows a validation test. It can be seen that the model response for the

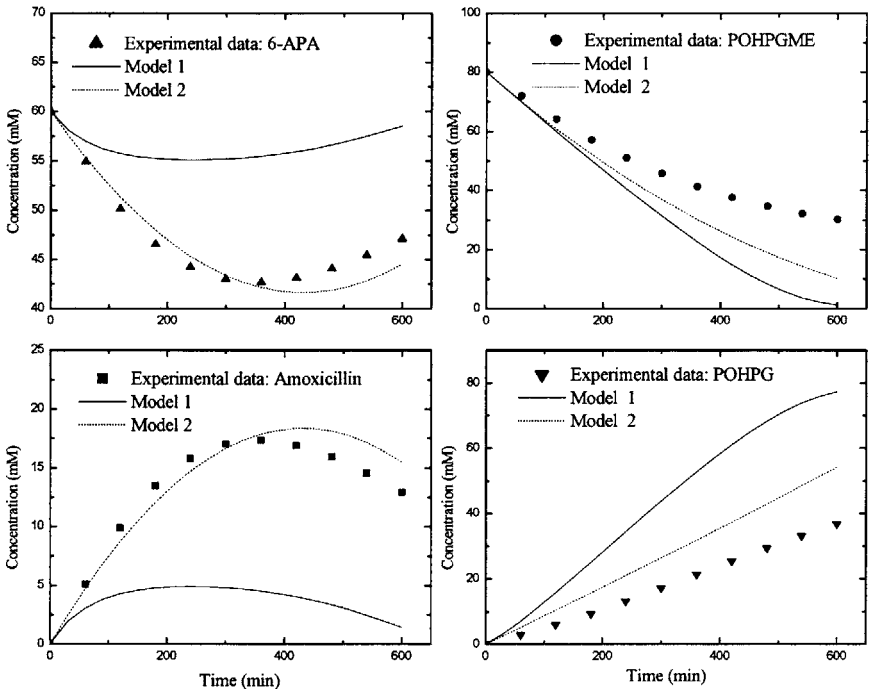


Figure 1 Synthesis of amoxicillin comparing models 1 and 2

Conditions were: pH 6.5, 25 °C, 1 g of 30 i.u./ml derivative, 80 mM POHPGME and 60 mM 6-APA.

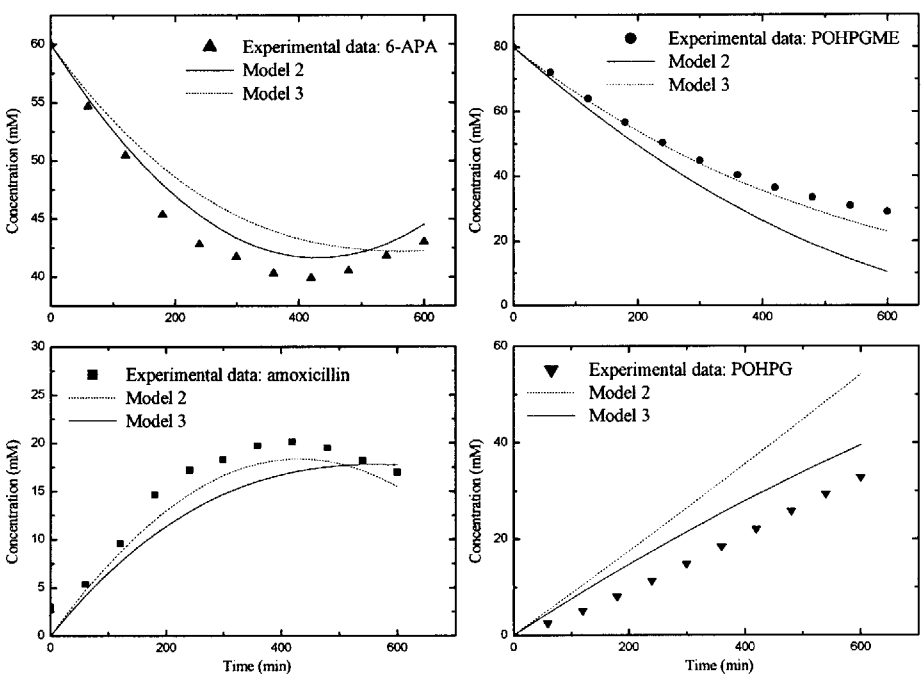


Figure 2 Synthesis of amoxicillin comparing models 2 and 3

Conditions were as in Figure 1.

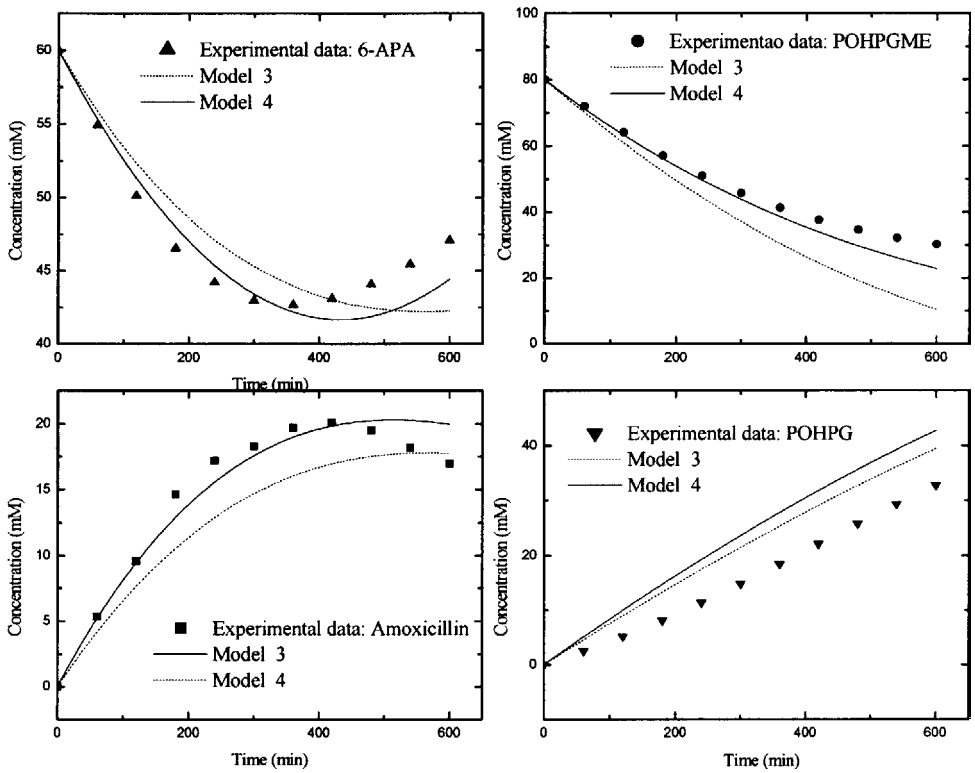


Figure 3 Synthesis of amoxicillin comparing models 3 and 4

Conditions were as in Figure 1.

POHPG profile was improved but that the representation of the amoxicillin profile deteriorated slightly.

Inhibitory effects of 6-APA on the hydrolysis of amoxicillin

In the view of the discrepancy in the literature about the inhibitory effect of 6-APA in amoxicillin synthesis and hydrolysis [4,11,27–29], studies were performed to discriminate the role of this compound. We considered in model 4 that 6-APA is a competitive inhibitor of amoxicillin hydrolysis. Nevertheless, it seems that the interaction between 6-APA and enzyme might be more complicated than that occurring between the other molecules [30].

Figure 3 (comparing models 3 and 4) shows that the assumption of competitive inhibition by 6-APA in antibiotic hydrolysis leads to a reasonable representation of the experimental synthesis data, both for amoxicillin and for POHPG profiles. The antibiotic profile has improved.

Inhibitory effects of methanol on the hydrolysis of amoxicillin and of POHPGME

Methanol has been used frequently to improve the yields in KCS of antibiotics catalysed by PGA, but results have not always been positive. Different hypotheses have been given to explain this result, such as recycling of the substrate [10] and enzyme conformational changes [7]. The effect of methanol on the enzymic reaction depends on the substrate [31,32] and even on the derivative used. Together with this practical interest, and from a strict point of view, methanol may be considered a by-product of the reaction. Thus we decided to include this interesting compound in our analysis.

Inhibition by methanol was found to be mainly of a non-competitive nature, at least in the range of concentrations used (up to 500 mM, equivalent to 1.6% v/v). Values of the inhibition constant for methanol were slightly smaller for the ester than for the antibiotic (1170 and 1563 mM respectively for pH 7.5 and 25 °C); pH does seem to alter the inhibition promoted by methanol. From these results, methanol should decrease the yields, because it favours the hydrolysis of the antibiotic compared with the ester. Therefore, the good results empirically obtained in many cases should be caused by other reasons. On the other hand, these very high values show that this effect may be not included in the model, at least for our range of concentrations. Results shown in Figure 4 reinforce this statement, since the inclusion of methanol inhibition (model 5) caused

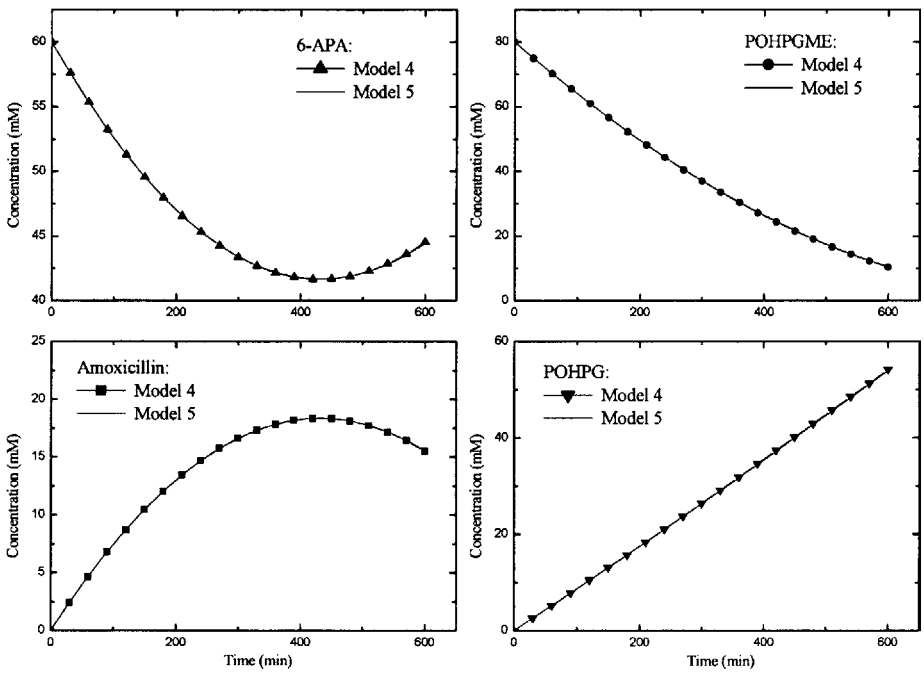


Figure 4 Simulated results of the synthesis of amoxicillin comparing models 4 (without methanol inhibition) and 5 (with methanol as a non-competitive inhibitor of amoxicillin hydrolysis)

Conditions were as in Figure 1.

no alteration in the profile of amoxicillin concentration when compared with results predicted by model 4.

Conclusions

The different inhibitions promoted by different substrates and products in the side reactions of KCS of amoxicillin may give some insight into the understanding of the KCS process. High concentrations of 6-APA and POHPGME at pH 6.5 favoured the ester versus the antibiotic hydrolysis, and therefore should have a positive effect on the final yields with respect to 6-APA. Methanol is a mild non-competitive inhibitor, with a greater effect on hydrolysis of the ester than on that of amoxicillin. The inhibitory effects detected in this work were included in a kinetic model, and in most of the cases this inclusion improved the model performance. Based on the results obtained, model 4 displayed the best performance when compared with experimental data, and can be used to represent amoxicillin synthesis under industrial operational conditions.

Acknowledgments

We thank CNPq, CAPES and FAPESP (Brazil) for the financial support that made this work possible. Hispanagar S. A. and Antibióticos S. A. are gratefully recognized by donation of supports, substrates and enzymes.

References

1 Sheldon, R. A. (1996) J. Chem. Technol. Biotechnol. **67**, 1–14
2 Louwrier, A. and Knowles, C. J. (1997) Biotchnol. Appl. Biochem. **25**, 143–149
3 Moody, H., Hogenboom, A., Lange, B., Heemskerk, D., Dooren, T. V., Boesten, W. and Roos, E. (2001) in Abstract Book of 10th European Congress on Biotechnology, p. 205, Sociedad Española de Biotecnologia (sebiot), Madrid, Spain
4 Ospina, S., Barzana, E., Ramírez, O. T. and López-Munguía, A. (1996) Enzyme Microb. Technol. **19**, 462–469
5 Kaasgaard, S. G. and Veitland, U. (1996) US Patent no. 5,525,483
6 Diender, M. B., Straathof, A. J. J., Van de Wielen, L. A. M., Ras, C. and Heijinen, J. J. (1998) J. Mol. Catalysis B Enzymic **5**, 249–253
7 Fernandez-Lafuente, R., Rossel, C. M. and Guisan, J. M. (1998) Enzyme Microb. Technol. **23**, 305–310
8 Isaka, K., Suga, N. and Ishimura, F. (1995) J. Ferment. Bioengin. **79**, 224–228
9 Duggleby, H. J., Tolley, S. P., Hill, C. P., Dodson, E. J., Dodson, G. D. and Moody, P. C. E. (1995) Nature (London) **373**, 264–268

10 Kasche, V. (1985) Biotechnol. Lett. **7**, 887–882
11 Kasche, V., Haufler, U. and Zöllner, R. (1984) Hoppe-Seyler's Physiol. Chem. **365**, 1435–1443
12 Fernandez-Lafuente, R., Rossel, C. M., Piatkowska, B. and Guisán, J. M. (1996) Enzyme Microb. Technol. **19**, 9–14
13 Schröen, C. G. H., Nierstrasz, V. A., Moody, H. M., Hoogschagen, M. J., Kroon, P. J., Bosma, R., Beeftink, H. H., Janssen, A. E. M. and Tramper, J. (2001) Biotechnol. Bioeng. **73**, 171–178
14 Rocchietti, S., Urrutia, A. S. V., Pregnolato, M., Tagliani, A., Guisán, J. M., Fernández-Lafuente, R. and Terreni, M. (2002) Enzyme Microb. Technol. **31**, 88–93
15 Fernandez-Lafuente, R., Rossel, C. M. and Guisan, J. M. (1995) J. Mol. Catalysis A Chemical **101**, 91–97
16 Hernández-Justiz, O., Fernandez-Lafuente, R., Terreni, M. and Guisan, J. M. (1998) Biotechnol. Bioeng. **59**, 73–79
17 Schroën, C. G. P. H., Nierstrasz, V. A., Bosma, R., Kemperman, G. J., Strubel, M., Ooijkaas, L. P., Beeftink, H. H. and Tramper, J. (2002) Enzyme Microb. Technol. **31**, 264–273
18 Blinkovsky, A. M. and Markaryan, A. N. (1993) Enzyme Microb. Technol. **15**, 965–973
19 Diender, M. B., Straathof, A. J. J., Van Der Does, T., Zomerdijk, M. and Heijnen, J. J. (2000) Enzyme Microbio. Technol. **27**, 576–582
20 Gonçalves, L. R. B., Fernandez-Lafuente, R., Guisán, J. M. and Giordano, R. L. C. (2000) Appl. Biochem. Biotechnol. **84–86**, 931–945
21 Schroën, C. G. P. H., Mohy Eldin, M. S., Janssen, A. E. M., Mita, G. D. and Tramper, J. (2001) J. Mol. Catalysis B Enzymic **15**, 163–172
22 Terreni, M., Pagani, G., Ubiali, D., Fernández-Lafuente, R., Mateo, C. and Guisán, J. M. (2001) Bioorg. Med. Chem. Lett. **18**, 2429–2432
23 Youshko, M. I., Van Langen, L. M., De Vroom, E., Moody, H. M., Van Rantwijk, F., Sheldon, R. A. and Svedas, V. K. (2000) J. Mol. Catalysis B Enzymic **10**, 509–515
24 Giordano, R. L. C. and Schmidell, N. W. (1992) in Atlas do XIII Simpósio Iberamericano de Catalisis, v. 01, pp. 643–646, Segovia, Spain
25 Segel, I. H. (1975) Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems, John Willey & Sons, New York
26 Marquardt, D. W. J. (1963) Soc. Ind. Appl. Math. **11**, 431–441
27 Balasingham, K., Warburton, D., Dunnill, P. and Lilly, M. D. (1972) Biochim. Biophys. Acta **276**, 250–256
28 Plaskie, A., Roets, E. and Vanderhaeghe, H. (1978) J. Antibiot. **31**, 783–788
29 Ospina, S. (1992) J. Chem. Biotechnol. **53**, 205–214
30 Gonçalves, L. R. B., Fernandez-Lafuente, R., Guisan, J. M. and Giordano, R. L. C. (2002) Enzyme Microb. Technol. **31**, 464–471
31 Kim, G. M. and Lee, S. B. (1996) J. Mol. Catalysis B Enzymic **1**, 201–211
32 Fernandez-Lafuente, R. F., Rossel, C. M. and Guisán, J. M. (1998) Enzyme Microb. Technol. **23**, 305–310

Received 24 January 2003/24 March 2003; accepted 11 April 2003
Published as Immediate Publication 11 April 2003, DOI 10.1042/BA20030016