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Review

Penicillin G acylase-based stationary phases: analytical applications

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Abstract

A review of Penicillin G Acylase (PGA)-based stationary phases is given, focusing on immobilisation methods, selection of immobilisation material and applications in chiral liquid chromatography. Two immobilization methods, namely "*in situ*" and "*in batch*" techniques, are described for the immobilisation of PGA on silica supports. Microparticulate and monolithic silica, both functionalized with aminopropyl- and epoxy-groups, were used in the development of the PGA immobilised enzyme reactor (IMER). The best results, in terms of PGA immobilised amount and enzyme activity, were obtained with the "*in situ*" immobilisation on epoxy monolithic silica.

The use of PGA columns as enzyme reactors for the preparation of 6-APA and for the production of enantiomeric pure drugs in a one-step reaction in described. The review also covers the application of PGA-columns as chiral stationary phases for the separation of acidic enantiomers.

An on-line chromatographic system based on the PGA-IMER combined with a switching valve to an analytical column is also described as a highly efficient tool to study the enantioselective hydrolyses properties of PGA. Finally a molecular modelling study is reported with the aim to give more insights into PGA-substrates interactions and to expand the application of these stationary phases as a chiral biocatalysts for pharmaceutical processes.

Keywords: Penicillin G Acylase; Chromatographic bioreactor; Enantioselective hydrolysis; Chiral stationary phases

1. Introduction

Biotechnology represents a powerful and versatile enabling technology for the delivering of clean industrial products and it is competitive with other technologies in terms of costs and risks [1].

The application of enzymes as catalysts for chemical synthesis is now well established and enzymatic reactions are used for the production of agrochemicals, fine chemicals and pharmaceuticals. In this field, the use of biological catalysts in the synthesis of pharmaceutical intermediates, drug metabolites and drug products is common [2]. The list of compounds prepared with the assistance of enzymes now includes anti-cancer, anti-viral, anti-infective, anti-psychotic, anti-arhythmic and cholesterol-lowering agents, calcium channel blockers, ACE inhibitors and many others [3,4].

The interest for biocatalysts lies clearly in the exploitation of the outstanding properties of the biocata-

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lysts with respect to chemoselectivity, regioselectivity and, especially, stereoselectivity for the production of enantiomerically pure compounds. In particular, biocatalytic resolutions make use of the selectivity of enzymes for one of the enantiomers of a chiral molecule, whereby one enantiomer of a racemate remains virtually untouched and the other enantiomer is converted into the desired enantiomerically pure product/intermediate.

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, difficult product recovery and the impossibility of re-using it continually. These disadvantages have been overcome by enzyme immobilization. The main technical advantage of immobilized enzymes is that they allow heterogeneous catalysis of enzymatic reactions; there are some other advantages, such as economic, ecological and toxicological ones. At present, immobilized biocatalysts are currently available from several companies [5]. However, the separation of the desired product from the other components (downstream process) still represents a critical point. Traditionally, biochemical 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 many applications have been described [6–11].

Many proteins (enzymes) have the ability to discriminate chiral molecules and chiral stationary phases (CSPs) based on immobilized enzymes represent an efficient and rapid method for the separation of enantiomers [11–13]. This direct chromatographic approach represent, for pharmaceutical industry, an attractive method for the easy and rapid supply of amounts of enantiomerically pure drugs for biological investigations.

Hydrolases are by far the most prominent group of enzymes used in production of fine chemicals by biocatalytic resolution. In particular, the enzyme penicillin G amidase (PGA, also called penicillin acylase) has been used for over 20 years in the production of 6-aminopenicillanic acid. Although this enzyme has been known for quite some time, only recently a synthetic application using PGA with substrates of pharmaceutical relevance other than β-lactam antibiotics has been published [14].

More recently PGA has been covalently immobilized on HPLC supports and the prepared analytical columns have been used as an enzyme reactor and as chiral stationary phase for the resolution of racemic mixture of enantiomers [15–18].

Aim of this review is to summarize the steps in the development of chromatographic bioreactors based on immobilized penicillin G acylase (PGA-IMER) and the trends in applications of a PGA-IMER for the analysis and production of single enantiomers of pharmaceutical interest.

2. Penicillin G acylase (PGA)

Biotechnological applications of penicillin acylases (PA) have emerged as a serious alternative to traditional chemical procedures for the manufacture of β-lactam antibiotics, small peptides and pure isomers from racemic mixtures. However, Penicillin acylases are involved mainly in the industrial production of semi synthetic penicillins.

Penicillin acylases are classified into three groups based on substrate specificity: PGA, penicillin V acylases (PVA) and ampicillin acylases [19–21].

PGAs catalyse the cleavage of the acyl chain of penicillins to yield 6-aminopenicillanic acid (6-APA) and the corresponding organic acid (Fig. 1). The in vivo role of PA has not yet been elucidated, although it has been suggested that PA could be involved in the metabolism of aromatic compounds in order to generate a carbon source.

PGA of *Escherichia coli* ATCC 11105 (EC 3.5.1.11) is the most widely studied. Its crystal structure is well

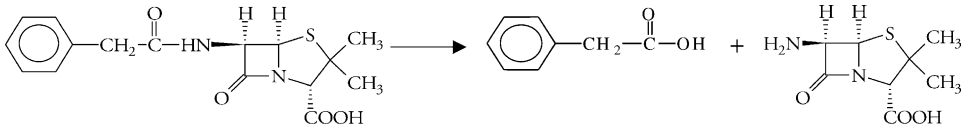


Fig. 1. Reaction scheme of penicillin G hydrolysis. The substrate is converted into phenylacetic acid (PAA) and 6-aminopenicillanic acid (6-APA).

known and many crystallographic data are accessible [22–24]. Mature *E. coli* PGA is a heterodimer with a 20.5 kDa A-subunit and 69 kDa B-subunit; the two monomer chains consist of 209 and 557 amino acid residues, respectively. Crystallographic studies have indicate that the two chains of the enzyme are closely intertwined and form a pyramidal structure that contains a deep cone-shaped depression at the bottom of a which is the active site.

PGA belongs to the newly recognized structural superfamily of N-terminal nucleophile (Ntn) amidohydrolases, characterized by an N-terminal nucleophile that acts as the catalytic residue [22,25–27]. A novel feature of the catalytic machineries of the Ntn amidohydrolases is that the nucleophile, which attacks the carbonyl carbon of the scissile amide or ester bond, and the base, which facilitates the attack by accepting the proton from the nucleophile, are located in the same N-terminal amino acid. In the case of PGA, the hydrolytic mechanism, as suggested by Duggleby et al. [22] involves the nucleophilic attack by the N-terminal 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. The acyl group binding site of PGA is a pocket made up of several hydrophobic residues, rendering the enzyme highly specific for the phenylacetyl group of penicillin G. However, PGA is capable of hydrolyzing a diverse range of amides with a general structure: R−CO−NH−R'. In particular the enzymatic activity has been associated with the acyl moiety, R (hence the term acylase is often used in preference to amidase) and it has been shown that PGA prefers hydrophobic groups. In contrast, R′ can be varied substantially, and the leaving group of the substrates has minimal effect on the hydrolysis rate [28].

The "relaxed" substrate specificity has led to a variety of new applications of this enzyme as biocatalyst, including resolution of alcohols, hydroxy-amino acids, amino acids, and for the deprotection of the phenylacetyl group in peptide synthesis.

Moreover, PGA can resolve racemic mixtures of chiral compounds and exhibits moderate to excellent stereochemical discrimination between corresponding enantiomers in the hydrolytic cleavage of the phenyl-acetyl group from α-aminoalkylphosphoric acids, α-, β- and γ-amino carboxylic acids, sugar, amines, peptides and esters of phenylacetic acid.

Despite the widespread use of these enzymes in asymmetric synthesis, the factors that determine their structural specifity and stereospecificity toward the types of new and non-natural substrate structures of most interest as chiral synthon precursors, remain poorly understood.

3. Development of stationary phases based on immobilized PGA

The development of robust immobilized biocatalysts is a major challenge in industrial biocatalysis and the carriers used to immobilise enzymes have been one of the important research field. In fact, it is well known that the chemical attachment of enzyme to a support can cause significant changes in the catalytic behavior of the enzyme [29].

The technology for PGA immobilization has been improved in the last decades and PGA has been covalently bonded to various supports [30–33]. More recently PGA was immobilized on microparticulate and monolithic silica supports [15–18]. Different immobilization reactions, various pore size and derivatized silica supports were considered in the preparation of the PGA stationary phases for the development of a chromatographic reactor.

3.1. PGA immobilized on microparticulate silica supports

Two immobilization methods, namely "in situ" and "in batch" techniques, were considered for the immobilization of PGA on silica supports.

In order to assess the "in batch" process, PGA was immobilized on loose aminopropyl silica chromatographic support following a procedure described in [34]. Before the packing procedure, the enzymatic activity of the obtained stationary phase was determined using penicillin G as substrate. The enzymatic activity, measured as described in [15] of the obtained solid support was 121.18 U/g (equivalent to 87.06 mg enzyme/g solid support). The enzymatically active PGA stationary phase was than 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. A loss of catalytic activity was observed suggesting that the packing process could have irreversibly compromised the enzymatic activity.

On the bases of these negative results, all the next columns were prepared with 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.

The "in situ" immobilization procedure [35] was used to prepare four PGA columns. PGA was covalently bound to activated amino propyl silica both via amino and carboxylic groups of the protein. PGA was bound to *N*,*N*′-disuccinimydil carbonate (DSC)-activated aminopropyl-pre-packed columns with different pore size (50 mm × 4.6 mm i.d., 5 μm particle size, 100 and 200 Å pore diameter, PGA-$NH_2$100 and PGA-$NH_2$200). It was also tried to bind PGA via a carboxylic group(s) using a water-soluble carbodiimide and *N*-hydroxysulfosuccinimide.

There are many protocols for protein immobilization described in literature but most of them may be difficult to perform. In this context, epoxy-activated supports are almost ideal matrices for performing very easy immobilization of proteins, this material is able to directly form very stable covalent linkages with different proteins groups (amino, thiol, and phenolic ones) under very mild experimental conditions. PGA was therefore immobilized on an epoxyde silica column (50 mm × 4.6 mm i.d., 5 μm particle size, 200 Å pore diameter) (PGA-epoxyde-200) following a method described in literature.

The reaction schemes considered for PGA immobilization are reported in Fig. 2.

The best support for PGA immobilization was selected on the bases of the bound amount of PGA, estimated by elemental analysis. The hydrolysis of penicillin G potassium salt has been used as a standard assay for the determination of the catalytic activity of the enzyme, measured as international units (U, equivalent to μmol of penicillin G hydrolyzed per minute), in the immobilized form [15]. PGA-column prepared via the carboxylic groups did not show any hydrolytic activity, therefore it is not included in the discussion.

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) for PGA-NH_2-100 and PGA-NH_2-200 respectively, as determined by elemental analysis. For the epoxy column the amount of immobilized enzyme was 184.13 mg/g solid support (1252.38 U/g solid support). The three columns were than tested as chiral stationary phases and as a combined reaction-separation systems.

The best results in terms of bound amount of PGA, enzymatic activity, enantioselectivity 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. The data will be discussed in paragraph 4.

3.2. PGA immobilized on monolithic silica supports

The attractive results achieved with Chromolith technology [36–38] and the recent application of monolithic supports to the development of bioreactors [39,40] have lead to consider this innovative chromatographic stationary phases for the extension of the studies on immobilized penicillin G acylase as bioreactor and chiral stationary phase. These innovative materials are based on a development of Nakanishi et al. [41] who used a new sol–gel process for the preparation of monolithic silica columns with a bimodal pore structure (i.e., with throughpores and mesopores). Due to their properties concerning the fast mass transfer between the substance within the eluent and the active sites inside the skeleton of the monolithic silica support, these materials seem to be an

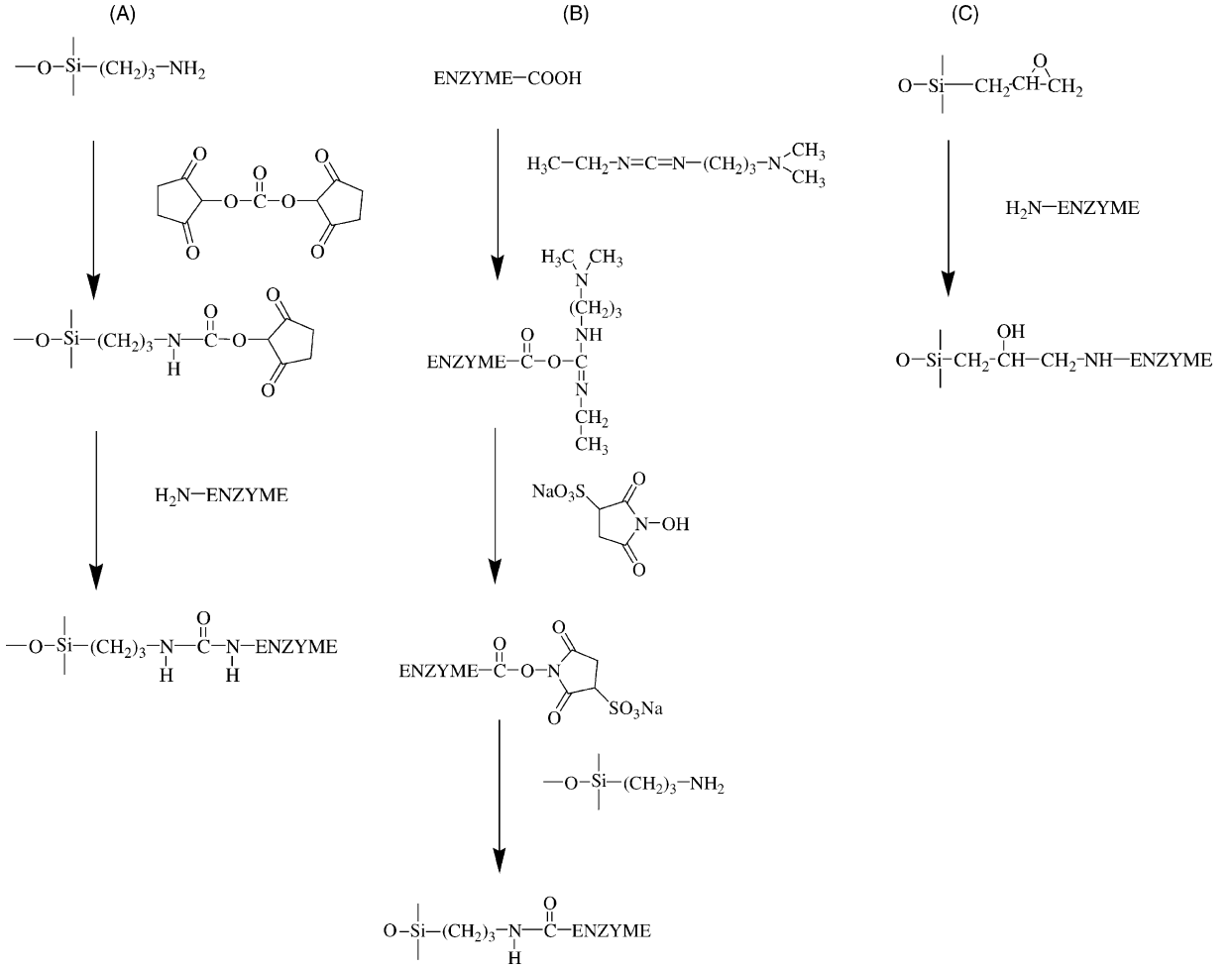


Fig. 2. Synthesis scheme for the preparation of PGA-based stationary phases. (A, C) Via the aminogroups of the enzyme; (B) via the carboxyl group of the enzyme.

ideal support for the immobilization of enzymes and the fast conversion of substrates. In contrary to conventional stationary phases in form of few micrometer particles, monoliths are made of a single piece of porous material. Pores are highly interconnected forming a channel network through which the mobile phase flows. Since the main transport mechanism is convection, mass transfer is significantly facilitated resulting in a flow independent characteristic. In contrast to conventional particle shape porous supports, where the overall behavior is determined mainly by diffusional resistance faced by molecule traveling from the mobile phase to the active sites and back, in the case of monoliths mass transfer resistance can be neglected under operating conditions. Consequently, the overall behavior of the immobilized enzyme reflects its true reaction kinetics. Therefore, such immobilized system is expected to allow higher throughput because of higher enzyme efficiency.

PGA was covalently immobilized on aminopropyl and epoxy monolithic-type HPLC silica support following the same reactions utilized for the microparticulate silica.

The amount of immobilized protein was determined by measuring the difference in protein quantity of the enzymatic solution used for immobilization before and

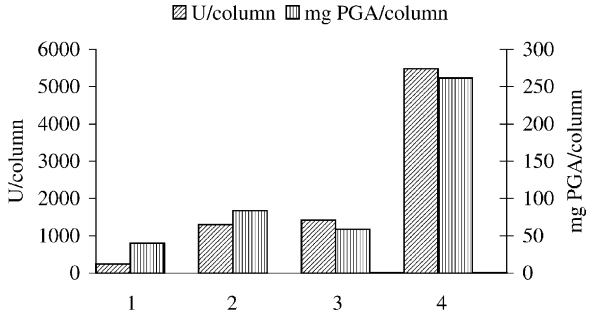


Fig. 3. Amount and activity of PGA immobilized on microparticulate and monolithic silica supports. (1) PGA immobilized on aminopropyl-microparticulate silica. (2) PGA immobilized on epoxyde microparticulate silica. (3) PGA immobilized on aminopropyl-monolithic silica. (4) PGA immobilized on epoxyde monolithic silica.

after the grafting procedure. Bradford Reagent was used as protein assay. The amount of immobilized units was calculated from the difference between the initial and the final enzymatic activity of the enzymatic solution applied to the column. The activity of PGA immobilized onto the monolithic support was calculated by deriving an *on-line* procedure, developed on purpose. For an exhaustive description of this procedure, see [17]. In brief, 20 μl of penicillin G samples in the concentration range to reach the saturation conditions were injected on the monolithic column. The species eluted were collected and analyzed off line on an analytical stationary phase for product quantitation. By linearizing the Michaelis Menten plot, the V_{max} value was estimated to calculate the amount of active immobilized enzyme. The amount of active immobilized enzyme was found comparable to the value of immobilized units, therefore as expected all the immobilized enzyme was found to be active after the immobilization procedure.

In Fig. 3 are reported the amount of immobilized PGA and the expressed units in both monolithic supports in comparison with the data obtained with microparticulate silica. As expected, the quantity of immobilized enzyme was higher on epoxy than aminopropyl-monolithic silica. It was also interesting to observe that the highest amount of immobilized PGA was obtained with the monolithic supports. Moreover, the enzyme activity per mg of immobilized PGA was higher for the monolithic-type columns.

4. Analytical applications of PGA-based stationary phases

The particular structure of PGA, presenting a highly selective pocket for the acyl moiety and a wide tolerance for substrates in the aminic subsite, offers a valuable tool for synthetic applications. Furthermore, the remarkable selectivity of PGA (both regio- and enantio-selectivity) can be successfully utilized in biocatalytic processes for the production of optically pure compounds.

PGA columns have been used as enzyme reactors for the preparation of 6-APA and for the production of enantiomeric pure drugs in a one-step reaction. In additions, the columns were tested as chiral stationary phases for the separation of acidic enantiomers.

4.1. PGA-based stationary phases as chromatographic bioreactor

An integrated reaction-separation chromatographic reactor should combine the hydrolytic activity of the enzyme towards the substrate with the separation of the products. It has been demonstrated that PGA immobilized on epoxy silica (200 Å pore size diameter) is able to completely hydrolyze penicillin G, the natural substrate and separate the products (6-APA and PAA) [15]. A representative chromatogram is given in Fig. 4. In order to extend the use of the chromatographic enzyme reactor to a semi-preparative scale it was interesting to study the influence of sample loading on retention factors and selectivity of the hydrolysis products. The experiments were carried out in the concentration range 1–10 mg/ml of penicillin G. 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. The results are summarized in Table 1. This behavior can be ascribed to a progressive rise in the hydrolytic velocity due to the increasing of substrate concentration [13,42]. 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.

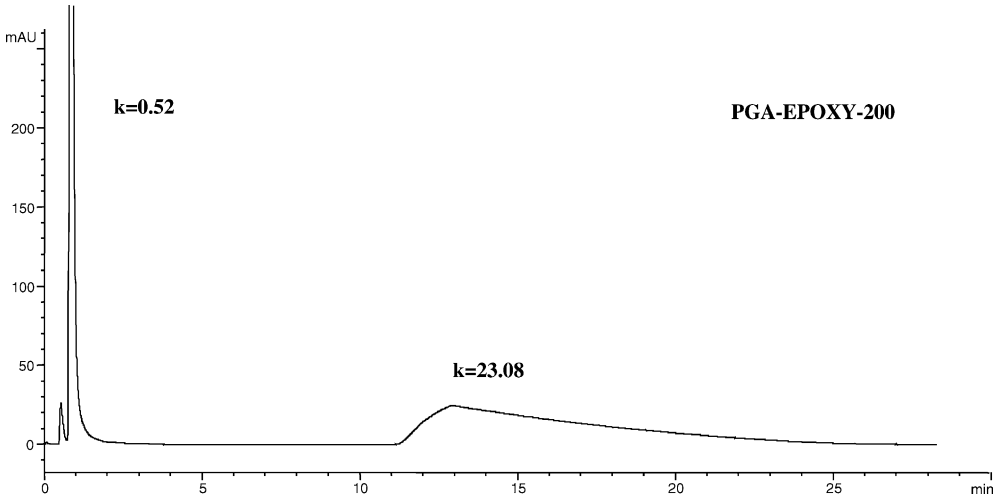


Fig. 4. Chromatograms of penicillin G (1 mg/ml) on PGA epoxy 200. Mobile phase: 50 mM KH_2PO_4, pH 7.0; flow-rate 0.8 ml/min; detection 225 nm. Original figure provided by [15].

The high enantioselectivity observed in free solution in the hydrolysis of *rac* α-methoxyphenylacetic methyl ester, in favor of the (*S*)-enantiomer [43] was also obtained with PGA immobilized on silica support. A solution of α-methoxyphenylacetic methyl ester was injected into the HPLC system and two peaks appeared in the chromatogram (Fig. 5) and identified as the *S* enantiomer of α-methoxyphenylacetic acid and the enantiomer of the non-hydrolyzed α-methoxyphenylacetic methyl ester.

These data encouraged the authors to consider other substrates to test the performances of PGA chromatographic bioreactor. The selection of the compounds was based on the consideration that it has been reported that phenoxy acetyl derivatives are also accepted as substrates [43]. For this reason the 2-(4-phenoxyphenoxy) propionic acid methyl ester and 2-(4-benzylphenoxy) propionic were injected onto the enzymatic column and in both cases four peaks were obtained (Fig. 6a and b). The peaks were identified for both substrates as the separated enantiomers of the acids and the enantiosepared not hydrolyzed methyl ester.

The investigation was also extended to *rac* ketoprofen, *rac* suprofen and *rac* mandelic acid methyl esters and to a series of racemic 2-aryloxyalkanoic acid methyl esters and isosteric analogues [17].

The hydrolysis of racemic 2-aryloxyalkanoic acid methyl esters was easily carried out with a PGA-based monolithic column. The flow characteristics of this support enabled the coupling of the enzymatic stationary phase with an analytical column by means of a switching valve. The developed chromatographic set up was successfully used as a highly efficient tool to study the enantioselectivity of ester hydrolyses catalyzed by PGA. The chemical structures together with the analytical results are reported in Table 2.

The total ester hydrolysis and the enantiomeric excess of the product were calculated with the intent of further delineating the factors determining structural specificity and stereospecificity.

As reported in Table 2, high reaction rates ($C >$ 40%) and enantioselectivity ($ee >$ 80%) were obtained for the compounds characterized by a methyl

Table 1
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

Original table provided by [15].

[a] Mobile phase: 50 mM KH_2PO_4, pH 7.0; flow-rate, 0.8 ml/min; detection, 225 nm.

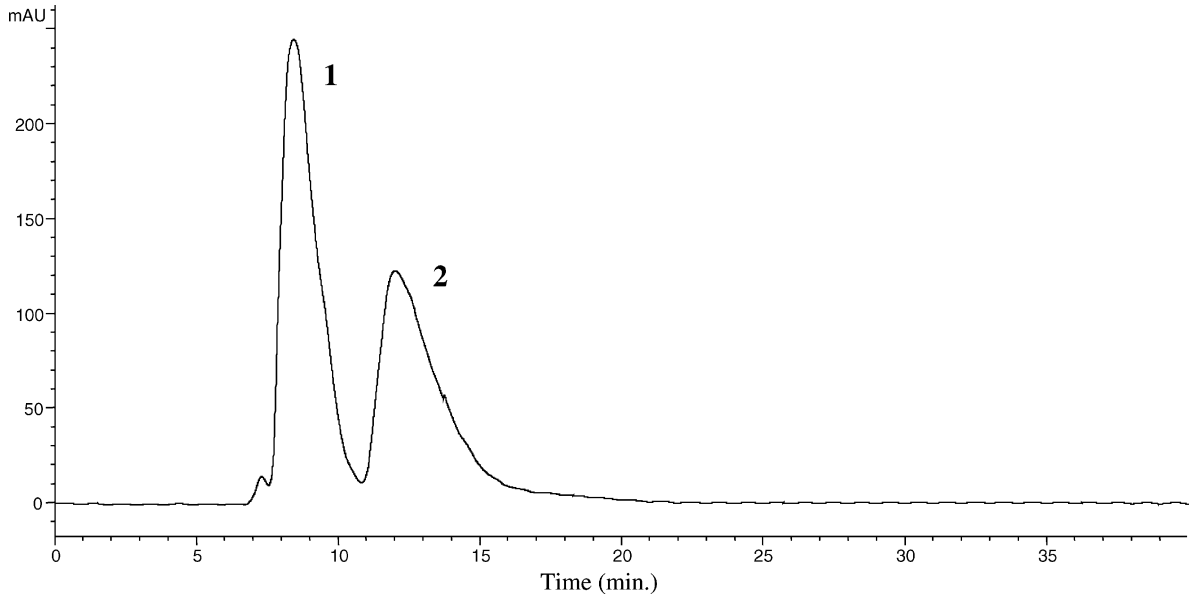
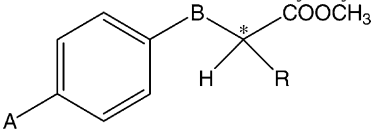


Fig. 5. Chromatogram showing the stereoselective hydrolysis of *rac* α-methoxy phenyl acetic acid methyl ester on the epoxyde column. Mobile phase: 50 mM KH_2PO_4, pH 7.0; flow-rate 0.1 ml/min; detection, 225 nm. Peak 1: single enantiomer of α-methoxy phenyl acetic acid. Peak 2: single enantiomer of the survived α-methoxy phenyl acetic acid methyl ester. Original chromatograms provided by [15].

group in the α-position and for the compound presenting a second aromatic ring on the asymmetric carbon. For this compound, a cleavage greater than 99.9% of the (*S*)-ester, corresponding to 47.9% conversion of the racemic ester, was observed. It is interesting to remark that a change of the substituent on the α-position from a methyl to an ethyl group led to a dramatic reduction in the conversion rate and *E*.

From these data, it was concluded that the substituent on the aromatic ring plays a role in the reaction rate and enantioselectivity. The compounds with the most hydrophobic and electron- withdrawing sub-

Table 2
Influence of solute structure on hydrolysis rate and enantioselectivity



Racemate	A	B	R	*C* (%)	R.S.D.%	*ee*[a]	*E*
1	Cl	O	CH_3	50.6	2.97	81.1 % (*S*)	23
2	Cl	O	C_2H_5	5.4	0.46	39.0 % (*S*)	2.3
3	Cl	O	C_6H_5	47.9	2.75	>99.9 % (*S*)	>200
4	Cl	S	CH_3	19.7	4.8	4.3 % (*S*)	1.1
5	Cl	NH	CH_3	6.7	2.2	66.4% (*S*)	5.3
6	Cl	CH_2	CH_3	10.5	0.64	38.2 % (*S*)	2.3
7	Br	O	CH_3	46.7	0.73	82.9 % (*S*)	23
8	F	O	CH_3	28.2	0.42	17.1 % (*S*)	1.5
9	CH_3	O	CH_3	22.7	0.25	85.3 % (*S*)	15

Data taken from [17]. C, conversion (%); ee, enantiomeric excess (%); *E*, enantioselectivity; R.S.D.%, experimental error ($n = 2$). See text for experimental details.

[a] Fast reacted enantiomer.

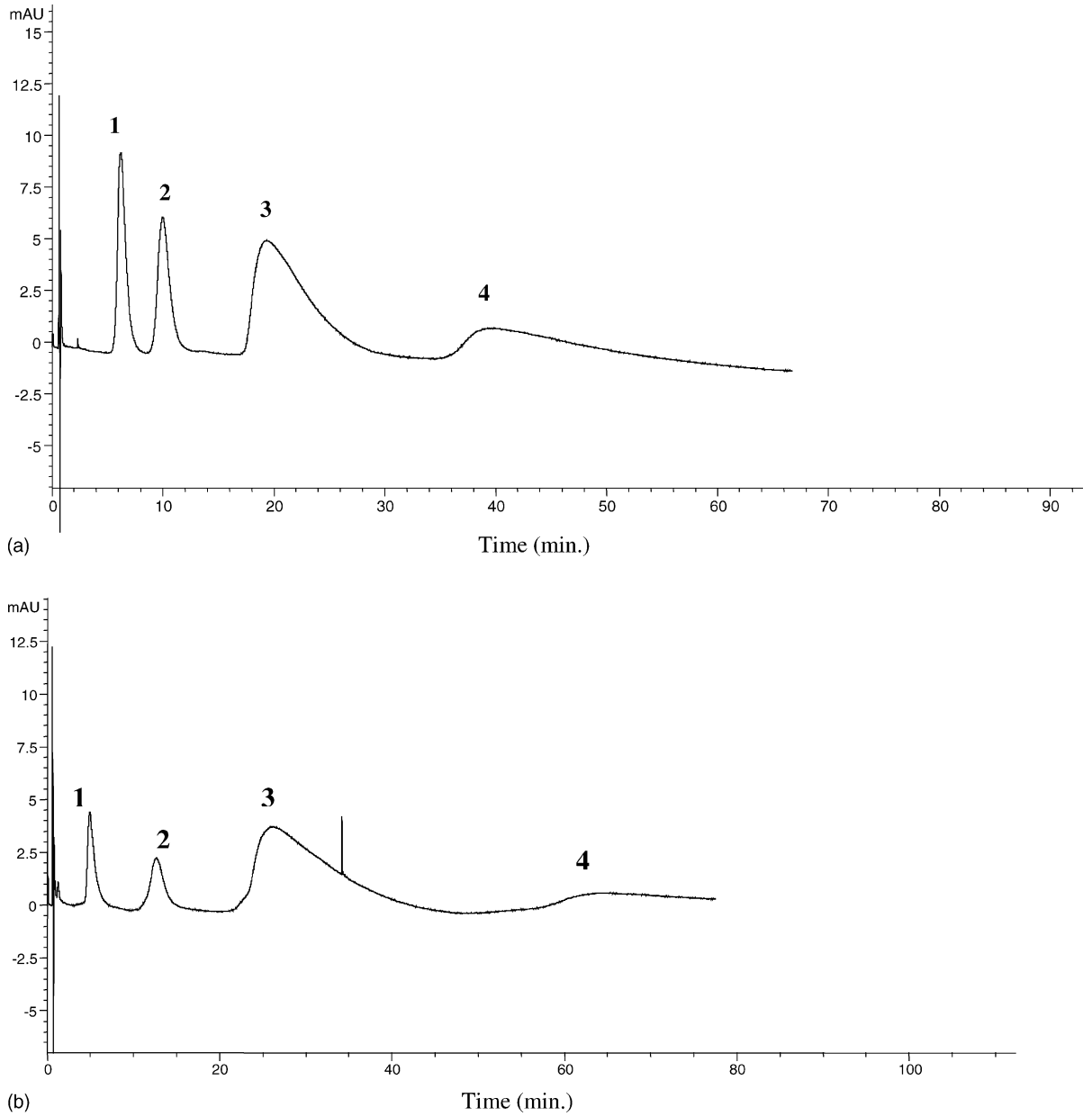


Fig. 6. (a) Chromatogram showing the hydrolysis of *rac* 2-(4-phenoxyphenoxy) propionic acid methyl ester on the epoxyde column. Mobile phase: 50 mM KH_2PO_4, pH 7.0; flow-rate 0.8 ml/min; detection, 225 nm. Peaks 1 and 2: enantiomers of 2-(4-phenoxyphenoxy) propionic acid. Peaks 3 and 4: enantiomers 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 mM KH_2PO_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. Original chromatograms provided by [15].

stituent were the most rapidly hydrolyzed with a high ee value. The presence of the oxygen in the α-position was also important for the catalytic activity, all the isosters presented a low conversion rate and/or a low ee value.

Molecular modeling can be a useful technique when studying the interactions between small organic molecules and large protein targets in order to predict selectivity or to explain experimental results. Therefore, in order to clarify the results of PGA enantioselectivity toward the aryloxyalkanoic acid methyl esters, computer modeling of enzyme-transition-state analogue complexes was used. The binding site of PGA has been found to consist of three major regions that are responsible for the hydrolysis reaction of the substrates by the enzyme [23,24]: the catalytic residue SerB1, the oxyanion hole (stabilizing the negative charge present on one of the oxygens of the tetrahedral intermediates by hydrogen bonding) formed by GlnB23, AlaB69, AsnB241 and a hydrophobic pocket which is able to accommodate hydrophobic groups. The first chemical step in PGA-catalyzed ester hydrolysis is the attack of the active site serine (SerB1) on the ester carbonyl forming a tetrahedral intermediate. Collapse of this tetrahedral intermediate releases the alcohol (Fig. 7a). In this study it has been assumed that the transition state involved in the formation or collapse of this first tetrahedral intermediate defines the selectivity of PGA toward aryloxyalkanoic acid methyl esters studied. To mimic this transition state, it was used the phosphonate linked to PGA shown in Fig. 7b. Indeed, researchers have shown that phosphonates mimic the transition state for ester hydrolysis well [44,45].

The molecular modeling study supported the experimental observations on structure-property relationships of esters processed by PGA. The reaction occurs well when a relatively small group such as methyl is present on the stereogenic center, preferentially in the *S* configuration. In the case of larger substituents, such as ethyl, the hydrolysis reaction is prevented, possibly due to steric hindrance within the active site. However, when a planar system is attached to the stereogenic center, the enzymatic hydrolysis reaction occurs well again, since the complex is stabilized by favorable stacking interactions between the planar ring and PheB71 and PheB146 side chains.

As regards substituents on the aromatic ring, hydrophobic and electronic properties seem to play an important role in the enzymatic process control. The bromo derivative 7, in fact, has a behavior similar to the chloro derivative 1, as expected; the analog 8 shows low conversion rate and enantioselectivity, possibly due to the poor hydrophobicity of the fluorine substituent. In the case of the analog 9, on the contrary, low conversion rate would be the result of the methyl group electron-donor properties that would weaken the π-stacking interaction formed between 9 and PheB24 aromatic rings. The isosteric replacement of the oxygen atom in the α position to the stereogenic center (analogs 4-6) affects the enzymatic hydrolysis too, producing lower conversion rate and enantioselectivity. One may speculate that the detrimental effect of the O/S/NH replacement originates from a less good accommodation of the *p*-chlorophenyl ring into the hydrophobic pocket of the enzymatic cavity due to the different stereo-electronic features of the isosteric substituents.

Molecular modelling studies gave interesting data that could expand the application of immobilized PGA as a chiral biocatalyst for the considered compounds and new processes.

4.2. PGA-based stationary phases as CSPs

Enzymes have gained most attention because of their stereoselectivity. Especially in pharmaceuticals the recent tendency to develop single stereoisomer drugs instead of racemates has helped tremendously to establish enzymes as tools in organic synthesis. Almost unbeatable in this context are *N*-acylases because of their perfect enantioselectivity and broad substrate tolerance.

Therefore in a preliminary screening the developed PGA columns were tested as chiral stationary phases against a wide range of racemic compounds (basic, neutral and acidic compounds) [15]. These initial experiments clearly indicated that immobilized PGA can be used as chiral selector only for acidic compounds, products of the enzyme catalyzed reactions; subsequently the applicability of the PGA-CSPs was evaluated by using a large number of closely related 2-aryloxyalkanoic acids, isosteric analogous and some 2-arylpropionic acids [16] (Tables 3 and 4). Fig. 8 reports the enantiomeric separation obtained for some compounds.

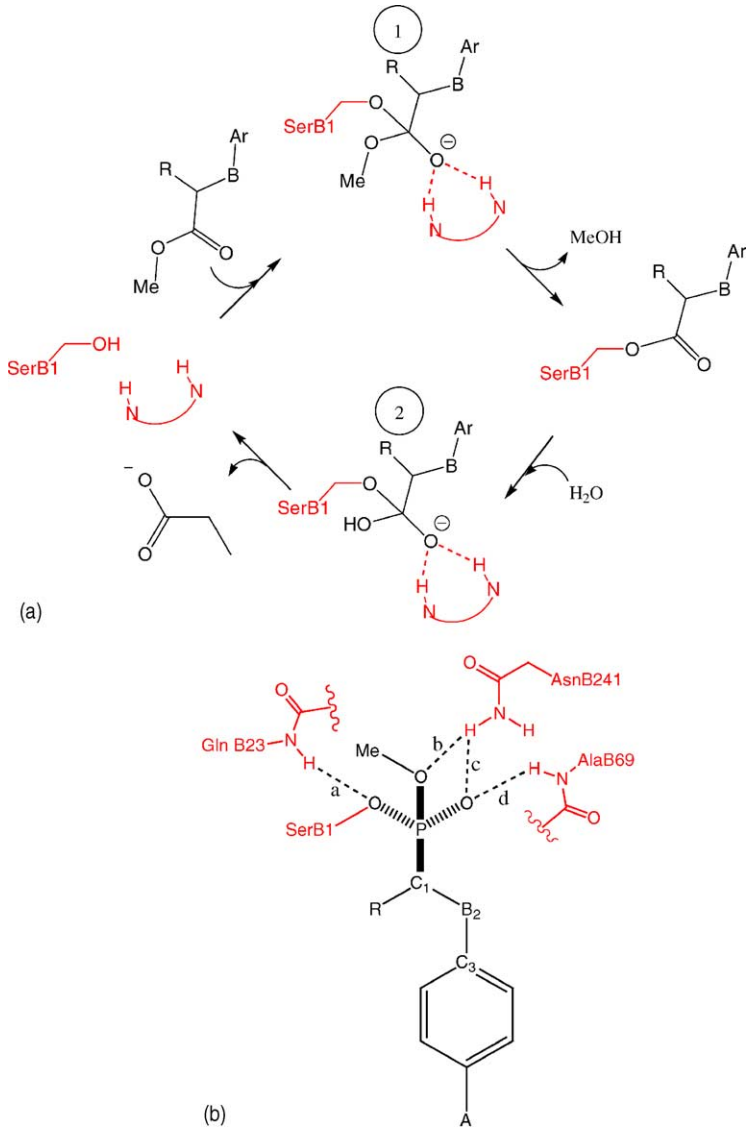
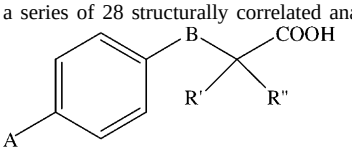


Fig. 7. Intermediates and their analogues in the PGA-catalyzed hydrolysis of esters. Amino acids correspond to those in PGA. (a) In the accepted mechanism for hydrolysis of esters, the catalytic serine (SerB1) attacks the ester once it binds in the active site. This attack forms the tetrahedral intermediate marked (1). Hydrogen bonds from two amide N-H's stabilize the oxyanion in this intermediate. Collapse of tetrahedral intermediate 1 releases the alcohol and forms an acyl enzyme intermediate. Attack of water on the acyl enzyme yields tetrahedral intermediate (2). Collapse releases the acid and regenerates the free enzyme. (b) A phosphonate mimics the transition states for formation and collapse of tetrahedral intermediate 1. Four hydrogen bonds, marked 'a'–'d', stabilize the charges. To be judged catalytically competent, the minimized structures must contain all four hydrogen bonds. Original figures provided by the authors [17].

The compounds were analyzed in constant chromatographic conditions. Tables 3 and 4 summarize the results obtained with the PGA-CSP: 27 out of 35 racemates were chiraly separated. From the large set of analytes considered it was possible to get qualitative information on the structural requirement to obtain retention and to observe enantioseparation. An increase of the lipophylicity of the substituent on the benzene ring led to an increase of the retention of the first eluted enantiomer while the observed enantios-

Table 3
Influence of solute structure on retention and enantioselectivity on a series of 28 structurally correlated analytes



A	B	R′	R″	k_1[a]	α
Cl	O	CH_3	H	0.37 (*R*)	26.4
Cl	O	C_2H_5	H	0.4 (*R*)	3.34
Cl	O	*n*-C_3H_7	H	0.92	1.68
Cl	O	*i*-C_3H_7	H	0.6	1.0
Cl	O	*n*-C_6H_{13}	H	6.43 (*R*)	1.96
Cl	O	C_6H_5	H	2.19 (*S*)	3.69
Cl	O	$C_6H_5CH_2$	H	3.99	1.14
Cl	S	CH_3	H	2.76 (*R*)	3.29
Cl	S	C_2H_5	H	1.75 (*R*)	4.4
Cl	NH	CH_3	H	1.84 (*R*)	2.84
Cl	CH_2	CH_3	H	2.92 (*S*)	4.06
Cl	CH_2	C_2H_5	H	1.68 (*S*)	3.30
Br	O	CH_3	H	1.27 (*R*)	3.78
Br	O	C_2H_5	H	0.93	2.09
F	O	CH_3	H	0.06 (*R*)	250.79
CH_3	O	CH_3	H	0.085 (*R*)	22.05
CH_3	O	C_2H_5	H	0.18	1.0
CN	O	CH_3	H	0.09	1.0
CH_3O	O	CH_3	H	0.07	1.0
CH_3CO	O	CH_3	H	0.10	1.0
C_6H_5CO	O	CH_3	H	5.56	2.58
C_6H_5O	O	CH_3	H	5.89	1.97
$C_6H_5CH_2$	O	CH_3	H	4.57	4.33
Cl	O	CH_3	C_6H_5	1.08	25.66
Cl	O	CH_3	*n*-C_3H_7	0.8	6.79
Cl	O	CH_3	C_2H_5	1.13	1.0
Br	O	CH_3	C_2H_5	1.18	2.48
Br	S	CH_3	C_2H_5	1.91	1.19

Original Table provided by [16]. Column 2. Mobile phase: 50 mM KH_2PO_4 (pH 7.0). Flow-rate: 0.8 ml/min.

[a] The configuration of the isomer corresponding to the first-eluted peak is given in parentheses.

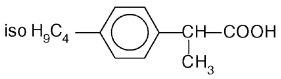
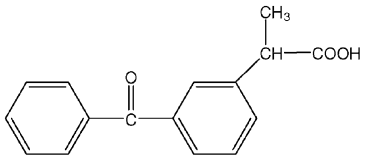
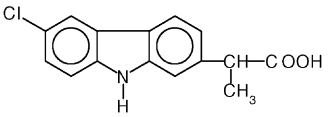
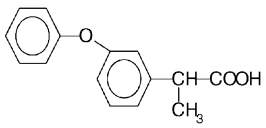
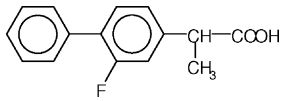
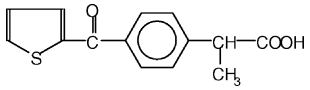
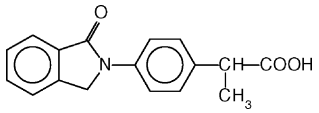
electivities did not correlate with the hydrophobicity of the analytes. If considering the dimensions of the benzene ring substituent of the same racemates, an increase of its size caused a progressive reduction in selectivity suggesting that the binding site at which enantioselective binding occurs is a chiral cavity with defined steric restrictions. When the bulkiness of the substituent reaches a critical dimension the solute might fit only partially into the cavity diminishing the ability of the enzyme to discriminate between the two enantiomer of the solute. Compounds with an additional aromatic ring in the A position were the most retained and the above consideration could not be extended to this group but it was possible to hypothesize that an additional hydrophobic interactions can be involved in the chiral recognition mechanism. The steric bulk on the stereogenic centre was also evaluated. In general, an increase in bulkiness of this substituent caused an increase in retention while enantioselectivity was reduced. When a second substituent was introduced in the α position the retention was almost unaffected while enantioselectivity increased. In particular the enantioselectivity increased dramatically with an aromatic ring in this position, indicating that hydrophobic interactions are strategic in the chiral recognition mechanism. As far as the influence of B substituent is concerned, an increase of the lipophilicity caused an increase in retention. The isosteric substitution of the ether oxygen atom with sulfur, amino or methylen groups had a slight effect on enantioselective retention.

The same considerations can be extended to the arylpropionic acids where a complete loss of enantioselectivity was observed for analytes with bulky (ibuprofen) and rigid aryl moiety (carprofen) that may lead to exclusion of the solute to the chiral binding site.

These result are in agreement with the crystallographic studies which suggest that non-polar contacts and conformational properties of PGA are the principal factors that govern enzyme interactions with ligands. It is apparent that the aromatic residues within the active site are the main contributors to substrate recognition and binding [24].

The influence of hydrophobic, electrostatic and hydrogen binding on chiral recognition was studied by varying the mobile phase composition (ionic strength, pH and type of organic modifier). The enantioselective retention could be controlled by the modification of the considered mobile-phase parameters [16]. The most important tool for affecting the enantioselectivity and retention was the mobile phase pH and interestingly the retention order of the enantiomers of some analytes could be controlled by this parameter. The increase of mobile phase ionic strength caused a decrease in retention while enantioselectivity was almost unaffected. The addition of an organic modifier to the mobile phase (i.e., methanol and 2-propanol) reduced retention and enantioselectivity [16].

Table 4
Retention and enantioselectivity on a series of seven non-steroidal antiinflammatory drugs

Racemate	Structure	k_1	α
Ibuprofen		1.16	1.0
Ketoprofen		1.59	4.0
Carprofen		125.84	1.0
Fenoprofen		4.26	3.89
Flurbiprofen		12.35	1.68
Suprofen		5.77	1.95
Indoprofen		46	1.55

Original table provided by [16]. Mobile phase: 50 mM phosphate buffer pH 7.0. Flow-rate 0.8 ml/min.

The involvement of the active site of the enzyme in the chiral recognition mechanism was proved by displacement studies that were carried out adding phenylacetic acid, a specific inhibitor of the enzyme, to the mobile phase [16]. A concentration dependent reduction of the retention factors of both enantiomers was observed and at high competitor concentration (0.1 mM) the stationary phase was unable to retain and to stereochemically resolve all test compounds. Therefore, the specific active site seems to be necessary for enantioselective discrimination and for enhanced retention.

The PGA-monolithic columns were also evaluated as chiral stationary phase in chromatography [18]. Separations of 14 racemic analytes were attempted on the epoxy monolithic PGA column and compared to data obtained with a PGA microparticulate silica column (50 mm × 4.6 mm i.d. 5 μm pore size) in the same chromatographic conditions (Table 5).

Surprisingly the resolution was higher on the microparticulate column, if considering that the amount of bound PGA was lower in this stationary phase. The worse resolution values on PGA-epoxy monolithic column are the consequence of the lower selectivity unlike the efficiency.

The k_1 values are higher on the monolithic column than on microparticulate one with few exception regarding analytes with two aromatic rings, while the retention factors of second enantiomer (k_2) present an opposite trend, leading to a reduction of selectivity on monolithic material. A possible explanation could be that the higher protein coverage on the monolithic

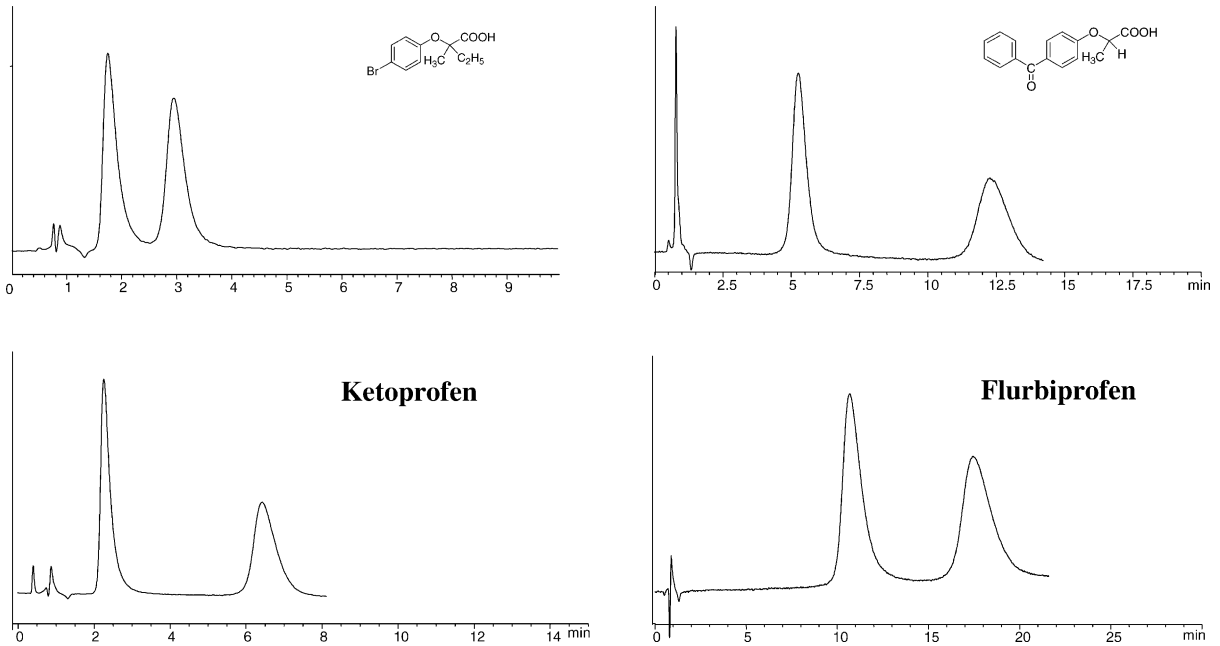
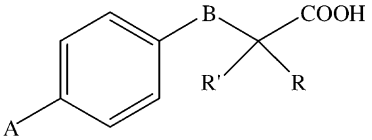


Fig. 8. Chromatograms of some racemates on PGA-column. Mobile phase: 50 mM phosphate buffer (pH 7.0). Flow: 0.8 ml/min. Original chromatograms provided by the authors [16].

Table 5
Chiral resolution of 14 racemates on microparticulate and monolithic columns



Racemate				k_1		k_2		α		R_s	
A	B	R	R′	C-1[a]	C-2[b]	C-1	C-2	C-1	C-2	C-1	C-2
Cl	O	CH_3	H	0.87	0.37	6.74	9.77	7.74	26.4	6.48	5.06
Cl	O	C_2H_5	H	0.88	0.40	1.45	1.34	1.64	3.34	1.37	1.71
Cl	O	C_6H_5	H	3.30	2.19	5.58	8.08	1.69	3.69	2.72	4.75
Cl	S	CH_3	H	2.38	2.76	4.91	9.08	2.07	3.29	3.98	5.37
Cl	S	C_2H_5	H	2.30	1.75	5.93	7.70	2.58	4.40	4.81	5.30
Cl	CH_2	CH_3	H	2.28	2.92	5.99	11.86	2.63	4.06	4.46	6.77
Cl	CH_2	C_2H_5	H	1.85	1.68	3.63	5.54	1.96	3.30	2.81	4.46
Br	O	CH_3	H	1.28	1.27	2.92	4.80	2.28	3.78	3.40	3.70
F	O	CH_3	H	0.32	0.06	7.11	15.05	22.15	250.80	8.09	9.47
CH_3	O	CH_3	H	0.39	0.09	1.14	1.87	2.94	22.05	1.53	1.48
C_6H_5CO	O	CH_3	H	3.81	5.56	7.44	14.35	1.95	2.58	3.70	4.82
Cl	O	CH_3	C_6H_5	2.82	1.08	15.55	27.71	5.52	25.66	6.39	16.24
C_6H_5O	O	CH_3	H	4.87	5.89	7.37	11.60	1.51	1.97	2.55	3.46
$C_6H_5CH_2$	O	CH_3	H	3.94	4.57	10.03	19.79	2.55	4.33	3.45	7.09

Data elaborated from [18].

[a] Monolithic column.

[b] Microparticulate column. Chromatographic conditions: mobile phase: 50 mM phosphate buffer, pH 7.0; flow: 0.8 ml/min; wavelength: 225 nm.

Table 6
Chromatographic parameters of rac-ketoprofen on PGA- monolithic column at different flow rates

Flow (ml/min)	k_1	k_2	α	R_s	As_1	As_2	N_1	N_2
0.4	2.5	5.45	2.18	3.42	0.39	0.47	541	555
0.6	2.48	5.38	2.17	3.38	0.36	0.41	535	525
0.8	2.53	5.39	2.13	3.27	0.33	0.37	543	500
1.0	2.56	5.52	2.15	3.26	0.34	0.41	513	489
1.2	2.55	5.48	2.16	3.16	0.35	0.41	495	454
1.5	2.53	5.48	2.16	3.06	0.36	0.42	457	420
2.0	2.52	5.42	2.16	2.87	0.38	0.46	412	372
3.0	2.52	5.48	2.17	2.64	0.46	0.51	335	313

Original table from [18]. Chromatographic conditions: mobile phase: 50 mM phosphate buffer, pH 7.0; wavelength: 225 nm.

support determines an increase of the aspecific interaction whereas, on the other hand, hindering the access to the specific catalytic site. In fact, as previously demonstrated, the retention mechanisms of the two enantiomers are different, i.e., the first is mostly retained by aspecific interactions while for the second the main role is played by the specific catalytic site. The different chromatographic behavior observed for analytes with two aromatic rings brings back to the above explanation as it was previously demonstrated [17] that the second aromatic ring is involved in an additional interaction which takes place in a binding area close to the specific catalytic site. As far as efficiency is concerned, the best results in terms of number of theoretical plates were achieved with PGA immobilized on monolithic support.

An important feature of monolithic supports is their ability to operate at high flow rate regardless of column back pressure, this is intrinsically not possible with particulate columns because by operating at flow rate higher than 1.0 ml/min would lead to a non-compatible enzyme column back pressure. Focus was therefore given to the evaluation of this column, with respect to speed of eluent flow. *Rac*-ketoprofen was used as a probe to demonstrate that PGA-monolithic columns can operate at high flow-rate without a significant loss in enantioselectivity (Table 6). The new developed chiral stationary phase successfully combined the well known chiral recognition properties of PGA and the unique properties concerning the flow behavior of silica monoliths. It was possible to obtain the complete enantioseparation of ketoprofen in 3 min analysis by operating at a flow rate of 3 ml/min. The fast enantioseparation achieved make the prepared supports very interesting for high-throughput separations.

5. Conclusions

This review has focused on the development and applications of a chromatographic column based on immobilized PGA. From the examples of applications mentioned here, it is clear that the PGA based-stationary phases are capable of producing substances with potential high purity (and consequently high added value) resulting from enzyme substrate specificity and stereospecificity. The molecular modelling studies gave more insights into PGA-substrates interactions and this information could expand the application of immobilized PGA as a chiral biocatalyst for pharmaceutical processes.

At present, analytical HPLC PGA-column can be used as a screening tool for studying the parameters that can influence the hydrolytic activity and the chiral biocatalysis. In the future, the development of stationary phases based on immobilized PGA for preparative purpose will answer the increased demand of pure enantiomers of new biologically active racemic compounds.

There is lot of improvement possible, however the promising results obtained with immobilized PGA bioreactor/chiral column have opened a new road for biocatalysis.

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