Immobilized penicillin G acylase as reactor and chiral selector in liquid chromatography

G. Massolini[a],*, E. Calleri[a], E. De Lorenzi[a], M. Pregnolato[a], M. Terreni[a], G. Félix[b], C. Gandini[a]

[a]*Department of Pharmaceutical Chemistry, University of Pavia, Via Taramelli 12, 27100, Pavia, Italy*
[b]*ENSCPB, Avenue Pey-Berland (BP 108), 33402, Talence, France*

Abstract

In this paper, the use of penicillin G acylase (PGA) as a biocatalyst and as a chiral selector is described. Penicillin G-acylase is an interesting enzyme used in the manufacture of semisynthetic antibiotics and, in particular, in the production of 6-APA by hydrolysis of penicillin G. Five PGA-based HPLC columns have been prepared by using two different silica supports by employing two immobilization methods, namely ''in situ'' and ''in batch''. The effects of the immobilization techniques and of different silica pore size on the catalytic properties of the enzyme as well as the applicability of the PGA-bonded stationary phases as chiral selectors for a number of chiral drugs have been investigated. The HPLC columns based on immobilized PGA combine the hydrolytic activity and the chiral recognition properties of PGA, therefore they have been used for the development of a combined reaction–separation system for chiral and achiral substrates. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Immobilized enzyme reactor; Chiral stationary phase, LC; Enantiomer separation; On-line hydrolysis; Penicillin G acylase; Enzymes

1. Introduction

The use of enzymes as catalysts for the synthesis of pharmaceutical products and for the stereoselective transformation of optically pure drugs has gained in popularity and the number of important applications in biocatalysis is growing rapidly [1].

Enzymatic catalysis avoids the need for environmentally harmful chemicals and can reduce the waste treatment costs. Furthermore, the reactions can be carried out in mild conditions, leading to increased yield by reducing side-products. As regards the preparation of pure enantiomers, the enzymatic reactions minimize problems of isomerization, racemization, epimerization and rearrangement that may occur during chemical processes.

Nevertheless, employing isolated enzymes in biocatalytic processes presents some important drawbacks, such as enzyme stability under operational conditions, difficult product recovery and the impossibility of reusing it repeatedly. Although these disadvantages have been overcome by enzyme immobilization, the separation of the desired product from the other components (downstream process)

*Corresponding author. Tel.: +39-382-507-383; fax: +39-382-422-975.

E-mail address: g.massolini@unipv.it (G. Massolini).

still represents a critical point. Traditionally, bio-chemical reactors are followed by separation units, designed with the aim of maximizing the yield and the purity of the product(s). Since the costs associated with the downstream process are usually high, alternative methods have been proposed and one interesting technological approach is the use of the integrated reaction–separation process; as this approach is the most time and cost-effective, it can be achieved by using chromatographic reactors. The importance of chromatographic bioreactors is increasing and applications of the integrated reaction–separation process have been described [2–10]. In addition, chiral stationary phases (CSPs) based on immobilized enzymes can represent an efficient and rapid method for the separation of enantiomers [5,6,10]. The use of enzyme based CSPs by pharmaceutical industry can represent an attractive approach for the easy and rapid supply of amounts of enantiomerically pure drugs for biological investigations.

Penicillin acylase (PGA) of *Escherichia coli* ATCC 11105 (EC 3.5.1.11) catalyzes the hydrolysis of penicillin G to phenylacetic acid (PAA) and 6-aminopenicillanic acid (6-APA) and it is well known for its industrial application in the production of this β-lactamic nucleus which is a building block in the synthesis of semi-synthetic penicillins [11,12]. The reaction scheme is reported in Fig. 1. The hydrolytic mechanism involves the nucleophilic attack by a serine residue on the acyl carbon of penicillin G, with the formation of a tetrahedral intermediate, evolving in a serial acyl enzyme and realize of free 6-APA. The acyl enzyme is then susceptible to attack by water to form a second tetrahedron intermediate which can in turn collapse to release the free phenylacetic acid [11].

The enzymatic reaction for the preparation of 6-APA is regio- and stereo-specific, it can be carried

out in mild conditions and it is more economical than the chemical process.

The enzymatic activity of penicillin G acylase has been associated with the phenacetyl moiety and it has been shown that hydrolysis takes place in a variety of phenacetyl derivatives of primary amines as well as alcohols according to their chirality and that phenoxy acetyl derivatives are also accepted as substrates [13–16].

The large production of PGA, its availability and the "relaxed" substrate specificity have led to a wide range of new applications of this enzyme as biocatalyst. Although PGA has been covalently bonded to various supports [17–20] and PGA-immobilized systems have been reported as useful tools for the synthesis of pure optical isomers [16,21–27], there are no publications dealing with PGA immobilized on silica HPLC particles for the development of an integrated reaction–separation chromatographic reactor.

This paper reports the development of an HPLC-compatible immobilized enzyme reactor containing covalently immobilized penicillin G acylase and its applications in an on-line HPLC system. The prepared HPLC–PGA columns were first used as an enzyme reactor for the preparation of 6-APA and then as chiral stationary phases for the separation of enantiomers and for the production of enantiomeric pure drugs in a one-step reaction.

2. Experimental

2.1. Reagents and materials

Penicillin G acylase crude extract from *E. coli* ATCC 11105 (EC 3.5.1.11) was kindly donated by Recordati (Milan, Italy) and used as received;
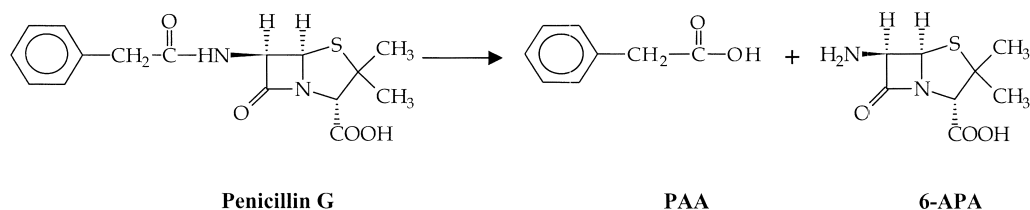

Fig. 1. Reaction scheme of penicillin G hydrolysis. The substrate is converted into phenylacetic acid (PAA) and 6-aminopenicillanic acid (6-APA).

penicillin G potassium salt, phenylacetic acid (PAA), (*R*)- and (*S*)-mandelic acid methyl esters, *rac* α-methoxyphenylacetic acid, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC), *rac* fenoprofen were purchased from Sigma (St. Louis, MO). (*S*)-mandelic acid, (*R*)-mandelic acid and sodium *N*-hydroxysulfosuccinimide (HSSI) were purchased from Fluka (Buchs, Switzerland).

Rac ketoprofen was kindly donated by S.I.M.S s.r.l (Incisa Valdarno, FI, Italy); *rac* ketoprofen methyl ester, *rac* suprofen methyl ester and *rac* α-methoxyphenylacetic acid methyl ester were synthesized in our laboratory. *Rac* 2-(4-phenoxyphenoxy) propionic acid and *rac* 2-(4-benzylphenoxy) propionic acid with their methyl esters were synthesized in our Department by Professor O. Azzolina.

Aminopropyl silica packing material (Nucleosil-5NH$_2$, 5 μm particle size, 100 Å pore diameter) was obtained from Macherey-Nagel (Düren, Germany). Kromasil 5 μm, 100 and 200 Å silica gel was a gift from Eka-Nobel (Bohus, Sweden). *N,N'*-disuccinimidyl carbonate (DSC) and 6-aminopenicillanic acid (6-APA) were purchased from Sigma–Aldrich (Milan, Italy). Potassium dihydrogen phosphate and dipotassium hydrogen phosphate used for the preparation of the mobile phases were of analytical grade and purchased from Merck (Darmstadt, Germany). Sodium perchlorate (NaClO$_4$) and acetonitrile were from Carlo Erba (Milan, Italy); perchloric acid 70% (HClO$_4$) was from BDH Italia (Milan, Italy). Water was deionized by passing through a Direct-Q™ (Millipore) system (Millipore, Bedford, MA).

2.2. Equipment

Chromatographic experiments were performed with two HPLC systems. One system consisted of a Hewlett-Packard HP 1100 liquid chromatograph (Palo Alto, CA, USA) with a Rheodyne sample valve (20-μl loop) equipped with a Hewlett-Packard HP 1100 variable-wavelength detector and a HP 1100 thermostat. The second equipment was a Hewlett-Packard HP 1050 liquid chromatograph with a Rheodyne sample valve (20-μl loop) equipped with a Hewlett-Packard HP 1050 variable-wavelength detector. Both systems were connected to a HPLC ChemStation (Revision A.04.01).

Titration was performed by means of 718 STAT Titrino from Metrohm Italiana (Saronno, VA, Italy).

2.3. Methods

2.3.1. Chromatographic conditions for PGA columns

The mobile phase used for the enzymatic columns was 50 m*M* phosphate buffer (pH 7.0). All the chromatographic experiments were carried out at room temperature and the column flow-rate, unless otherwise stated, was set at 0.8 ml/min. The UV trace was followed at 225 nm.

When not in use, the columns were stored at 4°C in a 0.01% (w/v) solution of sodium azide.

2.3.2. Chromatographic responses

The retention factor (*k*) was calculated using the equation $k = (t_r/t_0) - 1$, where t_r is the retention time of the analyte and t_0 is the retention time of an unretained compound; in this study t_0 was calculated from the first disturbance of the baseline after injection. The separation factor (α) was calculated using the equation, $\alpha = k_2/k_1$ where k_1 and k_2 are the retention factors for the first and last eluted enantiomers, respectively.

2.3.3. Identification methods

Identification of the analytes from the enzymatic columns was assessed by collecting the fractions corresponding to the eluted peaks and analysing them on an off-line chromatographic system with methods developed on purpose, as follows.

2.3.3.1. Identification of mandelic acid enantiomers together with their methyl esters (Method A)

The column used was a Chiralcel ODR-CSP (250×4.6 mm ID), Daicel Chemical Industries, Ltd., Daicel (Europa) GmbH (Duesseldorf, Germany). The eluent was 0.5 N NaClO$_4$–HClO$_4$ buffer (pH 2.0)-acetonitrile (90:10, v/v) and detection was performed at 225 nm. The analyses were carried out applying the following flow-rate gradient: 0.5 ml/min for the first 5 min and increasing the flow to 1.0 ml/min in 15 min. Under these chromatographic conditions, the relative chromatographic retentions of (*S*)- and (*R*)-mandelic acid were 2.14 and 2.23 respectively, corresponding to an observed separa-

tion factor of 1.04. The k_s for (S)- and (R)-mandelic acid methyl esters were 6.93 and 8.05, respectively and the observed α was 1.16.

2.3.3.2. Identification of α-methoxyphenylacetic acid enantiomers together with their methyl esters (Method B)

The column was a Chiralcel ODR-CSP (250×4.6 mm ID). The flow-rate was maintained at 0.8 ml/min and the detection was performed at 225 nm. The mobile phase was 0.5 N NaClO$_4$–HClO$_4$ buffer (pH 2.0)-acetonitrile (70:30, v/v). The retention factors of α-methoxyphenylacetic acid enantiomers were 0.85 and 1.01 corresponding to an observed separation factor of 1.18. The k_s for α-methoxyphenylacetic acid methyl ester enantiomers were 3.49 and 4.35 and the observed α was 1.25. A representative chromatogram showing the simultaneous enantioresolution of both enantiomeric pairs is reported in Fig. 2a.

2.3.3.3. Identification of penicillin G together with its hydrolysis products phenyacetic acid and 6-aminopenicillanic acid (Method C)

The column used was a 100 RP-18, 5 μm Li-Chrosher (125×4 mm ID), Merck (Darmstad, Germany). The mobile phase employed was 0.1 M phosphate buffer (pH 3.5)-methanol (60:40, v/v). Flow rate was 1.0 ml/min and the detector was set at 225 nm. The chromatographic retention factors were 0.136 for 6-aminopenicillanic acid, 2.75 for phenyacetic acid and 3.94 for penicillin G.

2.3.3.4. Identification of 2-(4-phenoxyphenoxy) propionic acid enantiomers together with their methyl esters (Method D)

The column was a Chiralcel ODR-CSP (250×4.6 mm ID). The flow-rate was maintained at 0.7 ml/min and the detection was performed at 225 nm. The mobile phase was 0.5 N NaClO$_4$–HClO$_4$ buffer (pH 2.0)-acetonitrile (50:50, v/v). The retention factors of 2-(4-phenoxyphenoxy) propionic acid enantiomers were 2.19 and 2.27, corresponding to an enantioselectivity of 1.03. The k_s for 2-(4-phenoxyphenoxy) propionic acid methyl ester enantiomers were 6.94 and 8.06 with an observed α of 1.16. The contemporary enantioresolution of both enantiomeric pairs is reported in Fig. 2b.

2.3.3.5. Identification of 2-(4-benzylphenoxy) propionic acid enantiomers (Method E)

The column was a AGP-CSP (100×4.0 mm ID) (Chromtech, Hägersten, Sweden). The flow-rate was maintained at 0.8 ml/min and the detection was performed at 225 nm. The mobile phase was 100 mM phosphate buffer (pH 6.5)-isopropanol (99:1, v/v). The retention factors of 2-(4-benzylphenoxy) propionic acid enantiomers were 9.91 and 12.94, corresponding to an enantioselectivity of 1.3.

2.3.3.6. Identification of 2-(4-benzylphenoxy) propionic acid methyl ester enantiomers (Method F)

The column was a AGP-CSP (100×4.0 mm ID). The flow-rate was maintained at 0.8 ml/min and the detection was performed at 225 nm. The mobile phase was 100 mM phosphate buffer (pH 6.5)-isopropanol (92:8, v/v). The retention factors of 2-(4-benzylphenoxy) propionic acid methyl ester enantiomers were 11.04 and 16.82, corresponding to an enantioselectivity of 1.52.

2.3.4. Immobilization techniques

The "in batch" and "in situ" immobilization techniques were considered in this study. The enzymatic activities of immobilized PGA were determined following the procedure described in Section 2.3.5 and the bound amount of PGA to silica gels was calculated by elemental analysis.

Aminopropyl silica loose packing material was used for the "in batch" immobilization of PGA according to a previously described method [28]. An additional final washing step with a 2 M NaCl solution was carried out in order to remove the possible adsorbed enzyme monitoring the washing fractions in respect to their enzymatic activity towards penicillin G.

After the washing process, the measured enzymatic activity of the obtained solid support was 121.18 U/g (equivalent to 87.06 mg enzyme/g solid support).

The active solid enzymatic support obtained was packed in a stainless tube (100×4.6 mm ID) by a classic slurry packing technique using 50 mM phosphate buffer (pH 7.5) as suspending medium and 50 mM phosphate buffer (pH 7.5)-isopropanol (85:15, v/v) as packing solvent.
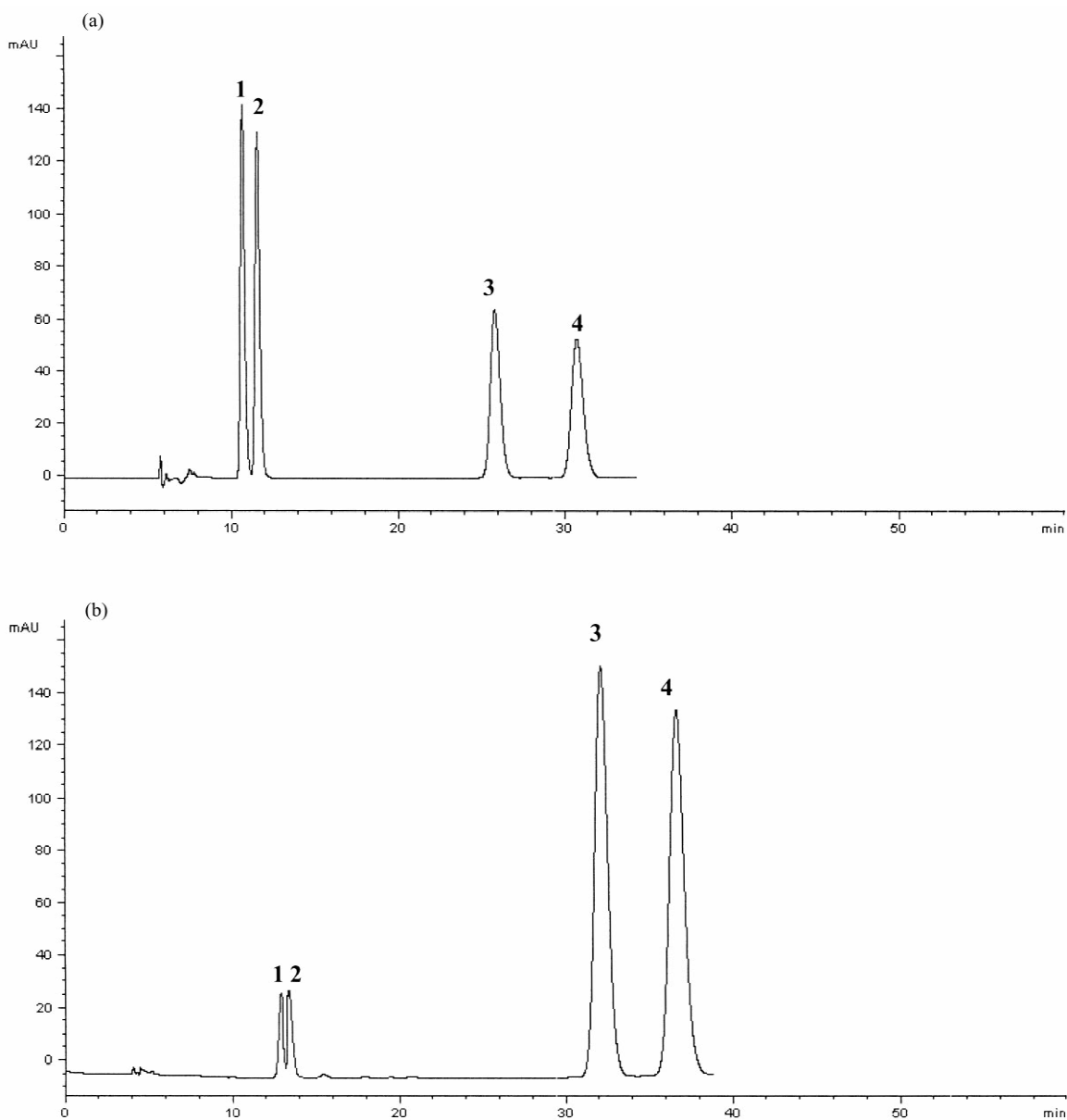
Fig. 2. (a) Chromatogram representing a standard of *rac* α-methoxy phenyl acetic acid (1 m*M*) and *rac* α-methoxy phenyl acetic acid methyl ester (1 m*M*). See Method B for chromatographic conditions. Peaks 1and 2: enantiomers of α-methoxy phenyl acetic acid; Peaks 3 and 4: enantiomers of α-methoxy phenyl acetic acid methyl ester. (b) Chromatogram representing a standard of *rac* 2-(4-phenoxyphenoxy) propionic acid (0.1 m*M*) and *rac* 2-(4-phenoxyphenoxy) propionic acid methyl ester (1 m*M*). See Method D for chromatographic conditions. Peaks 1and 2: enantiomers of 2-(4-phenoxyphenoxy) propionic acid; Peaks 3 and 4: enantiomers of 2-(4-phenoxyphenoxy) propionic acid methyl ester.

The "in situ" immobilization procedure [29] was also used to prepare four PGA columns.

The immobilization of PGA via the amino groups of the protein on to aminopropyl silica pre-packed columns, (50×4.6 mm ID, 5 μm particle size, 100 and 200 Å pore diameter), indicated as PGA-NH$_2$-100 and PGA-NH$_2$-200, was carried out as follows.

A total of 2.25 g of DSC were dissolved in 100 ml

acetonitrile and the resulting solution was continuously circulated for 18 h at 0.5 ml/min through the column previously equilibrated with the same solvent. The column was then washed at the same flow-rate, first with 60 ml acetonitrile and then with 60 ml of 1 mM phosphate buffer (pH 7.0). A total of 300 mg PGA were dissolved in 100 ml of 1 mM phosphate buffer (pH 7.0) and the enzyme solution continuously circulated at 0.3 ml/min. After 24 h the column was washed with 200 ml of 20 mM phosphate buffer (pH 7.0), 200 ml of a 0.5 M solution of NaCl and finally with 100 ml of a 0.2 M glycine solution to block the remaining activated groups and equilibrated with a 50 mM solution of phosphate buffer (pH 7.0). The amount of immobilized enzyme was 39.98 mg/g solid support (241.23 U/g solid support) and 70.11 mg/g solid support (495.39 U/g solid support) for PGA-NH$_2$-100 and PGA-NH$_2$-200, respectively.

PGA was also immobilized on aminopropyl silica via the carboxylic groups of the protein following a previously reported reaction scheme [30] on a pre-packed column (100×4.6 mm ID, 5 μm particle size, 100 Å pore diameter). In this procedure the column was first equilibrated with 50 mM phosphate buffer (pH 7.0) and then with a 4 mg/ml enzymatic solution in the same buffer. A total of 0.2 g EDC and 0.1 g HSSI were solubilized in the enzymatic solution and the resulting solution was continuously circulated for 24 h, 30°C at 0.5 ml/min through the column. The column was then washed with water at the same flow-rate and equilibrated with 50 mM phosphate buffer (pH 7.0).

The epoxyde column (50×4.6 mm ID, 5 μm particle size, 200 Å pore diameter), used for the "in situ" immobilization of PGA (PGA-epoxyde-200) was prepared following a previously described method [9]. The pre-packed column was first equilibrated with the coupling solution, i.e. 50 mM phosphate buffer (pH 7.5) containing 1.875 M ammonium sulfate. A total of 50 ml of coupling solution containing 150 mg of PGA have been filtered and applied to the column at 0.5 ml/min for 24 h. The column was back flushed every 30 min. Then the column was washed with 70 ml of 50 mM phosphate buffer pH 7.0 and with a 1 M solution of glycine to block the remaining epoxyde groups. The amount of immobilized enzyme was 184.13 mg/g solid support (1252.38 U/g solid support)

2.3.5. Determination of enzyme activity and enantioselectivity

The hydrolysis of penicillin G potassium salt has been used as a standard assay for the determination of the catalytic activity of the enzyme both in solution and in the immobilized form. The hydrolysis was carried out in 20 ml of a 100 mM penicillin G solution in 25 mM phosphate buffer (pH 7.5) and the phenylacetic acid produced was titrated with 0.1 M NaOH with automatic pH correction. The experiments were carried out at room temperature. The enzymatic activity was measured as international units (U), equivalent to μmol of penicillin G hydrolysed per minute.

The enantioselective enzymatic activity of PGA immobilized on loose material has been evaluated by measuring the initial hydrolysis rate of each enantiomer of methyl mandelate [13]. In this procedure 20 ml of a 20-mM solution of substrate in 25 mM phosphate buffer (pH 6.5) at 4°C were treated with 100 units of immobilized enzyme while the pH was maintained at the initial value by means of an automatic titrator with 100 mM NaOH. At 50% conversion the reaction mixture was filtered and the enantiomers of mandelic acid together with the enantiomers of non-hydrolyzed esters were directly analyzed by chiral HPLC (*Method A*).

3. Results and discussion

3.1. Immobilized PGA as combined reaction–separation system: influence of the immobilization technique

The optimal immobilizing technique is the first step in the development of a stationary phase, therefore the "in batch" and "in situ" protocols and the immobilisations via amino or carboxylic group(s) of the protein were considered for the preparation of silica supports based on immobilized PGA.

In order to evaluate the "in batch" process, PGA was initially immobilized on loose aminopropyl silica chromatographic support. Before the packing procedure, the enzymatic activity of the obtained stationary phase was determined in the pH range 5.0–7.5 following the procedure described in Section 2.3.5. The immobilized PGA maintained the sen-

Table 1
Effect of pH on the activity of PGA immobilized on loose aminopropyl silica

pH	Activity (U/g support)
5.0	18.6
6.5	70
7.0	86.2
7.5	121.2

sitivity to changes in pH (Table 1) that is an increasing of the enzymatic activity with pH [18].

The enantioselective enzymatic activity of immobilized PGA was investigated by using *rac* methyl mandelate as substate, a compound transformed at a high rate. The hydrolysis of the racemic mixture of methyl mandelate gave a low enantiomeric excess with (*R*) enantiomer hydrolyzed first (enantiomeric excess of ~16 at 50% conversion) as monitored with *Method A*, this result is in agreement with published data [13].

The enzymatically active PGA stationary phase was then packed into a stainless-steel column by the slurry packing technique and the prepared column was tested in respect to the hydrolytic activity towards penicillin G. The observed loss of catalytic activity suggested that the packing process could have irreversibly compromised the enzymatic activity.

These negative results led us to move to the ''in situ'' process. This method consists in the immobilization of macromolecules directly in a pre-packed activated column and it should avoid packing problems. Since the pore size of the base silica gels and the immobilization technique can influence retentivity and enantioselectivity of protein-based stationary phases [30,31], four different columns were prepared and used to study the catalytic activity and the enantioselective properties of immobilized PGA.

PGA was bound to aminopropyl silica with different silica pore size via amino (PGA-NH$_2$-100 and PGA-NH$_2$-200) or carboxylic group(s) of the protein. The studied enzyme was also immobilized on epoxy silica (PGA-epoxy-200).

Before performing the on-line catalytic experiments on the chromatographic system, the hydrolysis products of penicillin G (PAA and 6-APA) were individually injected onto PGA-columns in the chromatographic conditions selected for the hydrolytic

experiments (see Section 2.3.1); these analytes were eluted with the front solvent. Subsequently, the performances of the developed columns were compared towards their ability to hydrolyze penicillin G according to the scheme reported in Fig. 1. A solution of penicillin G (1 mg/ml in 50 m*M* phosphate buffer pH 7.0) was injected into the HPLC system.

A lack of the hydrolytic activity was observed with the PGA-column prepared via the carboxylic groups and therefore this column was not considered for further experiments. On the contrary the enzymatic activity of the columns prepared via the amino groups (PGA-NH$_2$-100, PGA-NH$_2$-200 and PGA-epoxy-200) was maintained. Two peaks were detected on these three enzymatic columns and the chromatographic parameters and profiles are presented in Fig. 3. The obtained peaks were identified by collecting the fractions corresponding to the two eluted peaks and analyzing them off-line by reversed-phase chromatography (*Method C*); the first eluted peak from the PGA-columns was identified as pure 6-APA and the second eluted peak as pure phenyl acetic acid.

PGA-NH$_2$-100, PGA-NH$_2$-200 and PGA-epoxy-200 columns combine the catalytic activity of the enzyme towards penicillin G with the on-line separation of the products. The best results in terms of bound amount of PGA, enzymatic activity and product separation selectivity were obtained with the PGA immobilized on epoxy silica, therefore this column was chosen to study the loading behavior for the hydrolysis of penicillin G.

3.2. Loading capacities of PGA-epoxyde-200 column

The influence of sample loading on retention factors and selectivity of the hydrolysis products was determined in the concentration range 1–10 mg/ml of penicillin G and the results are reported in Table 2. The separation of the hydrolytic products of penicillin G was complete up to 7 mg/ml and on high overloading (e.g., 0.2 mg of penicillin G on column) the retention of phenyacetic acid approaches that of 6-APA and this makes difficult the isolation of these compounds. This behavior can be ascribed to a progressive rise in the hydrolytic velocity due to
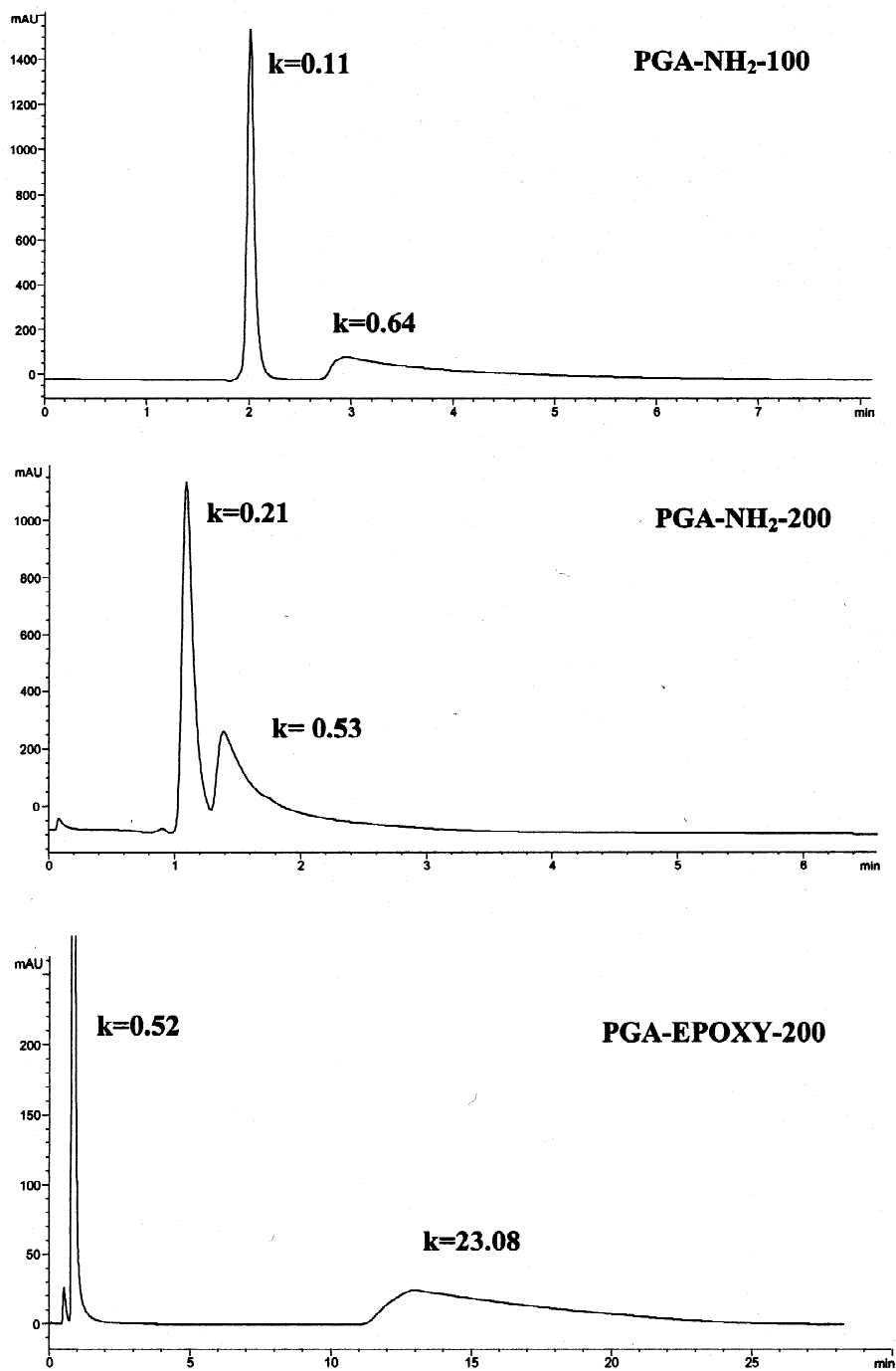
Fig. 3. Chromatograms of penicillin G (1 mg/ml) on PGA columns. Mobile phase: 50 m*M* KH$_2$PO$_4$, pH 7.0; flow-rate 0.8 ml/min; detection, 225 nm.

Table 2
Influence of the loading on the retention factors and selectivity of 6-APA and phenylacetic acid[a]

Concentration (mg/ml)	6-APA	PAA	α
1	0.52	23.08	44.11
2.5	0.53	13.90	26.11
5	0.53	6.47	12.11
6	0.53	4.52	8.50
7	0.53	3.12	5.87
10	0.53	1.50	2.83

[a] Mobile phase: 50 mM KH$_2$PO$_4$, pH 7.0; flow-rate, 0.8 ml/min; detection, 225 nm.

the increasing of substrate concentration as already shown with α-chymotrypsin [10,32]. This finding was lately confirmed by reducing the column temperature and therefore the hydrolytic velocity. Decreasing the temperature to 15°C an increase in selectivity, due to a rise in PAA retention factor, allowed the separation of the hydrolysis products up to 10 mg/ml substrate concentration.

3.3. Immobilized PGA as chiral selector and as combined reaction–separation system of chiral substrates and products

One of the aims of this study was the development of an appropriate immobilization procedure in order to show that immobilized PGA could be a useful chiral selector and a specific chiral biocatalyst. Five chiral compounds characterized by an aromatic ring and a carboxylic group in their structure were selected to study the enantioselective behavior of immobilized PGA.

It has been shown that retentivity is influenced by the amount of bonded macromolecules while enantioselectivity is not strongly affected [33,34], therefore in order to correctly compare the chromatographic properties of PGA-columns, the amount of immobilized enzyme was determined and the data are reported in Table 3. The chromatographic parameters (k, α and R_s) of the racemic mixtures tested together with the chemical structures of the compounds obtained with the three columns are presented in Table 3; the chromatograms of 2-(4-benzylphenoxy) propionic acid enantiomers analyzed

on the three columns are depicted in Fig. 4. The enantioselective properties of the stationary phases were also investigated analysing two other compounds, *rac* mandelic acid and *rac* α-methoxyphenylacetic acid. The enantiomers of these compounds were neither retained nor resolved by PGA-stationary phases therefore the results were not reported in Table 3.

As expected, the retention factors of the solutes increased with the amount of immobilised enzyme (k_s on PGA-epoxy-200 column > PGA-NH$_2$-200 column > PGA-NH$_2$-100 column). The best enantioselectivity was observed on PGA-epoxy-200 column while the enantioselectivity obtained on PGA-NH$_2$-200 support was lower than on PGA-NH$_2$-100 and PGA-epoxy-200 columns irrespectively to the bound amount of PGA.
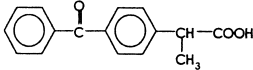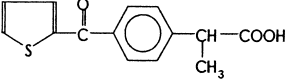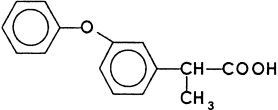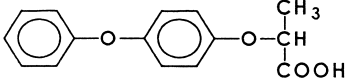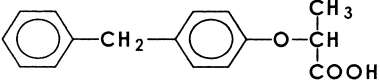
The data obtained in this part of the work seem to indicate that the immobilization on epoxy-activated silica column is the best way to obtain a stationary phase for the direct enantiomeric separations of acidic racemates and that PGA-epoxy-200 chiral stationary phase may be the best candidate for a scale-up designed for an on-line enzymatic production of enantiomerically pure acidic compounds.

A chromatographic combined reaction–separation system based on PGA-epoxy-200 column was therefore set-up and the stereoselectivity of PGA catalyzed reactions was studied by using different substrates.

Penicillin G acylase has been shown to reveal high enantioselectivity in the hydrolysis of *rac* α-methoxyphenylacetic methyl ester, which yields an enantiomeric excess higher than 98% in favour of the (*S*) form as reported in literature [13]. This analyte, whose acid is a well known chiral derivatizing agents used in NMR methods based on diastereotopicity, was used as a model compound.

A 1-mM solution of α-methoxyphenylacetic methyl ester was injected into the HPLC system and two peaks appeared in the chromatogram (Fig. 5). The two fractions corresponding to the elution of the peaks were collected and the fraction corresponding to the first eluted peak ($k = 0.15$), analyzed off-line on an ODR chiral column (*Method B*), contained one of the enantiomers of α-methoxyphenylacetic acid ($k = 0.85$) while the fraction corresponding to second eluted peak ($k = 0.64$) contained the enantiomer of

Table 3
Retention factors, enantioselectivity and resolution on PGA-columns[a]

Compound	Parameter	Immobilized amount		
		39.98 mg/g PGA-NH$_2$-100	70.11 mg/g PGA-NH$_2$-200	184.13 mg/g PGA-epoxy-200
Ketoprofen	k_1	1.05	2.72	2.82
	α	1.71	1.24	3.15
	R_s	3.8	1.15	3.3
Suprofen	k_1	1.49	3.26	6.12
	α	1.34	1.09	1.74
	R_s	2.64	0.28	4.05
Fenoprofen	k_1	0.54	2.53	4.97
	α	2.44	1.33	2.58
	R_s	1.87	1.26	1.80
2-(4-phenoxyphenoxy) propionic acid	k_1	0.87	2.82	7.28
	α	1.60	1.16	1.69
	R_s	1.70	0.70	3.09
2-(4-benzylphenoxy) propionic acid	k_1	0.97	3.32	6.66
	α	2.48	1.5	3.30
	R_s	4.18	1.77	6.56

[a] Mobile phase: 50 m*M* KH$_2$PO$_4$, pH 7.0; flow-rate 0.8 ml/min; flow-rate 225 nm.

the non-hydrolyzed α-methoxyphenylacetic methyl ester ($k = 4.49$).

It has been reported that phenoxy acetyl derivatives are also accepted as substrates [13] and for this reason the 2-(4-phenoxyphenoxy) propionic acid methyl ester and 2-(4-benzylphenoxy) propionic acid methyl esters were chosen as further test compounds. Solutions of ester's racemic mixture have been injected onto the enzymatic column and in both cases four peaks were obtained (Figs. 6a and b); the peaks were identified by reinjecting the eluent corresponding to each peak to a second HPLC system

using three enantioselective chromatographic methods (*Method D, E and F*). For 2-(4-phenoxyphenoxy) propionic acid methyl ester analyzed on PGA column, peaks with retention factors of 8.72 and 14.61 were identified as enantiomers of 2-(4-phenoxyphenoxy) propionic acid ($k = 2.01$ and $k = 2.09$ on Chiracel ODR column), peaks with retention factors of 29.32 and 60.93 were identified as enantiomers of the remaining not hydrolyzed 2-(4-phenoxyphenoxy) propionic acid methyl ester ($k = 6.56$ and $k = 7.63$ on Chiracel ODR column).

In the same way for 2-(4-benzylphenoxy) propion-

PGA-NH₂-100

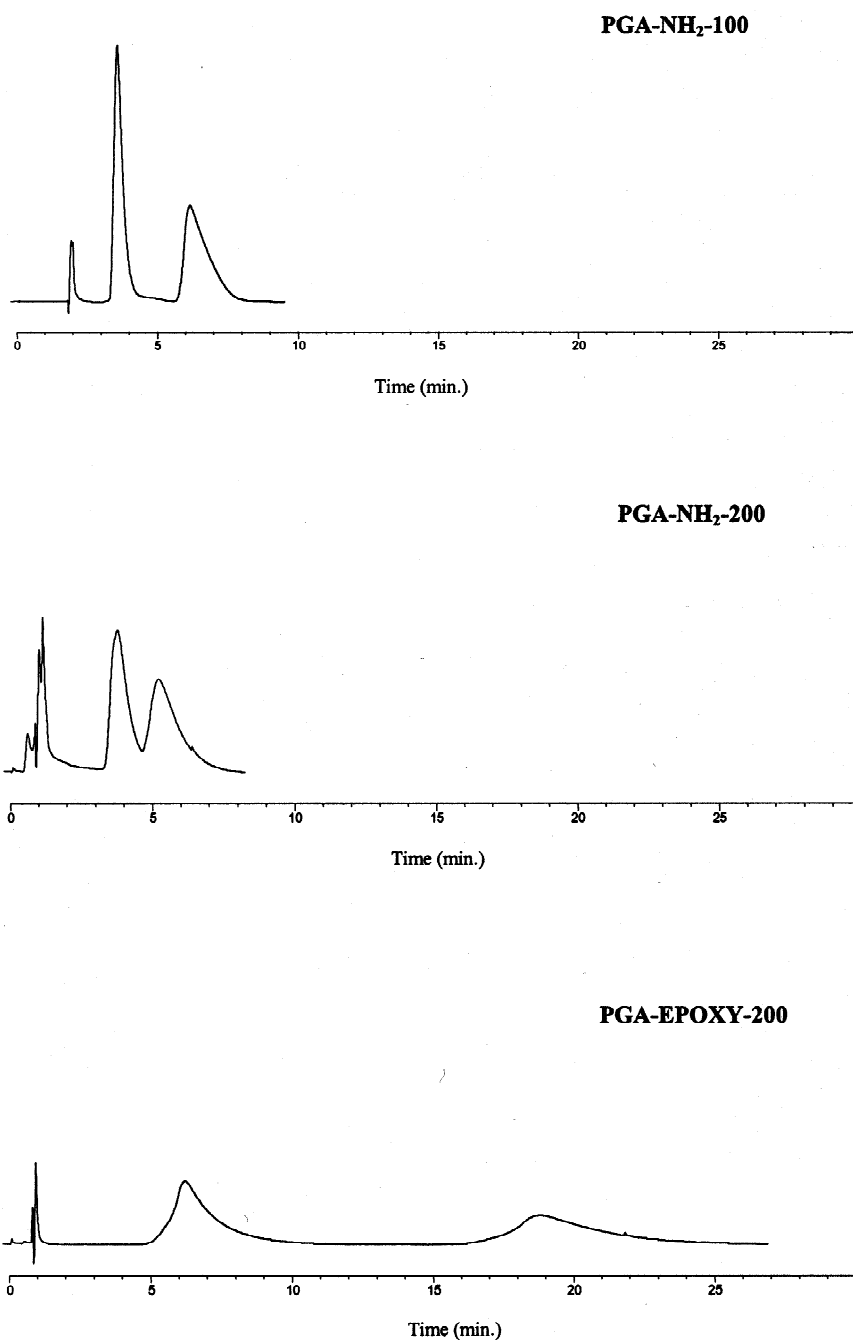**PGA-NH₂-200**

PGA-EPOXY-200

Fig. 4. Resolution of 2-(4-benzylphenoxy) phenyl acetic acid enantiomers on PGA columns.

ic acid methyl esters, peaks with retention factors of 6.82 and 19.05 were identified as enantiomers of 2-(4-benzylphenoxy) propionic acid ($k = 9.89$ and $k = 12.80$ on AGP column), peaks with retention factors of 40.32 and 100.43 were identified as enantiomers of the remaining not hydrolyzed 2-(4-benzylphenoxy) propionic acid methyl ester ($k = 11.10$ and $k = 16.93$ on AGP column).
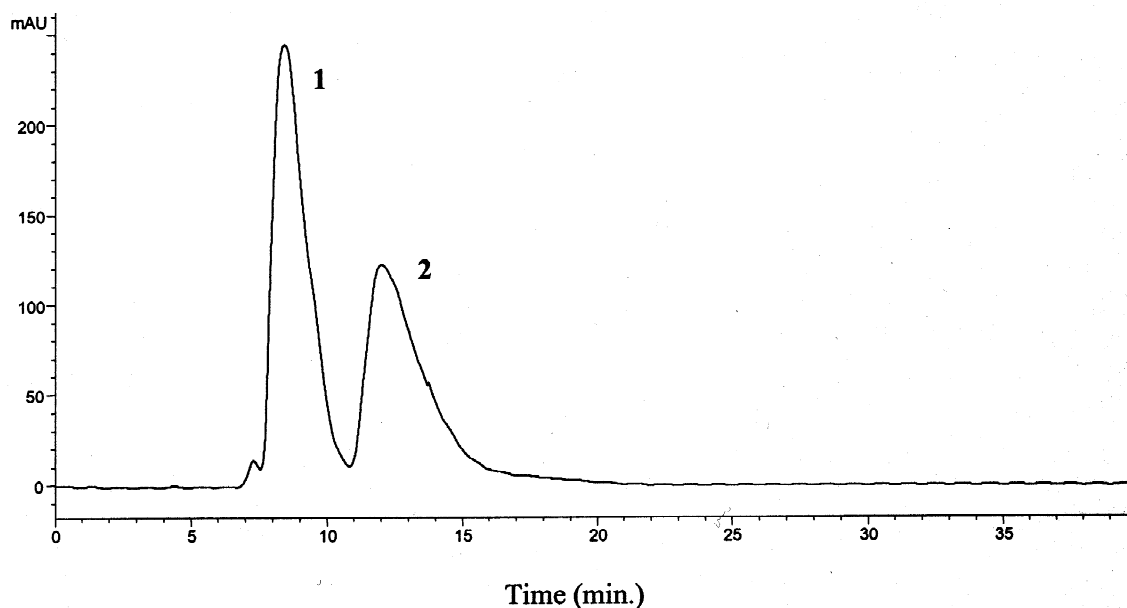
Fig. 5. Chromatogram showing the stereoselective hydrolysis of *rac* α-methoxy phenyl acetic acid methyl ester on the epoxyde column. Mobile phase: 50 m*M* KH_2PO_4, pH 7.0; flow-rate 0.1 ml/min; detection, 225 nm. Peak 1: single enantiomer of α-methoxy phenyl acetic acid; Peak 2: single enantiomer of the survived α-methoxy phenyl acetic acid methyl ester.

In order to study the influence of the substrate structure on the hydrolytic activity of immobilized PGA the investigation was extended to *rac* ketoprofen, *rac* suprofen and *rac* mandelic acid methyl esters. Under the chromatographic conditions used in these studies, *rac* mandelic acid methyl ester gave only one peak with low retention factor. The eluted analyte was identified as the racemic mixture of mandelic acid when analyzed on ODR column (*Method A*). On the contrary, when *rac* ketoprofen methyl ester was injected on PGA-column two peaks were detected and identified as unhydrolyzed esters. In the case of suprofen methyl ester neither hydrolytic activity nor enantiomeric resolution was observed.

These studies have shown that phenyl acetic structures are the ideal substrate for the hydrolytic activity of PGA also when the enzyme is immobilized on silica support. However, the developed PGA-columns can be used as combined reaction–separation system only for the preparation of α-methoxyphenylacetic acid enantiomers while the enantiomers of *rac* mandelic acid obtained during the chromatographic run are not resolved.

The hydrolytic activity towards phenyl acetic structures is still maintained when a second aromatic ring is introduced in the phenyl acetic structure if an oxygen atom in α-position is present. This was confirmed by the fact that ketoprofen and suprofen methyl esters were not accepted as substrate while the aryloxypropionic esters considered were hydrolyzed.

4. Conclusion

The low cost and the large availability of penicillin G acylase led us to choose this enzyme for the development of an integrated reaction–separation process on a HPLC stationary phase as an alternative technological approach for the production of compounds of pharmaceutical interest.

The preliminary results obtained show that the developed PGA-stationary phases are capable of producing substances with potential high purity (and consequently high added value) resulting from enzyme substrate specificity and stereospecificity such as 6-aminopenicillanic acid, enantiomers of 2-arylpropionic and 2-aryloxypropionic acids.

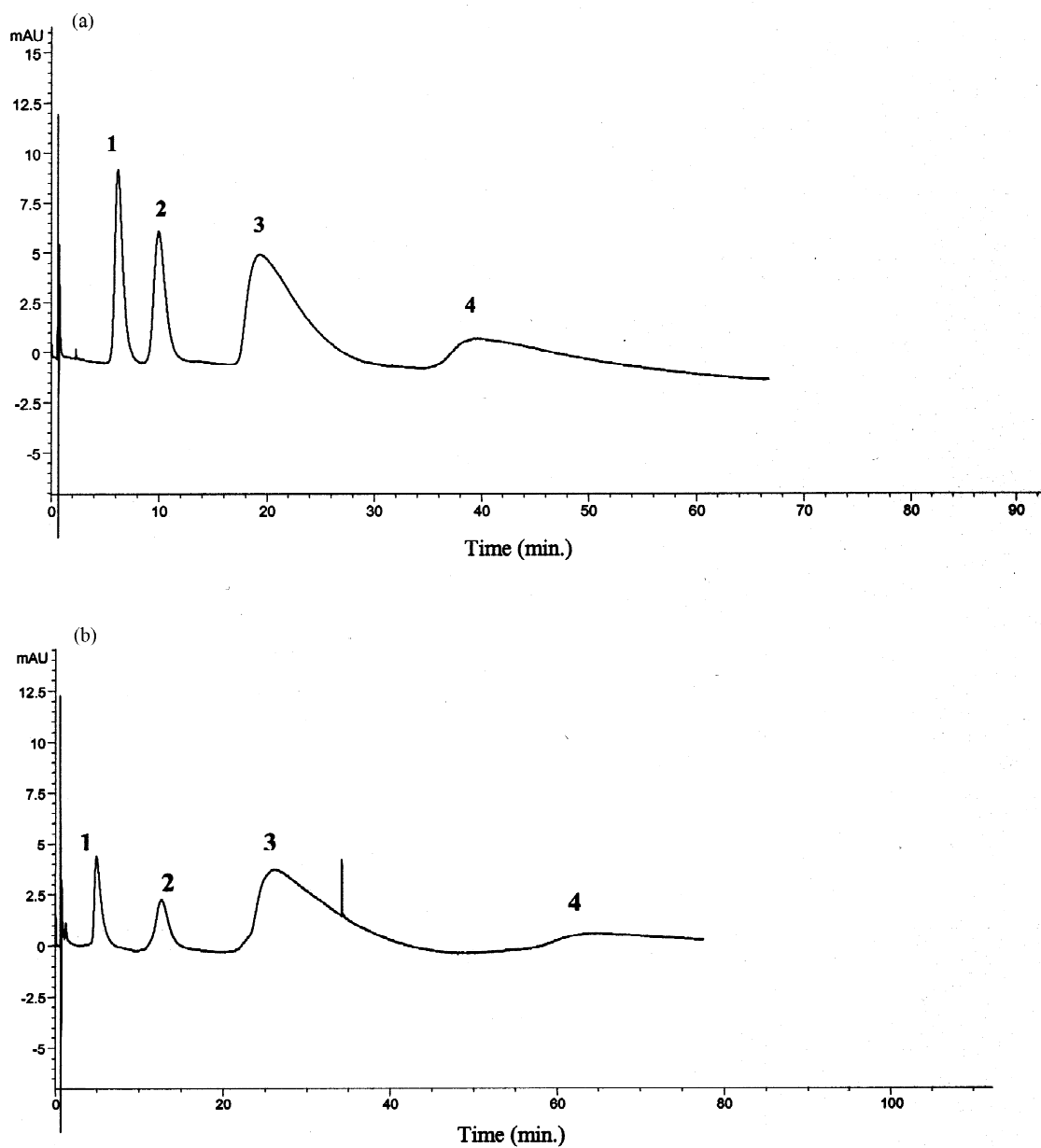As regards the structure demands for a good fit

Fig. 6. (a) Chromatogram showing the hydrolysis of *rac* 2-(4-phenoxyphenoxy) propionic acid methyl ester on the epoxyde column. Mobile phase: 50 m*M* KH$_2$PO$_4$, pH 7.0; flow-rate 0.8 ml/min; detection, 225 nm. Peaks 1 and 2: enantiomers of 2-(4-phenoxyphenoxy) propionic acid; Peaks 3 and 4: enantiomers of 2-(4-phenoxyphenoxy) propionic acid methyl ester. (b) Chromatogram showing the hydrolysis of *rac* 2-(4-benzylphenoxy) propionic acid methyl ester on the epoxyde column. Mobile phase: 50 m*M* KH$_2$PO$_4$, pH 7.0; flow-rate 0.8 ml/min; detection, 225 nm. Peaks 1 and 2: enantiomers of 2-(4-benzylphenoxy) propionic acid; Peaks 3 and 4: enantiomers of 2-(4-benzylphenoxy) propionic acid methyl ester.

into the enzymatic active site the results can be understood in terms of steric and/or polarity requirements of the ligand within the active site of the enzyme.

The HPLC column based on immobilized PGA combines the hydrolytic activity and the chiral recognition properties of PGA and it can be used both as an enzyme reactor and as a CSP with a simple chromatographic system and mobile phase. On the light of the increasing relevance of the preparation of enantiomers in an optically pure form, the wide availability of PGA and the easy setting up of the stationary phase make the developed process very interesting on a preparative scale for pharmaceutical applications. Moreover, the HPLC PGA-column can be used as a screening tool for studying the parameters that can influence the hydrolytic activity and the chiral biocatalysis.

Additional work in the optimization of the chromatographic performance and a more complete study on the chemical and physical stability of the developed stationary phases should be carried out in order to study in quantitative terms the enzymatic kinetic of immobilized PGA.

Acknowledgements

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