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Evaluation of a monolithic epoxy silica support for penicillin G acylase immobilization

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Abstract

In this work, a new type of penicillin G acylase (PGA)-based monolithic silica support was developed and evaluated for the chiral separation in HPLC. The preparation procedure consisted of two steps: preparation of an epoxy derivatized monolithic silica column and chemical modification of the epoxide groups with the enzyme chiral selector. The epoxy Silica-Rod column for the immobilization of PGA was prepared with the in situ modification process by using epoxy-silanes and the identification of the species bound to the surface was achieved by solid-state nuclear magnetic resonance. The enzyme was covalently immobilized to the surface of the derivatized monolithic column. The enantioselectivity and the performance of the developed column are discussed and compared to the corresponding experimental data obtained with a PGA-based microparticulate (5 μm) silica column.

Keywords: Chiral stationary phases, LC; Enantiomer separation; Monolithic columns; Protein immobilization; Penicillin G acylase; Enzymes

1. Introduction

Some years ago, monolithic materials were introduced as a new class of chromatographic supports. Different research groups have studied monolithic materials for use in HPLC [1–4]. Based on the nature of the material from which they are made, monolithic columns can be classified as organic polymer- or silica-based columns. The first monolithic columns were based on the use of organic polymers. Ross and Jefferson [5] were first to report the preparation of monolithic GC columns from polyurethane in 1970. Twenty years later, Zeng et al. [1] prepared materials within a chromatographic tube based on polyacrylamides and these columns were successfully applied to fast bioseparations. Frechet and co-workers [2] described the preparation of either polyacrylates or poly(styrene-co-divinylbenzene) in the presence of porogens leading to monolithic materials with a permanent macroporous structure and their chromatographic suitability was demonstrated with the separation of proteins.

However, the use of polymeric materials in HPLC has several disadvantages. Typically, the general drawback of these materials is a much lower efficiency (regard to plate numbers) compared to silica-based columns. Most polymers, especially those with low degrees of crosslinking, are known to swell or shrink in organic solvents. Such behaviour could have dramatic effects on the chromatographic performance of these monolithic columns and frequently leads to a lack of mechanical stability. Furthermore, the structure of porous polymers very often contains micropores, which negatively affect the efficiency and peak symmetry of columns. However, polymeric monolithic columns offer excellent biocompatibility, have a wide pH range and can be cleaned with caustic mobile phases. Polymeric monolith columns housed in convenient cartridge designs are available commercially.

Taking into account some of these drawbacks, porous inorganic monoliths offer the potential to be the column filling materials of choice for conventional applications just as it is the case in microparticulate packed HPLC columns.

Nakanishi and Soga [6] developed a new sol–gel process for the preparation of monolithic silica columns with a bimodal pore structure (i.e. with throughpores and mesopores). The method is based on the hydrolysis and poly-condensation of alkoxysilanes in the presence of water-soluble polymers. Minakuchi et al. [3] and Lubda et al. [4] demonstrated that this method allows the preparation of chromatographic columns with high efficiencies and low

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column backpressures. These improvements result from the independent control of the sizes of the silica skeleton and throughpores. Furthermore, it was demonstrated that the recently developed monolithic-type HPLC columns could be operated at high flow-rates while maintaining a high efficiency. The reproducibility of the preparation of the first commercially available monolithic silica columns, introduced by Merck in 2000, was investigated by Kele and Guiochon [7].

The attractive results achieved with Chromolith technology and the recent application of monolithic supports to the development of bioreactors [8,9] lead us to consider this innovative chromatographic support for the extention of our studies on immobilized penicillin G acylase (PGA) as chiral stationary phase.

Penicillin G acylase is mainly used in the industrial production of semisynthetic penicillins since it catalyses the cleavage of the acyl chain of penicillins to yeild 6-aminopenicillanic acid (6-APA) and the corresponding organic acid.

Penicillin G acylase is also useful for the resolution of alcohols, β-hydroxy-α-amino acids, β-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 [10].

In a previous investigation PGA was successfully immobilized on aminopropyl and epoxy conventional silica supports (5 μm) and it was shown that the use of epoxy silica gave the best immobilization yield [11]. The covalent immobilization of PGA on an aminopropyl monolithic-type HPLC silica support, creating an immobilized HPLC-enzyme reactor, has been already achieved [12]. Supported by our previous results, the next step was the development of an epoxy derivatized monolithic support in order to have an overview of the chiral recognition properties of PGA immobilised on both microparticulate and monolithic silica modified with aminopropyl and epoxide groups.

A new monolithic epoxy silica column was prepared with the in situ modification process by using epoxy-silanes. The characterisation of the monolithic silica support was achieved by solid-state nuclear magnetic resonance. Following, PGA was covalently bonded to the surface of the monolithic epoxy column and the immobilization efficiency was evaluated in terms of the enzymatic activity of the support after the immobilization procedure. The chromatographic performances of the obtained stationary phase were studied using some 2-aryloxyalcanoic acids and isosteric analogs, in which the absolute configuration has been shown to exert a strong influence on the biological activity (skeletal muscle membrane chloride conductance, prostaglandin-dependent platelet aggregation, peroxisome proliferation and, more recently, the anti-inflammatory activity and the PPARα-mediated hypolipidemic effects) [13].

The chromatographic results were compared to the corresponding experimental data obtained with a conventional microparticulate (5 μm) silica support whose preparation has been previously described [13].

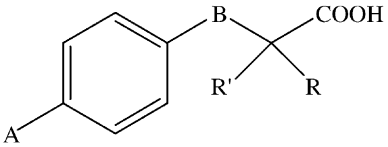
2. Experimental

2.1. Reagent and materials

Chromolith SpeedRod Si silica monolithic column (50 × 4.6 mm i.d.) cladded with polyether ether ketone (PEEK) was prepared as research samples at Merck (Darmstadt, Germany). Toluol, potassium dihydrogenphosphate and dipotassium hydrogenphosphate were of analytical grade and purchased from Merck. (3-Glycidoxypropyl)trimethoxysilane was from ABCR (Karlsruhe, Germany).

Penicillin G acylase crude extract from *Escherichia coli* ATCC 11105 (penicillin amidohydrolase, EC 3.5.1.11) was kindly donated by Recordati (Milan, Italy) and used as received; penicillin G potassium salt used as standard substrate for the determination of catalytic activity, phenylacetic acid (PAA) and Bradford Reagent were from Sigma (St. Louis, MO, USA). 6-Aminopenicillanic acid was purchased from Sigma–Aldrich (Milan, Italy). Water was deionized by passing through a Direct-Q (Millipore) system (Millipore, Bedford, MA, USA). The chemical structures of the fourteen racemic analytes used for the comparative study are given in Table 1. For the synthetic preparation of racemates 1–12 see [13]. Racemates 13 and 14 were synthesized in our Department by Professor O. Azzolina. *Rac*-ketoprofen was kindly donated by S.I.M.S. (Incisa Valdarno, FI, Italy).

Table 1
Chemical structures of the 14 racemates used in the compartive study



Analyte	A	B	R	R′
1	Cl	O	CH_3	H
2	Cl	O	C_2H_5	H
3	Cl	O	C_6H_5	H
4	Cl	S	CH_3	H
5	Cl	S	C_2H_5	H
6	Cl	CH_2	CH_3	H
7	Cl	CH_2	C_2H_5	H
8	Br	O	CH_3	H
9	F	O	CH_3	H
10	CH_3	O	CH_3	H
11	C_6H_5CO	O	CH_3	H
12	Cl	O	CH_3	C_6H_5
13	C_6H_5O	O	CH_3	H
14	$C_6H_5CH_2$	O	CH_3	H

2.2. Apparatus

The HPLC Pump used for the in situ modification was a L-7100 HPLC-Pump (Merck–Hitachi, Darmstadt, Germany). For the thermostatization during the modification, a L-7300 column oven was used. The specific surface area of the Silica-Rods was determined by the measurement of the nitrogen adsorption and desorption isotherms using the ASAP 2400 instrumentation from Micromeritics. The specific surface area was calculated according to the theory of Brunauer, Emmett and Teller (S_{BET}). The ^{13}C cross-polarized and magnetic angle spinning nuclear magnetic resonance (CP-MAS-NMR) spectra were obtained at room temperature using a Bruker AMX-300 Fourier transform NMR spectrometer.

Chromatographic experiments were performed with 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 system was 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. Chromatographic conditions

The mobile phase used for the PGA column was 50 mM 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 column was stored at 4 °C in a 0.01% (w/v) solution of sodium azide.

2.4. Chromatographic parameters

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. The plate number N was calculated as $N = 5.54\ (t_r/w_{0.5})^2$ where $w_{0.5}$ is the peak width at half peak height. Resolution is calculated from the equation $R_s = 2(t_2 - t_1)/(t_{w1} + t_{w2})$. Where t_1 and t_2 are retention times of the first- and second-eluted peaks, respectively, and t_{w1} and t_{w2} are the peak widths. The asymmetry factor (A_s) was calculated using the US Pharmacopeia (USP) method.

2.5. Preparation of the enzyme column

2.5.1. Preparation procedure of a surface modified Silica-Rod

The preparation of a Silica-Rod Epoxy column was carried out as given further. Prior to the reaction, one Chromolith SpeedRod Si silica monolithic column (50 × 4.6 mm i.d.), (50 mm in length containing approximately 0.25 g silica) encased in a PEEK plastic cover was dried for 5 h under vacuum at 100 °C. The surface modification of the dried Silica-Rod Si column was carried out with a solution of 12 μmol/m^2 (calculated based on 323 m^2/g specific surface area of the unmodified Silica-Rod) of 3-glycidoxypropyltrimethoxysilane in dry toluene by pumping the reaction solution through the monolith at a volumetric flow-rate of 0.2 ml/min. During the reaction, the Silica-Rod column was stored inside an HPLC column oven at 80 °C. In order to remove the unreacted amount of 3-glycidoxypropyltrimethoxysilane from the epoxy bonded Silica-Rod, the column was flushed for an additional 2 h with toluene at a volumetric flow-rate of 0.5 ml/min.

2.5.2. Calculation of the epoxy surface coverage

After drying the modified Silica-Rods the reaction process was verified on the basis of an elemental analysis of several parts of the Silica-Rods. The carbon content was measured by elemental analysis of an average value of 6%.

The surface coverage (α_{epoxy}) was found to be 2.9 μmol epoxy groups per m^2 unmodified silica. It was obtained using Eq. (1), which has been introduced by Jeroniec et al. [14].

$$\alpha_{epoxy}\ (\mu mol/m^2) = \frac{10^6}{S_{BET}[M_{Element} \times 100(n_C/P_C) - M_{Ligand}]} \quad (1)$$

where P_C is the percent carbon measured in the epoxy phase, $M_{Element}$ the mass of the calculated element and M_{Ligand} the molecular mass of the attached ligand, n_C the numbers of carbon atoms per bonded ligand, S_{BET} the specific surface area in m^2/g of the unmodified silica.

^{13}C CP-MAS-NMR spectroscopy was used to distinguish between the two different types of surface bonded groups, epoxy and diol groups (Fig. 1). According to literature [15–17], the chemical shift values found for the 5,6-epoxypropoxy-3-propyl-1-silyl-silica (the abbreviation used in chromatography is epoxy) and 5,6-dihydroxypropoxy-3-propyl-1-silyl-silica (the abbreviation used in chromatography is diol) modified silica gels are summered in Table 2.

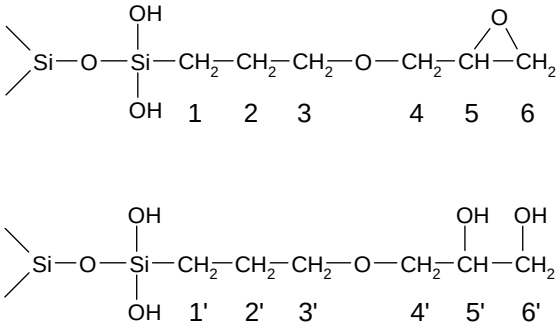


Fig. 1. Two possible structural elements at the surface of silica gel after the silanization with an epoxy silane.

Table 2
Chemical shift values found in literature [15–17] for the 5,6-epoxypropoxy-3-propyl-1-silyl-silica and 5,6-dihydroxypropoxy-3-propyl-1-silyl-silica modified silica gels

Resonances (ppm)	Carbon epoxy chain	Carbon diol chain
9	1	1′
22	2	2′
44	6	No
52	5	No
63	No	6′
71	3/4	3′/4′/5′

2.5.3. Immobilization procedure and column activity determination

The immobilization of PGA on epoxy monolithic support was carried out following a previously described method [11,18]. The amount of immobilized protein was determined by measuring the difference in protein quantity of the enzymatic solution used for immobilization before and after the grafting procedure. Bradford reagent was used as protein assay following the procedure described by Sigma. The column contained approximately 99 mg of immobilized protein.

The activity of PGA immobilized onto the monolithic support was calculated by using an on-line procedure previously reported [12]. In brief, 20 ml of penicillin G samples in the concentration range between 0.1 and 6 M were injected on the monolithic column. The species eluted were collected and analysed off line on an analytical RX-C_8 Zorbax stationary phase for product quantitation. The saturation level was found at 6 M substrate concentration. By linearizing the Michaelis–Menten plot, the V_{max} value was estimated to calculate the amount of active immobilized enzyme. The activity of the enzyme immobilized in the column was found to be 1531.1 U ± 169 S.D. One unit (U) corresponds μmol of penicillin G hydrolysed per minute.

3. Results and discussion

3.1. Preparation and characterization of the monolithic support

Generally, there are two ways to modify silica gels used as chromatographic stationary phases: the so-called batch reactions and the in situ method. The chemical modification of monolithic silica supports is, due to the longer diffusion passes through the diameter of the monolith, much harder to realize by using a batch procedure. For this reason the in situ modification process for monolithic silica supports was chosen.

The (3-glycidoxypropyl)trimethoxysilane was used for the surface modification of silica gel and the surface coverage was found to be 2.9 μmol epoxy groups per m^2 unmodified silica calculated on the basis of an elemental analysis. After the modification procedure the materials presented a mixed kind of epoxy and diol groups. This is the case even if we try to stop the surface reaction after the attachement of the epoxy groups to the surface by a reaction of the alkoxy groups of the silane with the so-called silanol groups of the silica gel. The reason for that might be found in small amounts of water bound to the surface or being introduced from the used chemicals of the reaction mixture. An additional danger of hydrolyzing the epoxy function arises from the silanol groups of the silica surface itself as they are of acidic nature and depending on the reaction conditions they are able to hydrolyse the epoxy function, too. Due to this it is important to determine the amount of epoxy groups using a second analytical method in addition to the elemental analysis.

A very powerful method for the characterization of compounds are those in the field of the nuclear magnetic resonance spectroscopy. One drawback of using NMR spectroscopy was the extremely low level of mobility of the groups anchored to the surface of solid stationary phase, leading to severe line broadening in the resultant NMR spectrum and therefore to a loss of information. The advent of the combined techniques of dipolar decoupling, cross-polarization (CP) and magic-angle spinning (MAS), however, has meant that “high-resolution” NMR spectra of solids can now be routinely obtained. During the last years this new very powerful method for the characterization of chemisorbed compounds, the solid-state ^{13}C and ^{29}Si CP-MAS-NMR spectroscopy, has been established due to an excellent development of different research groups [15–17]. ^{13}C CP-MAS-NMR spectroscopy was used to distinguish between the two different types of surface bonded groups, epoxy and diol groups (Fig. 1). It can be seen in Table 2, that the two different groups at the end position of the bonded chain do influence the absorption of the carbons 1-4, respectively 1′-4′ but not very much. The main peaks assigned to the diol and epoxy modification should be found at 44, 52 and 63 ppm. While the epoxy-silica can be detected by its resonance’s of C-5 at 52 ppm and of the C-6 at 44 ppm we should be able to distinguish between the epoxy and the diol modification if no resonance of the C-6′ neighboring hydroxy group at 63 ppm of the diol can be found.

The nearly absence of an absorption at 63 ppm in the ^{13}C CP-MAS-NMR spectra (Fig. 2) indicates that the prepared Silica-Rod derivative only contains a very low amount of diol carrying chains. Taking these results into account the epoxy modified Silica-Rod should be an ideal support for a further modification step using a reactive compound, as an enzyme or others, which is able to react with epoxy groups by forming a covalent bonding to the surface groups.

3.2. Evaluation of PGA immobilized on monolithic epoxy silica support

In Table 3 are reported the characteristics of the monolithic column in comparison with the microparticulate one.

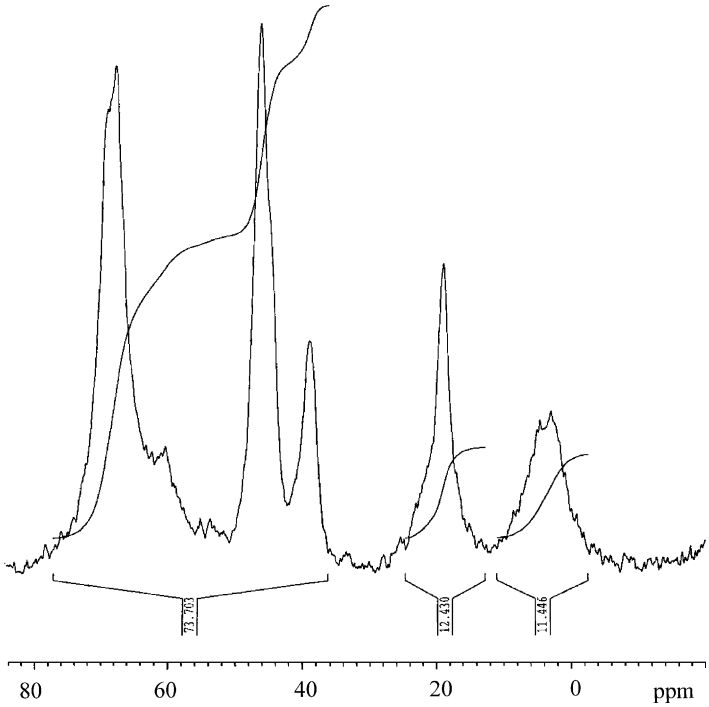


Fig. 2. ^{13}C CP-MAS-NMR spectra from the prepared Silica-Rod epoxy.

In both columns the activity of the support is related to the amount of immobilized enzyme, however, the immobilization yield was higher on the monolithic support.

It was interesting to observe an increase of the amount of bound PGA on epoxy monolithic support in comparison with the previously developed PGA-aminopropyl monolithic column [12]. This finding is in agreement with the results reported in our previous work [11] where the two immobilization methods on microparticulate silica were compared. The data obtained for the different stationary phases based on PGA immobilized on microparticulate silica [11] and monolithic silica support [12] clearly indicate that epoxy monolithic support gives the best results in terms of bound PGA.

3.3. Comparison of PGA immobilised on monolithic and particle epoxide silica supports as chiral stationary phases

The prepared enzymatic monolithic support was evaluated as chiral stationary phase in chromatography. Separations of 14 racemic analytes were attempted on the monolithic PGA column and compared to data obtained with a PGA microparticulate silica column (50 × 4.6 mm i.d., 5 μm pore size) in the same chromatographic conditions [13]. Table 4 shows the retention factors (k), enantioselectivity (α), resolution factor (R_s), peak asymmetry factor of the second-eluted enantiomer (A_{s_2}) and efficiency (N).

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 (racemates 11, 13 and 14), 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 support determines an increase of the aspecific interaction whereas, on

Table 3
Characteristics of the columns compared in this study

Column type	Dimension	Immobilized protein (mg per column)	Column activity (U per column)
Microparticulate (5 μm)	50 × 4.6 mm i.d.	67[a]	945.3[b]
Monolithic	50 × 4.6 mm i.d.	99	1573.5

[a] Calculated by elemental analysis [13].
[b] The hydrolysis of penicillin G potassium salt has been used as a standard assay for the catalytic activity of the enzyme in the immobilized form. See [11] for experimental conditions.

Table 4
Chiral resolution of 14 racemates on microparticulate and monolithic columns

Racemate	k_1		k_2		α		R_s		A_{s_2}		N_1		N_2	
	C-1[a]	C-2[b]	C-1	C-2	C-1	C-2	C-1	C-2	C-1	C-2	C-1	C-2	C-1	C-2
1	0.87	0.37	6.74	9.77	7.74	26.4	6.48	5.06	0.70	0.80	265	35	527	239
2	0.88	0.40	1.45	1.34	1.64	3.34	1.37	1.71	0.71	0.84	502	309	401	146
3	3.30	2.19	5.58	8.08	1.69	3.69	2.72	4.75	0.50	0.71	614	294	724	462
4	2.38	2.76	4.91	9.08	2.07	3.29	3.98	5.37	0.60	0.69	903	436	829	614
5	2.30	1.75	5.93	7.70	2.58	4.40	4.81	5.30	0.64	0.98	762	297	730	472
6	2.28	2.92	5.99	11.86	2.63	4.06	4.46	6.77	0.66	0.87	574	451	630	750
7	1.85	1.68	3.63	5.54	1.96	3.30	2.81	4.46	0.62	1.06	568	375	560	514
8	1.28	1.27	2.92	4.80	2.28	3.78	3.40	3.70	0.62	0.78	774	199	608	340
9	0.32	0.06	7.11	15.05	22.15	250.80	8.09	9.47	0.60	0.74	853	1848	471	441
10	0.39	0.09	1.14	1.87	2.94	22.05	1.53	1.48	0.59	1.13	774	366	791	30
11	3.81	5.56	7.44	14.35	1.95	2.58	3.70	4.82	0.59	0.74	792	552	705	599
12	2.82	1.08	15.55	27.71	5.52	25.66	6.39	16.24	0.47	0.68	271	146	471	555
13	4.87	5.89	7.37	11.60	1.51	1.97	2.55	3.46	0.70	0.86	817	527	858	581
14	3.94	4.57	10.03	19.79	2.55	4.33	3.45	7.09	0.39	0.92	434	482	292	646

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

the other hand, hindering the access to the specific catalytic site. Infact 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 [13]. The different chromatographic behaviour observed for analytes with two aromatic rings brings back to the above explanation as it was previously demonstrated [12] 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 immobilised on monolithic support although accompanied by slight tailing as shown in Table 4.

An important feature of monolithic supports is their ability to operate at high flow-rate regardless of column back pressure [19], 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. The influence of flow-rate on selectivity using ketoprofen as a probe was therefore investigated only for the

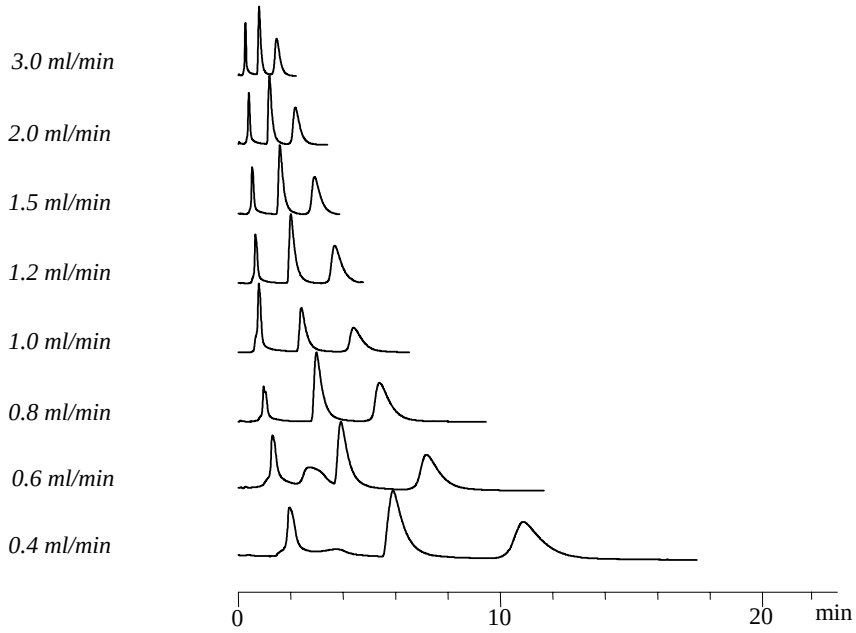


Fig. 3. Chromatographic separation of *rac*-ketoprofen on PGA–Silica-Rod column at different flow-rates. Sample: *rac*-ketoprofen; mobile phase: 50 mM phosphate buffer (pH 7.0).

Table 5
Chromatographic parameters of *rac*-ketoprofen on PGA-monolithic column at different flow-rates

Flow (ml/min)	k_1	k_2	α	R_s	A_{s_1}	A_{s_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

Chromatographic conditions: mobile phase: 50 mM phosphate buffer (pH 7.0); wavelength: 225 nm.

monolithic column. In order to avoid instability problems of the enzyme, the effect of linear velocity on the plate height was examined in the range 0.4–3.0 ml/min. Chromatograms obtained from the chromatography of *rac*-ketoprofen at different flow-rates are reported in Fig. 3. As can be seen from the Table 5, a decrease in column efficiency was observed increasing the flow-rate while selectivity was almost constant. The column maintained approximately 65% of plate number at 3.0 ml/min compared to 1.0 ml/min for both enantiomers.

To evaluate the efficiency of the column the Van Deemter plots (H versus u) for the separation of ketoprofen enantiomers is depicted in Fig. 4 together with the column back pressure.

The Van Deemter plots in the range studied showed a relatively sharp increase in H with the increase in linear velocity. The H value for the first eluted enantiomer linearly increases with the linear velocity (u) of the mobile phase. A different trend was obtained for the second-eluted enantiomer. Plate heights of 92.42 and 92.08 μm were observed at a speed of 0.43 and 0.86 mm/s (corresponding to 0.4–0.8 ml/min), respectively. Even at a flow-rate of 3.0 ml/min a plate height of 149.25 and 159.74 μm for the first and second-eluted enantiomer, respectively were obtained with a corresponding column back pressure of 91 bar.

The stability of the PGA monolithic stationary phase was investigated by using the *rac*-ketoprofen as a probe and compared with the data obtained with microparticulate column.

In Table 6 are reported the chromatographic parameters (retention factor and separation factor) obtained immediately after preparation of the columns and approximately after 50 analyses.

For both columns, a significant reduction in the retention factor of the second-eluted enantiomer and consequently of selectivity was observed. This behaviour can be easily ascribed to a partial denaturation of the enzyme in the immobilized form due to the stressing conditions of the research work, affecting more the second enantiomer, the one with specific interaction for the catalytic binding site.

It is interesting to observe that the decrease of k and α values is more evident for the particulate column suggesting that the higher operating pressure on these columns can lead to a reduction of enzyme stability.

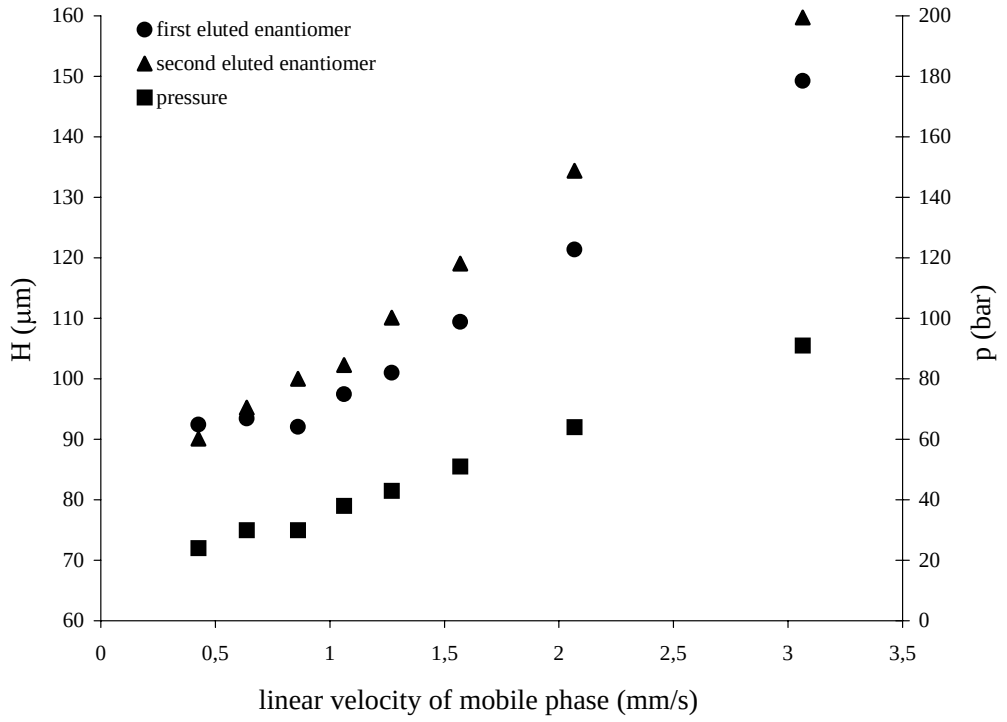


Fig. 4. Plots of column back pressure and plate height (H) vs. linear velocity of mobile phase (u). Sample: *rac*-ketoprofen; mobile phase: 50 mM phosphate buffer (pH 7.0).

Table 6
Stability of monolithic column using ketoprofen as test analyte[a]

	First analysis		After 50 analysis	
	Monolithic column	Microparticulate column	Monolithic column	Microparticulate column
k_1[b] ± R.S.D. (%)	2.62 ± 1.53	1.68[c]	2.53 ± 0	1.45[c]
k_2[b] ± R.S.D. (%)	6.14 ± 2.39	9.08[c]	5.41 ± 0.31	7.57[c]
α[b] ± R.S.D. (%)	2.34 ± 0.93	5.61[c]	2.14 ± 0.26	5.21[c]

[a] Chromatographic conditions: mobile phase, 50 mM phosphate buffer (pH 7.0); flow-rate, 0.8 ml/min; wavelength, 225 nm.
[b] Average, $n = 5$.
[c] Not calculated.

4. Conclusions

A new type of epoxy derivatized monolithic silica support was prepared. The chemically modified monolithic silica column was characterised in terms of epoxy groups attached to the surface. The new support was employed for the covalent grafting of penicillin G acylase and a higher immobilization yield was obtained if compared to coventional epoxy-silica material.

The new developed chiral stationary phase was successfully used for enantioseparations combining the well known chiral recognition properties of PGA and the unique properties concerning the flow behaviour of silica monoliths.

This work demonstrated that a PGA monolithic column can operate at high flow-rate without a significant loss in enantioselectivity. Consequently, faster enantioseparations can be achieved making the prepared supports of interest for high-throughput separations.

The information obtained from this study, especially if the amount of immobilized enzyme is considered, could extend the application of the described epoxy monolithic material to the development of chromatographic bioreactors.

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