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Review

# Drug affinity to immobilized target bio-polymers by high-performance liquid chromatography and capillary electrophoresis

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**Abstract**

This review addresses the use of high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) as affinity separation methods to characterise drugs or potential drugs–bio-polymer interactions. Targets for the development of new drugs such as enzymes (IMERs), receptors, and membrane proteins were immobilized on solid supports. After the insertion in the HPLC system, these immobilized bio-polymers were used for the determination of binding constants of specific ligands, substrates and inhibitors of pharmaceutical interest, by frontal analyses and zonal elution methods. The most used bio-polymer immobilization techniques and methods for assessing the amount of active immobilized protein are reported. Examples of increased stability of immobilized enzymes with reduced amount of used protein were shown and the advantages in terms of recovery for reuse, reproducibility and on-line high-throughput screening for potential ligands are evidenced. Dealing with the acquisition of relevant pharmacokinetic data, examples concerning human serum albumin binding studies are reviewed. In particular, papers are reported in which the serum carrier has been studied to monitor the enantioselective binding of chiral drugs and the mutual interaction between co-administered drugs by CE and HPLC. Finally CE, as merging techniques with very promising and interesting application of microscale analysis of drugs' binding parameters to immobilized bio-polymers is examined.


*Keywords:* Reviews; Drug-binding constants; High-throughput screening; Bio-polymers, immobilized

**Contents**



*Abbreviations:* ACE, affinity capillary electrophoresis; AChE, acetylcholinesterase; AGP, α1-glycoprotein; BGE, background electrolyte; BSA, bovine serum albumin; CE, capillary electrophoresis; CEC, capillary electrochromatography; CE-FA, capillary electrophoresis frontal analysis; CIM, convective interaction monolith media; DBH, dopamine β-hydroxylase; DTT, dithiothreitol; EDTA, etylenediaminetetraacetic acid; FA, frontal analysis; FAC, frontal affinity chromatography; GABA, γ-amino butyric acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Glut-P, silica-based matrix containing glutaraldehyde; GnT-V, *N*-acetylglucosaminyltransferase V; GS, glutamine synthetase; HD, Hummel–Dreyer method; HPALC, high pressure affinity liquid chromatography; HPFA, high-performance frontal analysis; HPLC, high-performance liquid chromatography; HSA, human serum albumin; IAM, immobilized artificial membrane; IMER, immobilized enzyme reactor; MS, mass spectrometry; MR-SP, multiple-receptor stationary phase; NAChRs, nicotinic acetylcholine receptors; NMDA, *N*-methyl-D-aspartate receptors; NSAID, non-steroidal anti-inflammatory drug; ODS, octadecyl silane; OXH, oxazepam hemisuccinate; PGA, penicillin G acylase; Pgp, P-glycoprotein transporter; PNMT, phenylethanolamine *N*-methyltransferase; QSRR, quantitative structure–retention relationship; SAP, serum protein amyloid P; TTR, transthyretin; UF, ultrafiltration; VACE, vacancy affinity capillary electrophoresis; VP, vacancy peak

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## 1. Introduction

Biological and behavioural activities like learning, memory, response to stress, pain addiction, feed behaviour, the immune response, cardiovascular function and many others are modulated or controlled by ligand–macromolecules interactions. Recent advances in understanding the chemistry of life particularly in the field of genomics, proteomics, molecular biology and molecular physics have shown that the interaction ligand–macromolecules such as receptors, enzymes, membrane proteins constitute the main physico-chemical mechanism by which living processes are controlled. Pharmaceutical research is oriented towards the development of new leads which can modulate these biological activities. New drugs discovery involves, as the first step, robust strategies for the ligand-based drug design that require careful consideration of the structural features and detailed analyses of biological activity of large series of compounds, including binding to specific targets, recognised as specific enzymes, receptors or membrane proteins [1]. The disclosure of potential drugs' activity is ultimately related to the affinity for a biological target which is reflected in the ligand binding constant for the target bio-polymer.

Moreover, the definition of drugs pharmacokinetic has to deal with transport protein binding to elucidate the drug free fraction that reaches the pharmacological target and the metabolising enzymes. Binding to human serum albumin (HSA) controls the free, active concentration of a drug, provides a reservoir for a long duration of action, and ultimately affects drug absorption, metabolism, distribution and excretion [2].

Drug activity and metabolism are, therefore, mediated by different types of interactions with specific biological targets and the esteem of these interactions may elucidate the drug mechanism of action, the tool to improve activity and specificity.

One approach to study drug or potential drugs binding to target bio-polymer is the affinity chromatography. Although this technique is primarily intended for isolation and purification of complex biological mixtures [3], it has been shown to be very powerful and predictive to monitor ligand–protein, substrate–enzyme, inhibitor–enzyme and ligand–receptor interactions [4–6].

Affinity chromatography involves essentially three steps: the immobilization of a target bio-polymer (enzyme, receptor, transport protein, etc.), the assessment of unchanged protein functions after immobilization, and the determination of ligand binding parameters, after the insertion of the immobilized protein in a separation system. The high information content contained in affinity chromatographic data reflects the fact that the fundamental processes of drug action, absorption, distribution, excretion and receptor activation, are dynamic in nature and, in this manner, similar to the basic mechanisms involved in chromatographic retention. Indeed, the same basic intermolecular interactions (hydrophobic, electrostatic, hydrogen bonding) determine the behaviour of compounds in both biological and chromatographic environments.

One of the advantages of this approach over the conventional methods is that it can reveal differences in binding of solutes in the presence of fast association–dissociation kinetics, due to the establishment of a large number of consecutive equilibria between free and bound ligands. The retention parameter for the injected solutes becomes then a direct measure of the relative amount of drug at equilibrium that is bound to the immobilized protein. Besides, the advantages of dealing with immobilized bio-polymers in comparison to traditional techniques are the increased stability (few hours for the free enzyme in solution, several weeks for the immobilized enzyme), the use of small amount of bio-polymer, the recovery for reuse, the reproducibility and the on-line high-throughput screening for enzyme inhibitors or for ligands.

The immobilization of protein has been widely investigated and a variety of covalent and non-covalent immobilization techniques have been developed for this purpose. These techniques have been used to successfully develop several immobilized chromatographic columns based upon carrier proteins such as human serum albumin (HSA) [7], enzymes such as α-chymotrypsin [8–12], lipase [13], mem-

brane protein [14,15], and receptor proteins such as nicotinic acetylcholine receptors [16]. All of the immobilized proteins retained their ability to bind and/or transport ligands. Thus, they could be used to determine pharmacological activities and as a rapid screen for isolation and identification of lead compounds candidates from complex biological mixtures.

Immobilization is a critical step which can easily deactivate the target bio-polymer. Concerning the enzymes, the development of a suitable on column assay to monitor the substrate catalytic conversion is essential to establish the activity after immobilization and then the activity reduction of already known or new inhibitors.

Besides affinity constants, affinity chromatography allows competitive protein binding interactions to be investigated to gain information on the specific ligand interaction sites on the immobilized bio-polymer [17,18]. This information can be exploited to underline the structural ligand features for specific binding and to rationally design site directed ligands.

This review deals with examples of affinity separation for evaluating drug–bio-polymer interactions by means of the two most widely used separation techniques: HPLC and CE. Concerning HPLC as separation method, the use of immobilized enzymes, receptors and transport proteins as immobilized bio-polymers for the determination of binding constants of specific ligands, substrates and inhibitors of pharmaceutical interest is reported.

However, for the sake of focus, the use of immobilized enzyme reactors in stereoselective analyses is not discussed in this paper.

Furthermore, dealing with the acquisition of relevant pharmacokinetic data, examples concerning human serum albumin binding studies are reviewed. Finally, CE as merging techniques with very promising and interesting application of microscale analysis of drugs' binding parameters to target protein is examined.

## 2. Drug binding by affinity HPLC

### 2.1. Enzymes as immobilized bio-polymers

Many drugs actually available on the market show their pharmacologically activity because they are enzyme inhibitors (i.e. clavulanic acid and β-lattamase, trimetoprim and dihydrofolate reductase, aciclovir and herpes simplex DNA polymerase, etc.) [1] and many diseases are still object of research for the development of new drugs as enzyme inhibitors. This is the case of Alzheimer's disease: for its treatment the only four drugs available on the market are acetylcholinesterase (AChE) inhibitors which act by elevating the level of acetylcholine in the central nervous system (tacrine, rivastigmine, donepezil and galantamine) [19]. In recent years, the investigation of AChE inhibitors has gained further interest, because it has been disclosed the involvement of the peripheral site of the enzyme in the β-amyloid aggregation process [20].

Numerous methods have been developed to assay enzyme activity, i.e. radiochemical and spectrophotometric or chromatographic methods. However, these methods require the use of radioactive substrates and/or large amount of purified enzyme, which is often produced by DNA recombinant technology and, therefore, highly costly. In addition, these methods require solubilised enzymes and tend to be affected by low reproducibility and activity variability due to different batches of enzymes. Moreover, they tend to be complicated and time consuming.

Enzyme immobilization is one approach that can be used to overcome these problems. Immobilized enzymes can be used in batch-wise experiments or packed into columns and used in a flow system as immobilized-enzyme reactors (IMERs). This approach does not require highly purified enzyme and decreases the amount of protein required. Moreover, immobilized enzymes can be reused

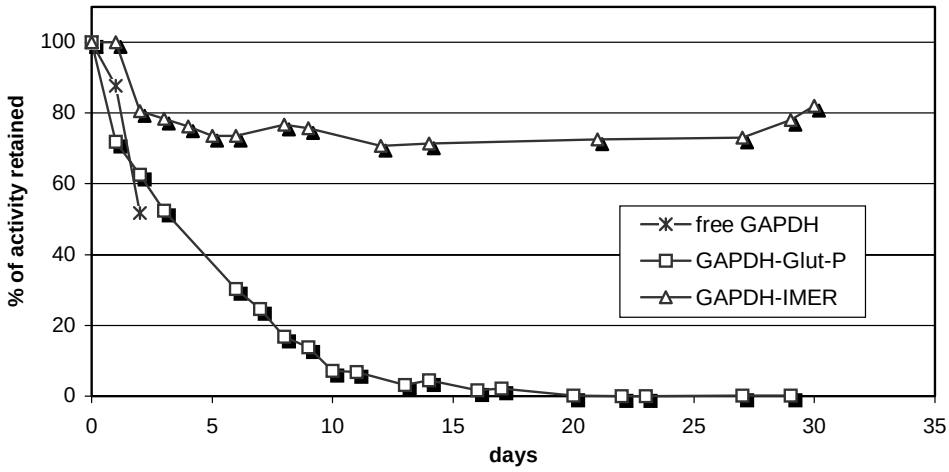


Fig. 1. The stability of free and immobilized GAPDH during storage in 0.1 mM triethanolamine–HCl, 1 mM sodium azide, 5 mM magnesium sulphate, 1 mM DTT, 1 mM EDTA and 10 mM $K_2HPO_4$. (reprinted from [33]).

Table 1
Recent applications for on-line HPLC-IMERs

| Immobilized enzyme | Target disease | Inhibitors | Substrate | References |
|---|---|---|---|---|
| Phenylethanolamine *N*-methyltransferase (PNMT) | Hypertension | Benzylamine, *N*-ethylmaleimide, *p*-chloromercuriphenylsulfonic acid, *S*-adenosyl-L-homocysteine, methyldopa | Normetanephrine, norepinephrine | [29,30] |
| Dopamine β-hydroxylase (DBH) | Hypertension | Captopril, fusaric acid | Tyramine, ascorbic acid | [31,32] |
| Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) | Tripanosoma diseases | Agaric acid | Glyceraldehyde 3-phosphate | [33] |
| Acetylcholinesterase (AChE) | Alzheimer's disease, myasthenia gravis, glaucoma | Tacrine, donepezil, rivastigmine | Acetylthiocholine, acetylcholine | [34–39] |
| Glutamine syntetase (GS) | Epilepsy, drug toxicity | Phenytoin, carbamazepine, Methionine sulfoximine, AMP | Glutamate | [40] |
| *N*-acetylglucosamininyl transferase-V (GnT-V) | Mammary, hepatocellular, pancreatic malignant tumours | Trisaccharides | UDP-*N*-acetylglucosamine | [41,42] |

multiple times following a simple washing procedure and usually they show an increased stability. This is of utmost importance especially for those enzymes that require over-expression, isolation and purification, being then usually available in a limited amount. This was the case of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which showed higher stability when it was immobilized and packed into an IMER (Fig. 1).

In the last few years, IMERs have been developed by numerous groups and have proven to be useful and economic alternative to conventional methods. These immobilized enzyme reactors have proven to be useful in the field of drug discovery and analysis. In particular, IMERs were prepared for the separation and identification of metabolites in drug metabolism research [21–25], for enantioselective analyses [8–13] and synthesis [13,21,26–28], as well as for the identification of substrate and inhibitors as drugs or potential drugs (Table 1). In addition, the immobilized enzyme stationary phases were also used to investigate the properties of enzyme inhibitors, including a qualitative assessment of the type of enzyme inhibition, i.e. competitive, non-competitive, etc. and a quantitative evaluation of inhibition constants ($K_i$).

### 2.1.1. Immobilization techniques

Enzymes can be covalently immobilized on a solid support by using either the in situ derivatisation technique or by the batch-wise method, followed by packing into a column. Various supports are commercially available or have been specifically developed for the immobilization processes. Among these, it is worth to mention several silica based derivatized matrices [11,43,44], monolithic chromatographic supports [45], and immobilized artificial membrane stationary phases [46–48]. In general, the immobilization of small biomolecules results easier because small proteins can be directly immobilized on the support; however, in the case of large molecules, the active centre of the enzyme could be no longer accessible at all or only to a limited degree. In these cases, an improvement can be achieved by introducing a spacer, which allow maximum enzymatic conversion. Moreover, immobilization protocols have to be carefully chosen in order to avoid alterations in the protein surface and obtain a highly stable linkage with the chosen support (e.g. secondary amino bonds, ether bonds, etc.) [11–13,24,26–28].

Multifunctional supports containing epoxy groups for covalent immobilization of proteins under mild experimental conditions are commercially available or can be easily prepared [43]. The covalent immobilization of proteins proceeds via their initial physical adsorption on the supports followed by a covalent linkage between some nucleophilic groups of the adsorbed enzyme (e.g. amino, thiol or hydroxy groups) and the dense layer of epoxy groups on the matrix. At the end of the immobilization process, unreacted epoxy groups can be blocked by reaction with very different thiol or amine compounds preventing further uncontrolled reactions between the support and the enzyme that could decrease its stability. AChE immobilization was performed by in situ derivatisation technique using a prepacked epoxide silica gel chromatographic column (Kromasil 200A-5 μm) [34]. The enzyme solution was recycled through the column for 24 h. The amino groups of lysine residues of the enzyme reacted with the epoxide groups to give a covalent bond carbon–nitrogen and a vicinal hydroxyl residue. The unreacted epoxide groups were deactivated by fluxing through the column 1 M glycine solution. The reaction yield in terms of immobilized enzyme was 25%. The stability of the immobilized enzyme was found to be maintained for more than 4 months with a daily use.

Monoliths are considered as a novel generation of stationary phases whose special feature is the fast separation and enzymatic conversion due to lack of diffusion resistance during mass transfer [49–51]. Moreover, the large through-pores of monolithic materials allow high-speed analysis and low back pressures and consequently enable the coupling of the enzymatic column with an analytical column. In particular, convective interaction media (CIM®) disks (12 mm in diameter and 3 mm thick) look promising for analytical application due to reduced time analysis and high enzyme efficiency [45]. Penicillin G acylase was covalently immobilized using the in situ method via the carboxylic groups on an aminopropyl monolithic support (Chromolith Performance $NH_2$, 10 cm × 0.46 cm (i.d.)), for the affinity study of some aryloxyalkanoic acid methyl esters [27,28].

Instead, immobilization by a batch wise technique involves essentially two steps: immobilization of the target enzyme onto the non-packed affinity support followed by packing of the enzyme–stationary phase into an empty column. The advantages of this technique is the performance of optimisation studies (pH, time of reaction, amount of support/enzyme ratio), using small amount of enzyme and matrix and at low cost. Using the batch wise method, a widely spread support for the immobilization of proteins and enzymes is a silica-based matrix, containing glutaraldehyde (Glut-P) [44]. Glut-P is a wide-pore silica that has been covalently clad with a hydrophilic polymer (poly(ethylenimine)) and then activated with glutaraldehyde. A Schiff base reaction is involved in the coupling of the ligand to the affinity medium. Immobilization of the selected enzyme onto this interphase results in formation of an amine–aldehyde Schiff linkage with Glut-P. This constitutes a useful model for a soluble form of an enzyme because the enzyme is situated outside the phase.

This support was successfully used by Markoglou and Wainer to develop on-line immobilized enzyme reactors containing enzymes involved in the synthesis of catecholamines, i.e. dopamine β-hydroxylase (DBH) [31,32] and phenylethanolamine *N*-methyltransferase (PNMT) to be used for new drug development [29,30] and to obtain a gliceraldehyde-3-phosphate dehydrogenase reactor (GAPDH–IMER) [33].

Non-covalent immobilizations are alternative modes, especially performed for resemble the biological environment in which the enzyme is located and for avoiding the involvement of the active site in the matrix link which may reduce overall catalytic activity.

The immobilization by hydrophobic entrapment is generally performed with immobilized artificial membrane (IAM) supports, which resemble one-half of a cellular membrane. IAMs are prepared by covalent binding of a monolayer of phospholipids to silica particles [46], thus mimicking the lipid environment of a fluid cell membrane on a solid support. The prototype IAM surface, 1-mystroyl-2-[(13-carboxyl)-tridecanoyl]-*sn*-3-glycerophosphocholine (lecithin-COOH, IAM.PC) has been modified in order to increase the chemical stability of the bonded phase and to avoid aspecific interactions. Several IAM stationary phases, in which residual groups have been end-capped, are currently commercially available, i.e. IAM.PC.MG in which end-capping has been performed with methyl glycolate, IAM.PC.DD (Drug Discovery) in which end-capping of the surface was performed using decanoic and propionic anhydride [47], IAM.PC.DD2, in which the residual amino groups are end-capped using C10 and C3 alkyl chains.

A comparison between IAM and Glut-P dopamine β-hydroxylase (DBH) immobilizations was performed in the work of Markoglou and Wainer [31,32]. DBH occurs in a soluble form and a form confined to the surrounding membrane. Therefore, DBH was non-covalently immobilized onto a IAM stationary phase in order to mimic the membrane bound of the enzyme. DHB was also covalently immobilized onto Glut-P. The DBH-Glut-P showed to display the characteristics of the solubilised enzyme. The two IMERs were shown to be stable and retained over 85% enzymatic activity for over a 3 months period of use.

Concerning non-covalent immobilization, biotin–avidin or streptavidin techniques are mostly used methods for oriented immobilization [52,53]. The binding of water soluble vitamin biotin to the egg white protein avidin or to its bacterial counterpart, streptavidin is accompanied by a vast decrease in free energy compared to that observed for other non-covalent interactions. This technique was used for the preparation of a *N*-acetylglucosaminyltransferase V (GnT-V) reactor (Fig. 2) [41]. Recombinant human *N*-acetylglucosaminyltransferase, overexpressed in many malignant tumours, was biotinylated by the biotinylation reagent sulfosuccinimidyl 6-(biotin-amido) hexanoate and adsorbed onto immobilized streptavidine in a microcolumn of porous polystyrene streptavidine beads. The obtained column was used for inhibition studies for the development of new drugs in the cancer therapy.

Bio-specific adsorption of biologically active proteins to immunosorbents prepared by use of suitable monoclonal or polyclonal antibodies can also combine the isolation of molecules with their oriented immobilization. Oriented immobilization of enzymes by antigen–antibody interaction result both in good steric accessibility of the active site and in an increased stability. One example is the immobilized multienzyme flow reactor containing acetylcholinesterase and choline oxidase [36] for determination of acetylcholine and

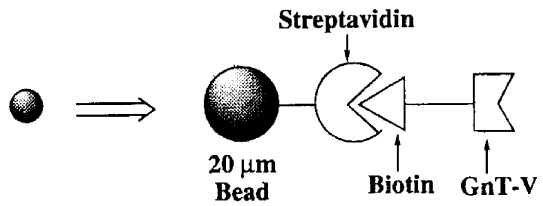


Fig. 2. Non-covalent immobilization of GnT-V (from [41], by permission of the Editor).

choline at femtomole levels. Immobilized antibody goat antimouse IgG, was used for the preparation of immunosorbent containing site directed monoclonal antibodies against both enzymes. This type of immobilization is based on the formation of suitable bio-specific complex which involve the hydrophobic clusters of the protein located on the surface, thus reducing the non-polar surface area, which contacting water, can lead to protein unfolding and inactivation. The advantages of this technique is the possibility of achieving good steric accessibility of active binding sites, prolonging protein stability.

*2.1.2. Determination of the immobilized enzyme activity*

The amount of immobilized enzyme does not necessarily correspond to the active immobilized enzyme. One aspect is the determination of the yield of immobilization in term of protein bound to the matrix and another is the definition of how many units have remained active. In order to define the immobilization yield, the UV absorbances of enzyme solution (usually at 280 nm) before and after the linking process are averaged. Colorimetric assays based on the differential colour change of a dye i.e. Coomassie Brilliant Blue in Bradford's assay [54] or cupric sulfate–tartrate/Folin-Ciocalteu reagent in Lowry's assay [55], in response to various concentration of protein can be also used to monitor the reduction of the protein amount in solution, during the immobilization procedure.

The evaluation of the enzyme activity after immobilization is the next step for checking the enzyme stability, which means verifying the enzyme structure has been maintained and quantifying the yield of immobilization, i.e. the amount of active units. This step is necessary to compare different types of immobilization, covalent or non-covalent, choosing the best approach which gives higher yields and improve enzyme stability. In many papers, IMERs were inserted in a HPLC system and, in order to determine the enzyme activity after immobilization, a known substrate was injected at increasing concentrations or flow through the enzyme reactor and the resulting product and remaining substrate directly determined. The chromatographic conditions needed to be optimised in term of pH of the mobile phase, ionic strength, nature of the buffer and flow rate, variables which affect specific enzyme catalytic activity. To determine the amount of immobilized enzyme, the rate of catalysis (amount of product per time unit in the defined chromatographic conditions: mobile phase, pH and flow rate) at increasing injected substrate amounts was plotted against the injected substrate concentration [28–34,40–42]. Lineweaver and Burk reciprocal plots [56,57] of $1/V$ and 1/[S] allowed to estimate the values of $K_m$ and $V_{max}$.

The active immobilized units per milligram of matrix are determined by the $V_{max}$ obtained by the Michaelis–Menten plot.

$$\text{Units/mg matrix} = \frac{(\Delta A/\text{min})_{max}}{\varepsilon(\text{mg matrix})}$$

Another approach to determine the active amount of immobilized enzyme is frontal affinity chromatography (FAC) [58].

Using this technique, the column capacity $B_t$, which is the amount of immobilized enzyme that can bind the ligand is determined by injecting increasing concentration of substrate and applying the following relationship:

$$V_x - V_0 = \frac{B_t}{(K_d + [X]_0)}$$

where $V_x$ is the elution volume of ligand $X$ and $V_0$ the void volume of the system which can be determined by injecting compounds that do not bind to the enzyme. The retention volume of a ligand decreases as its concentration increases, and $B_t$ and its dissociation constant $K_d$ can be determined.

This procedure has been used to characterise the capacity of *N*-acetylglucosaminyl transferase-V (GnT-V) affinity column. Selective monitoring of either the product or the substrate is attained by coupling HPLC with mass spectrometer and UV, the most frequently used detectors [41]. Conventional interfacing is made with a UV-Vis spectrophotometer. In this case, a co-elution of substrate and product will be detectable if they show different absorption spectra. On the other hand, mass spectrometry allows for a sensitive detection of compounds that break through the column and provides an extra dimension to the analysis, namely the *m/z* ratio. Moreover, due to the high sensibility, the requirement for the precious enzyme is greatly reduced and the demands on the amount of ligand are proportionally decreased.

Coupling FAC with mass spectrometry results an useful technique for high-throughput screening of combinatorial libraries, while simultaneously confirming the molecular weights of the inhibiting species. FAC–MS is a two steps method involving: (a) the use of a void volume marker and a ligand indicator to check if any strong ligand exists in a compound library. If the front of the elution curve for the indicator ligand shifts in the presence of a library, it can be concluded that at least one strong and competitive ligand is present in the library. (b) The second step is the direct FAC–MS analysis of the compound library to identify and rank the binding ligands [41].

If the product formed by the catalysis of the specific substrate is coloured, the UV-Vis detector can selectively monitor the rate of formation of the coloured specie, at the product absorbance wavelength. Alternatively, an in-line or post-column derivatisation technique can be applied in order to obtain a coloured product. The first approach has been used in the case of AChE-IMER [34]. The product of enzymatic AChE hydrolysis, thiocholine, does not present a significant chromophore for UV detection, therefore, the evaluation of enzyme activity after immobilization was performed by injecting acetylthiocholine with Ellman's reagent dissolved in the mobile phase. The amount of the resulting coloured adduct was selectively monitored fixing the detector wavelength at either 412 nm ($\lambda_{max}$) or at higher wavelengths, such as 445 nm, in order to reduce interference from

Ellman's reagent or from the disulfide side-product. The second approach has been applied to determine the activity retained on glutamine synthetase reactor (GS-IMER) [40]. The activity of the immobilized GS was determined using the method of Wellner and Meister. The eluent from GS-IMER was mixed by mean of a T-connector with a $FeCl_3$ solution and the output was monitored with a UV detector at 535 nm.

The rate of catalysis can be determined either monitoring the disappearance of the substrate or the appearance of the product of enzymatic reaction. The use of an analytical post-column connected in-line or of a column switching system can be useful to separate substrate and product and follow simultaneously the enzymatic conversion. GAPDH catalyses the oxidative phosphorilation of D-glyceraldeyde-3-phosphate into 1,3-diphosphoglycerate in the presence of NAD+ and inorganic phosphate. Its activity is monitored by following the conversion of the cofactor $NAD^+$ to NADH. Thus, the ability to separate $NAD^+$ and NADH is a crucial element in the GAPDH–IMER chromatographic system. The chromatographic efficiency of the GAPDH–IMER was not sufficient enough to separate $NAD^+$ from NADH and the activity of the GAPDH–IMER could not be directly determined. In order to separate $NAD^+$ from NADH, a monolithic RP18 column was placed in-line after the GAPDH–IMER. This column was chosen because it had enough efficiency and selectivity to separate the analytes and a low back-pressure, insuring that the immobilized enzyme was not unduly stressed [33].

Switching systems were used in the activity determination and chromatographic studies on the penicillin G acylase reactor (PGA-IMER) and PNMT-IMER. In the case of PGA-IMER, the hydrolysis of penicillin G potassium salt has been used as a standard assay for the determination of the catalytic activity of the enzyme in the immobilized form, coupling the enzyme column with a reversed-phase analytical column.

The substrate was loaded onto the enzyme column, the product and the unreacted substrate were switched to the analytical column and the conversion percentage was measured on the analytical column. Then, the enzymatic product enantiomeric excess (ee) was calculated off-line [28].

The activity of the PNMT-IMER was determined by connecting a Cyano and an ODS stationary phases by a switching valve. The substrate was injected onto the PNMT-IMER and the unreacted substrate and product were eluted and transported via a switching valve onto the analytical columns [30].

The construction of a coupled system was able to provide a number of approaches to basic research as well as to drug discovery. Two individual IMERs containing enzymes involved in the synthesis of catecholamine (DBH-IMER and PNMT-IMER) have been coupled in order to carry out the on-line synthesis of epinephrine from dopamine. Dopamine was injected onto the DBH-IMER and the reactants and products were eluted onto a phenylboronic acid column for on-line extraction. The products were transported via a switching valve to the PNMT-IMER where norepinephrine was converted into epinephrine and directed onto the analytical column for analysis. This system allowed the use of the IMERs individually or in combination [30].

### 2.1.3. Enzyme inhibitors affinity

By using immobilized enzymes, the determination of the enzyme inhibitors affinity can be expressed either by the determination of the $IC_{50}$ (concentration which reduces by half the product peak obtained at saturating conditions) and/or determining the $K_i$ by the Lineweaver and Burk plot. In Table 1, some examples of inhibition studies on immobilized enzyme inserted in HPLC systems are reported.

The $IC_{50}$ of inhibitors was assessed by using the immobilized enzyme column, by extrapolation from the inhibition curves. The inhibition curves were obtained by injecting

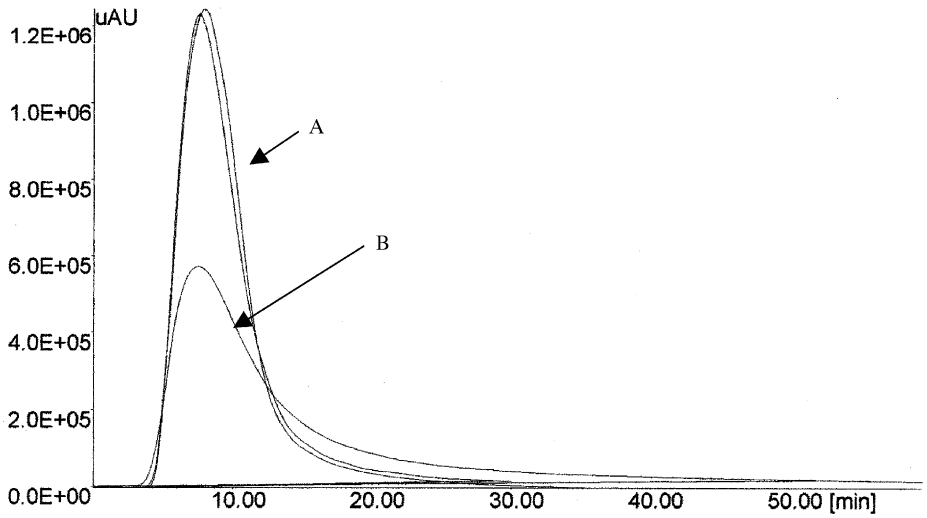


Fig. 3. Overlaid chromatograms related to the injection in the AChE-IMER of: (A) acetylthiocholine as substrate at saturating concentration alone and (B) with the addition of 9 μM donepezil (reprinted from [34]).

simultaneously both the substrate at a fixed saturating concentration as determined by the Michaelis–Menten plot and increasing concentration of inhibitors. Increasing reduction of the peak area of the product (i.e. inhibition of enzyme rate of hydrolysis) when compared to the area obtained by the sole substrate was observed for increasing inhibitor concentration (Fig. 3). The percent inhibition was plotted against inhibitor concentration to obtain the inhibition curves. The $IC_{50}$ values of known inhibitors tested on the enzyme columns were found correlated with the values obtained with the enzyme in solution [30,32–34].

The determination of $K_i$ of GnT-V inhibitors which is the dissociation constant for the enzyme–inhibitor complex was directly determined by frontal affinity chromatography and mass spectrometry [41,42], but it can also be determined using the Lineweaver and Burk plot, injecting together with increasing substrate concentration below the saturating conditions, increasing inhibitor concentrations, as it is used with the enzyme in solution, but using the areas of the chromatographic peaks. This was reported for glutamine synthetase [40].

The only problem which can merge in testing inhibitors is the deactivation of the IMER by analysing irreversible inhibitors and inhibitors' matrix aspecific interactions, which increase the concentration of inhibitors in the interstices of the IMER, reducing the enzyme activity recovery. The choice of chromatographic supports characterised by relatively large pores (200–300 nm) and short length, or better monolithic supports like CIM disk, help avoiding these problems.

The striking advantage of an HPLC in-line immobilized enzyme reactor is the possibility of automating the system accelerating the screening of hundreds of potential inhibitors in a short time, in a reproducible system.

### 2.2. Immobilized receptors and membrane proteins

In the past few years, stationary phases containing immobilized trans-membrane receptors and transporters have been developed for the on-line screening of complex mixtures in drug discovery. The phases have included immobilized nicotinic acetylcholine receptors (nAChRs) alone [16,59–62], or co-immobilized with the γ-amino butyric acid receptors (GABA) and *N*-methyl-D-aspartate receptors (NMDA) [63], G-coupled receptors [64], and estrogen receptors [16]. The drug transporter P-glycoprotein [65,66] and D-glucose transporter have also been immobilized [67,68]. Liquid chromatographic columns containing immobilized α3β4-nAChR or α4β2-nAChR subtypes have been prepared using cell line (α3β4-nAChR) or from solubilised rat forebrain (α3β2-nAChR). The nicotinic receptors were immobilized via hydrophobic insertion into the interstitial spaces of an immobilized artificial membrane (IAM) stationary phase or Superdex 200 gel beads [59,61] and frontal and zonal chromatography were used to characterise the activity of the immobilized nAChRs. Affinity

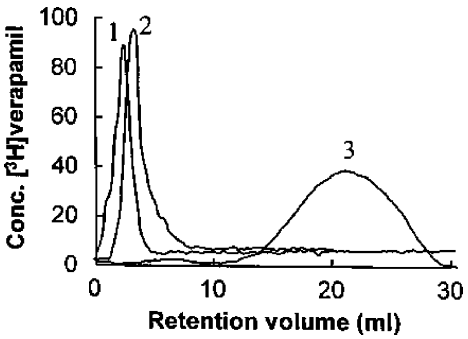


Fig. 4. Zonal affinity chromatographic profiles of 100 μl of 23.5 nM [3H]verapamil at a flow rate of 0.5 ml/min with 50 mM Tris–HCl, pH 7.4, buffer: (1) from Pgp-negative-IAM column; (2) from IAM particles column; and (3) from Pgp-IAM column (from [65], by permission of the Editor).

chromatography allowed to determine binding affinities ($K_d$ values) of compounds to the two different receptor subtypes and were used to identify both competitive and non-competitive ligands. The estrogen receptor was tethered to a hydrophilic stationary phase [16] and the membranes containing the P-glycoprotein transporter (Pgp) were coated on the surface of the IAM stationary phase or were reconstituted into the phospholipid bilayer of liposomes that were immobilized on Superdex 200 gel beads by using freeze-thawing methods [65,66]. The stationary phases were characterised using known ligands and substrates for the respective non-immobilized proteins. The results from zonal and frontal chromatographic experiments demonstrated that the stationary phases could be used to determine binding affinities (expressed as dissociation constants, $K_d$) and to resolve mixtures of ligands according to their relative affinities (Fig. 4). In addition, competitive ligand binding studies on the P-glycoprotein-based stationary phase have established that this phase can be used to identify and characterise competitive displacement and allosteric interactions. These studies demonstrate that immobilized-receptor phases can be used for on-line pharmacological studies and as rapid screens for the isolation and identification of lead drug candidates from complex biological or chemical mixtures.

The ability to developing a liquid stationary phase containing more than one functioning receptor, a multiple-receptor stationary phase (MR-SP) was investigated by Moaddel et al. [63]. Using solubilised rat forebrain, an MR-SP containing different members of the ligand-gated ion channel superfamily, including NMDA, $GABA_A$ and NCT (nicotinic receptors), was developed in order to perform on-line multiple-receptor screens. Frontal chromatography studies demonstrate that co-immobilized receptors might be useful in the investigation of overlapping affinities and new ligands might be readily screened for multiple binding properties or selective interactions.

### 2.3. Immobilized serum albumin

The binding of endogenous and exogenous ligands to human serum albumin (HSA) has been the object of many reviews and books [2,6,17,69–73]. Decisive advancement in the characterisation of the protein binding areas resulted from X-ray crystallography [74,75], spectroscopy [6,76] and the study of recombinant mutants of the protein [77–79]. However, high pressure affinity liquid chromatography (HPALC), i.e. the use of HPLC columns with HSA immobilized on the silica matrix, still represents a very well suited methodology for studying the drug binding process, allowing to determine the extent of drug binding as well as the enantioselectivity of the process [6]. By adopting this affinity method, referred as biochromatography, on HSA-based HPLC columns it is possible to detect small differences in drug binding affinities and in their modulation by experimental parameters or by the presence of competitors. Since the retention of an analyte is related to the extent of its protein binding at a specific site, and the resolution of a chiral analyte to the enantioselectivity of the process, chromatographic runs can be a direct probe for binding site specificity and stereoselectivity, predictors of drug–drug, drug–metabolite and drug–ion interactions, and measures of induced changes in HSA structure.

The free concentration of a drug can also be affected by pathological conditions, which can modify to a significant extent the binding properties of the carrier, resulting in important clinical impacts for drugs that have a relatively narrow therapeutic index. The binding to albumin is often highly specific, and two of these binding sites, Sites I and II according to the definition of Sudlow et al. [80], are better characterised and they are involved in the binding of an incredibly high number of classes of compounds. This is usually true at therapeutic concentrations, while higher drug concentrations should also involve other binding sites, with lower binding affinity and selectivity. However, a more dynamic picture of the protein, i.e. a flexible protein changing in shape depending on its surrounding, is a better model to fully explain albumin binding properties, including the stereospecific binding of chiral ligands [81].

Here, we will focus our attention to some relevant applications of the immobilized HSA columns, in characterising the binding sites on the protein and to give evidence of the changes in the binding properties of the protein arising by its reversible or covalent modification. For these applications, it is essential the selection of the anchoring procedure, in order to guarantee that the immobilized protein maintains the same binding properties as in solution [6,17,81–83]. Among the reported anchoring procedures, those employing covalent binding of the protein to a silica diol [7] and to a silica epoxy [83] matrixes were proved to mirroring the binding processes in solution. This was demonstrated by carrying out binding experiments on column and in solution on the same HSA–drugs systems [6].

The application of immobilized serum proteins as chiral selectors will not be discussed here and we refer to already published papers [7,83–86].

This part of the review mainly concerns the biochromatographic approach referred as zonal elution, where a narrow plug of solute is injected onto a column while the solute elution time or volume is monitored. Another widely used approach in biochromatography is represented by the frontal analysis. This method involves the continuous, rather than plug type, application of an analyte to a column; the result is essentially a titration of the number of active binding sites within the column.

#### 2.3.1. Quantitative assessment of drug binding

*2.3.1.1. Zonal elution.* Studies with the HAS-based column have demonstrated that chromatographic retentions observed on this column ($k'$), when using zonal elution approach, reflect the binding properties as well as the stereoselectivity of the free protein. For example, two series of compounds, benzodiazepines and coumarin derivatives were chromatographed on a HAS-based column and their retentions determined [87]. The percentage of binding of each compound to non-immobilized HSA was also determined using standard ultrafiltration technique. For each series of drugs, the results from the ultrafiltration studies, expressed as percentage of drug bound to HSA, were correlated with chromatographic retentions of the members of the series, expressed as $k'/(k'+1)$: $k'$, capacity factor, is defined as $(t_{drug} - t_0)/t_0$, where $t_{drug}$ is the retention time of the drug, and $t_0$ is the retention time of a non-retained solute. Excellent correlations were obtained between chromatographic retention and extent of albumin binding with correlation factors of 0.999 obtained for both the series. The estimation of the protein binding has been extended by other authors to forty structurally unrelated drugs and the correlation has been improved by using a quantitative structure–retention relationship (QSRR) approach [88].

The zonal elution data can give information about ligand binding sites on the protein. This methodology is used to develop quantitative structure–retention relationships (QSRRs) that describe the binding of drugs and their analogues to protein columns. The retention factors are measured for a large set of structurally related compounds under constant temperature and mobile phase conditions. The resulting data are then correlated to several factors that describe various structural features of the solutes. Then, information can be obtained on the forces involved in the drug–protein binding and an approximate description can be developed for the sites that are involved in these interactions [17,89–92]. Quite recently, a comprehensive study on the importance of plasma protein binding and the prediction of this molecular property on the basis of the drug structure has been reported [93].

*2.3.1.2. Frontal analysis.* Frontal analysis experiments with HAS-based columns have been mainly carried out to provide quantitative data on the drug affinity constants and to determine the amount of active protein anchored on the column [94–96]. The time or the volume of the breakthrough are measured for several concentrations of the drug [72]. The resulting data are then fitted to equations selected for the model under investigation, and double reciprocal plots are particularly useful for this purpose.

$$\frac{1}{m_{\mathrm{L\,app}}} = \frac{1}{K_{\mathrm{a}} m_{\mathrm{L}}[\mathrm{A}]} + \frac{1}{m_{\mathrm{L}}}$$

where $m_{\mathrm{L\,app}}$ is the apparent moles of analyte that are required to saturate the column and it is measured as the volume of the breakthrough; [A] the applied analyte concentration, $K_{\mathrm{a}}$ the association constant of the drug and $m_{\mathrm{L}}$ the total binding capacity of the immobilized protein.

According to the equation, a linear relationship should be obtained when $1/m_{\mathrm{L\,app}}$ is plotted versus 1/[A], if the analyte has a single type of binding site on the immobilized HSA. Once it has been determined which reaction model best describes a solute–protein system, the affinity and number of binding sites in the column for the solute can be determined from the best fit parameters for the experimental data. As an example, the above equation predicts, for a single site binding model, a linear relationship by plotting $1/m_{\mathrm{L\,app}}$ versus 1/[A], with a slope equal to $1/(K_{\mathrm{a}} m_{\mathrm{L}})$ and an intercept of $1/m_{\mathrm{L}}$. According to this model, the total binding capacity of a HSA column and the association constant of the drug can be obtained from the inverse of the intercept on the *Y*-axis, and by the ratio between intercept and slope, respectively. The possibility of determining simultaneously both the association constant and the active bound protein, makes the frontal analysis the methodology of choice when information is needed on the binding capacity of a column. Furthermore, frontal analysis allows highly accurate association constant measurements, since the values that it provides for $K_{\mathrm{a}}$ can be determined independently from the column binding capacity [72,96,97].

### 2.3.2. Determination of drug–drug protein binding interactions

These studies can be performed by displacement chromatography experiments, i.e. the study of the change of the chromatographic parameters in the presence of specific binding site modifiers in the mobile phase. The reversible or HSA binding of the modifier occurs, thus reproducing in vitro a possible model for studying binding competition phenomena of physiological relevance. There are a number of potential interactions between ligands that simultaneously bind to a protein. The various possibilities are: (1) the ligands bind independently and there is no interaction (independent binding); (2) the binding of one ligand facilitates the binding of the other (co-operative binding); (3) the binding of one ligand decreases the ability of the other compound to bind (anti-co-operative binding); (4) the two ligands bind competitively (non-co-operative binding) [70]. Drug–drug interactions at the HSA binding level can be readily observed and quantified using biochromatography, since the effect of one drug on the binding of the other is immediately reflected in a change in the chromatographic retention of the test solute.

When the analyte is injected using a mobile phase which does not contain the competitor, the retention is directly proportional to its binding affinity for the immobilized protein. When the competitor is added to the mobile phase, the magnitude and direction of the resulting changes in retention can be used to determine the binding site of the ligand and to indicate if cooperative, anti-cooperative or non-cooperative interactions have occurred [17,72,84,85].

The relationship between the $k'$ of the solute and the mobile phase concentration of the displacer can be expressed by the equation:

$$\frac{1}{(k' - X - Y)} = \frac{V_{\mathrm{M}} K_{\mathrm{C}}[\mathrm{C}]}{K_{\mathrm{L}} m_{\mathrm{L}}} + \frac{V_{\mathrm{M}}}{K_{\mathrm{L}} m_{\mathrm{L}}}$$

where $k'$ is the capacity factor; $V_{\mathrm{M}}$ the void volume of the column; $K_{\mathrm{C}}$ and $K_{\mathrm{L}}$ are equilibrium constants for binding of the displacer and analyte, respectively; $m_{\mathrm{L}}$ is moles of the analyte bound to the stationary phase; [C] the concentration of the displacer in the mobile phase; $X$ the residual $k'$ resulting from binding at sites on the protein unaffected by the displacer; $Y$ the non-specific chromatographic interactions [84].

The term $X$ is a constant that represents the portion of $k'$ resulting from the binding of the analyte to sites at which the displacer does not compete. If both the analyte and displacer bind at only one identical site on the immobilized protein, then $X = 0$ and a plot of $1/(k' - Y)$ versus [C] will produce a linear relationship with a slope of $(V_{\mathrm{M}} K_{\mathrm{C}}/K_{\mathrm{L}} m_{\mathrm{L}})$ and an intercept of $(V_{\mathrm{M}}/K_{\mathrm{L}} m_{\mathrm{L}})$. The value of $K_{\mathrm{C}}$, the binding affinity constant for the displacer, can be determined directly by calculating the ratio of the slope to intercept for this plot. The inverse of the slope gives $m_{\mathrm{L}}/V_{\mathrm{M}}$, which is the effective concentration of the binding sites in the column.

The term $Y$ is used to take into account the interactions that occur between the analyte and the stationary phase. This process produces a residual contribution to the retention that can be observed by carrying out competition experiments. In the condition of self-drug displacement, $X$ should be zero, by definition, and a large excess of the displacer should reduce the $k'$ of the solute to zero. But, this is not actually the situation, since a residual contribution to $k'$ remains even when a large excess of the same injected drug is added to the mobile phase. Self drug displacement experiments can be carried out by determining the capacity factor of a drug in the presence of increasing concentrations of the same drug in the mobile phase. As an example, in the case of diazepam (Fig. 5a), a constant value is reached for value of the [competitor]/[drug] ratio higher than 5, this behaviour demonstrating that a contribution remain to the retention as due to non-specific binding to the matrix. The residual

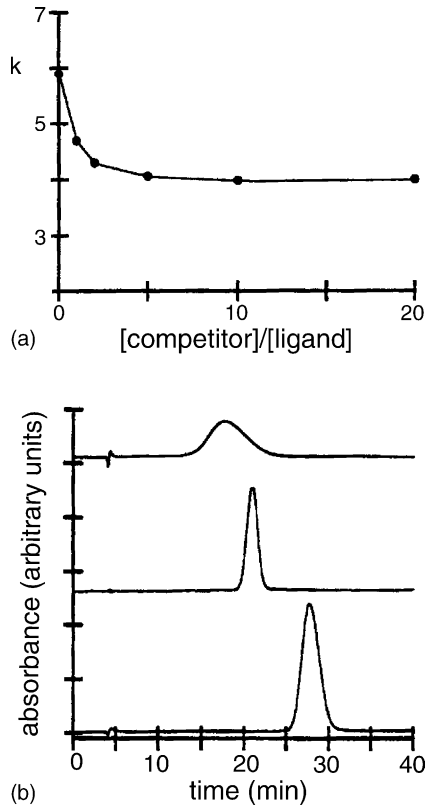


Fig. 5. (a) Self-displacement experiment with diazepam on HSA–HPLC: diazepam is either the ligand and the competitor. At large [competitor]/[ligand] ratios, the capacity factor (k) reaches a residual plateau; (b) chromatograms of diazepam injected on HSA–HPLC. The elution are under standard conditions (mobile phase: phosphate buffer 50 mM, pH 7.4, 0.6 ml/min, top and bottom trace), with a large excess of diazepam added to the mobile phase, [competitor]/[ligand]: 20/1 (middle trace), and after denaturing the anchored HSA (top trace). The retention of diazepam can be reduced but not to zero (from [84], by permission of the Editor).

retention is observable by comparing the chromatographic profile of the injected diazepam (Fig. 5b, bottom trace) with that obtained in the presence of a large excess of diazepam in the mobile phase (Fig. 5b, middle trace). It is interesting to observe that almost the same retention time we measured for diazepam in the presence of high concentration of the competitor in the mobile phase was obtained by denaturing the immobilized protein (Fig. 5b, top trace) [84]. The term *Y* is characteristic of each analysed compound and takes into account the interactions with the stationary phase other than binding areas (i.e. non-specific analyte–amino acid and solute–silica interactions). Thus, displacement chromatography data can be used to study pharmacologically relevant saturable interactions, and to distinguish between direct and indirect competition when co-binding of two drugs to the protein occurs [6,72,82–84,97–106]. Most of these studies have been carried out using as competitors drugs that bind to Sites I and II, like profens, benzodiazepines, phenylbutazone, warfarin, salicylate, and valproate. Displacement chromatographic studies, in addition to give information on the influence of a co-binding process on the affinity of a single drug, contributed significantly to better characterising the high affinity HSA binding areas. Furthermore, this methodology resulted useful to study the changes in the free fraction of a drug in pathological conditions. As an example, changes in the concentrations of endogenous factors have been invoked to justify the significant inhibition of the ketoprofen binding in hepatic patients. The use of bile acids as competitors demonstrated a direct competition with profens, the binding inhibition resulting enantioselective [107]. Quite recently, applications have been also reported on the role of metal ions on the affinity of a single drug [108]. In particular, the role of the $Mg^{2+}$ cation on anti-hypertensive molecule binding on HSA was studied.

Competitive binding experiments can be performed by frontal analysis quite in a similar manner to that described for zonal elution [72]. With frontal analysis methodology, an interaction between the analyte and the competitor leads to a changed breakthrough time for the analyte as the competing agent concentration is increased. A shift to higher or lower breakthrough times can be observed, depending on the type of the interaction occurring with the system under investigation. As an example, the competitive binding of sulphamethazole and salicylate for HSA has been studied by low-pressure chromatography [109]. It is worth mentioning that frontal analysis methodology needs much higher amount of the analyte to be studied with respect to the zonal elution, and the single enantiomers have to be analysed separately, when the drug is chiral.

### 2.3.3. Enantioselective binding

The application of biochromatography to study the binding of chiral drugs to HSA is of particular relevance. The peculiar advantage of using HSA-based stationary phase is that the single enantiomers of a chiral drug can be simultaneously investigated by zonal elution any time the racemate is resolved on the column. This is also possible when the drug is stereochemically unstable, i.e. the single enantiomers cannot be isolated. On this basis is possible not only to give evidence of the enantioselectivity of the binding but also to rationalize the enantiodiscrimination process by using different modifiers or displacers as binding site probes. In practice, enantioselectivity can arise from: (i) a different affinity of the two enantiomers in the binding to the same binding site; (ii) the binding of the single enantiomers to different binding sites; (iii) a multiple site binding which differently involves the two enantiomers.

The simplest situation is observed when the two enantiomers bind to the same binding site where the displacer does, but their affinity is different for that specific binding site. In this case, a displacement of both the enantiomers will be observed, the greater displacement occurring for the lesser-bound enantiomer, if the displacement is simply competitive in nature. This is the case of arylpropionic acids when lithocholic acid is used as the displacer. Indeed, increasing concentrations of lithocholic acid in the mobile phase determines a significant reduction of the retention

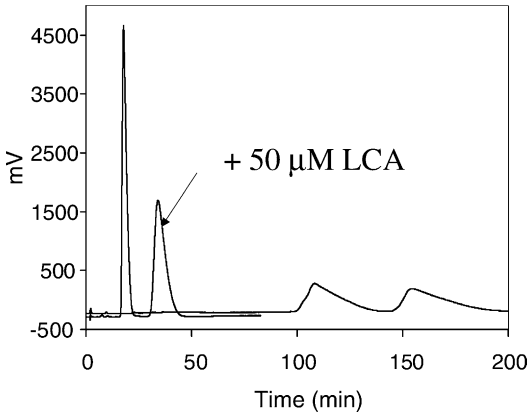


Fig. 6. Enantioselective analysis of *rac*-naproxen on a HSA-based column: chromatographic profiles in the absence and the presence of 50 μM lithocholic acid (from [110], by permission of the Editor).

and an increase of the enantioselectivity [110]. As an example, the chromatographic profiles of *rac*-naproxen in the absence and the presence of 50 μM lithocholic acid (Fig. 6) are shown. The capacity factors of both the enantiomers decreased to about 80%, the less retained enantiomer being more affected by the modifier, while enantioselectivity increased significantly (40%).

A different behaviour is obtained if the two enantiomers bind to different binding sites. A very clear example of this type of enantiodiscrimination is that reported for benzodiazepines. In the case of oxazepam hemisuccinate (OXH), the more retained enantiomer, (*S*)-OXH, is displaced either by (*S*)- or (*R*)-ibuprofen, as expected assuming a direct competition phenomenon [82]. On the contrary, the retention of the less retained enantiomer, (*R*)-OXH does not change significantly in the presence of concentrations of the interacting drug, either (*R*)- or (*S*)-ibuprofen, up to 25 μM.

The different behaviour of the two enantiomers of oxazepam hemisuccinate in the presence of the selective modifier in the mobile phase, clearly demonstrates that the (*S*)-OXH and both the displacers [(*R*)- or (*S*)-ibuprofen] bind to a specific site on the protein. On the contrary, (*R*)-OXH does not bind at the same site as its antipode. Thus, the two enantiomers of oxazepam hemisuccinate show an independent binding on the protein, i.e. they bind at different sites, and determines the observed enantioselectivity.

When two enantiomers bind to different binding sites on HSA, also specific allosteric effects can display enantioselectivity. This is the case of the cooperative allosteric interaction selectively determined by the interaction of (*S*)-lorazepam hemisuccinate and the (*S*)-enantiomer of warfarin. HSA is known to display enantioselectivity for warfarin, which bind at Site I, and for benzodiazepine derivatives, which binds at Site II, and this is reflected by the capability of HSA-stationary phases to resolve the lorazepam derivatives [111]. The presence of (*S*)-warfarin in the mobile phase determines a huge increase of the chiral resolution, due mainly to a huge increase of the retention time, and then of the affinity of (*S*)-enantiomer of lorazepam hemisuccinate. The phenomenon appeared to be related only to one of the warfarin enantiomers, as the addition of (*R*)-enantiomer did not change the retention parameters of both lorazepam hemisuccinate enantiomers.

Multiple site binding represents the more common situation in drug binding to HSA and biochromatography technique has been employed for characterising many pharmacologically relevant interactions. As an example, the dose requirements of *rac*-warfarin, widely used for oral anti-coagulant therapy, is critical, and adverse effects have been reported. In particular, this was true in the case of co-administering drugs competing for binding Site I, which is known to be the primary warfarin high affinity binding site on HSA. Using biochromatography, it was determined that (*S*)-warfarin has a greater binding affinity than the (*R*)-isomer and that only the (*S*)-enantiomer produces a cooperative allosteric interaction with (*S*)-lorazepam hemisuccinate. In addition, thermodynamic studies have suggested differential binding of (*R*)- and (*S*)-warfarin, even though displacement chromatography data obtained using the complementary enantiomer as the competitor indicated a common binding site for the two enantiomers [97]. However, different mechanisms can be distinguished for the two enantiomers of warfarin, if the non-saturable interactions of the ligand with the stationary phase are taken into account in the displacement experiments [83]. In particular, (*R*)-warfarin was completely displaced by salicylate at a [salicylate]/[(*R*)-warfarin] molar ratio >15, while only a (*S*)-warfarin reduction of retention time was observed with the same [salicylate]/[(*S*)-warfarin] molar ratio.

The results suggest that high concentration of salicylate might potentiate the anti-coagulant activity of warfarin mainly through an increase of the free (*R*)-warfarin levels which, however, is the less potent anti-coagulant enantiomer. Other NSAIDs, such as bucolome [112], have been shown, by using ultrafiltration methods, that can potentiate the anti-coagulant activity of warfarin more than salicylic acid, owing to larger increasing effect on free (*S*)-warfarin level.

Enantioselective binding of chiral drugs to HSA can be investigated also by frontal analysis, but obviously the single enantiomers are needed because they are analysed independently [72,95,97]. This surely constitutes a limitation in the applicability of the methodology any time the single enantiomers are not available in discrete quantities or the stereochemical stability is low in the experimental conditions adopted.

### *2.3.4. Covalent drug binding to HSA*

Most drugs undergo some degree of reversible binding to HSA, a process that may often have significant effects on the overall activity profile of the compounds. A site-oriented approach to drug binding may successfully describe the majority of the experimental findings [6]. However, changes in the protein structure can occur upon binding and this pro-

cess can determine significant changes in the binding properties of HSA at any single binding site [6]. In some cases, the ligands bind covalently to HSA and these non-enzymatic post-translational modification reactions have been demonstrated to occur in physiological conditions. The interest in the non-enzymatic post-translational modification of HSA resides also in the immunology consequences of the covalent binding as demonstrated in hypersensitivity to penicillin [103]. Changes in the binding properties of the modified protein can be studied in vitro by biochromatography, comparing to the retentions of drugs upon the HSA column before and after the modification. Examples are reported concerning the covalent derivatisation of single amino acids of the protein with aspirin, at $Lys_{195}$ [113], and with ethacrynic acid, at $Cys_{34}$ [114].

Changes in the binding properties could arise also from changes in the conformation of the protein because of the presence of the competitor in the mobile phase at high concentration [6].

## 3. Electromigration methods for the estimation of binding constants

In the last decade, capillary electrophoresis (CE) has gained wide approval as separation technique providing useful alternative to the well established chromatographic approaches. Also, in the field of interactions drugs–proteins, CE offers valid contributions especially concerning the estimation of association constants and on the nature and location of the binding site(s). Most of the developed CE methods in this area, are directly derived from the implementation of the existing HPLC techniques, however, CE can also put itself as a specific answer to the increasing demand of new methodologies in the investigation of the drug–macromolecule interactions. Actually, in CE, the interacting species can be introduced freely in solution; regarding the study of bio-polymers, this approach can preserve the structure of the biomolecules from denaturation and conformational change. On the other hand, adsorption of proteins to the inner capillary surface can occur and the minimization of this detrimental effect has to be considered (Tables 2 and 3) [71].

Different CE-based techniques can be succesfully applied to determine apparent equilibrium constants (association constants, binding constants and dissociation constants) as summarized in recent papers [115–118]. Mainly applied are: the frontal analysis (FA) method, Hummel–Dreyer (HD) method, the affinity capillary electrophoresis (ACE) method, the vacancy peak (VP) method and the vacancy affinity capillary electrophoresis (VACE) method; the latter considered as specifically performed by CE approach. The more recent literature offers poor applications of HD method [119]; VP and VACE methods have been discussed and compared with other CE methodologies [115–118]. Specifically, VACE, which is a relatively new methods, has been applied to study the binding between the glycopeptide vancomycin and the dipeptide *N*-Ac-D-alanyl-D-alanine [120]. Further, Erim and Kraak applied VACE technique in the evaluation of the displacement of model drug warfarin from human serum albumin (HSA) and bovine serum albumin (BSA) by furosemide and phenylbutazone [121]. Very recently, Tanaka and Terabe reviewed the CE methodologies suitable to the binding constant estimation by introducing some updated techniques [122]. It is proved that capillary electrophoresis frontal analysis (CE-FA) and ACE are undoubtedly the most widely applied approaches. Herein is reported a focus on the recent publications dealing with CE-FA and ACE methods for the study of interactions of drugs to macromolecules.

Table 2
Recent applications of CE-FA for the estimation of binding constants

| Bio-polymer | Ligand | References |
|---|---|---|
| HSA | Donepezil | [125] |
| | β-Blockers | [126] |
| | Miscellaneous of drugs | [132,133] |
| | Warfarin | [133,134,136] |
| | Flurbiprofen | [133,134] |
| | Verapamil | [136] |
| | Propranolol | [136] |
| | Sulfathiazole | [137] |
| | Phenylbutazone | [133,137] |
| | Phenytoin | [137] |
| | Diclofenac | [137] |
| | Ketoprofen | [137] |
| | Bilirubin | [137] |
| AGP | β-Blockers | [126] |
| | Miscellaneous of basic drugs | [132] |
| | Disopyramide | [135] |
| | Verapamil | [135] |
| Lipoproteins (LDL, HDL) | β-Blockers | [126] |
| | Nilvadipine | [127,128] |
| | Verapamil | [128] |
| | Propranolol | [129] |
| | Oxybutin | [130] |
| Transthyretin | Miscellaneous of neutral, basic and acid drugs | [161] |
| Phospholipids | Verapamil | [131] |
| | Propranolol | [131] |

### 3.1. Frontal analysis

Capillary electrophoresis frontal analysis (CE-FA) can be successfully applied to the estimation of binding constants of ligands to receptor by a simple and automated way; actually, similarly to the well established high-performance frontal analysis (HPFA) coupled with HPLC, CE-FA has been applied to the estimation of binding constants of drugs to different plasma proteins by CE [123]. As a general consideration, given a quickly established equlibrium between drug [D] and protein [P] in a reversible binding, CE-FA al-

Table 3
Recent applications of ACE for the estimation of binding constants

| Receptor | Ligand | References |
|---|---|---|
| HSA | Ketoprofen | [155] |
| | Quinidine | [155] |
| | Naproxen | [155] |
| | Imipramine | [155] |
| | Clofibrate | [155] |
| | Ibuprofen | [154,155] |
| | Warfarin | [154] |
| | Suprofen | [154] |
| | Flurbiprofen | [154] |
| | Porphyrin | [157–159] |
| BSA | Enediynes | [156] |
| AGP | Disopyramide | [160] |
| | Remoxipride | [160] |
| Transthyretin | Flurbiprofen | [153] |
| | Miscellaneous of neutral, basic and acid drugs | [161] |
| Cyclophilins | Bradykinin | [166] |
| Vancomycin | Peptides and derivatized peptides | [150–152,165] |
| Bovine carbonic anhydrase | Arylsulfonamides | [150–152] |
| Apoprotein | Kedarcidine (enediyne) | [164] |
| Amyloid P component (SAP) | Glycosaminoglycans | [162] |
| Antithrombin | Heparin | [163] |
| Anionic polydispersed polymer (PLGA-BP) | Angiopeptin, triptorelin and other peptides | [169] |
| DNA binding protein (from *E. coli*) | Single-stranded DNA | [167] |

lows the evaluation of the free drug [$D_f$]. From the determined [$D_f$] and the known total drug concentration in the sample, the bound drug concentration can be derived.

In the simplest condition of binding stoichiometry 1:1 between drug and protein the related equlibrium constant will be:

$$D_f + P_f \rightleftharpoons DP \quad (1)$$

and

$$K = \frac{[DP]}{[D_f][P_f]} \quad (2)$$

where [$P_f$] is the concentration of free protein.

The application of Scatchard analysis to Eq. (2) gives a linear plot with a slope corresponding to ($-K$) [116,118,122,124].

$$\frac{[DP]}{[D_f]} = -K[DP] + K[P_f] \quad (3)$$

The experimental set-up is simply performed: briefly, the capillary is filled with a running buffer in which the drug–protein complex is injected as a large sample plug. If the free drug at the established equilibrium can be selectively detected, its quantitation will be achieved. Representative electropherogram of the CE-FA analysis shows a typical frontal peak (Fig. 7) with a visible plateau due to the free drug (specifically the acetylcholinesterase inhibitor donepezil [125]) and a second peak representing the complex drug–protein. By the analysis of samples constituted by a mixture of protein (at a constant amount) and drug (at different concentrations), the free drug [$D_f$] at the equlibrium in each mixture can be derived. The requirements which have to be met for the correct application of CE-FA are that the mobility of the drug is different from the mobility of the complex, moreover, protein and complex should have approximately the same mobility [115–118]. The described method enables to determine binding capacity of drugs to serum proteins which are scarce and difficult to obtain such as lipoproteins [126–130], and phospholipid liposomes [131]. However, due to the great interest in the evaluation of the drug binding versus the two more represented plasma proteins several papers have been recently published about CE-FA using HSA [126,132–134] and $\alpha_1$-glycoprotein (AGP) [126,132,135].

The binding constants of single enantiomers of a racemic ligand to the receptor can be estimated by CE-FA; actually two enantiomers possessing different association to the protein exhibit different [$D_f$] which can be determined by the frontal analysis in the presence of suitable chiral selector in the running buffer. By means of this approach Ding et al. studied the interaction between verapamil enantiomers and HSA using trimethyl-β-cylodextrin as chiral selector [136].

The use of cyclodextrins in CE-FA analysis was also introduced to increase the difference in the mobility between plasma protein and drugs; actually, the assessment of binding to plasma protein by anionic drugs can suffer for the poor mobility differences between the protein and the drug. Ishihama et al. first introduced the use of cyclodextrins for the selective tuning of the mobilities of HSA and a serie of six anionic drugs [137].

One of the major advantage in the use of CE-FA is the small consumption of the sample subjected to the analysis; a promising application for clinical use has been proposed for the determination of the free bilirubin in the jaundiced serum in the neonatal management of hyperbilirubinaemia [138,139].

### 3.2. *Affinity capillary electrophoresis (ACE)*

ACE is commonly referred as a technique, where specific or non-specific interactions between components of an electrophoretic system result in mobility variations. On this rational, widely studied are the interactions between proteins and ligands represented by small chiral molecules. By supplementing a conventional background electrolyte (BGE)

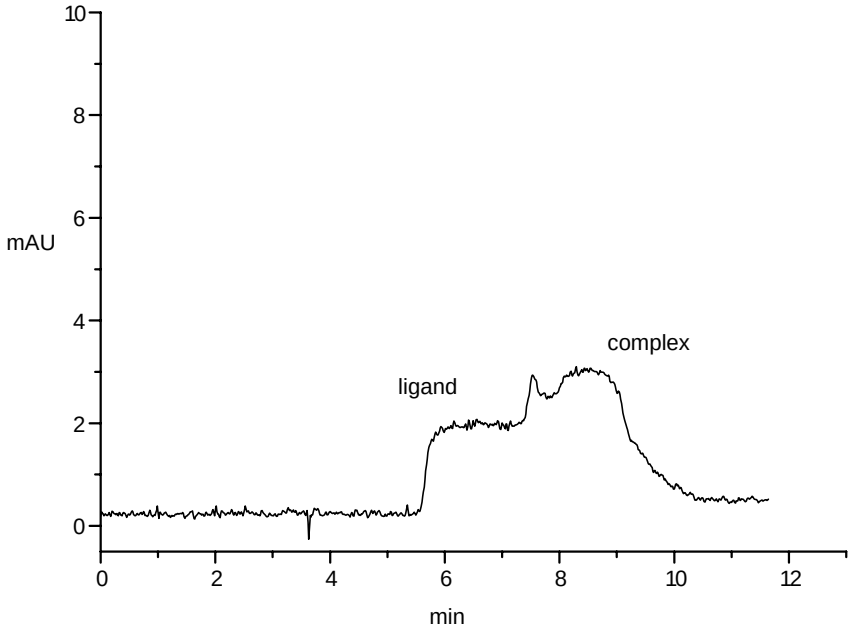


Fig. 7. Electropherograms in CE frontal mode of incubated mixture of HSA (600 μM) and donepezil (200.5 μM). Conditions: fused silica capillary (48.5 cm total length, 50 μm internal diameter); running buffer constituted of a pH 7.4 phosphate solution (50 mM); hydrodynamic injection at 50 mbar (50 s). The applied voltage was 10 kV and the temperature was constant at 25 °C. Detection UV at 320 nm.

with a protein, the resulting buffer can gain enantiorecognition ability. Due to the background UV absorption of protein solutions, the "partial filling" techniques (Valtcheva et al. [140]), can be considered one of the most useful approach to perform ACE with spectrophotometric detection. Alternatively, the immobilization of proteins within a gel structure or the use of capillary electrochromatography (CEC) have been successfully applied and reviewed [141–144].

Under the term ACE, are also included the approaches to investigate binding interactions using ligands (and receptors) that may be free in solution. Without immobilization, the concentration of the interacting molecules in the running buffer can be favourably controlled; the reliability of this approach makes ACE one of the most applied methods to the estimation of non-covalent binding and association drug–macromolecule.

In particular, ACE methods are based on the mobilty shift assay [71,115–118,122,144–148]; the drug (protein) is dissolved in the buffer and a small amount of protein (drug) is injected. The change in mobility of the injected compound (i.e. the macromolecule or receptor, R) is related to the concentration of the ligand L (drug) and a $\Delta\mu_{RL}$ can be evaluated:

$$\Delta\mu_{RL} = \mu_{RL} - \mu_R$$

where $\mu_R$ and $\mu_{RL}$ are the mobilities of the receptor in the absence and the presence of the ligand L. By variation of the concentration of L it is possible to express the magnitude of $\Delta\mu_{RL}$ as a function of the concentration [L] to obtain a Scatchard plot with the assumption of an association receptor–ligand 1:1. The linear isotherm in its *x*-reciprocal form is:

$$\frac{\Delta\mu_{RL}}{[L]} = K\,\Delta\mu_{RL}^{max} - K\,\Delta\mu_{RL}$$

where $\Delta\mu_{RL}^{max}$ is the mobilty of the receptor in the conditions of saturation by the ligand L [146–149].

A disadvantage of the described method could be related to the fluctuation of the electro-osmotic flow that strongly affects the migration parameters. However, normalization of the migration can be easily obtained by injecting samples containing a neutral non-interacting marker for the determination of mobility ratio; adequate correction of mobility parameters by considering factors accounting for the variations of viscosity of running buffer were also considered [150–152]. The importance of the relative mobilities has recently been underlined by Galbusera et al. in the study of interaction flurbiprofen–transthyretin using very low concentration of protein [153].

Among the investigated macromolecules by ACE, HSA occurs in several applications; it was reported a screening on the various binding sites on HSA by placing specific displacing agents into the running buffer, to examine the location of solute binding sites on this protein [154]. A specific study about subdomain IIIA of HSA has been proposed by Sowell et al. using near-infrared dye-displacement capillary electrophoresis to determine the association constants of a series of drugs (ketoprofen, ibuprofen, quinidine, naproxen, imipramine and clofibrate) known to bind to this specific site [155]. A recent application to bovine serum albumin (BSA) has been provided about the determination of binding con-

stants of novel enediyne compounds with antitumoral activity [156].

Hematoporphyrin derivatives, used as photosensitizing drug in photodynamic therapy of cancer were studied as a potential ligand to HSA by Ding et al. based on 1:1 molecular association [157,158]. Furthermore, the opportunity to have multiple sites of interaction between HSA and porphyrins was also evaluated by Zhang et al. which proposed equations to describe a two sites interaction [159]. Among plasma proteins, human AGP was studied by ACE using a partial filling technique to determine the binding constants of the enantiomers of disopyramide and remoxipiride [160]. A variety of other important proteins and peptides were studied by ACE methods: association constants of transthyretin (TTR) with natural ligand (thyroxine) and pharmaceutical compounds such as flufenamic acid, were estimated by ACE and compared with the results obtained using CE-FA analysis and parallel ultrafiltration experiments (UF) [161].

Among biologically important macromolecules, heparin and glycosaminoglycans were considered in ACE studies. Estimation of the extent of the interaction with specific proteins and peptides such as the human serum protein amyloid P component (SAP) [162] and anti-thrombin [163] was extensively studied. Furthermore, system employing glycosaminoglycans, specifically heparin towards fibronectin, was considered as a model for the validation of an ACE procedure successively applied to the study of interaction between apoprotein and the enediyne agent kedarcidine [164]. Vancomycin, a glycopeptide antibiotic, was investigated as a model receptor, knowing its ability to bind peptides with the C-terminal sequence. Tetrapeptides from libraries by combinatorial chemistry were simultaneously subjected to estimation of binding constants using ACE hyphenated with MS detection [165]. The molecular recognition between Vancomycin and small peptides was also extensively studied by the research group of Gomez and co-workers using the conventional UV detection mode [150] and partal filling technique [151]; a further implementation of ACE in the evaluation of binding constants of Vancomycin was provided by the use of multiple-step technique. In this approach, the solution of receptor Vancomycin (at fixed concentration; sample solution) was injected and electrophoresed in a sequence of steps (e.g. 8–10 steps), in which the buffer solution contained increasing concentration of ligand [152]. A confirmation of the validity of the proposed studies was obtained by the application of these techniques to examine the interaction of the enzyme carbonic anhydrase B and arylsulfonamides [150–152].

Among the enzymes studied by ACE, cyclophilins, known as members of peptidyl-prolyl *cis*/*trans*-isomerases, was chosen as a receptor; the characterisation of its binding to peptide ligands (bradykinin), was performed by Kiessig and Thunecke [166]. Very recently, the suitability of ACE methods for the study of biomolecular interactions has been extended to the estimation of association constants protein–DNA [167], antibody–antigen [168] and charged polymers–peptides [169].

## 4. Conclusions

Affinity separation methods, such as HPLC and CE, result versatile tools to determine binding constants of drugs or active compounds to pharmaceutical biological targets, offering the opportunity of characterising the ligand binding site. The papers revised in this review demonstrated that immobilized bio-polymers (receptors, enzymes, serum carrier, membrane proteins) can be used for HPLC on line pharmacological studies and as rapid screen for the isolation and identification of lead drug candidates from complex biological or chemical mixtures. Besides, different CE-based techniques can be successfully applied to determine apparent equilibrium constants (association constants, binding constants, dissociation constants) using very small amount of bio-polymer and ligand. Moreover, application of affinity separation methods are of particular relevance in monitoring interactions between exogenous or endogenous compounds in binding to HSA. These interactions can determine a significant change of the free fractions of active compounds in serum, with a related significant modification of their pharmacological and/or toxicological activities.

Thus, in conclusion, the use of affinity separation methods are valid alternatives for high throughput screening of compound libraries, for the development of new drugs and for their safe use, the activity of a drug not being fully understood without understanding its binding to target proteins.

## Appendix A. Nomenclature

| | |
|---|---|
| $B_t$ | column capacity (i.e. amount of immobilized enzyme) |
| D | drug |
| $D_f$ | free drug |
| $IC_{50}$ | inhibitor concentration which reduces 50% of enzyme maximum velocity |
| $k'$ | capacity factor |
| $K_a$ | association constant |
| $K_d$ | dissociation constant |
| $K_i$ | inhibition constant |
| L | ligand |
| $m_{L\,app}$ | apparent moles of analyte that are required to saturate the column |
| $m_L$ | total binding capacity of the immobilized protein |
| P | protein |
| R | receptor |
| $t_0$ | chromatographic retention time of a non-retained solute |
| $t_{drug}$ | chromatographic retention time of the drug |

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