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Review

Immobilized receptor- and transporter-based liquid chromatographic phases for on-line pharmacological and biochemical studies: a mini-review

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Abstract

This review addresses the synthesis and characterization of two different types of receptor-based liquid chromatographic supports, one based upon a trans-membrane ligand gated ion channel receptor (the nicotinic acetylcholine receptor) and the other a soluble nuclear receptor (the estrogen receptor). In addition, studies with the P-glycoprotein transporter are also reported. The nicotinic receptor was immobilized via hydrophobic insertion into the interstitial spaces of an immobilized artificial membrane (IAM) stationary phase, the estrogen receptor was tethered to a hydrophilic stationary phase and the membranes containing the Pgp transporter were coated on the surface of the IAM stationary phase. The stationary phases were characterized 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's) and to resolve mixtures of ligands according to their relative affinities. In addition, competitive ligand binding studies on the P-glycoprotein-based stationary phase have established that this phase can be used to identify and characterize 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.

Keywords: Reviews; P-glycoprotein; Estrogen receptor; Nicotine receptor

Contents

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1. Introduction

The fundamental processes of drug action, absorption, distribution, excretion and receptor activation are dynamic in nature and have much in common with the basic mechanisms involved in chromatographic distribution. Indeed, the same basic intermolecular interactions {hydrophobic, electrostatic, hydrogen bonding} determine the behavior of chemical compounds in both biological and chromatographic environments [1].

The relationships between basic pharmacological processes and chromatography have been emphasized by the inclusion of a wide variety of proteins as active components of chromatographic systems, the creation of protein-based stationary phases. The extreme complexity of biological systems limits rational design of an individual chromatographic system that would directly mimic a total biological system. However, construction of the right protein-based stationary phase can readily yield a great amount of diversified, precise and reproducible data about key aspects of that system [2]. The possibilities are only limited by the ability to create and use unique phases.

The theoretical approach to the use of protein-based stationary phases as a probes of ligand-protein and protein–protein interactions is ''Quantitative Affinity Chromatography'', and this technique has been extensively reviewed, e.g. Ref. [3]. The immobilization of proteins has also been widely studied and a variety of covalent and non-covalent immobilization techniques have been developed [4]. In addition, the experimental approaches to the isolation and purification of receptors from biological matrices have been extensively studied and discussed (see for example references cited in Ref. [5]).

Recent publications have demonstrated that these techniques can be used to immobilize transporter proteins such as the D-glucose transporter [6,7] and P-glycoprotein transporter [8,9], carrier proteins such as human serum albumin [10], and receptor proteins such as nicotinic acetylcholine receptors [11,12]. All of the immobilized proteins retained their ability to bind and/or transport ligands and could be used to determine pharmacological activities. Thus, it is conceivable to envision the creation of a wide variety of immobilized-receptor phases.

This report addresses this possibility through a review of the synthesis and characterization of two different types of receptor-based liquid chromatographic supports, one based upon a trans-membrane ligand gated ion channel receptor (the nicotinic acetylcholine receptor) and the other a soluble nuclear receptor (the estrogen receptor). In addition, studies with the P-glycoprotein transporter are also reported. The results from 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.

2. General experimental approach

2.1. Chromatographic studies

Two classical chromatographic techniques are used in Quantitative Affinity Chromatography, frontal elution and zonal elution. Both methods provide information on binding affinities and equilibrium or kinetic constants. The choice between one or the other is usually based upon the availability of the target protein and/or the test ligands as well as the required analytical speed and throughput.

2.1.1. Frontal chromatography

Radio-labelled markers were used in these studies and the elution profiles monitored with an on-line flow scintillation detector. A 50-ml sample superloop was used to apply a series marker concentrations to obtain elution profiles showing a front and plateau regions. The chromatographic data was summed up in 1-min intervals and smoothed using the Microsoft Excel program with a 10-point moving average [13].

2.1.2. Zonal chromatography, LC-mass spectrometry

The immobilized receptor or transporter column was connected on-line to a PE-SCIEX API-100 mass spectrometer and a variety of marker and test compounds were migrated through the column. A variety of ligand concentrations (1–10 μM), different ionic strengths (0–10 mM ammonium acetate) and various temperatures (4–37°C) were tested.

2.2. Calculation of dissociation constants using frontal chromatography

A series of marker compound (M) concentrations and competitive ligands (CL) are added to the mobile phase and passed through the receptor and transporter columns. Using the frontal chromatograms, the association constants of M, K_M, and the test drug, K_{CL}, as well as the number of the active and available binding sites of immobilized receptors, P, were calculated using the following equations, Eqs. (1) and (2) [3,11–13]:

$$(V_{max} - V)^{-1} = (1 + [M]K_M)(V_{min}[P]K_M)^{-1} + (1 + [M]K_M)^2(V_{min}[P]K_MK_{CL})^{-1}[drug]^{-1} \quad (1)$$

$$(V - V_{min})^{-1} = (V_{min}[P]K_{EB})^{-1} + (V_{min}[P])^{-1}[M] \quad (2)$$

In the above equations, V is the retention volume of M; V_{max}, the retention volume of M at the lowest concentration and in the absence of drugs; V_{min}, the retention volume of M when the specific interaction is completely suppressed. The value of V_{min} is determined by running M in a series of concentration of drugs and plotting $1/(V_{max}-V)$ vs. $1/[CL]$ extrapolating to infinite [CL]. From the above plot and a plot of $1/(V-V_{min})$ vs. [M], dissociation constant values, K_d, for M and CL can be obtained.

3. Immobilized nicotinic acetylcholine receptor stationary phases (NR-SP) [11,12]

Studies with the NRs have demonstrated that transmembrane receptors can be solubilized and then immobilized on an IAM stationary phase. The resulting receptor columns can be used to investigate ligand–receptor interactions and the differences between NR subtypes. This technique can also be used for rapid screening of compounds from a combinatorial pool where the compounds can be readily classified according to their retention volumes or K_d values. This technique may facilitate exploration of ligand–receptor interactions, determination of ligand–ligand binding interactions, identification of differences between receptor subtypes and construction of quantitative structure–activity relationships. Key aspects of these studies are presented below.

3.1. Nicotinic acetylcholine receptors (NR): background

NRs comprise a ligand gated ion channel superfamily [14] and are found in a variety of settings and subtypes including skeletal muscle (muscular NRs) and brain (neuronal NRs) [15]. These receptors are pentameric membrane proteins consisting of α-, β-, γ-, and δ-subunits.

Multiple forms of neuronal NRs exist in the brain, sensory systems and autonomic ganglia [15]. To date, 12 gene products have been identified (α2–α10 and β2–β4), α2-6 form functional receptors with other α and β subunits and α7–10 form functional gated ion channel without any β subunits (homo-oligomers) and hetero-oligomers between themselves [15,16]. The α8 subunit, is only found in chicks; α7 is found in abundance in human hippocampus, and α9 is located throughout the brain [15].

In humans, the α4β2 receptor subtype has become of particular importance, because of its putative role in short term memory [17]. This subtype is found predominantly in the rat forebrain. Nicotine and cytisine bind with high affinity to this receptor subtype and, it has become a target for therapeutic cognition enhancement in Alzheimer's disease patients. These receptors are of particular interest because of their possible therapeutic application for the treatment of Alzheimer's disease, Parkinson's disease, Tourette's syndrome, ulcerative colitis and the treatment of nicotine addiction [16].

3.2. Preparation of the immobilized α4β2 and α3β4 NR-stationary phases (NR-SP)

Columns were prepared from solutions containing the NR α4/β2 and NR α3/β4 receptor subtypes. In these studies rat whole forebrain (α4β2) or cells from a stably transfected KXα3/β4 cell line expressing the α3/β4 NR subtype were suspended in Tris–HCl [50 m*M*, pH 7.4], homogenized for 30 s with Brinkmann Polytron, and centrifuged at 40 000 *g* for 10 min at 4°C. The pellet was resuspended in 6 ml of 2% deoxycholate (rat whole forebrain) or 2% cholate (transfected cells) in Tris–HCl [50 m*M*, pH 7.4] and stirred for 2 h. The mixture was centrifuged at 35 000 *g* for 30 min, and the supernatant containing NR-deoxycholate and NR-cholate solution, respectively, was collected and used to prepare the stationary phase.

The immobilized artificial membrane (IAM-PC) liquid chromatographic stationary phase (Regis Chemical Company, Morton Grove, IL, USA) was used as the support. The IAM support was suspended in 4 ml of detergent solutions containing NR subunits or subtypes. For the immobilization of one NR subtype, the mixture of IAM-detergent-receptor was stirred for 1 h at room temperature. The suspension was dialyzed for 24 h at 4°C. The IAM with immobilized NRs was then washed, centrifuged and the solid collected.

About 63 mg protein isolated from the membrane of transfected (α3β4) cells and 14 mg of protein prepared from rat brain (α4β2) tissues were respectively immobilized per gram of IAM support, Table 1. Receptor binding assays using the marker ligand [^{3}H]-epibatidine ([^{3}H]-EB) showed that the NR binding activities were retained after the immobilization procedure, Table 1.

3.3. Chromatographic studies

3.3.1. NR-SPs can be used to determine binding affinities of NR ligands

The α3/β4 subtype was used in the LC study of nicotinic ligand binding using frontal chromatographic techniques [^{3}H]-EB as a marker ligand and with a LC on-line radioactivity detector to monitor the elution profiles [11]. The retention volumes of [^{3}H]-EB were 23 ml at the concentration of 60 p*M* (Fig. 1 profile A). This retardation was primarily due

Table 1
The activity of immobilized nicotinic acetylcholine receptor subtypes (α4/β2 NR and α3/β4 NR) as compared to the non-immobilized receptors expressed as specific binding and receptor density per gram protein

Sample	Specific binding (%)	NR density (nmol/g protein)
α4/β2 NR-detergent solution[a]	62	0.14
α4/β2 NR-IAM[a]	49	0.81
α3/β4 NR-detergent solution[b]	100	8.57
α3/β4 NR-IAM[b]	97.8	5.09

The test ligand was [^{3}H]-epibatidine (see Ref. [11] for experimental details).

[a] Prepared from rat forebrain with detergent deoxycholate.

[b] Prepared from transfected cells with detergent cholate.

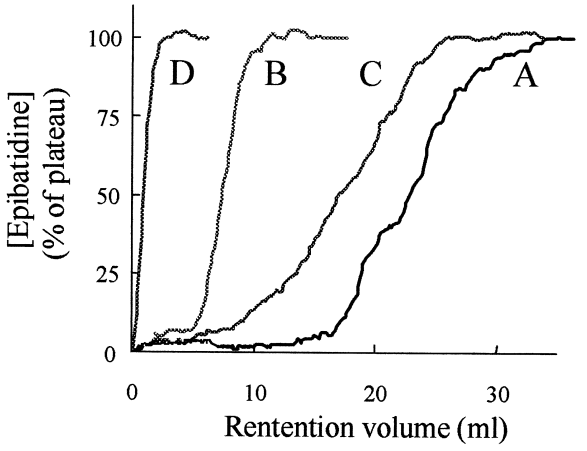

Fig. 1. Example of the elution profiles of [^{3}H]-EB on α3/β4 NR-IAM stationary phase (0.5×1.7 cm). 60 p*M* [^{3}H] EB (A), 450 p*M* [^{3}H]-EB (B), and 60 p*M* [^{3}H]-EB in the presence of 60 n*M* (−)-nicotine (C) and 1000 n*M* (−)-nicotine (D) were included, respectively, in eluent A: 50 m*M* Tris–HCl, pH 7.4. Flow rate, 0.4 ml/min (order of peaks from left to right: DBCA). {Reprinted from Ref. [11]}.

to the specific binding to saturable sites of the receptors as indicated by a decrease in retention volume to 8 ml when the concentration of [^{3}H]-EB was increased to 450 p*M* (Fig. 1, profile B). The binding of [^{3}H]-EB to the α3/β4 NR-SP could be reduced in competitive displacement experiments using known α3/β4 NR ligands in the mobile phase. For example, the retention volume of 60 p*M* [^{3}H]-EB decreased from 23 to 18 ml when a 60 n*M* concentration of the NR-ligand (−)-nicotine was added to the mobile phase (Fig. 1, profile C) and fell to 0.9 ml when the (−)-nicotine concentration was increased to 1000 n*M* (Fig. 1, profile D).

The decreases in retention volumes of [^{3}H]-EB relative to mobile phase concentrations of a displacer reflect the binding affinity of the displacer for the receptor (see Section 2.2). Using this technique, the relative affinities of nicotinic ligands for the α3/β4 NR could be readily classified by determining the concentrations required to decrease the retention volumes of [^{3}H]-EB to a predetermined level. The relative affinities of these ligands determined by this method were: (±)-epibatidine (K_d: 0.27±0.05 n*M*)> A85380 (K_d: 17.2±0.5 n*M*)>(−)-nicotine (K_d: 88±33 n*M*)>carbachol (K_d: 1280±30 n*M*)> atropine (K_d: 14 570±2600 n*M*). The rank order of these values showed a good correlation (r^2=0.9991) with the rank order of the K_d values measured by standard binding assays, Table 2. The low affinity of atropine (K_d: 17 200 n*M*) is also consistent with literature values.

Table 2
Binding affinities of nicotinic receptor ligands, expressed as dissociation constants (K_d) at the α3/β4-NR receptor

Ligand	Frontal chromatography K_d (n*M*)	Binding assays K_d (n*M*)
Epibatidine	0.27±0.05	0.38±0.07
A85380	17.2±0.5	73.6±6.3
(−)-Nicotine	88±33	475±52
Carbachol	1280±30	3839±276
Atropine	14 570±2600	

The data was obtained using frontal chromatography on an immobilized α3/β4-NR stationary phase and binding assays using membrane homogenates from a cell line expressing α3/β4-NR (see Ref. [11] for experimental details).

3.3.2. The NR-SP can be used to assess the differences between NR subtypes and subunits

Binding of [^{3}H]-EB was also measured in zonal format on the columns containing α3 subunits only, β4 subunits only, a mixture of the two cell types, or α3/β4 NRs [12]. The observed retentions of [^{3}H]-EB on α3 NR-SP (peak 1, Fig. 2A), β4 NR-SP (peak 2, Fig. 2A) and α3+β4 NR-SP (peak 3, Fig. 2A) were low, and no significant changes in the retention volumes were observed when (−)-nicotine was included in the mobile phase, Fig. 2B. [^{3}H]-EB was retained on the IAM column containing the immobil-

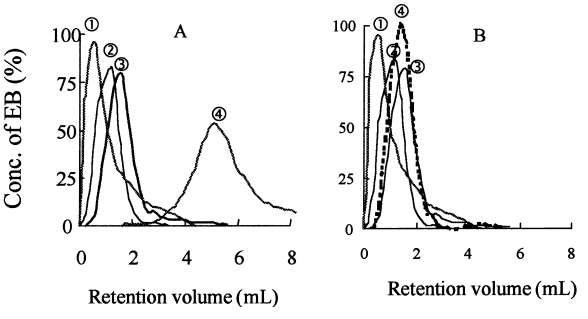

Fig. 2. Elution profiles of [^{3}H]-EB (0.5 n*M*) in zonal chromatography based on the α3 NR column (Peak 1), the β4 NR column (Peak 2), the α3+β4 NR column (Peak 3) and the α3/β4 NR column (Peak 4). Mobile Phase: Tris–HCl buffer [50 m*M*, pH 7.4] (A) No (−)-nicotine in the mobile phase. (B) 1 μ*M* (−) nicotine present in the mobile phase. {Reprinted from Ref. [12]}.

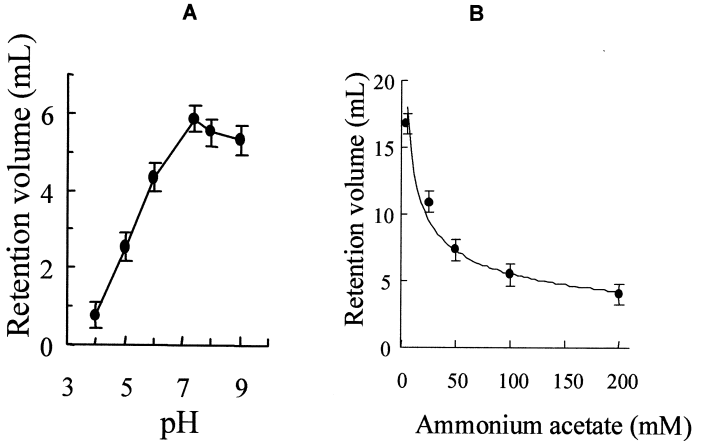

Fig. 3. The effect of mobile-phase composition on the retention volumes of [^{3}H]-EB on an α3/β4-NR column in frontal chromatography. Where: (A) The effect of pH from pH 3–7 {ammonium acetate buffers [50 m*M*]} and pH 7–9 {Tris–HCl buffers [50 m*M*]}; (B) The effect of the ionic strength. The error limits bars represent the maximum differences in retention volumes between runs ($n=2$) at a given pH and ionic strength, respectively. {Reprinted from Ref. [12]}.

ized α3/β4 NR-IAM (peak 4, Fig. 2A). The retention volume was decreased when (−)-nicotine was included in the mobile phase, peak 4 (dash line) Fig. 2B. The data demonstrates that there was no specific binding to the separate NR subuntis and that there was no self-assembly when the subunits were mixed. Specific binding occurred only with the intact NR.

3.3.2.1. The NR-SP can be used to examine the NR-ligand binding mechanism. The effect of mobile phase ionic strength and pH on the binding affinities of [^{3}H]-EB were determined with a α3/β4 NR-SP column [12]. The retention volumes increased when the pH of mobile phase was increased from pH 4.0 to pH 7.0 and remained constant between pH 7.0 to 9.5 (Fig. 3A). The retention volumes of [^{3}H]-EB were higher at low ionic strength (5-m*M* ammonium acetate) and decreased as the ionic concentration of the mobile phase increased (Fig. 3B). The data demonstrated that in the NR-SP, the ligand and receptor interacted via ionic interactions, which is consistent with previous studies [14].

4. Estrogen receptor stationary phase {ER-SP}

A stationary phase containing an immobilized estrogen receptor was developed. In these studies, the estrogen receptor was the human estrogen receptor ligand binding domain and in this presentation it will be designated as the ER. The ER was immobilized onto a novel stationary phase with a silica backbone. Columns containing this stationary phase have been used to determine the K_d of the ER, with results shown to be within an order of magnitude of the non-immobilized ER. An on-line LC–mass spectrometer screening system has been developed to determine the binding affinities of estrogen agonist/antagonists. Using this system, it has been demonstrated that the retention times of these ligands are related to their binding affinities for the ER.

4.1. Estrogen receptors: background

Estrogens, xenoestrogens, phytoestrogens, androgens and xenoandrogens, have gained increased importance in recent years due to their roles in reproductive defects, decreased quality and quantity in sperm counts, reduced fertility in general, and an increase in endocrine-related cancers (ovarian, breast, prostate and testicular cancers in humans) [18]. It has also been associated with a remarkable diversity of adverse effects, including abnormal reproductive defects in alligators, induction of the protein vitellogenin in fish and egg shell thinning [18]. Consequently, these xenobiotics are often re-

ferred to as ''endocrine disruptors'', and are synthetic or natural compounds that interfere with the endocrine system function.

The most common mechanism of action apparently results from the ability of xenobiotics to bind to and either activate or inactivate nuclear hormone receptors. These interactions often occur in competition with endogenous hormones. Generally, the receptors affected are members of the steroid hormone receptor superfamily, which includes receptors for estrogens, androgens, progestins, and retinoids.

Many of the xenobiotics identified to date have limited structural similarities. Examples are organochlorine pesticides such as DDT, aldrin, dieldrin, polychlorinated biphenyls (PCBs), methoxychlor, dioxans and furans; alkyl phenol polyethoxylate (APEs), phthalates and synthetic chemicals such as diethylstilbesterol. Detailed structure–activity relationship (SAR) studies have not been carried out for each class of compounds. It is difficult to a priori predict estrogenic and/or androgenic activity based solely on chemical structure [19].

Since, the adverse effects of the endocrine disruptors reflects the receptor agonist or antagonist activities of these compounds, any high throughput screening (HTPS) approach must be able to identify these pharmacological properties. ''Functional'' screens, such as promoter-reporter based assays, can be difficult or time consuming [20]. A more effective approach is to identify compounds that can bind the appropriate receptors using a HTPS and then to determine the functional nature of the isolated compounds (agonist, antagonist) in secondary screens. Clearly, there is a need for new test systems for the evaluation of the hormonal disruption potential of compounds in humans [21] and we have developed a novel stationary phase with immobilized estrogen receptor α in a liquid chromatographic column format for use in the HTPS screening for estrogenic properties.

4.2. Preparation of the ER-SP

The human estrogen receptor ligand binding domain (aa 302–595) {ER} was expressed and purified as follows. The plasmid was transformed into the BL21 codon+bacteria. The bacteria was grown in standard LB Broth to an optical density at $\lambda = 600$ of ~1.5. The bacteria was harvested by centrifugation and then frozen at −80°C until further purification. The bacteria pellets were lysed in an urea/HEPES lysis buffer by sonication and clarified by centrifugation and filtration. The lysate was loaded onto a 5 ml Ni–NTA nickel affinity column that was pre-equilibrated with the urea/HEPES lysis buffer. The Ni–NTA column selectively bound proteins with the 6-Histidine-tag. The non-tagged proteins washed off the column with the urea/HEPES buffer. The estrogen receptor was refolded on the column by gradually changing buffer to PBS buffer.

The ER obtained by this method was immobilized onto a column (4 mm ID×2 cm) via coordinating complex covalently linked to a silica backbone with a hydrophilic spacer.

4.3. Chromatographic studies with the ER-SP

The ER-SP was connected to an on-line flow scintillation detector and frontal chromatographic experiments were conducted using 18 ml samples of 0.5 n*M* [^{3}H]-Estradiol ([^{3}H]-E_2) supplemented with a range of concentration of cold E_2 (0.5–7.5 n*M*). The experiments were run at room temperature for 97.5 min at a flow-rate of 0.2 ml/min and the signal was recorded every 6 s.

The non-immobilized ER binding affinity (K_d) for E_2 was determined to be 1.2±0.3 n*M* using a scatchard plot. This is in agreement with the previously reported K_d of 1.49±0.16 n*M* obtained for a bacterially expressed fusion protein with protein A and the ER [22]. The K_d of E_2 was also calculated for the ER-SP using frontal chromatographic methods. The elution volume data was analyzed using a one-site binding equation: $Y = B_{max}\ [E_2]_{total}/(K_d + [E_2]_{total})$ and the calculated K_d was 7.4±1.9 nM.

The ER-SP column was connected on-line to a mass spectrometer and six known estrogen receptor ligands were chromatographed. The studies were run at 37°C using 10 μM of ligand, an ammonium acetate buffer [1 m*M*, pH 7.4] as mobile phase and a flow-rate of 0.2 ml/min. The observed retention times were consistent with the reported ER binding affinities, i.e. low affinity binding ligands (16-α-hydroxyestrone, 2-hydroxyestrone and tamoxifen) had retention times less than 10 min, a moderate ligand (genistein) had a retention time of 20 min, and high affinity ligands (4-hydroxytamoxifen and ICI 182, 780) had retention times greater than 90 min.

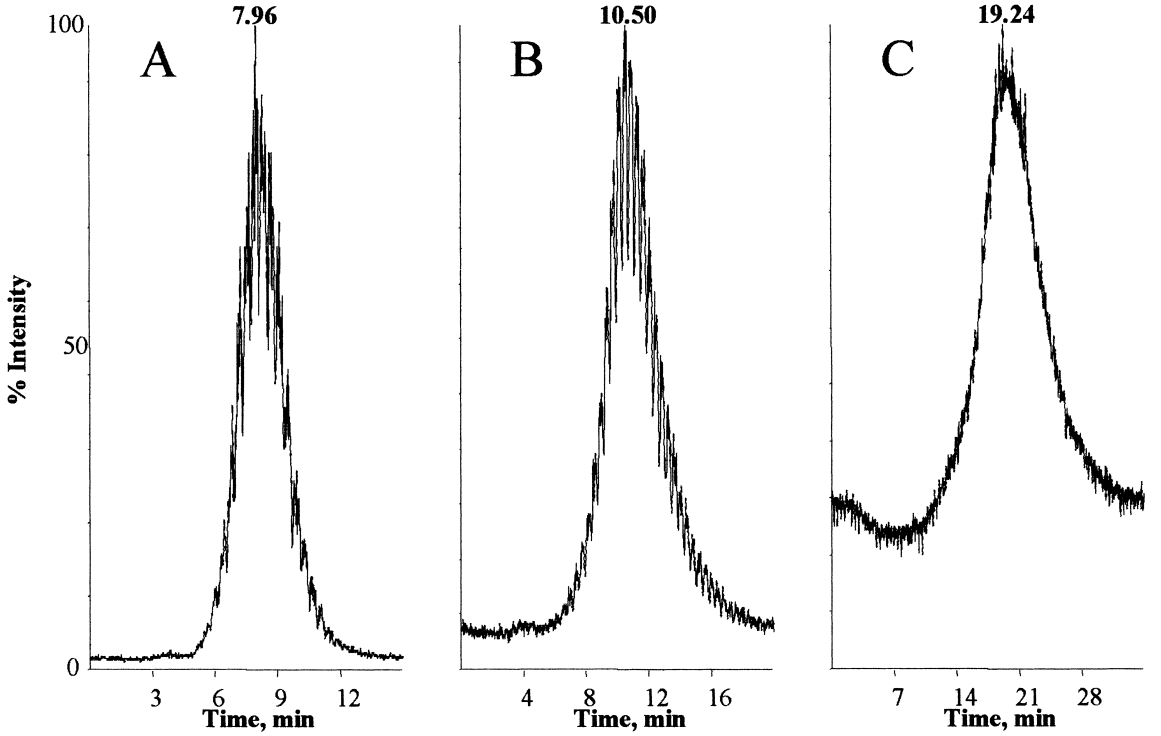

Fig. 4. The chromatographic retention of 16-α-hydroxyestrone (A), 2-hydroxyestrone (B) and genistein (C) on a liquid chromatographic stationary phase containing immobilized hER-LBD. The mobile phase consisted of ammonium acetate [1.0 m*M*, pH 7.4] and the chromatography was carried out at 37°C with a flow-rate of 0.2 ml/min using on-line mass spectrometric detection operating in a single ion monitoring mode.

Representative chromatograms for 16-α-hydroxyestrone, 2-hydroxyestrone and genistein are presented in Fig. 4.

5. P-glycoprotein-based stationary phase [8,9]

An immobilized Pgp liquid chromatographic stationary phase (Pgp-SP) has been developed. Chromatographic studies with the Pgp-SP demonstrate that retention on this phase correlates with Pgp substrate binding as determined by classical filtration binding assays. The observed binding is Pgp-specific, highly sensitive to changes in the protein's tertiary conformation caused by Pgp interactions with substrates and ATP and reflects changes occurring in the functional cycle of Pgp. Thus, Pgp-affinity chromatography represents a promising tool for a quick and reproducible evaluation of potential Pgp substrates and/or inhibitors and a useful probe of the transport mechanism. The data obtained through this approach provides new information on Pgp's mechanism of action, including evidence of binding sites for verapamil and for cyclosporin A that are distinct from the ones for vinca alkaloids. The data directly support a model of Pgp's action where these substrates can bind to distinct, though often allosterically connected regions.

5.1. P-glycoprotein (Pgp), background

P-glycoprotein (Pgp) is a 170 kDa cell membrane protein, and a member of the ATP binding cassette (ABC) superfamily of transport proteins. This superfamily includes the multidrug resistance-associated protein (MRP1), the canalicular multispecific anionic transporter (cMOAT, or MRP2), the breast cancer

resistance protein (BCRP), and the cystic fibrosis transmembrane conductance regulator (CFTR) [23,24]. Pgp is an efflux drug transporter whose substrates include anticancer drugs such as the anthracycline antibiotics and vinca alkaloids [25], steroids [26], verapamil [27], peptides [28] and quinolines [29]. This broad substrate specificity has not been definitively explained and represents a central question of Pgp biology.

Several indirect and direct models for Pgp activity have been proposed [30]. The most popular model is the membrane vacuum cleaner mechanism in which Pgp binds its substrate from the inner leaflet of the plasma membrane and releases it into the extracellular fluid [31]. In a related mechanism, Pgp activity has been described as a flippase ''that transports its substrates from the inner to the outer leaflet of the plasma membrane'' [32].

The number of binding sites on the Pgp molecule has not been determined. There is evidence for the existence of multiple binding sites as some substrates bind to Pgp in a mutually non-competitive manner [33,34]. Other data suggesting multiple binding sites includes synergistic activity on Pgp's ATPase activation [35], substrate discriminating effect of specific Pgp mutations [36,37] and differential effect of chemosensitizers on the photoaffinity labeling at two different locations on the Pgp molecule [38].

Pgp has been isolated from cells that overexpress the transporter through solubilization in a variety of detergents followed by purification using a combination of anion-exchange and affinity chromatography [39–41]. The isolated protein was then reconstituted into proteoliposomes prepared from a lipid mixture of *Escherichia coli* bulk phospholipid either by the detergent dilution method [39] or by detergent dialysis followed by Sephadex-G50 chromatography [41]. In the proteoliposomes prepared by either method, >90% of Pgp was reconstituted with an inside-out orientation, i.e. ATP-binding and cytoplasmic domains exposed to extravesicular medium [41]. The reconstituted Pgp could be used to study and characterize drug-stimulated ATPase activity as well as ATP-dependent transport. Using this approach, the effect on [^{3}H]-vinblastine ([^{3}H]-VBL) accumulation in the proteoliposomes {a measure of transport} of the Pgp inhibitors verapamil and daunorubicin could be measured [41] and the effect of verapamil on the ATPase kinetics {K_m and V_{max}} could also be determined [39].

5.2. Preparation of immobilized-Pgp liquid chromatographic stationary phases (Pgp-SPs)

Pgp-positive membranes were obtained from the MDA435/LCC6MDR1 cell line prepared from the MDA435/LCC6 cell line by transduction with a retroviral vector carrying MDR1 cDNA {Pgp}. The cultured cells (8×10^7 cells) were harvested in 10 ml of buffer (Tris–HCl [50 m*M*, pH 7.4], 50 m*M* NaCl, 2 μ*M* Leupeptin, 2 μ*M* phenylmethanesulfonyl fluoride and 4 μ*M* pepstatin). The suspension of cells was homogenized for 2×30 s. The homogenate was centrifuged first at 1000 *g* for 10 min, the pellets were discarded and the supernatant was collected and centrifuged at 150 000 *g* for 30 min. The membrane pellets were collected, resuspended in 6 ml solubilization solution (Tris–HCl, [50 m*M*, pH 7,4], 500 m*M* NaCl, 15 m*M* CHAPS, 2 m*M* DTT, 10% glycerol) for 3 h at 4°C.

The solubilized Pgp-membranes were mixed with 100 mg of IAM stationary phase and stirred for 1 h at room temperature. The suspension of Pgp-IAM was then dialyzed against dialysis buffer (150 m*M* NaCl, Tris–HCl [150 m*M*, pH 7.4], 1 m*M* EDTA, 1 m*M* benzamidine) for 36 h at 4°C. The immobilized Pgp-stationary phase (Pgp-SP) was then packed into a HR5/5 glass column (0.5×0.8 cm). Protein assay showed that for one milliliter of bed volume, about 170 mg proteins were immobilized on IAM column.

5.3. Chromatographic studies with the Pgp-SP

5.3.1. Comparison of substrate binding to IAM, Pgp-IAM, Pgp(negative)-IAM

The membranes from the Pgp-negative cells were immobilized on the IAM stationary phase and chromatographic columns were packed with either the Pgp-IAM, Pgp(negative)-IAM or IAM particles only. Zonal chromatographic studies were carried out using [^{3}H]VER as the solute. The results are presented in Fig. 5 and indicate that no specific retention was observed on the IAM and Pgp(negative)-IAM stationary phases while [^{3}H]VER was specifically retained on the Pgp-IAM.

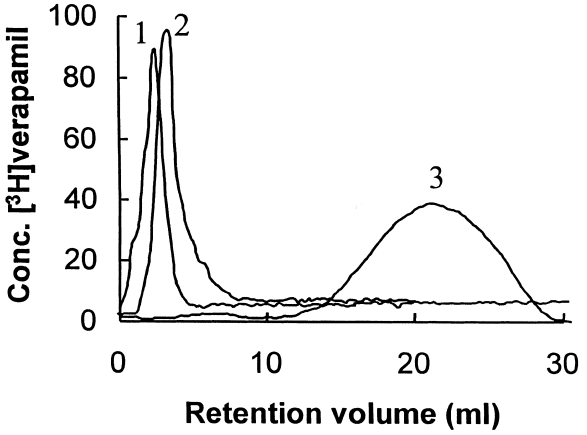

Fig. 5. Zonal affinity chromatographic profiles of [^{3}H]verapamil where: (1) obtained on a stationary phase containing Pgp-negative membranes; (2) obtained on the bare chromatographic support; (3) obtained on a stationary phase containing Pgp-positive membranes. {Reprinted from Ref. [9]}.

5.3.2. Calculation of binding affinities of Pgp substrates

The Pgp-IAM column was connected on-line to a flow scintillation monitor and all chromatographic experiments were conducted at room temperature using a flow-rate of 0.5 ml/min.

The tritiated ligands, either vinblastine ([^{3}H]-VBL), verapamil ([^{3}H]-VER) or cyclosporine A ([^{3}H]-CsA) were applied to the Pgp-IAM column in sample volumes of 25–50 ml. The solutions containing the marker ligands were supplemented with range concentrations of either cold ligand. Elution profiles were obtained showing front and plateau regions, and the observed elution volume data were used for calculation of ligand dissociation constants (K_d).

The K_d's of the Pgp substrates VBL, VER, CsA and doxorubicin were determined by frontal affinity chromatography. The values are shown in Table 3. The K_d's of VBL and doxorubicin were measured to be 23.5±7.8 nM and 15.0±3.2 μ*M* and are consistent with literature values [34].

The K_d of VER was determined to be 54.2±4.6 μ*M* and is higher than the previously reported 0.45±0.05 μ*M* [34] and 0.6±0.18 μ*M* [33]. On the Pgp-SP, VER was not displaced by VBL but VBL was displaced by VER. The data is consistent with the existence of two VER binding sites on Pgp. At one site, a low affinity site for VER, both VER and VBL bind while at the other site, a high affinity site for VER, only VER binds. The existence of two independent binding sites on Pgp has been previously postulated [33–38].

Table 3
The binding affinities of ligands for Pgp, expressed as K_d values, obtained using frontal affinity chromatography on the immobilized Pgp-IAM stationary phase as compared to binding affinities obtained from membrane filtration experiments (see Ref. [9] for experimental details)

Drugs	K_d [a]	K_d [b]
Vinblastine (n*M*)	23.5±7.8	37.0±10
Verapamil (μ*M*)	54.2±4.6	0.45±0.05
Doxorubicin (μ*M*)	15.0±3.2	31±7.3
Cyclosporine A (n*M*)	62.5±5.6[c]	18±3.6
	97.9±19.4[d]	

[a] These values were measured in the present work by frontal affinity chromatography with immobilized Pgp-IAM.
[b] These values are from Ref. [34].
[c] Obtained by displacing [^{3}H]vinblastine.
[d] Measured with 3 m*M* ATP in the running buffer.

When CsA was used as the displacer of the [^{3}H]-VBL marker ligand, the calculated K_d value for CsA was 97.9±19.4 n*M*, as compared to the previously reported value of 18.0±3.6 n*M* [34], Table 3. The displacement of [^{3}H]-VBL by CsA indicated that CsA specifically and competitively bound to immobilized Pgp. However, when [^{3}H]-CsA was used as the marker ligand, frontal chromatography with [^{3}H]-CsA alone in the running buffer produced a low retention volume, 7.8 ml and no detectable specific retention, Table 4. This indicated that under the experimental conditions, [^{3}H]-CsA did not specifically bind to immobilized Pgp.

The contradiction between the data obtained with [^{3}H]-VBL as the marker versus the data obtained with [^{3}H]-CsA alone in the running buffer was eliminated when VBL was added to the running buffer. The addition of 50 and 100 n*M* VBL to the running buffer produced classical frontal chromatograms for [^{3}H]-CsA and increased the retention volume to 15.7 and 18.8 ml, respectively, Table 4.

The results from the studies with [^{3}H]-VBL and [^{3}H]-CsA as the marker ligands indicate that the addition of VBL to the running buffer may produce a co-operative allosteric interaction in the binding process between [^{3}H]-CsA and the immobilized Pgp.

Table 4
Retention volumes of [^{3}H]vinblastine, [^{3}H]verapamil and [^{3}H]cyclosporine A obtained when no ATP was present in the running buffer, after 3 m*M* ATP was added in the running buffer and after 50 n*M* and 100 m*M* cold vinblastine (VBL) were added to the running buffer without ATP (see Ref. [9] for experimental details)

Retention volume (ml)				
Ligands	No ATP	3 m*M* ATP	50 m*M* VBL	100 m*M* VBL
[^{3}H]vinblastine	32.1	8.4	11.0	9.5
[^{3}H]verapamil	34.2	5.9	34.1	34.0
[^{3}H]cyclosporine A	7.8	17.5	15.7	18.8

This suggests that the immobilized Pgp has retained its conformational mobility and that the binding of VBL to Pgp opened up the site at which CsA binds. CsA can bind to Pgp and displace VBL through competitive and/or anti-cooperative allosteric interactions. One explanation for these results is that the VBL induced CsA binding site is contiguous with or part of the VBL site. CsA binding to the induced site on Pgp does not directly compete with VBL for the same site, but inhibits VBL binding through steric interactions.

5.3.3. The effect of ATP on chromatographic retention on the Pgp-IAM

Pgp is a member of the ATP-binding cassette superfamily and ATPase activity plays a role in substrate transport [42,43]. Thus, it should be expected that the addition of ATP to the running buffer would change the chromatographic properties of the immobilized Pgp chromatographic system. In this study, the addition of 3 m*M* ATP to the running buffer increased the retention volume of [^{3}H]-CsA from 7.8 to 17.5 ml, produced a classical frontal chromatogram for [^{3}H]-CsA and permitted the calculation of an K_d value of 62.5 n*M*, Table 4. These results indicate that the addition of ATP to the running buffer produced a cooperative allosteric interaction that increased the binding affinity of Pgp for CsA.

The presence of ATP in the running buffer produced the opposite effect on the retention volumes of [^{3}H]-VBL and [^{3}H]-VER. When [^{3}H]-VBL was the marker ligand, the addition of 3 m*M* ATP reduced the observed retention from 32.1 to 8.4 ml, Table 4, Fig. 6. The reduction in [^{3}H]-VBL retention volume was accompanied by an apparent loss of specific retention (i.e. there was a loss of a frontal plateau, see Fig. 6) suggesting an anti-cooperative allosteric interaction. This possibility is supported by the results of VBL displacement experiments. The addition of unlabelled VBL to the running buffer, in the absence of 3 m*M* ATP also decreased the retention volume of [^{3}H]-VBL, Table 4. However, the effect of VBL on [^{3}H]-VBL retention differs from the effect observed with ATP, since specific frontal chromatographic curves were observed after the addition of 50 and 100 n*M* VBL. Thus, the decreased retention was due to competitive displacement of [^{3}H]-VBL by VBL. The ATP induced reduction in the retention volume for [^{3}H]-VER, 34.2–5.9 ml, coupled with the loss of specific retention, also indicate that the primary site of VER

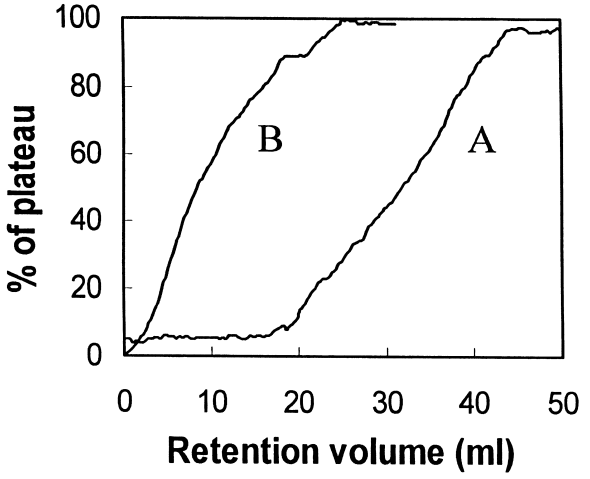

Fig. 6. Frontal affinity chromatographic profiles of [^{3}H]vinblastine on a Pgp-stationary phase where: (A) obtained with a mobile phase of Tris–HCl [50 m*M*, pH 7.4]; (B) obtained after the addition of 3 m*M* ATP to the mobile phase of Tris–HCl [50 m*M*, pH 7.4]. {Reprinted from Ref. [9]}.

binding to the Pgp molecule was affected by an anti-cooperative allosteric interaction.

The observation of ATP-induced conformational shifts in the immobilized Pgp molecule is consistent with the results from previous studies. An infrared spectroscopy study has shown that Pgp changes conformation a first time when ATP is added, and then again when a substrate is added in the presence of ATP [44]. The changes in affinity for VBL and CsA that we observed following the addition of ATP most probably reflects the second of the above conformational changes, or the situation that immediately follows substrate binding and ATPase activation.

6. Conclusions

Most receptors and drug transporters are transmembrane glycoproteins whose function is to transform external stimuli into intracellular responses. In addition, there are receptors that interact with steroids and other hormones, which are soluble DNA-binding proteins [5]. Within these groups, there are a variety of families and subtypes. Each one presents a unique set of issues relative to their isolation and immobilization. In this review, we have addressed the synthesis and characterization of two types of receptor-based liquid chromatographic supports, one based upon a transmembrane ligand gated ion channel receptor (NR) and the other a soluble nuclear receptor (ER). The differences in the receptors necessitated distinct experimental approaches. In the case of the NR, immobilization was achieved via hydrophobic insertion into the interstitial spaces of an IAM stationary phase, thereby mimicking the receptor's cellular environment. With the soluble ER, the best approach involved tethering to a hydrophilic stationary phase. In this manner, the conformational freedom of the molecule was optimized.

In addition to the preparation of the receptor-based stationary phases, this review has addressed the creation of a Pgp-SP. Pgp is a 170 kDa cell membrane protein that acts as an efflux drug transporter. The transmembrane nature of the Pgp molecule necessitated a third immobilization approach. In this case, membranes containing the Pgp transporter were coated on the surface of the IAM stationary phase.

Each of the three stationary phases was characterized using known ligands and substrates. The results from zonal and frontal chromatographic experiments demonstrated that each stationary phase could be used to determine binding affinities (expressed as dissociation constants, K_d's) and to resolve mixtures of ligands according to their relative affinities. In addition, competitive ligand binding studies on the Pgp-SP have established that this phase can be used to identify and characterize competitive displacement and allosteric interactions. These studies demonstrate that immobilized-receptor and immobilized-transporter 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.

7. Nomenclature

IAM	Immobilized artificial membrane stationary phase
SAR	Structure–activity relationships
HTPS	High-throughput screening
NR	Nicotinic acetylcholine receptor
NR-SP	Immobilized nicotinic receptor stationary phase
EB	Epibatidine
ER	Estrogen receptor
ER-SP	Immobilized estrogen receptor stationary phase
E_2	Estradiol
Pgp	P-glycoprotein
Pgp-SP	Immobilized P-glycoprotein stationary phase
ABC	ATP binding cassette superfamily
VBL	Vinblastine
VER	Verapamil
CsA	Cyclosporin A

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