Journal of Chromatography B, 875 (2008) 20–29

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Review

Penicillin G acylase as chiral selector in LC and CE: Exploring the origins of enantioselectivity☆

G. Massolini*, C. Temporini, E. Calleri

Department of Pharmaceutical Chemistry, University of Pavia, Via Taramelli 12, 27100 Pavia, Italy

ARTICLE INFO

Article history:
Received 4 April 2008
Accepted 31 May 2008
Available online 12 June 2008

Keywords:
Penicillin G acylase
Enantioselective discrimination
HPLC
CE
Molecular modelling
Review

ABSTRACT

The review examines the most recent achievement of immobilized Penicillin G acylase (PGA) as chiral stationary phases (PGA-CSP) for the separation of acidic enantiomers. Particular attention is paid to the influence of structural variations of a large number of analytes on retention and enantioselectivity with a special emphasis on advances in the elucidation of the chiral recognition mechanism. Docking calculations and molecular dynamic simulations are discussed to rationalize the origins of enantioselective behaviour. The review also touches the chiral behaviour of PGA in partial filling CE. The CE results, obtained using PGA in free solution, suggest that the immobilization procedure used in the development of PGA-CSPs did not alter the enantioselective properties of the enzyme.

Contents

1. Introduction

Chirality operates at every level of material existence, in fact all amino acids in proteins are 'left-handed', while all sugars in DNA and RNA, and in the metabolic pathways, are 'right-handed'.

As a consequence enantiomers of a chiral drug may display different chemical and pharmacological behaviour as a result of stereoselective interaction with optically active biological macromolecules present in living systems [1,2]. Thus, the pharmaceutical industry has concentrated its attention on the development of single optically active isomers in order to obtain safer, better tolerated and more efficacious drugs. To prepare and analyze enantiomerically pure synthetic drugs pharmaceutical companies have needed to develop new chiral technology tools. Today, the availability of new asymmetric synthesis and powerful analytical and preparative chiral separation methodologies have made it possible to develop and to produce single-enantiomer drugs [3–5].

The ideal way to obtain pure drug enantiomers would be enantioselective synthesis. This is not always practicable and in some case very complicated due to the complexity of the chiral molecules. The regio- and stereoselectivity of enzymes for one of the enantiomers of a chiral molecule can be exploited to obtain the desired enantiomerically pure product/intermediate. In particular, reactions with redox enzymes or hydrolases have been used [6,7].

Hydrolases are by far the most prominent group of enzymes used in the production of fine chemicals by biocatalytic resolution. As an example, the enzyme penicillin G amidase (PGA, also called penicillin acylase) has been used for over 20 years in the production

☆ This paper is part of the special issue 'Enantioseparations', dedicated to W. Lindner, edited by B. Chankvetadze and E. Francotte.

* Corresponding author. Tel.: +39 0382 987383; fax: +39 0382 422975.
E-mail address: g.massolini@unipv.it (G. Massolini).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.05.051

of 6-aminopenicillanic acid (6-APA). In addition, this enzyme can be used for the preparation of small peptides and pure isomers of chiral nucleophiles compounds [8–10].

However, stereoselective synthesis for the production of single-enantiomer is expensive and time-consuming, particularly in the early stages of the drug development.

As an alternative to asymmetric synthesis, the direct resolution of enantiomers by liquid chromatography and more recently by chiral capillary electrophoresis and capillary electrochromatography [11–17] can be used to generate limited quantities of pure enantiomers and to rapidly determine the enantiomeric composition of chiral molecules. Each of the available methods has its limitations, advantages and potential applications in the pharmaceutical industry. Currently, enantioselective HPLC based on chiral stationary phases (CSP) is one of the most widely utilized technique and the recent advances in CSPs performances allow reliable, robust and efficient resolution of chiral molecules. Nevertheless, a successful chiral separation is often not predictable based on analyte and CSP structures, thus there is heavy reliance and strong encouragement for the design and development of new chiral stationary phases.

The ability of proteins/enzymes to bind drugs stereoselectively has been exploited for the chromatographic and capillary electrophoretic separation of a wide range of chiral pharmaceutically active molecules [18,19].

In our laboratories, as a part of ongoing researches directed to the development of new enzyme-based CSPs, PGA has been covalently immobilized on HPLC supports and the prepared analytical columns have been used as an enzyme reactor and as a chiral stationary phase for the resolution of racemic mixtures of enantiomers [20–23].

A first review focusing on immobilization methods, selection of immobilization material and applications in chiral liquid chromatography of PGA was published in 2004 [24]. Subsequently, we extended our studies on PGA-CSP in order to find a chiral recognition model allowing predictions with respect to separability, qualitative magnitude of enantioresolution and elution order.

The present review is focused on recent studies carried out on PGA as chiral selector both in HPLC and CE with a special emphasis on computational studies for the elucidation of the stereoselective molecular recognition mechanism.

2. PGA

Penicillin G acylase (PGA, penicillin amidohydrolase E.C. 3.5.1.11) is widely present in bacteria, yeast and fungi. The biological role of PGA is obscure, even though it has been suggested to play a role as a scavenger enzyme with an ability to detach the phenylacetyl group for use as a carbon source [25].

PGA is an industrially important enzyme mainly used in the production of semi-synthetic penicillins and cephalosporins, which represent the most widely used group of antibiotics. PGA acts on the side chain of penicillin G and cephalosporin G to produce the β-lactam intermediates 6-amino penicillanic acid (6-APA) and 7-amido cephalosporanic acid (7-ACA), with phenyl acetic acid (PAA) as a common by-product (Fig. 1) [26,27]. The enzyme possesses an acyl-binding site, which accommodates a phenylacetic acid group or derivatives, and a nucleophile-binding site which can bind 6-APA/7-ACA or another nucleophile. The enzyme is able to hydrolyze diverse range of amides (R–CO–NH_2–R′), the substrate specificity is determined mainly by the acyl moiety phenylacetic acid (R), while R′ can be varied. The acyl specificity of PGA is restricted to aromatic molecules and has been investigated mainly with substrates containing phenylacetyl, phenylglycyl, mandelyl, pyridylacetyl or other arylacetyl moieties [28].

PGA has been used in several biotechnological applications (i.e. peptide synthesis and racemic resolution) due to the wide range of nucleophiles recognized as substrates (amines, alcohols, etc.) [29–31].

PGA belongs to the newly recognized structural superfamily of N-terminal nucleophile amidohydrolases which have no catalytic triad but an N-terminal serine that is activated by bringing water molecule. The X-ray structures of PGA (wild-type and mutant)

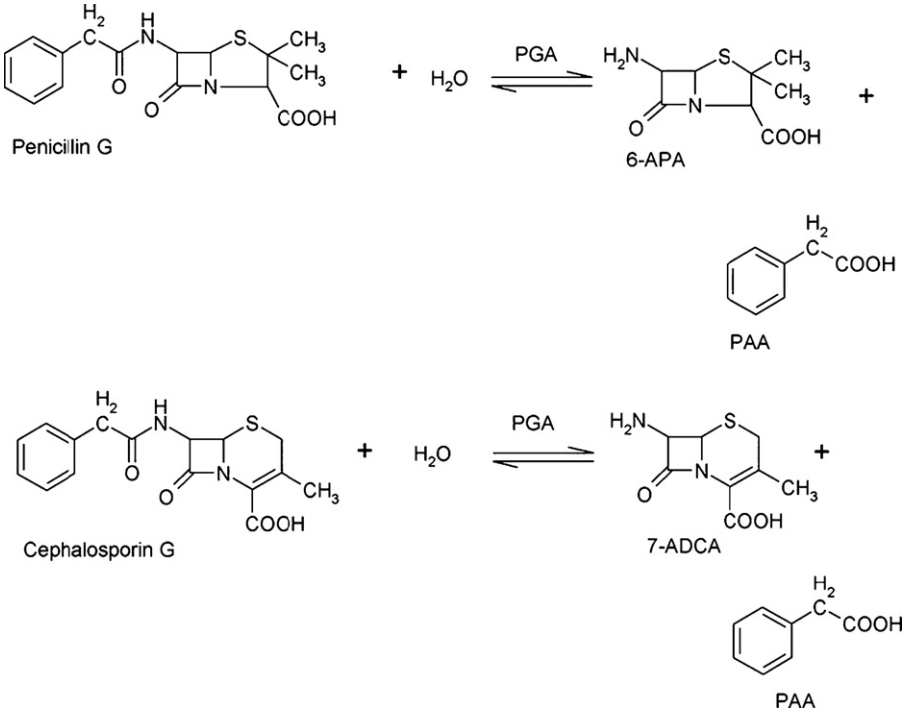


Fig. 1. Enzymatic conversion of penicillin G and cephalosporin G into 6-APA and 7-ADCA leaving phenyl acetic acid as common side product [27].

complexes with different ligands indicate the presence of a single roughly conical groove, the hydrophobic part of which accommodates the phenylacetyl moiety of the ligands [32]. The more polar aminic moiety resides in the open part of the groove, surrounded by several polar amino acid residues [28]. In detail, the binding site of PGA has been found to consist of three major regions that are responsible for the ligand recognition by the enzyme: the catalytic residue SerB1, the oxyanion hole (stabilizing the negative charge present on the ligand carboxylate group) formed by GlnB23, AlaB69, AsnB241 and a hydrophobic pocket which is able to accommodate lipophilic groups [32,33].

The key role played by PGA in pharmaceutical field has lead to the production of this enzyme by several microbial species such as *Escherichia coli*, *Bacillus megaterium*, *Arthrobacter viscous* and *Kluyvera citrophila* [23]. The enzyme mostly used in industrial processes is penicillin G acylase from *E. coli*, ATCC 11105 (EC 3.5.1.11) [34].

PGA from *E. coli* is a heterodimer: sub-unit a is composed by 209 amino acids, while sub-unit b by 557 amino acids. The active site is at the bottom of a conic depression formed by residues of the two sub-units, which are tightly intertwined. The crystal structure of the enzyme complexed with different side chain ligands has been reported in different papers [33,35–39].

3. PGA as chiral selector in liquid chromatography

In the first review, we have described the different immobilization reactions, the various pore size and derivatized silica supports that we have considered for the development of PGA stationary phases [24]. After these studies, it was concluded that the immobilization of PGA on epoxy monolithic silica gave the best chromatographic performances. The binding of PGA on activated silica has been accomplished *via* the free amino groups of lysine residues at the enzyme's surface. A scheme of the synthesis is reported in Fig. 2.

Immobilized PGA resulted to be an excellent chiral selector for the direct enantiomeric separation of acidic aromatic compounds with the (*S*)-enantiomer being more strongly bound [20,21,23]. In particular, 28 2-aryloxyalkanoic acids and isosteric analogues were analyzed and 22 racemic mixtures were resolved, same is the case with high separation factors.

The information derived from these chromatographic data sheds light on the stereoselective binding characteristics of PGA. It was hypothesized that the acid solutes enter into the hydrophobic active site and they are positioned in this cavity by their interactions with a positively charged group present close to the entrance of the binding pocket. A cationic region placed at the edge of the cavity is supposed to form an electrostatic bond with the acidic solute followed by the insertion into the PGA cavity of the molecule with its hydrophobic moiety. Electrostatic interactions between compounds and basic residues of the enzyme might be the main binding force, while hydrophobic interactions contribute to the stabilization of the analyte/enzyme complex and to enantioselectivity.

Moreover, the chiral cavity presents a defined steric restriction and we have demonstrated that there is a critical dimension of the molecules in order to obtain enantioselectivity. A schematic representation of the binding site and binding interactions derived by chromatographic studies is given in Fig. 3.

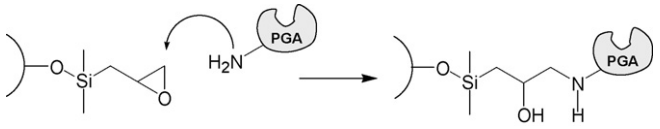


Fig. 2. Synthesis scheme for immobilization of penicillin G acylase on epoxy functionalized silica.

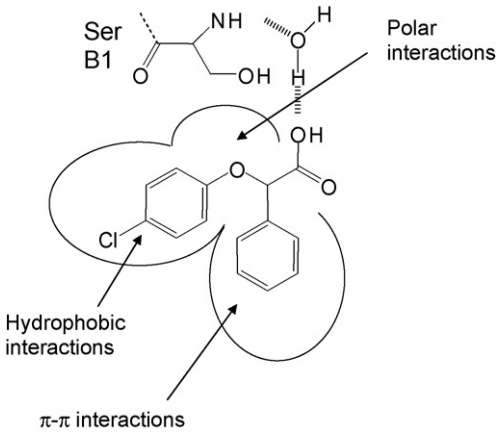


Fig. 3. Structural features of the binding site of acidic compounds on PGA as postulated on the basis of chromatographic data.

Starting from these guiding data, a large number of new compounds were prepared in order to systematically investigate the influence of the structure variation of the analytes on enantioselectivity, as well as to demonstrate the decisive enantiodiscriminating features on PGA-CSP.

A series of 2-aryloxy-2-arylacetic acids (Set 1; compounds 2–16, Table 1) together with a thioisostere derivative was designed staring from the observation that only the enantiomers of one analyte 2-(4-chloro-phenoxy)phenyl acetic acid (reported in Table 1 as compound 1), presented a reversed elution order (*S*:*R*) [40].

The impact of different electron-withdrawing and/or -donating substituents on both phenoxy and phenyl rings on enantioselectivity was evaluated (derivatives 3, 5–10, 14 and 15) as well as the influence of the bulkiness of the substituent on the chiral center (derivatives 11–13 and 16). Compound 2 was synthesized to test the PGA enantioselective behaviour toward the unsubstituted phenoxyphenylacetic acid.

On the basis of the obtained chromatographic results, it was supposed that H-bonds or dipole–dipole interactions between the polar groups of PGA and the carboxylic group or ethereal oxygen of the analytes contribute to the stabilization of the analyte/enzyme complex and to enantioselectivity. In fact, the substitution in compound 3 of the ethereal oxygen with the more lipophilic sulfur atom, in spite of the expected increased retention, does not induce any significant effect on the enantiodiscrimination process.

In addition to these polar interactions, the charge–transfer interactions may also play a role in the chiral recognition mechanism, as confirmed by the experimental data indicating an increase in retention and enantioselectivity for those compounds bearing electron-withdrawing substituents on the phenoxy and/or phenyl moieties.

PGA-CSP fails in the enantiodiscrimination of analytes characterized by the presence of an electron-donating substituent on the phenoxy or the phenyl ring whose increased π-electron density would weaken the π–π interactions as confirmed by the low retention factors of these compounds.

Representative chromatograms of compounds with α value higher than 2 are reported in Fig. 4.

For further characterization of chiral recognition profile of PGA-CSP, a second set of compounds was synthesized and analyzed on PGA column (Set 2, compounds 17–34, Table 1) [41].

The set included some derivatives characterized by the presence into the side chain of a phenyl ring (compounds 17–24) or a further

Table 1
Chemical structure of 2-aryloxy-2-arylacetic acids and chromatographic parameters obtained for racemates analyzed on PGA-CSP [40,41]

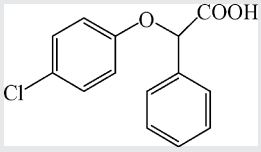
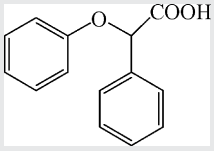
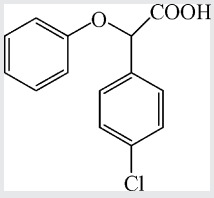
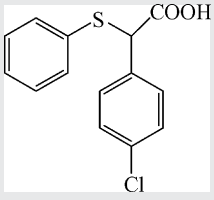
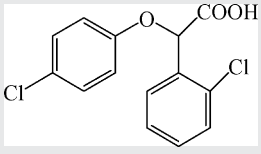
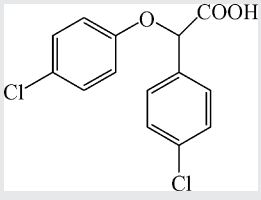
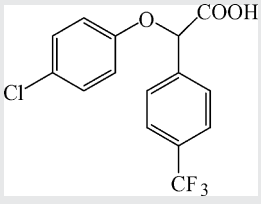
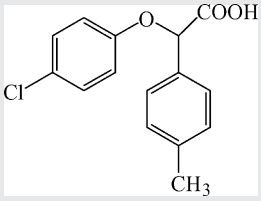
Compound		k_1	α	Rs
Set 1				
1		1.35	2.24	2.16
2		0.36	1.00	–
3		1.12	1.21	0.47
4		2.22	1.19	0.43
5		2.52	3.55	3.76
6		5.58	1.00	–
7		12.27	1.58	1.84
8		2.47	1.00	–

Table 1 (*Continued*)

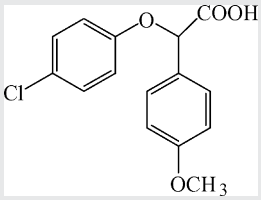
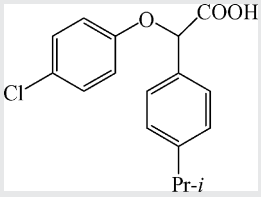
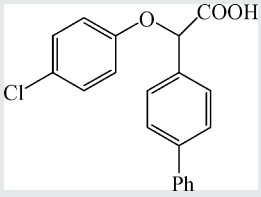
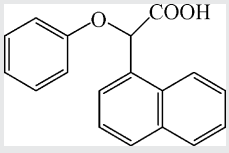
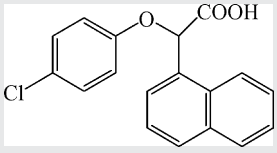
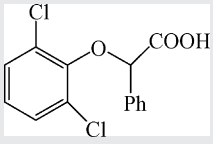
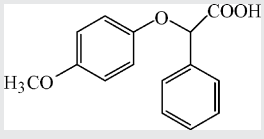
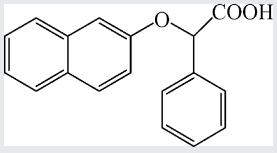
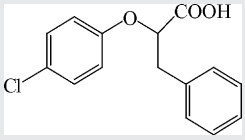
Compound		k_1	α	Rs
9		1.90	1.11	0.47
10		7.78	1.00	–
11		40.66	1.14	0.54
12		4.57	1.92	2.50
13		8.57	2.81	3.49
14		0.87	1.00	–
15		0.53	1.00	–
16		4.64	1.17	0.51
Set 2				
17		1.087	1.00	–

Table 1 (*Continued*)

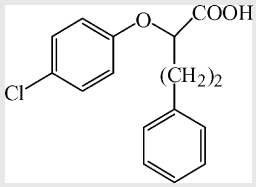
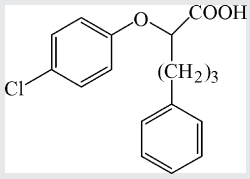
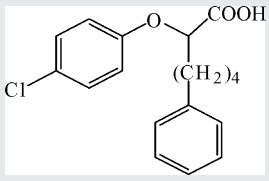
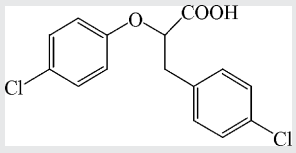
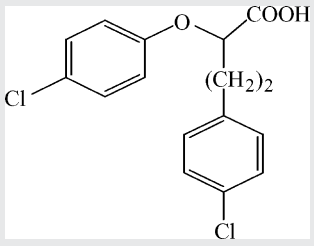
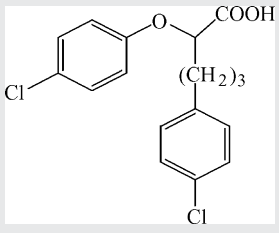
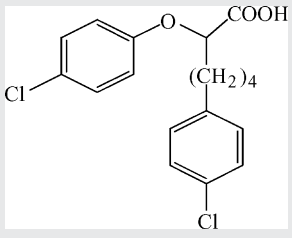
Compound		k_1	α	Rs
18		2.16	2.90	1.10
19		9.58	1.00	–
20		10.00	1.92	2.50
21		5.81	2.30	2.50
22		13.35	3.30	4.87
23		56.01	1.00	–
24		78.44	1.48	1.30

Table 1 (*Continued*)

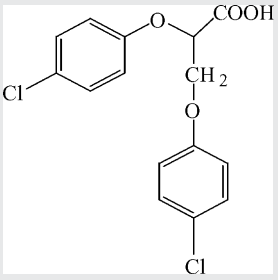
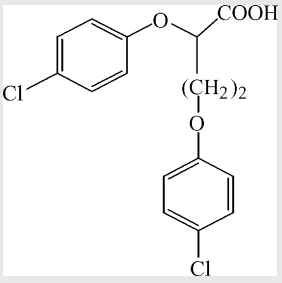
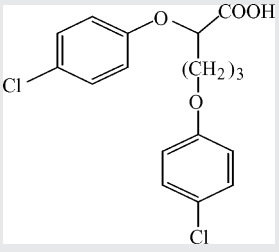
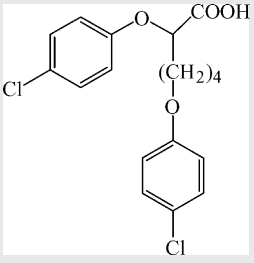
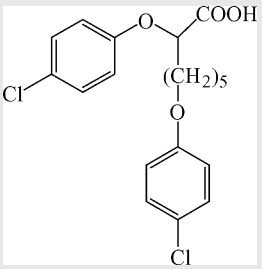
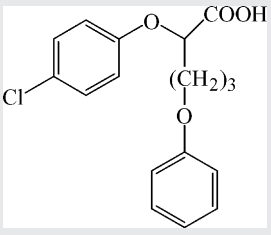
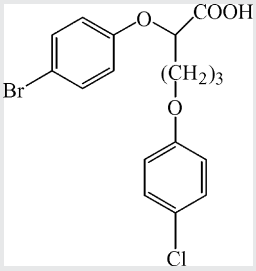
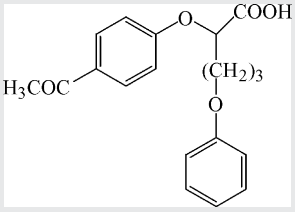
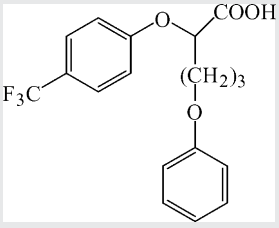
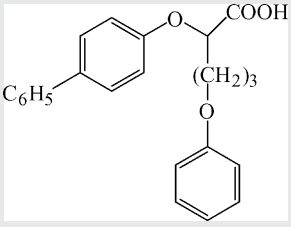
Compound		k_1	α	Rs
25		7.40	1.00	–
26		18.65	2.04	2.06
27		45.11	1.00	–
28		112.73	1.00	–
29		66.70	1.00	–
30		22.74	1.24	0.77

Table 1 (*Continued*)

Compound		k_1	α	Rs
31		14.06	1.16	0.68
32		7.02	1.40	1.23
33		11.04	1.20	0.81
34		25.07	1.20	1.10

phenoxy group (compounds 25–30) at a different distance from the chiral center. Moreover, half of these compounds had an additional chlorine atom in the *para*-position of the side chain benzene ring, whereas different atoms or groups with different size, shape and electronic properties were considered for the *para*-position of the phenoxy group directly linked to the chiral center.

From the interpretation of the experimental evidence, it is clear that the length of the chain is not the most significant structural parameter for the interaction with the binding site, while the substituent on phenoxy ring plays a more important role. However, the retention is strongly influenced by the lengthening of the carbon chain: up to four C atoms the retention increase while the chain with five carbon atoms leads to a reduction of retention. These data emphasize again the strict steric requirements within the analyte and the chiral selector. As regards the enantioselectivity, better resolution was obtained for compounds with two carbon atoms in the alkyl chain. Two illustrative chromatograms are reported in Fig. 5. In particular, the presence of oxygen molecule (compound 26) strengthened the interaction between the first eluting enantiomer and the chiral selector, while PGA and the second enantiomer interaction was weaker if compared to compound 22. The peaks of both enantiomers are broader as compared to the enantiomers of racemate 22, which indicates slower interaction kinetic.

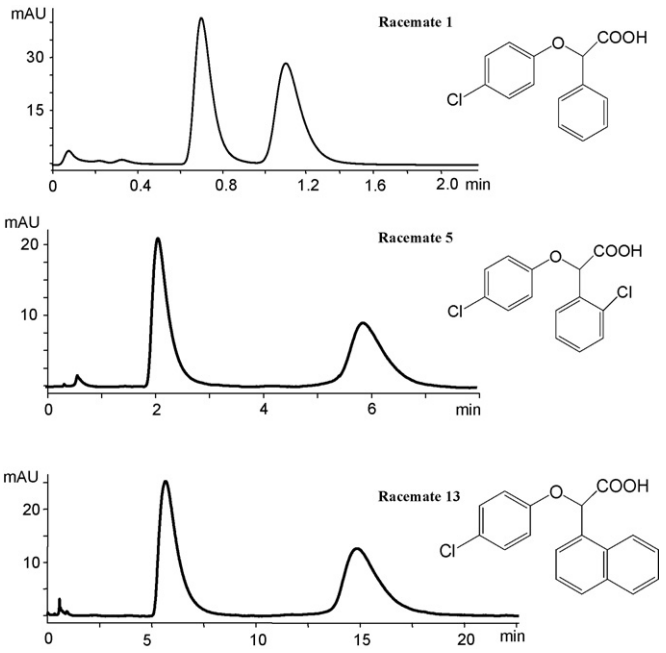


Fig. 4. Chromatograms of racemates 1, 5, and 13 (Table 1) on PGA-column. Mobile phase: 50 mM phosphate buffer (pH 7.0); flow: 1.5 ml/min [40].

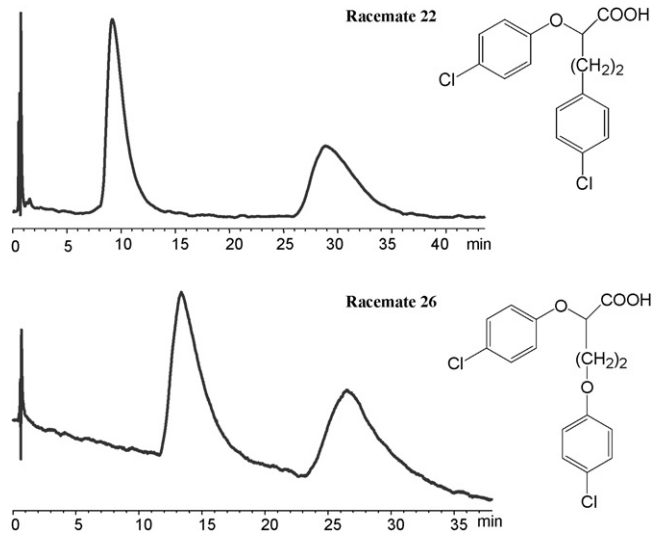


Fig. 5. Chromatograms of racemates 22 and 26 (Table 1) on PGA column. Mobile phase: 50 mM phosphate buffer (pH 7.0). Flow: 1.5 ml/min [41].

4. PGA as chiral selector in capillary electrophoresis

CE is a versatile technique of high speed, sensitivity and low running costs and it is an elective choice for resolving enantiomers, as reflected in literature reviews on the topic [42–45].

The chiral resolution is usually conducted by adding a chiral selector in the background electrolyte (BGE) to discriminate between the two enantiomers (i.e. the direct method). The number of chiral selectors available for CE is quite large and continues to increase. Among the chiral selectors, proteins can be favourably applied in CE also as free solution pseudostationary phases [46–49].

The major advantages of CE approach are the possibility to dissolve the proteins in the BGE, thus avoiding immobilization procedure, and the minor amount of enzyme consumption. On the

other hand, when UV-absorbing chiral selectors are present in the BGE strong interferences with the detection of the analyte can happen. To overcome this drawback the partial filling technique can be applied [50].

The interesting enantioselectivity exhibited by PGA for acidic compounds led us to investigate PGA as chiral selector in free solution CE also [51]. For the development of a reliable enantioselective method based on PGA, the use of coated capillaries was found to be essential in order to (i) minimize the adsorption of the protein by the capillary wall, (ii) suppress the EOF avoiding both selector and analytes to migrate in the same direction, and (iii) obtain adequate reproducibility.

To satisfy these requirements, pullulan, a homopolysaccharide consisting of maltotriose and maltotetraose units with both α-(1 → 4) and α-(1 → 6) linkages was used for the first time in the generation of a permanent coating of the inner capillary [52]. The coverage of the free silanol groups by the developed coating significantly reduced the EOF in a wide pH range and showed long-term stability.

After the development of the suitable capillary coating, the goal of the work was the application of PGA as chiral selector in free solution CE.

In chiral CE, as in HPLC, enantioseparation is controlled by the difference in binding constants of the enantiomers to the chiral selector; however, a significant role is also played by the differences between mobility of complexed and free solute [48,49,53]. In particular, using the partial filling technique, the availability of a system with suppressed EOF can allow the opposite migration direction of protein and solutes, thus favouring the enantioseparation. Since the p*I* of PGA from *E. coli* is reported to be 5.9 [54] and the pK_a of the analytes can be estimated to be around 4, the most favourable conditions in obtaining enantioresolution were achieved using running buffers in a restricted pH range. In particular, pH 5.5 was selected as optimum for all the experiments.

Moreover, in partial filling CE, the length of the chiral selector zone strongly affects the enantioresolution and it has to be carefully selected.

For this purpose, a PGA solution was introduced into the capillary by pressure and the time of loading was varied, in different experiments, from 60 to 140 s in order to obtain progressively increased chiral selector plug lengths. The optimum plug length was obtained with 120 s.

The influence of PGA concentration was also explored; as expected by increasing PGA concentration a progressive increase of enantioseparation was achieved.

The optimized conditions were used for the analysis of some chiral acidic compounds: 100 mM phosphate buffer (pH 5.5) containing 240 mM PGA using a partial filling of 120 s at 50 mbar at a constant temperature of 30 °C and voltage of 20 kV in negative polarity mode (anodic detection).

As shown in Fig. 6, for some of the tested racemates, the enantioseparation and/or the peak shape was not optimal because general electrophoretic conditions were applied without a specific optimization; nevertheless, good versatility of the proposed enantioselective method can be observed. Furthermore, the availability of single-enantiomer of some of the studied compounds allowed the migration order to be determined.

It was interesting to observe that for 2-(4-chlorophenoxy)-phenylacetic acid (compound 1) the *S*-enantiomer was the first migrated peak; this behaviour confirmed the evidence of HPLC studies [21] where the elution order *S*/*R* was demonstrated. The elution order *S*/*R* of 2-(4-chlorophenoxy)-phenylpropionic acid enantiomers was first established with CE experiments and than confirmed by HPLC analysis. These results, obtained using PGA in free solution, suggest that the immobilization procedure used in the development of PGA-CSPs did not alter the enantioselective properties of the enzyme.

In this work, the reliability of the proposed CE method for quantitative chiral analysis was also demonstrated at the impurity level.

5. PGA-CSP computational studies

Computational studies can be used to understand the chiral discrimination mechanism of stationary phases based on immobilized proteins as well as other chiral selectors [55–58].

The knowledge of structures of enzyme/ligand complexes is indispensable (although not necessarily sufficient) to clarify the molecular details of the binding mechanism and to understand the selectivity of the binding recognition process. Therefore, in order to get a better comprehension of the molecular recognition process between PGA and the considered acidic analytes, a molecular modelling (MM) study was undertaken [59].

Using MM it is possible to predict the chiral discrimination position and the energy of the complex enantiomer-chiral selector. From the energy, the elution order of the enantiomers can be derived.

The 3D-structure of PGA solved through X-ray crystallography [32] was used to dock both (*R*)- and (*S*)-isomers of selected compounds. Interestingly, it was found that the (*S*)-enantiomers of almost all compounds establishes several recurring polar interactions with SerB1, SerB386 and ArgB263.

As an example, Fig. 7 reports the binding mode of compound 3 whose chemical structure is reported in Table 1. Similar bindings pose for the (*R*)-enantiomers as well as the same set of interactions were established, with the exception of the H-bond between the ligand ether oxygen and the ArgB263 side chain. Hence, the loss of such interactions might rationalize the chiral selecting behaviour displayed by PGA towards the analyzed training set. Such a finding strongly agrees with previous data indicating the crucial role of ArgB263 in the PGA ligand specificity and stereoselectivity [27].

However, docking was not helpful when trying to explain the unexpected behaviour of compound 1, which displays a reversed elution order *S*:*R*. In the great majority of the separated compounds, the (*S*)-enantiomers adapt themselves in the stereoselective binding site establishing strong charge transfer interactions between their phenyl ring substituted with electron-withdrawing groups (compounds 3–5 and 7) or additional aromatic rings (compounds 11–13) and PheA146, PheB24 and PheB71. On the other hand, the predicted binding mode for the (*R*)-enantiomers places the phenoxy moiety in the same position occupied by the phenyl ring in the (*S*)-ligands, engaging the same sort of interactions. In compound 1, the electron-withdrawing chlorine substituent is present on the phenoxy moiety while the phenyl ring is not substituted, thus it could be hypothesized that only the (*R*)-enantiomer is able to establish strong charge transfer interactions with the enzyme. This might explain the reversed elution order found for this compound.

On the other hand, the docking study was able to explain the lack of enantioselectivity observed for the acetic acid derivatives in which the phenoxy and phenyl groups bear the same substituent in the same position. In fact, for both enantiomers of these compounds, AutoDock calculated two isoenergetic binding modes in which the carboxylate group still remained anchored to the binding site interacting with SerB1 and SerB386 side chain, whereas the phenoxy and phenyl groups were interchangeable. As a result, PGA is unable to discriminate between the two isomers of compounds 2 and 6. It was also concluded that compounds bearing bulky groups (compound 10) on the phenyl ring or more than one substituent on the phenoxy portion (compound 14) cannot adapt themselves to the stereoselective binding site of PGA, thus the enantiomers are not efficiently separated by the enzyme.

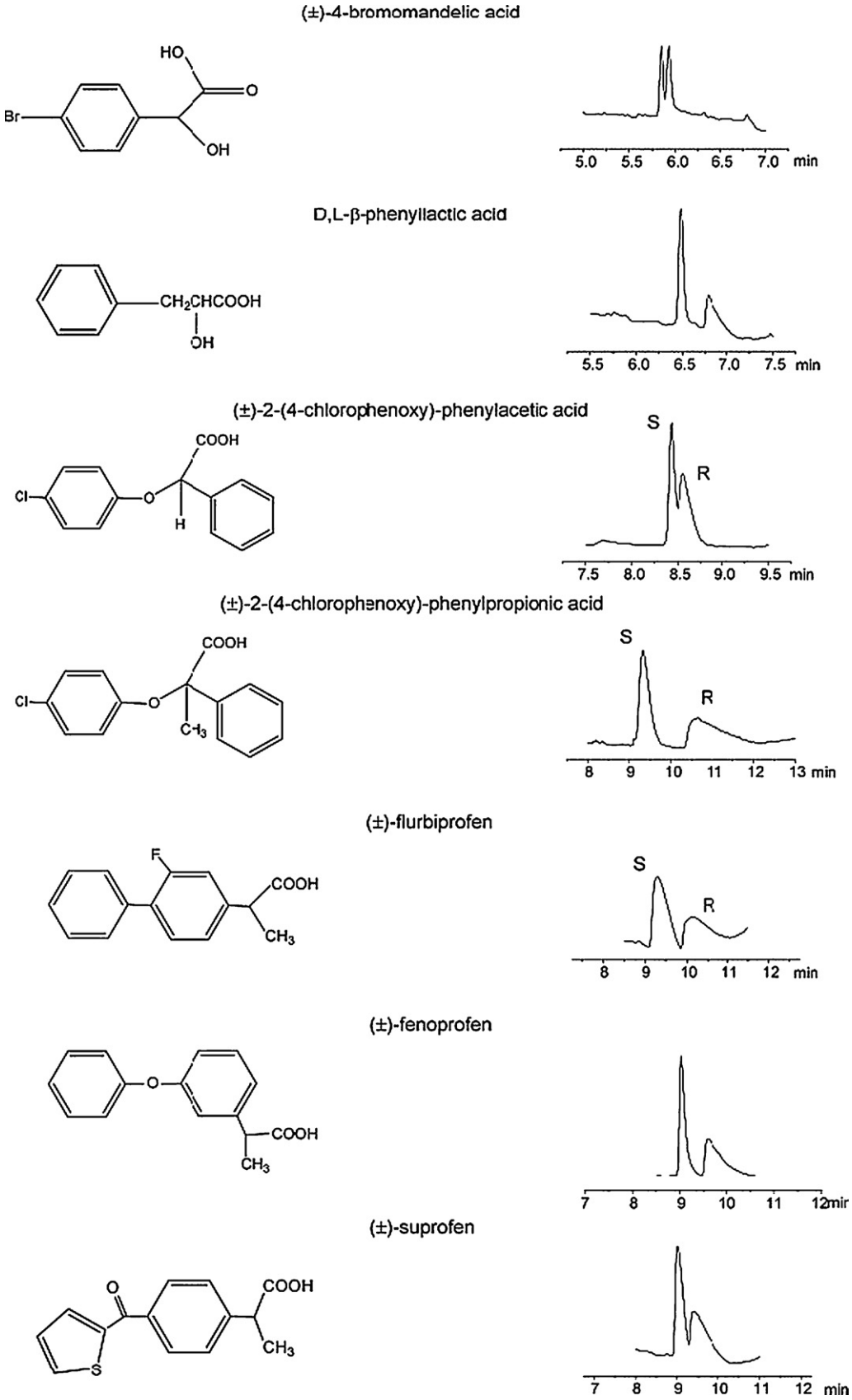


Fig. 6. Structures and CE enantioseparations of chiral acidic compounds using PGA as the chiral selector in partial filling conditions [51]. The migration order (*S*/*R*) was indicated only for the compounds for which one single-enantiomer was available. *Conditions*: 100 mM sodium phosphate BGE (pH 5.5) supplemented with PGA at 240 mM. Hydrodynamic injection of the analytes (0.1 mg/mL) at 50 mbar for 3 s.

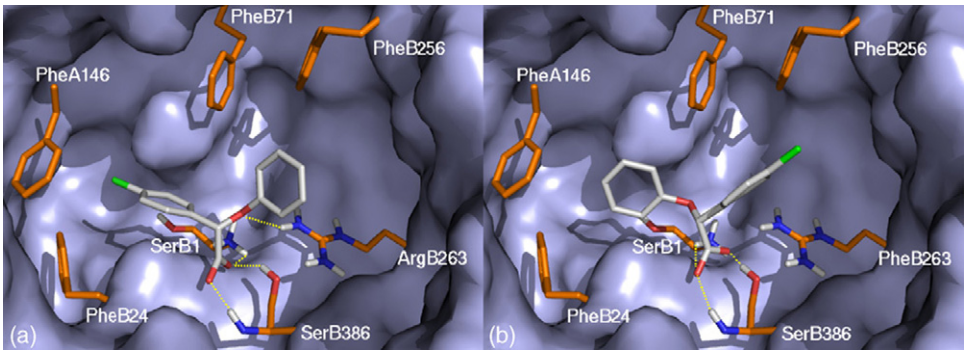


Fig. 7. Binding mode of compound (*S*)-3 (a) and (*R*)-3 (b) within PGA. For clarity reasons, only interacting residues are displayed. Hydrogen bonds between ligand and protein are shown as dashed yellow lines. Ligand (white) and interacting key residues (orange) are represented as stick models, while the protein as a light grey Connolly surface [59]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Peculiar results were obtained when docking was performed on both *R*- and *S*-isomers of compound 29, bearing a carbon chain of five C atoms. No convergence towards a single binding position was observed. From a visual inspection of the ligand/PGA complex, it was clear that the increased steric hindrance of the five C chain prevents both enantiomers from adapting themselves into the stereoselective PGA binding, thus explaining the reduction of the observed k_1 value in comparison to the homologous ones (racemates 25–28). From these data, it seems clear that the steric hindrance plays an important role on retention and chiral separation. This is in line with observations made by Chilov in a recent molecular modelling study [60].

Moreover, computational study has underlined the importance of the numerous charge–transfer interactions established between the ligands and the enzyme. It was interesting to note that charge–transfer interactions have a different strength depending on the electron-withdrawing or -donating substituent (compare k_1 of 8 versus 7 and 1 versus 15). In fact, electron-withdrawing substituents decrease the π-electron density in aromatic rings and subsequently the important π-electron repulsion, whereas electron donating substituents disfavour a π–π interaction, augmenting the π-electron density within the rings and, as a consequence, the π-electron repulsion. The importance of charge–transfer interactions in the predicted binding poses was confirmed by the experimental data indicating an increase in retention of both (*R*)- and (*S*)-enantiomers for those compounds bearing electron-withdrawing substituents on phenyl and/or phenoxy. Indeed, compound 2, bearing no substituents on phenyl and phenoxy rings, is less retained by PGA-CSP than structurally similar compounds 1, 3 and 6.

To evaluate the effect of the receptor plasticity on ligand binding together with the influence of the water solvation, molecular dynamic (MD) simulations were performed. This computational approach revealed to be really helpful in elucidating the molecular basis of the observed enantioselectivity of PGA towards the selected compounds. In fact, from an accurate analysis of MD results it seems clear that polar interactions provide most of the overall affinity of these compounds for PGA and are responsible for the enantiospecificity of the enzyme.

All these studies suggested that the predictive power of our computational approach cannot be assessed unequivocally. However, the qualitative consistency of both docking and MD simulations results with the chromatographic data suggests that the proposed approach could be used to shed light on the reasons of the observed enantioselective binding behaviour of PGA-CSP.

6. Conclusions

Recent results obtained using PGA as chiral selector in liquid chromatography and capillary electrophoresis have been presented. Chromatographic data have been used to extend our understanding of chiral recognition mechanism governing the enantioselective binding of acidic aromatic compounds and PGA-CSP while the chiral resolution of acid compounds successfully achieved in CE can extend the application of PGA as chiral selector.

By employing a combination of chromatographic and molecular modelling experiments an effort was made to highlight the origin of the observed enantioselectivity. In particular, the combination of molecular docking and molecular dynamics studies have brought up intimate details of PGA binding mechanism. The presented information can be helpful for the refinement of PGA as chiral selector and forms the basis for rational protein engineering efforts aimed at improving the enantioselectivity of PGA. This can lead to the development of a tailoring chiral selector for a specific group of molecules.

References

[1] Y. Zhang, D. Wu, D.B. Wang-Iverson, A.A. Tymiak, Drug Discov. Today 10 (2005) 571.
[2] H.Y. Aboul-Enein, I.W. Wainer (Eds.), The Impact of Stereochemistry On Drug Development and Use, Chemical Analysis Series, vol. 142, J. Wiley & Sons, New York, 1997.
[3] N.M. Maier, P. Franco, W. Lindner, J. Chromatogr. A 906 (2001) 3.
[4] E.R. Francotte, J. Chromatogr. A 906 (2001) 379.
[5] O. Mcconnell, et al., Chirality 19 (2007) 658.
[6] U.T. Bornscheue, R.J. Kazlauskas, Hydrolases in Organic Synthesis: Regio- and Stereoselective Biotransformations, Wiley-VCH, Weinheim, 2006.
[7] B.G. Davis, V. Boyer, Nat. Prod. Rep. 18 (2001) 618.
[8] P. Rasor, E. Voss, Appl. Catal. A: Gen. 221 (2001) 145.
[9] M. Arroyo, I. de la Mata, C. Acebal, M. Pilar Castillon, Appl. Microbiol. Biotechnol. 60 (2003) 507.
[10] L.M. van Langen, F. van Rantwijk, V.K. Švedas, R.A. Sheldon, Tetrahedron Asymm. 11 (2000) 1077.
[11] N.M. Maier, W. Lindner in, E. Francotte, W. Lindner (Eds.), Chirality in drug Research—Methods and Principles in Medicinal Chemistry, vol. 33, Wiley-VCH, Weinheim, 2006, p. 189.
[12] M. Laëmmerhofer, F. Svec, J.M.J. Freèchet, W. Lindner, Trends Anal. Chem. 19 (2000) 676.
[13] B. Preinerstorfer, M. Lämmerhofer, Electrophoresis 28 (2007) 2527.
[14] S. Andersson, S.G. Allenmark, J. Biochem. Biophys. Methods 54 (2002) 11.
[15] Ganapathy, Subramanian (Eds.), Chiral Separation Techniques A Practical Approach, third ed., Wiley-VCH, Weinheim, 2006.
[16] G. Gubitz, M.G. Schmid, Biopharm. Drug Dispos. 22 (2001) 291.
[17] J. Haginaka, J. Pharm. Biomed. Anal. 27 (2002) 357.
[18] M.C. Millot, J. Chromatogr. B 797 (2003) 131.
[19] J. Haginaka, J. Chromatogr. A 906 (2001) 253.
[20] G. Massolini, E. Calleri, E. De Lorenzi, M. Pregnolato, M. Terreni, G. Felix, C. Gandini, J. Chromatogr. A 921 (2001) 147.

[21] E. Calleri, G. Massolini, F. Loiodice, G. Fracchiolla, C. Temporini, G. Felix, P. Tortorella, G. Caccialanza, J. Chromatogr. A 958 (2002) 131.
[22] G. Massolini, E. Calleri, A. Lavecchia, F. Loiodice, D. Lubda, C. Temporini, G. Fracchiolla, P. Tortorella, E. Novellino, G. Caccialanza, Anal. Chem. 75 (2003) 535.
[23] E. Calleri, G. Massolini, D. Lubda, C. Temporini, F. Loiodice, C. Caccialanza, J. Chromatogr. A 1031 (2004) 93.
[24] E. Calleri, C. Temporini, G. Massolini, G. Caccialanza, J. Pharm. Biomed. Anal. 35 (2004) 243.
[25] E. Merino, P. Balbas, F. Recillas, B. Becerril, F. Valle, F. Bolivar, Mol. Microbiol. 6 (1992) 2175.
[26] C.F. Sio, W.J. Quax, Curr. Opin. Biotechnol. 15 (2004) 349.
[27] A.K. Chandel, L.V. Rao, M.L. Narasu, O.V. Singh, Enzyme Microb. Technol. 42 (2008) 199.
[28] M. Guncheva, I. Ivanov, B. Galunsky, N. Stambolieva, J. Kaneti, Eur. J. Biochem. 271 (2004) 2272.
[29] A. Liljeblad, L.T. Kanerva, Tetrahedron 62 (2006) 5831.
[30] C. Fuganti, C.M. Rosell, S. Servi, A. Tagliani, M. Terreni, Tetrahedron Asymm. 3 (1992) 383.
[31] C. Carboni, H.G.T. Kierkels, L. Gardossi, K. Tamiola, D.B. Janssenc, P.J.L.M. Quaedfliega, Tetrahedron Asymm. 17 (2006) 245.
[32] C.E. McVey, M.A. Walsh, G.G. Dodson, K.S. Wilson, J.A. Brannigan, J. Mol. Biol. 313 (2001) 139.
[33] S.H. Done, J.A. Brannigan, P.C. Moody, R.E. Hubbard, J. Mol. Biol. 284 (1998) 463.
[34] R.C. Giordano, M.P.A. Ribeiro, R.L.C. Giordano, Biotechnol. Adv. 24 (2006) 27.
[35] H.J. Duggleby, S.P. Tolley, C.P. Hill, E.J. Dodson, G.G. Dodson, P.C. Moody, Nature 373 (1995) 264.
[36] W.B.L. Alkema, C.M.H. Hensgens, E.H. Kroezinga, E. de Vries, R. Floris, J.M. van der Laan, B.W. Dijkstra, D.B. Janssen, Protein Eng. 13 (2000) 857.
[37] W.B.L. Alkema, A.J. Dijkhuis, E. de Vries, D.B. Janssen, Eur. J. Biochem. 269 (2002) 2093.
[38] W.B.L. Alkema, A.K. Prins, E. de Vries, D.B. Janssen, Biochem. J. 365 (2002) 303.
[39] W.B.L. Alkema, E. de Vries, R. Floris, D.B. Janssen, Eur. J. Biochem. 270 (2003) 3675.
[40] G. Massolini, G. Fracchiolla, E. Calleri, G. Carbonara, C. Temporini, A. Lavecchia, S. Cosconati, E. Novellino, F. Loiodice, Chirality 18 (2006) 633.
[41] C. Temporini, E. Calleri, G. Fracchiolla, G. Carbonara, F. Loiodice, A. Lavecchia, P. Tortorella, G. Brusotti, G. Massolini, J. Pharm. Biomed. Anal. 45 (2007) 211.
[42] G. Blaschke, B. Chankvetdaze, J. Chromatogr. A 875 (2000) 3.
[43] A. Amini, Electrophoresis 22 (2002) 3107.
[44] M. Blanco, I. Valverde, Trends Anal. Chem. 22 (2003) 428.
[45] P.T.T. Ha, J. Hoogmartens, A. Van Schepdael, J. Pharm. Biomed. Anal. 41 (2006) 1.
[46] D.K. Lloyd, S. Li, P. Ryan, J. Chromatogr. A 694 (1995) 285.
[47] D.K. Lloyd, A.-F. Aubry, E. De Lorenzi, J. Chromatogr. A 792 (1997) 349.
[48] J. Haginaka, J. Chromatogr. A 875 (2000) 235.
[49] Y. Tanaka, S. Terabe, J. Biochem. Biophys. Methods 48 (2001) 103.
[50] L. Valtcheva, J. Mohammad, G. Pettersson, S. Hjertén, J. Chromatogr. 638 (1993) 263.
[51] R. Gotti, E. Calleri, G. Massolini, S. Furlanetto, V. Cavrini, Electrophoresis 27 (2006) 4746.
[52] B. Chankvetadze, M. Saito, E. Yashima, Y. Okamoto, J. Chromatogr. A 773 (1997) 331.
[53] B. Chankvetadze, Chem. Soc. Rev. 33 (2004) 337.
[54] A. Bossi, M. Cretich, P.G. Righetti, Biotechnol. Bioeng. 60 (1998) 454.
[55] N.M. Maier, S. Schefzick, G.M. Lombardo, M. Feliz, K. Rissanen, W. Lindner, K.B. Lipkowitz, J. Am. Chem. Soc. 124 (2002) 8611.
[56] C. Czerwenka, M.M. Zhang, H. Kählig, N.M. Maier, K.B. Lipkowitz, W. Lindner, J. Org. Chem. 68 (2003) 8315.
[57] K.B. Lipkowitz, J. Chromatogr. A 694 (1995) 15.
[58] K.B. Lipkowitz, J. Chromatogr. A 906 (2001) 417.
[59] A. Lavecchia, S. Cosconati, E. Novellino, E. Calleri, C. Temporini, G. Massolini, G. Carbonara, G. Fracchiolla, F. Loiodice, J. Mol. Graph. Model. 25 (2007) 773.
[60] G.G. Chilov, O.V. Stroganov, V.K. Svedas, Biochemistry (Moscow) 73 (2008) 56.