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Review

Survey of binding properties of fatty acid-binding proteins Chromatographic methods

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Abstract

Fatty acid-binding proteins (FABPs) are members of a super family of lipid-binding proteins, and occur intracellularly in vertebrates and invertebrates. This review briefly addresses the structural and molecular properties of the fatty acid binding proteins, together with their potential physiological role. Special attention is paid to the methods used to study the binding characteristics of FABPs. An overview of the conventional (Lipidex, the ADIFAB and ITC) and innovative separation-based techniques (chromatographic and electrophoretic methods) for the study of ligand–protein interactions is presented along with a discussion of their strengths, weak points and potential applications. The best conventional approaches with natural fatty acids have generally revealed only limited information about the interactions of fatty acid proteins. In contrast, high-performance affinity chromatography (HPAC) studies of several proteins provide full information on the binding characteristics. The review uses, as an example, the application of immobilized liver basic FABP as a probe for the study of ligand–protein binding by high-performance affinity chromatography. The FABP from chicken liver has been immobilized on aminopropyl silica and the developed stationary phase was used to examine the enantioselective properties of this protein and to study the binding of drugs to FABP. In order to clarify the retention mechanism, competitive displacement studies were also carried out by adding short chain fatty acids to the mobile phase as displacing agents and preliminary quantitative structure–retention relationship (QSRRs) correlations were developed to describe the nature of the interactions between the chemical structures of the analytes and the observed chromatographic results.The results of these studies may shed light on the proposed roles of these proteins in biological systems and may find applications in medicine and medicinal chemistry. This knowledge will yield a deeper insight into the mechanism of fatty acid binding in order to indisputably show the central role played by FABPs in cellular FA transport and utilization for a proper lipid metabolism.
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1. Introduction

The multitude of functions of fatty acids (FA), as membrane phospholipid constituents, metabolic substrates, precursors for signaling molecules and mediators for gene expression, together with their relatively low aqueous solubility, strongly implies that specific and efficient mechanisms must exist to transport and target these compounds between and within cells [1,2]. It was proposed that intracellular fatty acid binding proteins (FABPs), identified 30 years ago, played an important role in cellular FA transport and utilization [3,4]. However, the large number of structurally distinct FABPs, combined with differing binding affinities, specificity and tissue expression suggest that these proteins play more complex roles. FABP may have an indirect effect on cellular processes by modulating the concentration of unesterified FA and other lipid mediators [5].

Generally, equilibrium dialysis and ultrafiltration are the reference methods for the evaluation of protein binding [6]. However, the hydrophobic nature of FA complicates the determination of binding affinities and a wide range of different techniques have been developed to measure ligand-binding. Lipidex assay, Acrylodal labeled intestinal fatty acid binding protein assay, isothermal titration calorimetry and nuclear magnetic resonance techniques have been established to determine fatty acid binding affinities [7]. Owing to the disadvantages reported in all the existing methods, a continuous effort to find better, faster and more convenient methods for the determination of the binding properties is necessary. Liquid chromatography and capillary electrophoresis are the latest techniques for examining ligand-binding affinities [8].

Biological processes of absorption, distribution, excretion and receptor activation are dynamic in nature as are the solute's distribution processes in chromatography. The same basic intermolecular interactions determine the behavior of chemical compounds in both biological and chromatographic environments.

This review will examine the characterization and the binding properties of FABPs as well as their physiological role. A survey of the various approaches that can be used for ligand–FABP binding studies will be presented, with emphasis being placed on high-performance liquid chromatographic methods. Typical results of the chromatographic method applied to liver basic fatty acid-binding protein will be reported to illustrate how the chromatographic approach has been used to study protein interaction with ligands and drugs.

2. FABPs background

2.1. Structural characteristics

FABPs belong to a family of low molecular mass proteins (127–132 amino acids corresponding to a molecular mass of 14–15 kDa), exhibiting high affinity binding constants for small endogenous and exogenous lipophilic ligands [9]. Despite insufficient and non-conclusive evidence for the physiological role of FABPs, most of the information reported over the last several decades suggests potential FABP functions. A complete overview of the proposed roles of FABPs can be found in the literature [10,11]. It has been suggested that FABPs are involved in the uptake and metabolism of fatty acids, in the maintenance of cellular membrane fatty acid levels, in intracellular trafficking of these substrates, in the modulation of specific enzymes of lipid metabolic pathways and in the modulation of cell growth and differentiation [12,13]. A current understanding of the molecular mechanism of cellular uptake and transport of long fatty acids is reported in Fig. 1.

FABPs were isolated from many different tissues of mammalian and non-mammalian species and classified into the following different types according to their primary structure and the tissue from which they were initially found: heart (H-FABP), liver (L-FABP), intestinal (I-FABP), adipocyte (A-FABP), myelin (M-FABP) and brain (B-FABP) FABPs [5]. There is a proposed nomenclature [14] for the use of the general abbreviation, X-FABPc where X is the predominant tissue type and c indicates a cytoplasmic compartment. Some types (L-FABP, H-FABP) are present in more than one type. This suggests that these proteins have evolved separately in order to fulfill different physiological functions. FABPs

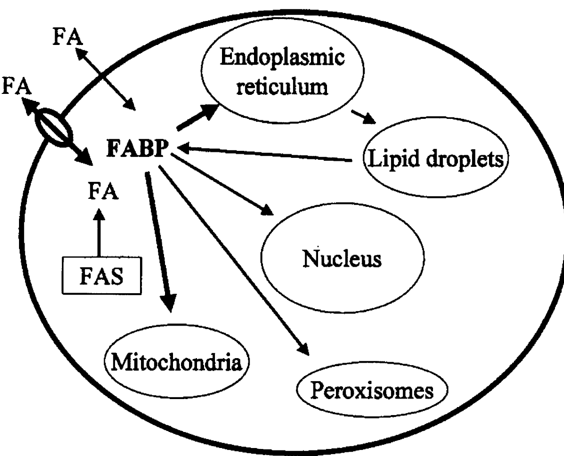


Fig. 1. Intracellular transport of long-chain fatty acids. Influx and/or efflux of fatty acids across the plasma membrane takes place by either protein-mediated or diffusive processes. Cytosolic transport to, or from, various intracellular organelles, including the mitochondria, endoplasmatic reticulum, peroxisomes, nucleus and lipid droplets, may be mediated by the FABPs. Abbreviations: FA, fatty acid; FABP, fatty acid-binding protein; FAS, fatty acid synthetase. Reprinted from Storch and Thumser [11].

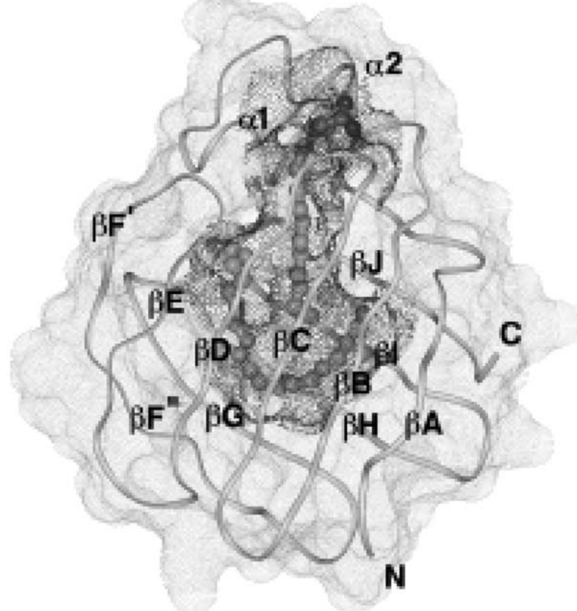


Fig. 2. The crystal structure and cavity location of L-FABP. Helices (top) are denoted by αI and αII: the 10 anti-parallel β-strands are labeled βA through βJ. The two oleic acid molecules bound to L-FABP in the crystal structure are shown by the green and red spheres. Reprinted from Thompson et al. [68].

from different tissues in a given species show a rather low degree of sequence homology (20–30%). However, as regards FABPs isolated from the same tissue type of different species, even if distant in evolution, sequence identity is considerably greater (70–80%).

Biochemical and biophysical studies have been performed on ligand binding and on the conformational and structural characteristics of FABP. However, the study of their three-dimensional structures is more sophisticated than the knowledge of the functions of FABPs. In particular, the understanding of FABP structures has shed light onto the mechanism of fatty acid binding, transport and release in enterocytes. The rate at which these events occur appear to have significant physiological consequences: deficiencies in or malfunctioning of FABPs may be involved in the aetiopathology of several diseases such as diabetes, hyperlipidemia, obesity, arteriosclerosis and cardiac hypertrophy [2].

The three-dimensional structures of several members of the FABP family have been determined by X-ray diffraction and/or NMR. The first FABP structure determined was the rat I-FABP [15]. The crystal structures of several other FABPs including human heart FABP [16], bovine heart FABP [17], bovine myelin FABP [18] and murine adipocyte FABP [19] have also been determined. In contrast, only three FABP solution structures, namely bovine heart FABP [20–22], I-LBP [23] and human intestinal FABP [24] have been determined by NMR spectroscopy. The representative structure of liver FABP is reported in Fig. 2.

A common structural motif was found in all FABPs: the tertiary structure consists of 10 antiparallel β-strands (A–J) and two short antiparallel α-helices (αI and αII), positioned over one end of the β-structure. This arrangement implies the formation of a large internal cavity, partially filled with ordered water molecules, that serves as a binding pocket for the ligand molecules. The cavity volume of individual FABPs ranges from 300 to 700 Å. Ligand movements to and from the cavity occur via a small opening. It has been postulated that this "portal region" is located between α-helix II and the two turns connecting β-strands βC and βD as well as βE and βF [25]. Hodson and Cistola [26] have suggested that the portal is dynamic in nature and exhibits conformational flexibility to allow ligand access to the cavity.

This arrangement implies the formation of a relatively large internal cavity where the FA is bound. Although most of the amino acid side chains lining the fatty acid binding cavity are hydrophobic, the cavity also contains a large number of polar side chains. In all FABPs, the carboxylate group of the fatty acid is coordinated to the protein through a network of ionic–hydrogen bonding interactions involving several polar amino acid side chains and water molecules, within the binding cavity. The large variability in amino acids present in the cavity is consistent with the wide range of amino acids identified in FABPs (20–70%). However, the interaction between the carboxylic group of the fatty acid and an arginine moiety is a common binding motif for FABPs. In rat I-FABP the arginine moiety is Arginine 106, in liver type FABP Arg 122 and in heart FABP Arg 106 and 126, the latter being more important for binding in H-FABP.

2.2. Binding characteristics

FABPs are capable of binding with high affinity long fatty acids such as palmitate, stearate, oleate, linoleate, arachidonate and linolenate. Recent studies into the equilibrium binding of FA to FABPs from different tissues have revealed that the affinities for fatty acids differ for FABP and fatty acid type with the dissociation constants ranging from about 2 to 1000 nM.

The binding affinities varied with FABP type in the following order: brain≈myelin≈heart>liver>intestine>adipocyte. Some FABP binding constants varied considerably according to their different FA typology. In general, affinities decrease with decreasing chain length and increasing double bond number. However, the ADIFAB method of determining FA-binding affinities revealed several differences between some saturated and some unsaturated FAs, but not other FABPs [27].

Differences in ligand specificity have been observed. I-FABP and H-FABP are specific binders of fatty acids, whereas L-FABP binds to more bulky, hydrophobic ligands such as lysophospholipids, bile acids, eisanoids and some drugs (Table 1). L-FABP has a unique stoichiometry among the FABPs, since it can bind a molar ratio of two fatty acids [28,29]. A review of the structural differences within the FABP family and the inherent consequences of their binding interactions with fatty acids has recently been published [7].

Table 1
Potential ligand of liver fatty acid-binding protein

Compound	Structure
Fatty acids (oleate)	
Acyl-CoAs (palmitoyl CoA)	
Eicosanoids (5-HPETE)	
Lysophospholipids (lysophosphatidyl-lethanolamine)	
Carcinogens (methylsulfone PCB)	
Anti-Coagulants (warfarin)	
Heme	

3. FABP binding assays

Advances in FABP research should integrate structural information with ligand specificity and stability in order to identify which ligand binds best to which FABP type.

Equilibrium dialysis and ultrafiltration represent two common methods that have been traditionally used to evaluate the binding of drugs or endogenous compounds to biopolymers. These methods often present drawbacks such as a difficulty in detecting low levels of unbound drug, undesirable drug adsorption onto membrane and leakage of bound drug, which may cause a considerable over-estimation of unbound drug concentrations. Due to the low hydro-solubility of FAs, these methods are not used for FABP binding studies. Different methods for determining fatty acid binding affinities have therefore been established.

In the following section an overview of the most commonly used conventional methods and innovative chromatographic techniques will be presented.

3.1. Conventional methods in the assessment of ligand–protein interactions

A wide variety of techniques and strategies have been used to probe FA interactions with FABPs. But to date, the Lipidex, the ADIFAB and ITC assays are the most commonly used techniques.

The Lipidex assay, developed by Glatz and Veerkamp [30], involves the separation of unbound FAs from those FABP-bound by using a hydroxyalkoxypropyl-derivative of Sephadex G-25 with hydrophobic properties (Lipidex 1000) at 0 °C. In this assay, delipidated FABPs are equilibrated in buffer with radiolabeled FA; Lipidex is then added to bind unbound FAs. The Lipidex/fatty acid complex is sedimented by centrifugation, and radioactivity is determined in the supernatant, representative of fatty acid bound to FABP.

The dissociation constants are determined from a Scatchard plot, which also establishes the total protein concentration and the ratio of unbound to bound FA. One of the problems presented by this assay is that the physical separation of FAs bound to Lipidex material or FABP is slow because they move from the latter to the former. Therefore, the value for fatty acids bound to FABP becomes too low. Cooling to 0 °C introduces a second uncertainty because dissociation constants change with temperature. Since this method does not operate in a real equilibrium, K_d values are generally considered too high. This assay has generally yielded affinities for long-chain FA in the mM range and does not reveal substantial differences based on FABP or ligand type. However, it is applicable to low solubility ligands [31].

The Acrylodan labeled intestinal fatty acid binding protein (ADIFAB) assay was developed in order to avoid some of the problems arising from the Lipidex methodology [32]. ADIFAB is a fluorimetric method that utilizes a rat intestinal fatty acid binding protein modified with fluorescent acrylodal at Lys^{27} in α-helix I. ADIFAB is a patented assay that measures levels of unbound free FAs in an aqueous solution in a simple, one-step procedure. It furnishes not only binding constants, but also thermodynamic parameters and binding kinetics. The essential technical set-up is a fluorescence spectrophotometer.

In the absence of unbound free FAs, the ADIFAB probe fluoresces at 432 nm upon excitation at 386 nm. In the presence of free fatty acids, the emission shifts to the green and peaks at 505 nm in a concentration dependent manner with the binding of fatty acid. The ratio of fluorescence at 505 and 432 nm is a sensitive marker for fatty acid binding.

In the ADIFAB assays, as in the Lipidex assays, the choice of ligands is mostly restricted to FAs.

The binding of a broad range of natural as well as xenobiotic ligands to FABPs, can also be examined by a third method first applied by Miller and Cistola [33], and known as isothermal titration calorimetry (ITC). ITC measures the heat of interaction between a FABP and a ligand in equilibrium. In an ITC experiment, one binding partner (usually the ligand) is titrated in small aliquots into a solution containing the other binding partner (FABP). The heat required to maintain the temperature inside the sample cell constant is measured and provides the raw data of the ITC experiment. Integration of the peaks, obtained after each ligand addition, corresponds to the heat released or adsorbed, and this represents the apparent binding enthalpy. However, the need for an expensive microtitration calorimeter and large protein quantities are required.

All these methods have been applied and dissociation constants have been derived for orthologus and paralogous FABPs [7]. The K_d values of L-FABP are reported in Table 2.

As shown, the data are not always concordant. Therefore, more simple and reliable methods are needed.

3.2. Separation-based methods for the study of ligand–protein interactions

Chromatographic and electrophoretic separation systems are able to overcome some of the problems presented by conventional methods of binding analysis. Separation-based methods provide the opportunity to study the binding interactions of all types of ligands. Several new developments or improvements in separation-based methods for the study of ligand–protein interactions have been made in recent years. Various formats for such methods, including the use of both soluble and immobilized proteins have been described.

3.2.1. Chromatographic techniques based on soluble proteins and drugs

There are several chromatographic methods that can be used to directly analyze the binding of ligands and proteins in solution [34–36]. Many methods are based on columns that contain a size-exclusion or internal surface reversed-phase (ISRP) support, which provide a means for the resolution of low to intermediate molecular-mass drugs from proteins or ligand–protein complexes. Such supports can be used in three general formats: zonal elution, which includes direct drug and protein separation techniques, peak-splitting measurements, the use of proteins as mobile phase additives, frontal analysis and the vacancy techniques, including both the Hummel–Dreyer and vacancy peak methods.

Previous reviews by Hage provide an exhaustive description of each technique supported by a discussion of the strengths, weaknesses and potential application of each method [8,37].

3.2.2. Chromatographic methods based on immobilized proteins

The use of an immobilized protein in a chromatographic system for the study of biomolecular interactions is known as "biochromatography" or high-performance affinity chromatography (HPAC), which offers an alternative experimental approach for both quantitative and qualitative determination of reversible solute-protein binding [38]. HPAC utilizes bio-polymers that have been covalently or non-covalently immobilized onto a high-performance liquid

Table 2
Comparison of binding data for orthologous L-FABP obtained by the ADIFAB, ITC and Lipidex assay (K_d values are given in μM)

Fatty acid	Rat			Mouse	Human			Bovine		
	ADIFAB	ADIFAB	LIPIDEX	ITC	ADIFAB	ITC	LIPIDEX	ITC	ITC	LIPIDEX
SA	9 ± 1		2.6	265 ± 4	23	1900 ± 20		95 ± 8		
PA	23 ± 2	7.2 ± 1			60					
OA	9 ± 2	2.6 ± 0.1	1.77 ± 0.8	21 ± 8	15	6 ± 4	0.89 ± 0.03	47 ± 13	260 ± 10	0.24
DHA	23			13 ± 1	19	3 ± 1		52 ± 35		
LA	29 ± 3	10.1 ± 0.1	1.9		57					
AA	48 ± 9	12.0 ± 0.1			100				540 ± 70	
LNA	69 ± 12	27.8 ± 0.3			240					

Data taken from the literature [7,86]. Abbreviations of fatty acids: SA, stearic acid; PA, palmitic acid; OA, oleic acid; DHA, docosahexanoic acid; LA, linoleic acid; AA, arachidonic acid; LNA, linolenic acid.

chromatography support and employs the experimental technique of HPLC. A general overview of the experimental approaches used in analytical affinity chromatography was described by Chaiken [39].

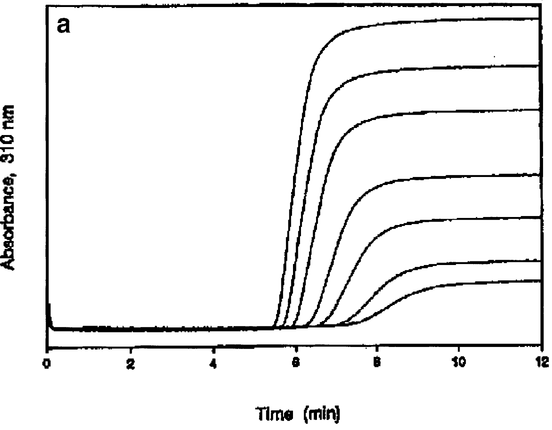
In this technique, the target protein is immobilized onto an LC support and the chromatographic retention, obtained from the resulting stationary phase, reflects the binding properties of the free protein. Once the binding of a known compound has been determined from the chromatographic retention, this compound can be included as a standard in the determination of the binding capacity of another compound under investigation. This method is very sensitive, fast, precise and ideal for automation. The extreme complexity of biological systems limits the rational design of a single chromatographic system that directly mimics a given biological system. On the other hand, chromatography is a unique method that can readily give a vast amount of diversified, precise and reproducible data. The possibility of reusing the same ligand preparation for multiple experiments is another advantage of using HPAC for binding studies. Therefore, only a relatively small amount of protein is needed for a large number of studies, thus helping to achieve good levels of precision by minimizing run-to-run variations. The relationships between biochemical processes and LC have been emphasized by the incorporation of the biomolecules as active components of chromatographic systems including serum proteins [40], receptors [41,42] and transporters [43–47] producing stationary phases where the activity of the biomolecule in its immobilized form was retained.

There are several different approaches in HPAC that can be used to examine the binding of small solutes with immobilized biopolymers; however, zonal elution and frontal analysis are the most widely used. There are a number of reviews to which the reader is directed as an introduction to this field of studies [8,37,48].

Frontal analysis can be used to measure the affinity constant between an immobilized protein and a ligand, the number of solute binding sites in the column and the type of binding (single site or multisite binding). In this technique, a solution with a known concentration of a given analyte (A) is continuously applied to a column containing a fixed amount of immobilized protein (P). As the protein becomes saturated, the amount of analyte eluting from the column gradually increases forming a characteristic breakthrough curve. Typical chromatograms of frontal analysis experiments are reported in Fig. 3a. If fast association and dissociation kinetics are present in the system, then the mean position of this curve can be related to the applied analyte concentration, protein quantity and association constants for the system. The results obtained in a frontal analysis experiments can be examined by Klotz's double-reciprocal plot:

$$1/m_{\mathrm{Papp}} = 1/K_{\mathrm{a}} m_{\mathrm{P}}[A] + 1/m_{\mathrm{P}} \quad (1)$$

For a system with a single binding site, Eq. (1) predicts that a plot of $1/m_{\mathrm{Papp}}$ (apparent moles of solute required to reach the mean position of the breakthrough curve) vs. 1/[A],



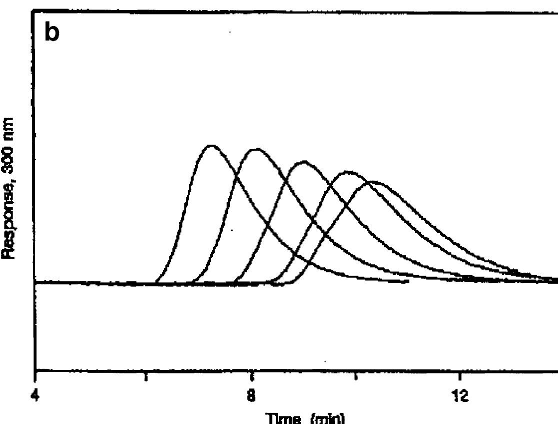


Fig. 3. (a) Chromatograms for the frontal analysis of various concentration of *R*-warfarin (increasing from right-to-left) applied to an immobilized human serum albumin column (reprinted from [34]). (b) Typical zonal elution chromatograms for the injection of *R*-warfarin onto an immobilized human serum albumin column in the presence of various concentrations (increasing from right-to-left) of a competing agent [34].

will give a straight line with a slope of $1/K_{\mathrm{a}} m_{\mathrm{P}}$ and an intercept of $1/m_{\mathrm{P}}$. The value of K_{a} (association constant for the binding of A to P) can be determined directly by calculating the ratio of the intercept to the slope from this plot.

One disadvantage of frontal analysis is the relatively large amount of solute that is required for each study. However, it also has a distinct advantage over zonal elution affinity chromatography in that it can simultaneously provide information on both the solute association constant and the total number of its binding sites in a column. This feature makes frontal analysis valuable in monitoring the stability of affinity columns during their use in long-term studies. In addition, the same feature makes frontal analysis a method of choice for accurate association constant measurements between a solute and an immobilized protein, since the resulting K_{a} values are essentially independent of the number of binding sites present in the column [8].

Zonal elution is the most common method used in HPAC to study solute–protein interactions; it easily provides an estimation of relative binding, measurements of affinity and number of binding sites, indirect determination of the

location and structure of binding sites. Zonal elution experiments with immobilized protein columns can be used to provide quantitative and qualitative information on binding and displacement processes [49]. In this technique, a known concentration of a competitor (C) is continuously applied to the column containing an immobilized protein (P) while injections of a small amount of a solute (A) are performed. Typical zonal elution chromatograms are reported in Fig. 3b

If C and A compete at a single site on P, and A does not bind to any other site on the matrix, then Eq. (2) describes the retention of A while [C] is varied.

$$1/k'_{\mathrm{A}} = K_2 V_{\mathrm{m}}[\mathrm{C}]/K_3 m_{\mathrm{P}} + V_{\mathrm{m}}/K_3 m_{\mathrm{P}} \quad (2)$$

In this equation, k'_{A} is the capacity factor for a solute A, V_{m} is the void volume of the column, [C] is the concentration of the competitor applied to the column, K_2 is the affinity constant of C for the protein and K_3 is the affinity constant of A for P.

For a system with single-site competition, this equation predicts that a plot of $1/k'_{\mathrm{A}}$ vs. [C] will give a linear relationship. By calculating the ratio of the slope to the intercept for such a plot, the value of K_2 can be directly obtained. This equation allows the measurement of the association constant for C at the site where C and A compete.

Zonal elution affinity chromatography has been commonly applied to ligand–protein studies for the qualitative examination of the displacement of ligands from proteins by other solutes.

The same experimental set-up can be used to highlight other ligand–ligand competition at a single site. In co-operative mechanisms, the binding of one compound added to the eluent induces a reversible change in the structural conformation of the protein, which enables a second compound (an injected test solute) to bind at a different site. The increased affinity of the injected test solute will be reflected by an increase in the retention factor.

On the contrary, when a ligand binds to a protein, the conformational change induced results in a decreased ability to bind other ligands in the anti-co-operative binding. Finally, independent binding can be observed when the addition of a ligand to the eluent has no effect on the retention of the injected solute.

3.2.3. Electrophoretic methods based on soluble proteins

Like chromatography, electrophoresis can be used as a tool to study solute–protein interactions. Many of the approaches already discussed in chromatography have been applied in electrophoresis (i.e. zonal elution, frontal analysis or vacancy techniques). Affinity capillary electrophoresis (ACE) is a relatively new technique which has recently been developed and documented [50–53].

3.2.4. Electrophoretic methods based on immobilized proteins

There are many previous studies into biomolecular interactions based on ligands immobilized on traditional gel electrophoresis supports. The overall approach is similar to that used for immobilized proteins in LC, which now uses an electric field instead of a pressure gradient to elute the applied sample. Gel-based electrophoretic systems with immobilized ligands (lecitins, antibodies, sugars, enzyme inhibitors and co-factors) have been used for many years. Nevertheless, more needs to be done on the use of immobilized ligands in CE for drug–protein studies. Furthermore, the work that has been carried out focussed mainly on the use of CE for chiral separation rather than on the direct assessment of drug–protein binding [54–56].

4. Chemometrics in the assessment of ligand-interactions

A combination of affinity chromatography and chemometrics was demonstrated to provide relevant information on the binding sites and binding characteristics of macromolecules. In particular, zonal elution data can be used to derive quantitative structure/retention relationships (QSRRs) for the binding of ligands to immobilized proteins [57–60]. Chromatography on an immobilized protein produces a substantial amount of precise and reliable binding-related data, which are necessary for chemometric analysis. The chromatographic retention mechanism can be identified with the help of multivariate regression equations, which relate the molecular structure of analytes to their retention parameters determined by biomolecule-containing LC systems. The derived QSRRs are interpreted in terms of the structural requirements of a specific binding site on the biomacromolecule, and provide an insight into the mechanism of molecular recognition.

Since all proteins are inherently chiral, protein-based LC stationary phases have been used as chromatographic stationary phases for the separation of enantiomers (chiral stationary phases, CSPs) [61–66] and as probes to study the properties (topography) of binding site(s) of the immobilized protein in relation to chiral solute. A large amount of chromatographic data has been used in quantitative structure-enantioselective retention relationship (QSERR) studies geared to explain and to predict enantioseparations on these stationary phases. A blend of these studies has demonstrated that the stereochemical resolution achieved on these columns reflects the binding properties and stereoselectivity of the free protein. In particular, the QSERR approach has been extensively used to investigate the stereoselective binding mechanisms and to obtain in-depth knowledge of the characterization of the binding site of two important physiological proteins: human serum albumin and a_1-acid glycoprotein.

The goals of QSERR studies are the possibility to predict the retention and enantioselectivity of a non-analyzed solute, and to elucidate the chiral mechanism operating in a given chromatographic system. If statistically significant QSERRs are found, and they account for differences in the

retention of individual enantiomers, then specific information on molecular mechanisms of separation, operating on a given chiral stationary phase, can be extracted.

If different structural descriptors have to be employed to describe the retention coefficients of the first and second eluting enantiomer, then two separate but highly enantiospecific binding sites are postulated. However, even though accompanied by different regression coefficients, the QSERR with the same set of structural descriptors for individual enantiomer series indicates a single binding site. Different magnitudes of regression coefficients for the first and second eluting enantiomers reflect differences in isomeric fitting to a common binding site.

5. Application of high-performance affinity chromatography to Lb-FABP

It has been shown that the binding constants and displacement phenomena of immobilized proteins closely match those observed for the proteins in solution. HPAC has therefore been used to investigate the binding characteristics of a subfamily of liver FABP, i.e. liver basic fatty acid-binding protein (Lb-FABP).

5.1. Lb-FABP background

Liver fatty acid binding protein belongs to the intracellular fatty acid-binding protein family. Like other family members, its functions are thought to include lipid uptake and transport, the regulation of lipid metabolism and cellular protection by keeping the concentration of free cytosolic fatty acids below toxic levels [67]. Details on the physiological significance of LFABP can be found in the review by Glatz and van der Vusse [14].

Livers of fish, amphibians, reptiles, birds, and mammals differ in the nature and number of their FABPs. Mammalian L-FABP have been thoroughly characterized [68]. The primary structure of four LFABPs (rat, human, cow and pig) has been reported, and they have an amino acid profile that is between 79 and 90% identical [69].

In terms of size and lipid-type, L-FABP ligand specificity is broad. The binding stoichiometry is unique to the family, 1 mol of L-FABP can bind 2 mol of long fatty acids. For larger ligands, the ratio is 1:1. The results of X-ray crystallographic studies on L-FABP and oleic acid indicate that the L-FABP ligand binding cavity is significantly larger compared to that of other family members whose structures are known. Because of the magnitude of the cavity, two oleic acids or a large ligand can be accommodated. One of the bound fatty acids is completely internalized in a bent conformation and its carboxylic group interacts with an arginine and two serines through an extensive hydrogen-bonding network. The orientation of the second ligand molecule adopts a rather linear shape so that the hydrocarbon chains of the two fatty acids interact. The carboxylic end sticks out of the fatty acid portal on the surface of L-FABP and is exposed to the solvent. Titration calorimetry has demonstrated that most of L-FABP's affinity for fatty acids is derived from an entropic contribution, thus suggesting that hydrophobic properties play an important role in ligand binding to L-FABP. The interaction between the internalized arginine(s) and carboxylic end of the ligand probably plays a major role in the enthalpic contribution to the binding energy. Arg^{122} of L-FABP is homologous with one of these arginine moieties in several other fatty acid-binding proteins.

The L-FABPs described are not the only FABPs characteristic of this organ. Some years ago, a new type of liver FABP, with an unusually high isoelectric point was discovered, purified and crystallized from chicken liver. This liver basic FABP (Lb-FABP) type was also found in the liver of other vertebrates such as toad [70,71], iguana [72], fish [73–75] and frogs [76]. This protein has not been detected in the liver of mammals. Therefore, Lb-FABPs have become a new subfamily of the liver FABPs, which are different from the better known mammalian liver FABP. One major difference is the number of fatty acid binding sites present in the two subfamilies; two sites in L-FABP and only one in Lb-FABP.

Lb-FABPs have a rather low level of sequence homology with mammalian L-FABP, even though sequence similarity within this group is very high (70%).

Chicken liver fatty acid binding protein is the only well characterized protein of this group. The protein contains 125 amino acid residues that correspond to a molecular mass of 14 094 [77] and presents an isoelectric point of 9.0 [78]. The primary structure of Lb-FABP has confirmed the expected sequence similarity to other members of this group.

Its structural features have been established by X-ray crystallography to 2.7 Å [79], CD [80], fluorescence, NMR [81] and ^{13}C NMR [82]. The overall architecture of chicken Lb-FABP appears very similar to that of rat intestinal FABP. Moreover, this protein seems to have a binding site capable of accommodating only one FA molecule, which occupies a higher position within the cavity compared to the primary binding site in rat L-FABP. As stated previously, an arginine moiety is always present in the binding site. However, chicken liver basic FABP has no arginine moiety in position 106. Electrostatic interactions may still occur between the carboxylic group of the FA and Arg^{120}, which occupies a position similar to that of Arg^{126} in human muscle protein. The ^{13}C NMR studies support the idea that FA binding takes place in the anionic form and indicates that the polar head of the fatty acid is near the portal and solvent-accessible.

5.2. Biochromatographic and chemometric studies on the Lb-FABP stationary phase

Lb-FABP was discovered, purified and characterized by Monaco and co-workers [78]. A collaborative work was undertaken in order to investigate the binding properties of

Lb-FAB by the synthesis of an Lb-FABP stationary phase. There are other reasons to explain the interest in the development of a Lb-FABP-based liquid chromatographic support: Lb-FABP is a basic protein and up until then the proteins used as chiral selectors were mostly acidic (α_1-acid glycoprotein, ovomucoid, human serum albumin), it presents a specific binding region, it is structurally characterized and it has possible physiological roles, which have not been fully examined to date.

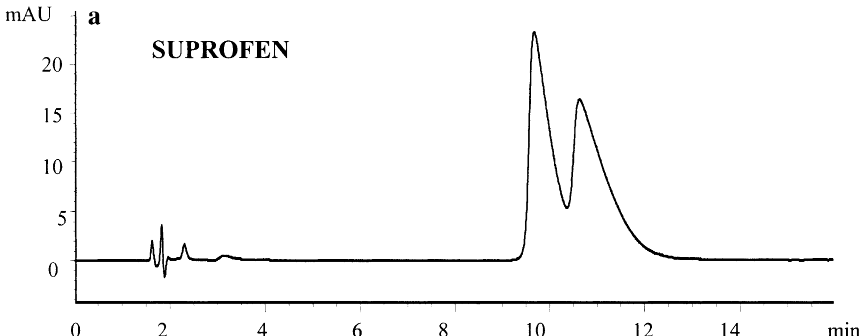
The preparation of a stationary phase based on immobilized Lb-FABP was carried out in order to use a chromatographic approach in the examination of enantioselective properties of this protein and in the study of the binding of ligands to Lb-FABP [83].

5.2.1. Preparation of Lb-FABP column

Lb-FABP was purified from chicken liver by slight modifications of a previously described method. Immobilization was performed on Nucleosil-5 NH_2-silica previously activated with *N*,*N*-disuccinymidyl carbonate. The obtained Lb-FABP stationary phase was then packed in a stainless steel column (100×4.6 mm I.D.). Two different Lb-FABP stationary phases were prepared with different purification and immobilization processes. The amount of immobilized protein was different in the two columns, 51.7 mg/g silica for column A and 21.77 mg/g silica for column B as calculated by elemental analysis.

5.2.2. Column applicability as a chiral stationary phase

CD spectroscopy studies have indicated that the positioning of the FA in a specific chiral cavity is responsible for the optical activity observed [80]. The potential chiral discriminating properties of the newly-developed stationary phases were therefore assessed by evaluating the retention and enantioselectivity of a large number of racemic drugs. Enantiomers of basic and neutral compounds were poorly retained and not resolved. On the contrary, we obtained the resolution of the enantiomers of some aryl- and aryloxypropionic acids (suprofen, flurbiprofen, 2-(3-ethylphenoxy) propionic acid, 2-(3-phenylphenoxy) propionic acid, 2-(3-phenoxyphenoxy) propionic acid and 2-(2,6-dimethylphenoxy) propionic acid) [83]. This finding was not unexpected considering that the protein presents a specific binding site for acidic compounds such as natural fatty acids. Fig. 4 shows examples of chromatograms with the chiral separation of some 2-aryl-propionic acids and 2-aryloxy-propionic acids. The enantioselective performance of the two columns was compared. Resolution was higher in the stationary phase with the higher amount of bound Lb-FABP. It was interesting to observe that there



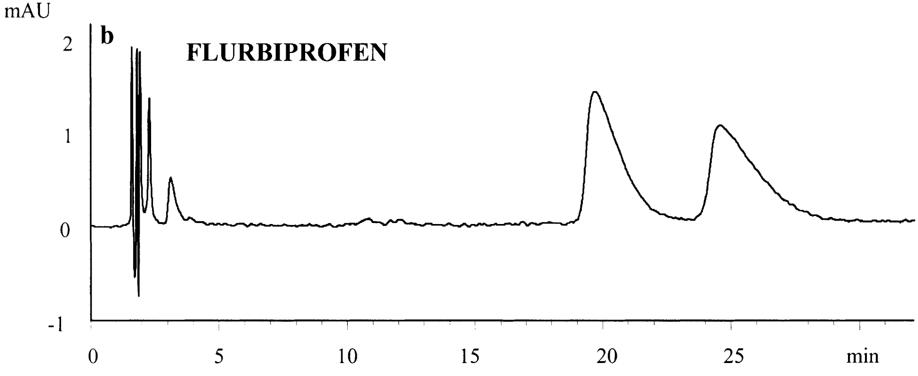
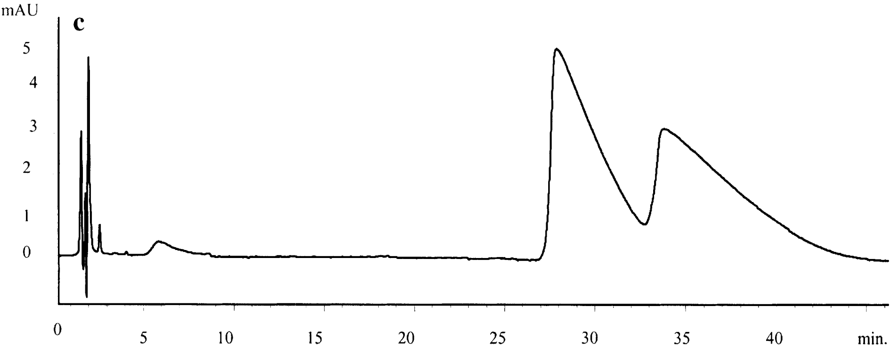


Fig. 4. Resolution of suprofen, flurbiprofen, 2-(4-phenylphenoxy)-propionic acid and 2-(4-phenoxyphenoxy)-propionic acid on the bL-FABP stationary phase. Experimental conditions are given in Massolini et al. [83]. Original chromatograms provided by the authors.



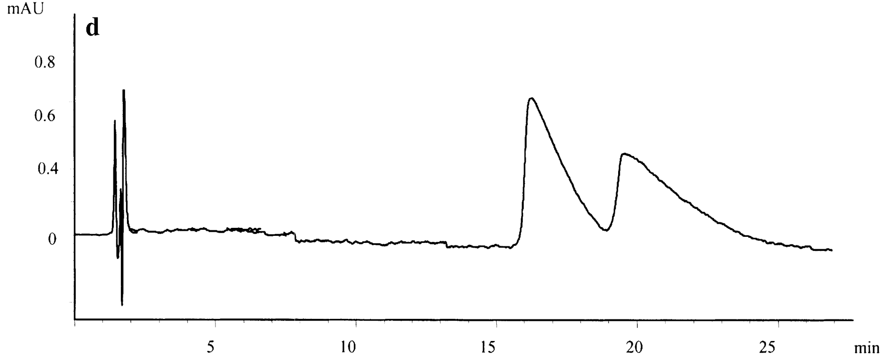


Fig. 4. (*Continued*).

was an enhanced retention of some tested compounds in the stationary phase with low quantities of immobilized Lb-FABP. This finding was ascribed to improved aspecific interactions of the acidic analyte in column B with unreacted aminopropyl groups since no blocking of these groups was carried out.

Good stability was demonstrated for the developed stationary phases. The column was found to be stable for more than 250 analyses before observing a significant reduction in enantioselectivity.

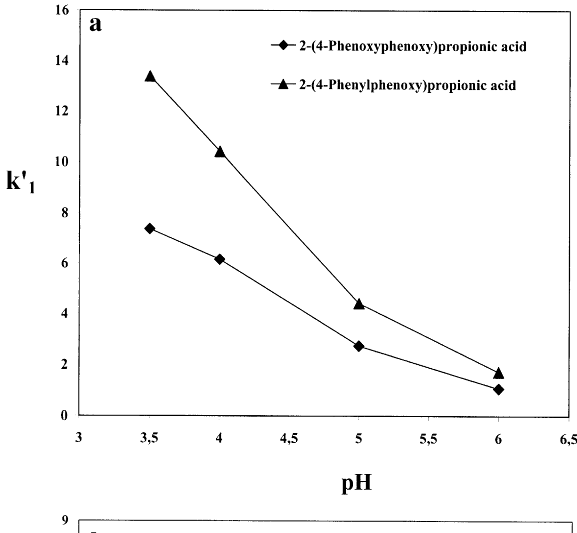
5.2.3. Influence of pH and organic modifiers on retention and chiral resolution

Solvent pH can affect the interactions between protein and ligand by influencing electrostatic interactions, or by changing the protein's conformation at its binding site. Solvent polarity is another factor that can influence interactions between proteins and ligands. These studies can be easily performed with HPAC. Both mobile phase pH and the presence of an organic modifier appear to influence retention and enantioselectivity on Lb-FABP columns.

The influence of mobile phase pH was tested in the 3.5–6.0 range. Two sets of racemates were considered for this study: a group of 2-aryloxy-propionic acids with a pK_a around 3 and a group of 2-aryl-propionic acids whose pK_a is between 4 and 5. For all the analytes considered, maximum retention was observed at pH values close to the pK_a, giving rise to characteristic bell-shaped curves for the 2-aryl-propionic acids. Differently, the retention factors of 2-aryloxy-propionic acids decreased with an increasing pH and in accordance with the lower pK_a values.

As far as enantioselectivity is concerned; the best resolutions were obtained at pH values where the interactions between the acids and the protein appear to be stronger. The graphs in Fig. 5 illustrates an example of the effects of pH on retention. These results are concordant with the data reported in literature on the influence of the pH on optimal binding affinity.

Adjusting the solvent polarity by adding a small amount of organic modifier (in order not to denature the protein) can alter solute–protein binding by destroying non-polar interactions or eliciting changes in the protein structure. Different percentages of two organic modifiers, methanol and acetonitrile, have been used to modulate retention and selectivity. An increase in methanol concentration in the mobile phase results in the reduction of retention and enantioselectivity, confirming the importance of hydrophobic interactions in the enantioselective retention. The addition of methanol to the mobile phase resulted in better selectivity values than those obtained with the same percentage of acetonitrile.



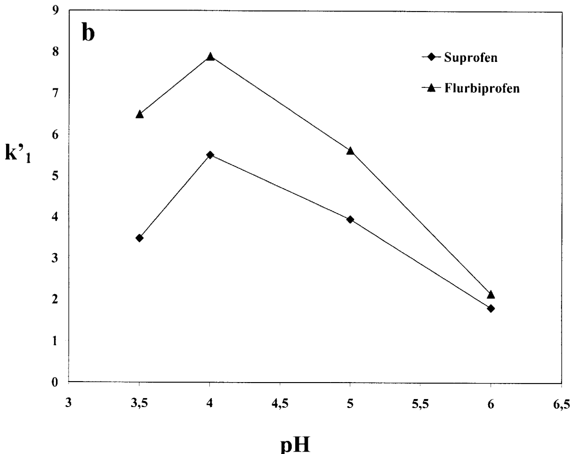


Fig. 5. (a) Effect of pH on the retention of the first eluted enantiomer of 2-(4-phenylphenoxy)-propionic acid and 2-(4-phenoxyphenoxy)-propionic acid. (b) Effect of pH on the retention of the first eluted enantiomer of suprofen and flurbiprofen. Original graphics provided by the authors [83].

5.2.4. Retention mechanism studies

In order to demonstrate whether a specific binding site is involved in the observed enantioselective retention, the aryloxy-propionic acids previously analyzed were converted into their methyl ester derivatives and chromatographed on the Lb-FABP column. While retention was significantly reduced, selectivity was lost for all the esters. The ionization status of FAs and amino acid residues is implicated in protein–ligand binding, thereby sustaining the fact that electrostatic interactions at the binding site are involved in the chiral recognition mechanism. It was interesting to observe that the esters with two aromatic rings in their structure are still retained with retention factors higher than 1.0. This suggests that hydrophobic interactions are involved in the retention of these analytes, and this is consistent with the lipophilic ligand binding nature of FABP.

The same compounds were also selected to perform preliminary quantitative structure–retention relationship (QSRR) studies using multiparameter regression analysis. QSRR correlations were used to describe the nature of the interactions between the chemical structures of the analytes and the observed chromatographic results.

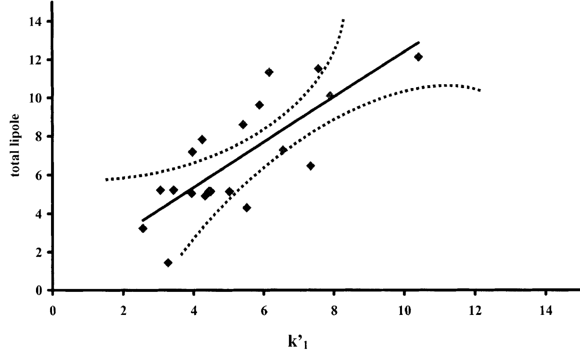


Fig. 6. Correlation between the observed capacity factors and the total lipole [83]. Original figures provided by the authors [83].

The effects of the solute structure on observed chromatographic retentions were investigated. The authors correlated the chromatographic retention factors of first and second eluted enantiomers with hydrophobicity (total lipole) and molecular volume (ellipsoidal volume) of the analytes. Statistically significant correlations were observed in both series of enantiomers. Retentions increased with increasing hydrophobicity, indicating that the retention of acidic compounds appears to correlate with the expected order of the hydrophobicity of the molecule (Fig. 6). The observations derived from the relationships between molecular volume and retention led to the conclusion that the binding site at which the two enantiomers bind is a chiral cavity with steric restrictions. No correlation was found between ellipsoidal volume and retention. However, by restricting the correlation to the most retained compounds, a significant correlation was found. Retention is decreased by increasing the ellipsoidal volume. The correlation between retention factors and volume of the less retained compounds was not significant. The structure of these compounds is small enough to allow free access to the binding site.

Based on multiple linear regression analysis, retention parameters of the first- and the second-eluting enantiomers were described by structural descriptors obtained from molecular modeling. The best relationships between the retention data of both enantiomers and structural descriptors, i.e. total lipole accounting for lipophilicity and HOMO for electrostatic interactions, were developed.

The following equations were obtained:

$$\log k'_1 = 0.13\mathrm{HOMO} + 0.025\mathrm{TL} + 2.04$$

$$n = 13,\ R = 0.91,\ F = 24.46,\ s = 0.068,$$
$$\mathrm{C.V.\ (predictiveindex)} = 0.7034$$

$$\log k'_2 = 0.14\mathrm{HOMO} + 0.032\mathrm{TL} + 2.2$$

$$n = 13,\ R = 0.92,\ F = 27.79,\ s = 0.07,\ \mathrm{C.V.} = 0.75$$

It was possible to conclude from these equations that hydrophobic interactions are predominant in the retention mechanism and this is consistent with the hydrophobic character of the protein-binding site. However, electrostatic interactions play an important role as accounted for by the positive sign of HOMO energy

5.2.5. Zonal elution studies

The developed Lb-FABP column was also used to perform zonal elution studies. This approach was applied to examine the competition between short chain fatty acids (*n*-butyric acid, *n*-hexanoic acid and *n*-octanoic acid) and acidic analytes. Long chain fatty acids were not considered in this study because of their low solubility in the mobile phase. However, more recent developments regarding the modification of HPAC methods, including the addition of solubilizing agents to the mobile phase, seem to overcome solubility problems [84,85]. The presence of a displacing agent in the mobile phase produced a concentration dependent reduction of the capacity factor and enantioselectivity and the extent of the reduction increased as the length of the chain increased. A typical zonal elution experiment is shown in Fig. 7 where the change in retention of the first-eluting enantiomer of 2-(4-phenoxyphenoxy) propionic acid (the injected solute) is being examined on an immobilized bFABP column as various amounts of *n*-butyric, *n*-hexanoic and *n*-octanoic acids are added to the mobile phase. The retention of the injected analytes shifts to lower values as the competitor concentration is increased, indicating a competitive mechanism during the binding of these solutes to Lb-FABP. The displacement effect increased as the chain length of the displacing agent increased. This is consistent with the higher affinity of long chain fatty acids for FABPs.

By elaborating the data according to Eq. (1), a linear correlation was found only with *n*-butyric acid as a displacer. A simple competitive model does not apply for hexanoic and octanoic acid.

It was also interesting to observe a concentration-dependent reduction in the enantioselectivity when carboxylic acids were added to the mobile phase, confirming the involvement of the specific ligand binding site in enantioselective recognition.

5.3. Information obtained from the Lb-FABP stationary phase

The developed Lb-FABP column was used to obtained more information about the enantioselective binding mechanism of the protein. These studies reveal that hydrophobic interactions are predominant in the retention mechanism, electrostatic interactions are also important for the stabilization of the analyte–protein complex. These conclusions were confirmed by quantitative structure–retention relationship studies. The source of the enantioseparation appears to be in the structure of the binding site on the Lb-FABP molecule. This is illustrated by the fact that the enantioselectivity was lost when acidic (FA) displacing agents were added to the mobile phase.

The mechanism proposed from our chromatographic results agrees with the data reported in the literature on the characteristics of the FABP binding site. An arginine moiety is placed in the large binding pocket where acidic solutes

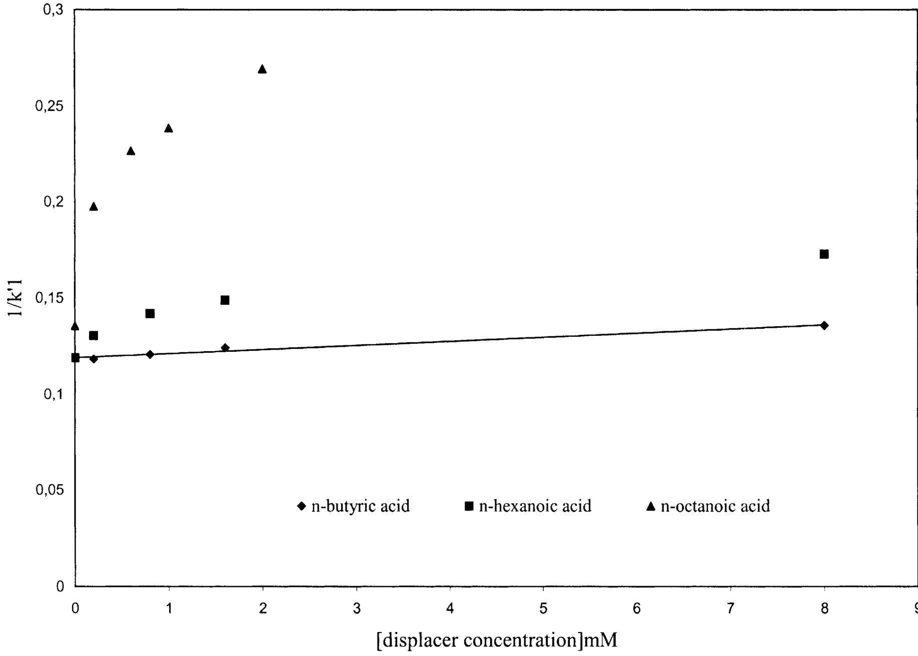


Fig. 7. Zonal elution plots of $1/k$ of the first-eluting enantiomer of 2-(4-phenoxyphenoxy)-propionic acid versus *n*-butyric acid, *n*-hexanoic acid and *n*-octanoic acid concentration, according to the relationship for competitive displacement from a single site. Original figure provided by the authors [83].

attach; the presence of a positive charge on the edge of the hydrophobic cavity (Lys or Arg[55]) is the driving force that attracts the negatively charged FAs. The ligands are drowned down deeper into the cavity by electrostatic interactions with the positively charged moieties. This cavity must be large enough so that when the directing electrostatic charge is not present (acids converted into esters or higher pH), the acids can still enter the cavity and be retained. The acids may orientate themselves in a number of random positions thereby eliminating the possibility of enantioselective discrimination. The proposed mechanism is consistent with the effects observed in the displacement studies. It can be assumed that the small *n*-butyric acid molecule can only compete with solutes for the electrostatic interaction with the arginine moieties by obtaining a clean single site competitive interaction profile. *n*-Hexanoic acid and *n*-octanoic acid with larger hydrophobic tails can compete for both the electrostatic and hydrophobic binding sites achieving a more efficient reduction in retention and enantioselectivity, albeit with a more complicated mechanistic relationship.

Van der Waals hydrophobic interactions have been observed between the hydrocarbon chain of the lipid and side chains of the protein interior.

6. Conclusions

In this review, an overview of high-performance affinity chromatography methods, and the structure and function of FABPs were presented. The possibility of studying the affinities of FABPs with a series of drugs using a fast and reliable HPAC method has been reported. The review has underlined the capacity of HPAC methods in the study of a wide array of solute–protein systems and in the evaluation of association constants, the number of protein binding sites, the role played by the various forces involved in solute–protein interactions, and the location and properties of the binding sites.

Interest in FABPs has produced more than 1000 research papers since their discovery nearly 30 years ago. Despite this large number of studies on their three-dimensional structure, FA-binding characteristics and tissue incidence of FABPs, as well as the mystery surrounding the physiological role of these proteins has not been completely revealed. The application of HPAC with immobilized FABPs as probes for drug- and ligand–protein binding can shed light onto the proposed roles of these proteins in biological systems.

The application of FABP information is not just an academic endeavor, it has promising prospectives in medicine and medicinal chemistry. FABPs can be targets for the treatment and diagnosis of medical conditions. In fact, deficiency and malfunctioning of FABPs may play a role in the pathologenesis of diseases like cancer, diabetes, obesity and atherosclerosis.

On the basis of the great potential of chromatographic techniques and the growing interest in fatty acid-binding proteins, it is expected that HPAC with immobilized FABPs will become increasingly important in biochemical and pharmaceutical research.

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