We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900
Open access books available

185,000
International authors and editors

200M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



14

Affinity Chromatography as a Tool for Quantification of Interactions Between Drug Molecules and Their Protein Targets

Piotr Draczkowski, Dariusz Matosiuk and Krzysztof Jozwiak
Faculty of Pharmacy with Division of Medical Analytics, Medical University of Lublin
Lublin
Poland

1. Introduction

Separations in affinity chromatography are based on specific biological interactions between (bio)molecules and in many aspects resemble processes by which these species interact in a living organism. The technique is widely used in biomedical sciences to separate and detect certain molecules based on their defined specificity to other (bio)molecules immobilized on a stationary phase. Moreover,it can also be used for quantitative determination of affinity interactions and their physiological and pharmacological role in a living system. In both cases the same basic physico-chemical effects (hydrophobic interactions, electrostatic, hydrogen bonds etc.) lead to description of the equilibrium state between unbound, free-floating molecules and those forming a complex with a target. This allows the use of affinity chromatography system as a model for analyzing interactions that normally occur in human body. This approach was suggested by analytical chemists over 50 years ago, e.g. Soczewinski and Bieganowska stated in 1969:

"... If the body is regarded as an extremely complex chromatographic system, in which the blood plays the role of the developing solvent, a certain parallelism can be expected between the behaviour of drugs and their chromatographic parameters in common "simple" partition systems."

Therefore, affinity chromatography is currently successfully employed in medicinal chemistry projects for detailed characterization of interactions between drug molecules and their protein targets. This type of liquid chromatography is referred to as analytical or quantitative affinity chromatography (QAC).

In most applications the assay is performed in HPLC systems using a column with a protein immobilized on the surface of the stationary phase. Relatively simple and rapid procedures (time of a single assay average between 5 to 15 min.), ability to multiple use of the same column without significant loss of properties of immobilized protein (Jozwiak et al., 2004) and the possibility to automate the analysis process, make this technique a promising method for screening and determination of relative affinities of a series of analyzed drug molecules. Loun & Hage (1996) reported that theirs column was stable even to 500-1000 injections of an analyte. Above mentioned advantages of this method additionally increase

reproducibility of obtained results. Thus, affinity chromatography is considered by many medicinal chemists as good alternative to tedious *in vitro* tests using cell cultures (Jozwiak et al., 2004). It also allows to reduce time needed to perform the assays (Jozwiak et al., 2005).

Special procedures were developed in order to transform retention of a substance or it elution profiles to characterize the interaction between a drug and the target protein. Therefore, various protocols can be used to characterize binding equilibrium constants, the kinetics of drug-protein complex association/dissociation, relative amount of bound drug, number of binding sites in the system or forces responsible for complex formation. Simultaneous application of more than one active substance on the column allows defining interactions between them during binding to immobilized target. It is also possible to determine the affinity of various isomeric forms of the drug to immobilized protein, which is extremely important in a pharmaceutical research (Chen & Hage, 2006). Data obtained by QAC show high correlation with derived from reference methods and can be further used in studies of quantitative structure - activity relationships (QSAR) (Markuszewski & Kaliszan, 2002; Jozwiak et al., 2004).

This chapter reviews current aspects of application of QAC including basic issues of protein immobilization on the surface of the stationary phase, advantages and disadvantages of zonal and frontal elution techniques and vast information which can be provided by competition displacement in QAC studies. The last subsection provides short review of application of these techniques in medicinal chemistry investigations.

2. Immobilization of target proteins on the surface of the stationary phase

QAC assay requires a column where one of the partner of the drug-protein complex is immobilized on the surface of the stationary phase. Both, drug molecules and protein targets may be immobilized on the chromatographic bead particles. The latter option seems to be more versatile as it allows to investigate different types of active substances without having to change the column bead. It directly permits to compare properties of a series of substances on the basis of obtained chromatograms. The way in which the ligand is attached to its support is a key factor in any type of affinity chromatography. Immobilization methods for soluble cytosolic proteins are well established (Taylor, 1991; Turková, 1999; Kim & Hage, 2006; Scheil et al., 2006) and they are based mainly on chemical or physical mechanisms. Physical methods include protein adsorption (physical or ionic adsorption) or protein entrapment within insoluble gel matrix through which only small drug molecules can diffuse. The advantage of these methods is relatively small perturbation of the protein native structure, on the other hand immobilisation forces are weak and promotes the leak of adsorbed protein from the support during use, especially while temperature, pH or ionic strength is changed. Chemical immobilization methods mainly include protein attachment to the stationary phase by covalent bonds or cross-linking reactions. Covalent linkage may alter the native tertiary structure of the protein and cause a change in drug binding properties, but the target associates strongly with the support preventing the desorption phenomenon and increasing a column lifetime. The functional groups that usually take part in this binding are amino, epoxy, carboxyl, sulfhydryl, hydroxyl, diol and phenolic groups which according to the mode of linkage lead to a wide variety of binding reactions such as diazotization, amide bond formation, arylation, Schiff's base formation and amination

(Girelli & Mattei, 2005). Another type of immobilization method is biospecific adsorption. It uses the binding between the ligand of interest and a secondary ligand attached to the support. Although a variety of secondary ligands can be used for this purpose, two of the most common are avidin and streptavidin for the adsorption of biotin-containing compounds, and protein A or protein G for the adsorption of antibodies (Akerstrom & Bjorck, 1986; Wilchek & Bayeras, 1990; Bayer & Wilchek, 1996; Wilchek & Bayeras, 1998; Page & Thorpe, 2002, as cited in: Kim & Hage, 2006).

Various supports are commercially available or have been specifically developed for the immobilization processes, including silica based derivatized matrices (Narayanan et al., 1990; Ruhn et al., 1994; Mateo et al., 2000) and monoliths (Josic & Buchacher, 2001; Lebert, 2008). An important factor is a structure of the support used in preparation of affinity column, since it determines accessibility of protein active sites to substrates. The ideal support must be inert, stable and resistant to mechanical strength, so it can retain its tertiary structure, and this ensures the substrate accessibility to interact with the active sites. Other physical properties, such as porosity, pore size distribution and charge are also important, because they influence the kinetic process (Girelli & Mattei, 2005). Variety of available methods of protein connection with chromatographic beads, gives the opportunity to select and optimize right immobilization method for binding of our protein of interest. It should be noted that the immobilized ligand must as close as possible imitate the behaviour which it exhibits in natural conditions. The method chosen for protein binding should not disrupt the structure crucial for drug binding and provide proper orientation to eliminate any negative steric interactions. Proper immobilization allows retaining activity of the protein on the column, and even its conformational mobility (Beigi et al., 2004), which allows to study allosteric interactions (Chen et al., 2004).

In last years new techniques were developed to immobilize membrane proteins on the surface of chromatographic bead particles to describe the nature of interactions between drug molecules and target receptors. This is very valuable from pharmacological point of view considering that the membrane and transmembrane receptor proteins are targets for almost 75% of current pharmaceuticals (Landry & Gies, 2008). The first membrane protein that was analysed using QAC was glucose transporter present on the surface of red blood cells (GLUT1) (Yang and Lundahl, 1995). Two immobilization techniques were used in this system: in first proteoliposomes or cytoskeleton depleted membrane vesicles containing GLUT1 were immobilized in the pores of size exclusion chromatography beads (Superdex) (Gottschalk et al., 2000) by technique of repeated freezing - thawing (Lundqvist et al., 1998). In the second approach whole cells were immobilized on the surface of beads with positively charged groups (Zeng et al., 1997) or columns with wheat germ lectin agarose gel beads (Gottschalk & Lundahl, 2000). Currently, there are many reports in literature describing the columns with different immobilized receptors and membrane transporters. Most of them use silicon particles with immobilized phospholipids (IAM) (Pidgeon & Venkataram, 1989) which connect parts of the cell membranes of tissues showing high expression of the receptor or cultured cells transformed with receptor gene. It is known as cellular membrane affinity chromatography: CMAC, or CMC. Using this method columns with immobilized nicotinic receptor (nAChR) subtypes α3β4, α4β2, α3β2, α4β4, α7 and subunits β4 and α3 (Zhang et al., 1998; Wainer et al., 1999; Moaddel et al., 2005; Moaddel et al., 2008) purinergic receptors, both acting as ion channels (P2X family) (Trujillo et al., 2007) as well as G-protein coupled receptors (P2Y family)

(Moaddel et al., 2007), the P2Y-like receptor GRP17 (Temporini et al., 2009), β2 adrenergic receptor (Beigi et al., 2004) or the μ and κ opioid receptors (Beigi & Wainer, 2003) were constructed. Multi-receptor columns containing nAChRs, γ-amino-butyric acid receptors (GABA) and N-methyl D-aspartate receptors (NMDA) (Moaddel et al., 2002) were also developed. Kitabatake and co-workers (2008) constructed multi-receptor column and confirmed presence of two types of nAChRs: α7 nAChR and heteromeric nAChRs but also GABA, and NMDA receptors on the surface of 1321N1 and A172 astrocytoma cell lines. The results indicate that the columns can be used to characterize binding affinities of small molecules to each of the receptors, and that this approach can be used to probe the expression of endogenous membrane receptors. With similar immobilization technique beads with transmembrane transport proteins such as P-glycoprotein (Zhang, 2000), human organic anion transporter protein (hOAT1 and hOAT2) (Kimura et al., 2007) and human organic cationic transporter protein (hOCT1) were prepared (Moaddel et al., 2005).

Alternative method was used to immobilize α1 adrenergic receptor (Yu et al., 2005) or muscarinic receptor (Yuan et al., 2005). In this case, the membrane fragments with an interesting protein were subjected to adsorption on the surface of silica particles under vacuum and with use of ultrasounds. Since the phospholipid bilayer fragments show a spontaneous ability to connect, they enfold bead particles forming compact, durable coating of cell membrane. Same approach was used to construct multi-receptor column for the simultaneous determination of drug interactions with the purinergic, P2Y1 and histamine 1 receptors (Moaddel et al., 2010) and multi-receptor column prepared using the glioma cell membranes in order to identify the types of receptors on the surface of these cells by their specific ligands (Kitabatake et al., 2008).

Immobilization of membrane proteins on the inner surface of silica capillaries (open tubular column) is also applied. For this purpose, fragments of cell membrane containing membrane protein bind non-covalently with a capillary using the avidin-biotin pair. Capillaries with immobilized P-glycoprotein (Moaddel et al., 2004), and recently with immobilized cannabinoid receptors (CB1/CB2) (Moaddel et al., 2011) were developed using this approach.

When a native structure of a target protein cannot be obtained during immobilization a drug can be used as an immobilized ligand. However, in this situation any steric interactions should be eliminated by connecting drug molecule to the bead using a spacer with adequate length and hydrophobicity, in order to secure free access to the binding site on the protein molecule. It is important not to connect the drug using functional groups that participate in binding to the target protein. Application of QAC with immobilized drug molecules is particularly useful for identifying biomolecules targets for substances with known therapeutic effect. It is also used as a pre-clinical method of detection of undesired interactions with other system biomolecules (Guiffant et al., 2007).

3. Elution techniques

3.1 Zonal chromatographic studies of drug-protein binding

One method of QAC is a technique known as the zonal elution technique. It was first used by Dunn and Chaiken (1974) as modified low-pressure liquid chromatography method used to investigate the retention of *Staphylococcus* nuclease on the column with immobilized

thymidine-5'-phosphate-3'-aminophenylphosphate. Zonal elution method can also be applied using a standard HPLC apparatus equipped with temperature control unit. This method involves injection on the column small volume of analyte solution and then isocratic elution with mobile phase, which usually has a composition and pH reflecting the physiological conditions. Compared to frontal elution (described in subsection 3.2) small amount of analyte is needed to perform the assay in zonal format. Detection is carried out on-line, however there are applications with off-line detection when chromatographic systems has low efficiency (Dunn & Chaiken, 1974).

In result of the analysis the retention factor also called capacity factor is determined. It is expressed by formula $k = (t_r/t_m) - 1$, where t_r is the retention time of the test substance, and t_m is the column dead time. Comparing the value of k for different substances, we can determine their relative affinity for the immobilized protein. Typical chromatogram obtained from zonal chromatography studies is shown in Figure 1. Studies on the affinity of benzodiazepines and related coumarins done by Noctor and colleagues using immobilized human serum albumin (HSA) (Noctor et al., 1993, as cited in Bertucci et al., 2003) have shown a strong correlation (r = 0.999) between the percentage of bounded drug measured by the standard method of ultrafiltration, and the data obtained from chromatographic studies expressed as $k/(k + 1)$.

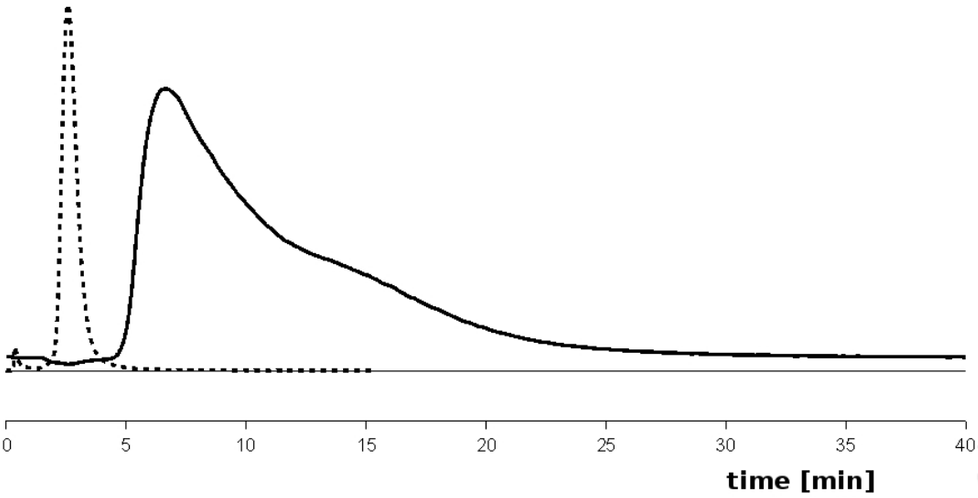


Fig. 1. Typical chromatogram obtained from zonal AC. Comparison of elution peak profiles of ketamine (solid) and negative control phenylbutazon (dashed line) on the column with immobilized nicotinic receptor (nAChR), subtype α3β4. Adapted with permission from Jozwiak, K., Ravichandran, S., Collins, J. R., & Wainer, I. W. (2004). Interaction of noncompetitive inhibitors with an immobilized α3β4 nicotinic acetylcholine receptor investigated by affinity chromatography, quantitative-structure activity relationship analysis, and molecular docking. *J Med Chem*, Vol. 47, No. 16, pp. 4008-4021. Copyright 2011 American Chemical Society.

Similar results were reported by Cheng et al. (2004). In this case, the working curve between literature values of % drug bounded to HSA (by either ultrafiltration or dialysis method) and $k/(k + 1)$ factor determined by chromatography method showed good linearity with the

coefficient of 0.96, which is acceptable considering diversity of drugs tested and the variety of %binding data resources used.

According to the Equation 1 (Chaiken, 1987, as cited in Cheng et al., 2004):

$$k = \frac{K_a m_L}{V_M} \tag{1}$$

compound retention factor is closely related with equilibrium constant of binding reaction to immobilized protein target. This allows to define an order relative binding strength of different compounds by comparing their retention factors (Xuan & Hage, 2005) In Equation 1, V_M is the void volume of the column (i.e., the elution volume of a non-retained solute), m_L is the moles of active binding site and K_a is association equilibrium constant for the injected solute at this site .

Zonal elution technique is also used to determine the forces that play a fundamental role in the formation of drug-protein complex. Changing assay conditions (e.g., pH, ionic strength, content of organic modifier) allows to determine which factors affect the most binding reaction. For example, retention dependence on mobile phase pH indicates a considerable contribution of Coulomb interactions in the binding of a drug. In turn, addition of organic modifier can accelerate the elution of analyte by disturbing the hydrophobic interactions (Hage & Chen, 2006). It also allows to examine the change in binding of drugs when standard physiological system conditions will be changed as a result of pathological lesions (Basiaga & Hage, 2010).

Temperature studies allow to define changes in enthalpy and entropy of interactions between the drug and immobilized protein. It follows from the equilibrium constant depending on temperature, which can be described by the Equation 2:

$$\ln K_a = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} \tag{2}$$

where ΔH express a change of enthalpy and ΔS entropy change in a place of interaction, R is the gas constant and T is absolute temperature. So if the system meets the assumption that the number of binding sites (m_L) does not change with temperature, and this is a single-site binding then $\ln K_a$ plotted against $1/T$ should be linear with a slope equal to $-\Delta H/\mathrm{R}$, and intercept to ΔS. The total energy change can be calculated using Equation 3 (Kirkwood & Oppenheim, 1961, as cited in Yang & Hage, 1993):

$$\Delta G = -RT\ln\left(K_a\right) \tag{3}$$

The method, however, requires earlier determination of K_a at given temperature, for example, by conducting self-competition studies with test compound at different temperatures. It is also possible to designate the enthalpy and entropy of binding directly from the value of retention factor. In this case, if a binding has a single-site character, the plot of $\ln k$ against $1/T$ is linear with a slope equal to $-\Delta H/\mathrm{R}$ and the intercept equal to $[\Delta S/\mathrm{R} + \ln(m_L/V_M)]$. However, the calculation of the value of ΔH and ΔS requires the prior determination of concentration of binding sites for the analyte (m_L/V_M) (Yanda & Hage, 1993).

3.2 Frontal affinity chromatography (FAC) in drug-protein interaction investigations

Another commonly used method of determining the drug-target protein affinity is the frontal technique. Although this elution format is practically no longer used in analytical applications of chromatography, it is still successfully applied in QAC assays, and has some advantages over zonal elution methods. For the first time it was used in 1975 by Kasai and Ishii. In contrast to zonal elution, a test substance is applied continuously on the column as an addition to the mobile phase in specified concentration. The result is essentially a titration of active sites within the column. As the mobile phase flows through the column, the analyte saturates binding sites on the immobilized protein and we can observe a gradual increase of the amount of unbound analyte leaving the column. This produces a vertical rise in the chromatographic trace, called breakthrough curve which ends or plateaus when the immobilized target is fully saturated. Initial, relatively flat portion of the chromatographic traces represents the non-specific and specific binding of the tested compound to the cellular membranes and the target. Inflection points of breakthrough curves shift to shorter breakthrough times (volumes) as the ligand concentration increases (see Figure 2).

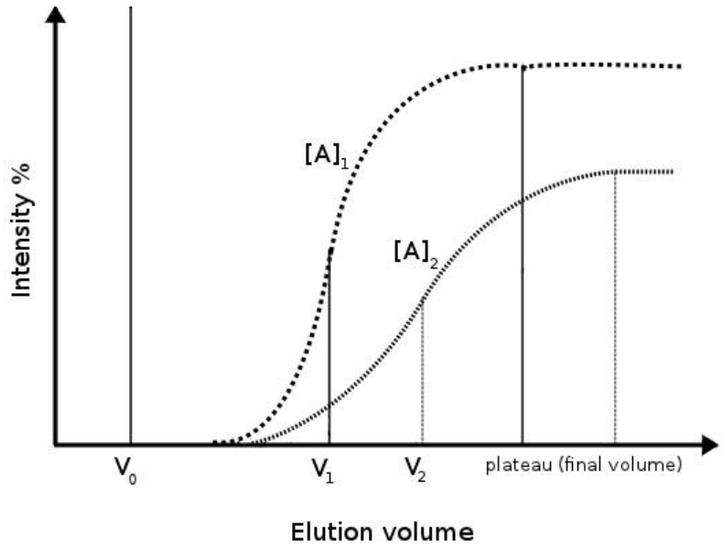


Fig. 2. Typical breakthrough curves of two analyte concentration $[A]_1$ (higher) and $[A]_2$ (lower). V_o represents breakthrough volume of the ligand in the absence of the binding event.

Extent and asymmetry of obtained chromatographic profile are related to the analyte-target protein binding kinetics. Measuring the breakthrough times for several concentrations and fitting the results to equations based on various reaction models allow to characterize a nature of binding affinity and the amount of immobilized target on a column. This is accomplished by plotting number of apparent moles of analyte required to reach the mean point of the breakthrough curve ($1/m_{Lapp}$) versus $1/[A]$ (where $[A]$ is applied analyte concentration). According to Equation 4 (Loun & Hage, 1992), in case of single site reaction, plot ($1/m_{Lapp}$) versus $1/[A]$ should give a linear response (see Figure 3) with a slope equal to $1/(K_A m_L)$ and an intercept of $1/m_L$. Dividing the intercept by the slope allows to obtain information about equilibrium binding constant of analysed interaction.

$$m_{Lapp} = \frac{m_L K_A [A]}{1 + K_A [A]} \text{ or } \frac{1}{m_{Lapp}} = \frac{1}{K_A m_L [A]} + \frac{1}{m_L} \quad (4)$$

A similar approach, but using a combination of both nonlinear and linear fits, can be used for more complex systems that involve multisite interactions (Jacobson et al., 1993; Tweed et al., 1997, as cited in Hage & Chen, 2006).

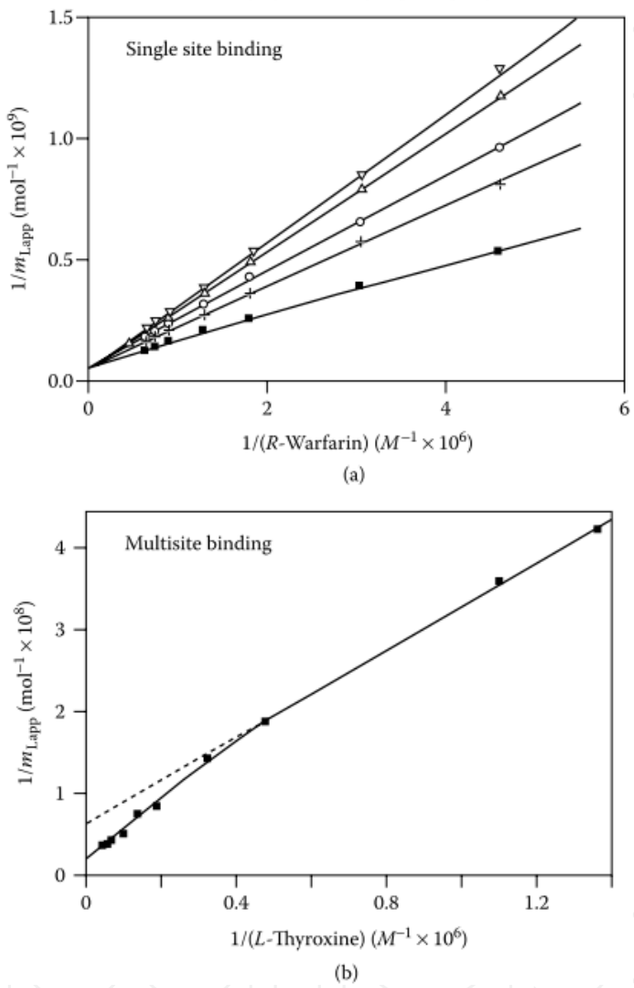


Fig. 3. Examples of double-reciprocal frontal analysis plots for systems with (a) single-site binding and (b) multisite binding. Reprinted with permission from: Loun, B., & Hage, D. S. (1994). Chiral separation mechanisms in protein-based HPLC columns. 1. Thermodynamic studies of (*R*)- and (*S*)-warfarin binding to immobilized human serum albumin. *Anal Chem*, Vol. 66, No. 21, pp. 3814-3822; Tweed, S. A., Loun, B., & Hage, D. S. (1997). Effects of ligand heterogeneity in the characterization of affinity columns by frontal analysis. *Anal Chem*, Vol. 69, No. 23, pp. 4790-4798. Copyright 2011 American Chemical Society.

The simplest binding event, involving the interaction of a ligand with a single type of binding site can also by described by Equation 5. There may be multiple, equivalent sites in a given target molecule but the model assumes their independence.

$$V - V_0 = \frac{B_t}{[A]_0 + K_d} \tag{5}$$

In this basic FAC equation (5), two variables are present: $[A]_0$ (ligand infusion concentration) and V-V_0 (breakthrough volume V for the ligand corrected by the breakthrough volume of the ligand in the absence of the binding event V_0). This simple equation indicates that once B_t (corresponding to the dynamic capacity of the affinity column for the ligand) and the concentration of the analyte are known, the dissociation constant can be determined from a single measurement of its V-V_0. In order to determine B_t, various concentrations of the compound are pumped through the column and the corresponding V-V_0 values are measured. The analysis of changes in V-V_0 versus $[A]_0$ by means of Lineweaver–Burk type double reciprocal plot or standard nonlinear regression analysis, B_t (equal to the reciprocal of the y intercept) and dissociation constant K_d (in M, expressed as negative reciprocal of the x intercept) are obtained (Calleri et al., 2009). Reliable measurement of V_0 requires a suppression of specific binding or application of a saturating ligand concentration. In case of membrane proteins it can be done by measuring retention of marker using a column constructed with membranes from cells that do not express the target protein (Moaddel et al., 2005). Other approach that can be employed to differentiate between specific and non-specific interactions is parallel chromatography system in which analysed compounds are simultaneously applied on a column with immobilized membranes containing target protein (experimental) and on a column with immobilized membranes from the same cell line that does not express the target protein (control). The assumption is that all of the non-target interactions between a test compound or protein and the cellular membranes will be the same for the control and experimental cell lines and will be reflected in the chromatographic retention on the control column. Then the difference in compound retention between control and experimental column will reflect only the specific binding. This system may be applied in both, zonal and frontal chromatographic studies (Baynham et al., 2002, as cited in Moaddel & Wainer, 2006).

In frontal QAC connected with mass spectrometry (FAC-MS), ligand is sequentially infused at increasing concentrations, but with no washing steps between infusions. This allows to determine binding parameters in a single experiment. This is accomplished by infusing a FAC–MS column sequentially starting with the lowest of a series of concentrations of analyte. For this approach, referred to as a modified staircase, the summed concentrations ($[A]_0$+y) refer to initial concentration of the ligand for the first step of the staircase but for the second step of the staircase, it will be the sum of the initial concentration plus the concentration of the second step. Similarly, the concentration of the ligand for the third step of the staircase will be the sum of the initial, second and third steps, and so on for the remaining concentrations. A plot of $[A]_0$+y versus reciprocal breakthrough volume supports the determination of B_t and K_d by linear regression analysis. This type of assays referred to as direct measurements might not always be advantageous, especially if each tested ligand required unique mass spectrometric conditions. (Chan et al., 2003; Slon-Usakiewicz et al., 2005). Indirect methods will be described further (see subsection 3.3) since they require usage of known competitive marker ligand. Figure 4 illustrates an ideal chromatogram that would arise from the application of this procedure. Note that successively higher void marker (a compound that has no affinity for the immobilized protein target and gives the same elution front whether the target protein is present in the column or not) concentrations are applied as well, to ensure accurate measurement of V_0.

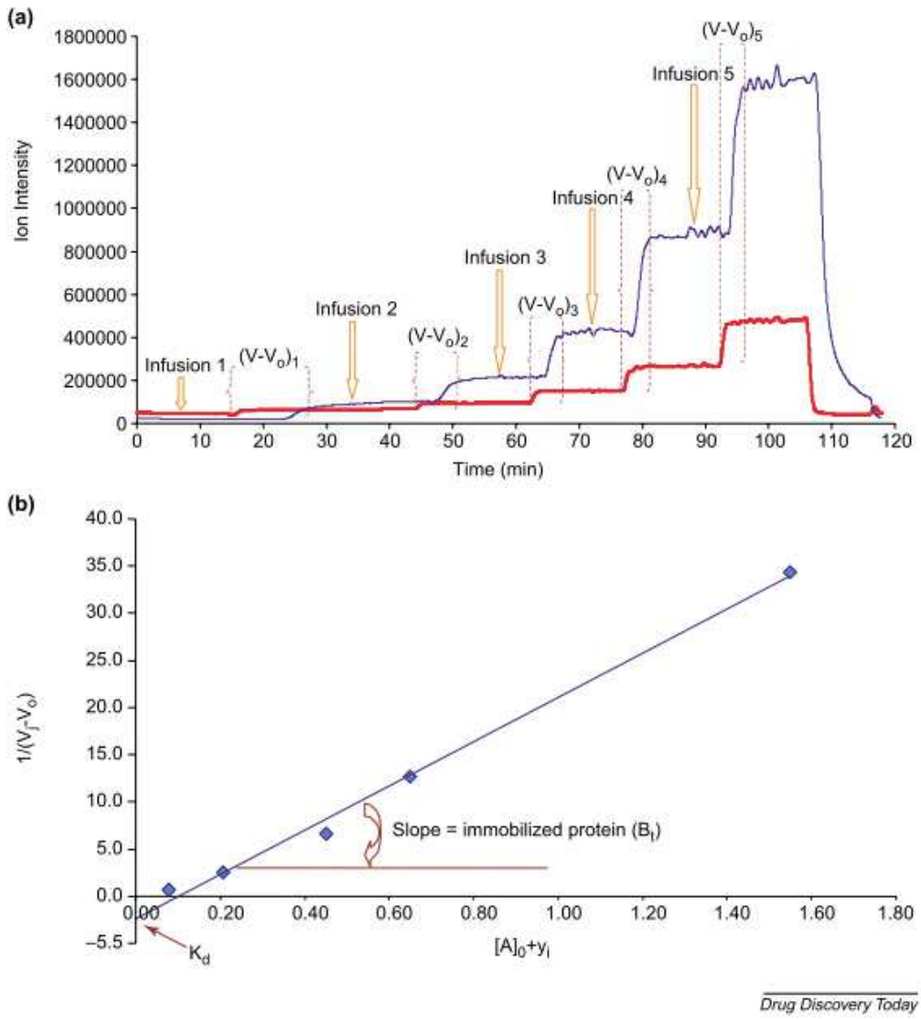


Fig. 4. Depiction of a typical 'modified staircase' experiment to evaluate K_d for a protein target (immobilized in the column) and a small molecule ligand. (a) The ligand (blue) is infused at increasing concentrations starting from an initial (i) to a final (j) concentration along with a void marker (red). (b) The reciprocal of the breakthrough volumes, $1/(V_j-V_0)$, are plotted against the summed ligand concentrations, $[A]_0+y_j$, to produce a linear correlation from which the K_d value can be determined from the y intercept. The total amount of immobilized protein (B_t) in the column is then obtained from the slope . Reprinted from *Drug Discovery Today*, Vol. 10, No. 6, Slon-Usakiewicz, J. J., Ng, W., Dai, J. R., Pasternak, A., & Redden, P. R. (2005). Frontal affinity chromatography with MS detection (FAC-MS) in drug discovery, pp. 409-416, Copyright 2011, with permission from Elsevier.

The association constant measured from frontal chromatography can be directly related to the retention factor obtained from zonal elution chromatography using the same column. Kim & Wainer (2008) reported a linear relationship (r^2 = 0.9993, n = 7) between the standard association constants from frontal analysis and retention factors from zonal elution using reference drugs analysed on a column with immobilized HSA. This standard plot was later used for rapid determination of association constants of various drugs which show low to

medium binding affinity to HSA. Determination of association constants was as fast as 1.5 min and showed a high correlation to equilibrium dialysis or ultrafiltration. The combination of frontal and zonal chromatography for determining association constants showed several advantages, one being rapid determination of association constant of drug to HSA.

Other notable advantages include an ease of automation and simultaneous ability to distinguish association constants of chiral compounds at the same time. Authors suggested that the same approach could be used for studying interaction of other drugs and proteins and should further improve overall drug screening process.

There are several advantages of frontal over zonal chromatographic method. In the FAC technique, the dynamic capacity of the affinity column for the ligand and the dissociation constant for the interaction can be obtained from a single set of experiments. In case of zonal elution technique to determine number of binding sites a separate assay or self-competition studies are required This makes this approach valuable in characterizing the properties of a column and in obtaining accurate measurements of binding affinity and activity.

The direct methods described above are applicable for a broad K_d range from submicromolar to low millimolar. The lower limit to K_d determination is dictated by the detectability of a given ligand, and while this is both compound- and detector-dependent. Zonal chromatographic approach requires a protein stationary phase concentration much greater than the applied ligand concentration to ensure the experiment is conducted in the linear region of the binding isotherm. Thus consumption of target protein is increased and miniaturization capabilities are lower. Smaller column leads to lower amount of immobilized protein but also require lower ligand concentrations and this challenges the detectability of the ligand. From the same reasons application of zonal chromatography is limited to low and mid-micromolar K_d range because either strong (and thus difficult to remove from a column) or weak (and thus not well retained) interactions can lead to low concentrations of ligand at the detector (Chan et al., 2003).

Frontal elution technique is particularly useful in studies on solvent and temperature influence on drug interaction with target molecule. As it was mentioned earlier, analyte retention shifts may be due to alterations in either the affinity or number of binding sites. In frontal chromatography this is not a problem, since data on both affinity and activity are provided in the same experiment. However, frontal techniques need to use much larger amount of analyte (Hage & Chen 2006).

For more theoretical information and practical considerations about frontal and zonal elution techniques see comprehensive chapters about QAC in book "Handbook of Affinity Chromatography" (2006) edited by prof. David S. Hage.

3.3 Competition displacement studies

QAC allows to study relationships between different drugs interacting with the same target protein by observing the effect of the addition of one compound on the retention of the second. In zonal technique, the retention of a drug is measured in specially prepared mobile phase with addition of constant, known concentration of competitive agent. Consecutive

injections of an analyte can be applied to a series of mobile phases with increasing concentration of competitive agent and changes in the retention as a function of competitor concentration allow describing its interaction with test analyte. In case of direct single site competitive interactions we should see direct, linear dependence of reciprocal of analyte retention factor ($1/k$) on concentration of competitor added to the mobile phase *[I]*. Increasing value of $1/k$ (drop in the retention) with an increase of additive concentration suggest positive competition of both molecules on the binding site. If this relationship is nonlinear, and the increase of competitive reagent in the mobile phase decreases the retention of the analyte (increasing $1/k$), this suggests that there is negative allosteric interaction between them or multisite competitive interaction. The nonlinear nature of the dependence $1/k$ on *[I]* characterized by an increase in analyte retention as higher concentrations of compound are added to the mobile phase, indicates a positive allosteric interaction (Hage & Chen, 2006). Examples of such plots representing interactions above mentioned are shown in Figure 5.

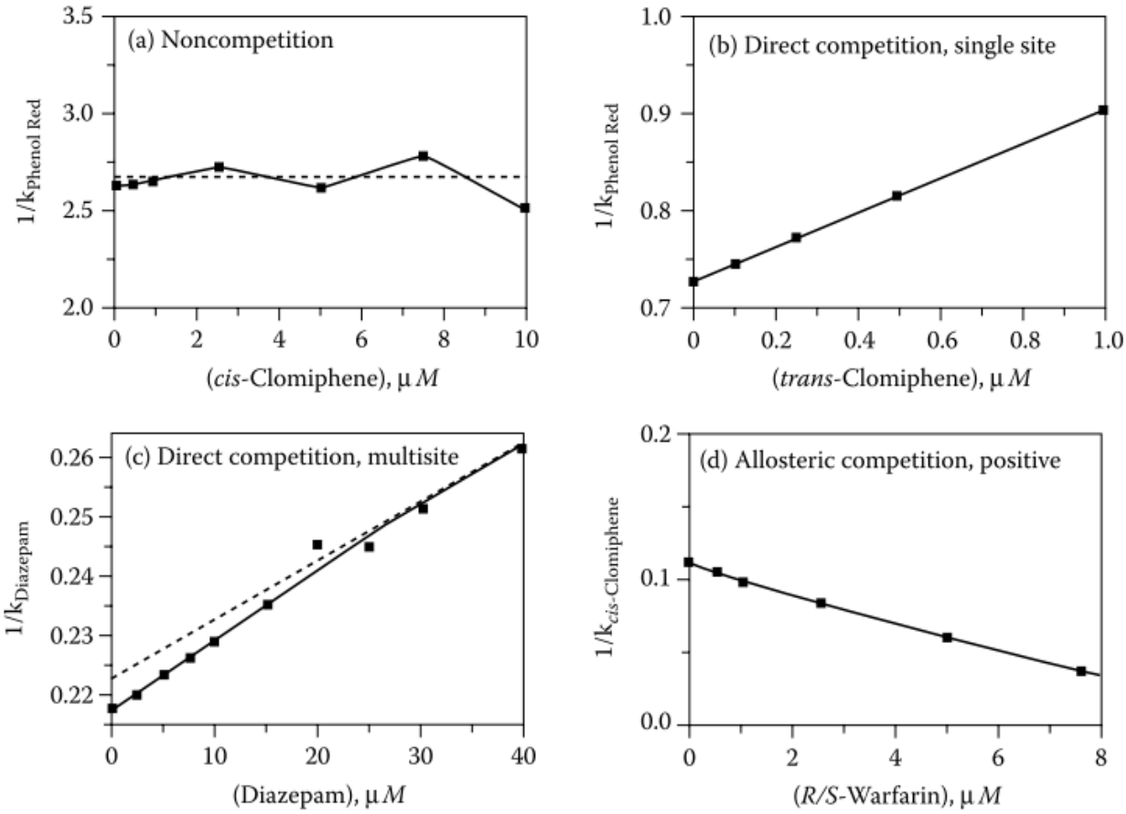


Fig. 5. Reciprocal plots prepared for analyte and competing agents with various types of competition on immobilized HSA columns . Reprinted from *Journal of Chromatography B*, Vol. 768, No. 1, Hage, D. S. (2002). High-performance affinity chromatography: a powerful tool for studying serum protein binding, pp. 3-30, Copyright 2011, with permission from Elsevier.

When the interaction is limited to a single binding site on the protein molecule, and analysed substances show no other interaction with the stationary phase, the Equation 6 describes the observed retention:

$$\frac{1}{k} = \frac{K_I V_M [I]}{K_a m_L} + \frac{V_M}{K_a m_L} \tag{6}$$

In this equation, the V_M determines the volume of eluted substances when they are not interacting with the ligand (the column dead volume) m_L describes the number of moles of binding sites for the analyte (A) and competitive agent (I), *[I]* the concentration of added competitive agent, and K_a and K_I binding equilibrium constants, respectively (Hage et al., 2009). In this case, the ratio of the slope of a graph of $1/k$ dependence on *[I]* to its intercept will determine the K_I. Determination of K_a requires separate measurement of the concentration of binding sites in the column (m_L / V_M). Similar approach can be used to define binding affinity of multisite and allosteric interaction by choosing the equation best describing the shape of the dependence $1/k$ on *[I]* (Hage & Chen, 2006; Joseph & David 2010). In case of simple allosteric interactions this Equation 7 will have the form (Chen & Hage, 2004):

$$\frac{k_0}{(k_0 - k)} = \frac{1}{(\beta_{I \to A} - 1)} \left(1 + \frac{1}{K_{IL}[I]} \right) \tag{7}$$

In this equation, k_0 defines the retention factor of an analyte without addition of compound I in the mobile phase, and the K_{IL} equilibrium binding constant of I with the immobilized protein. In the case of allosteric interactions between compound A and I, the presence of compound I leads to increase or decrease in the binding of substance A, which in turn changes the equilibrium binding constant (K_{AL} to K'_{AL}). This change is represented in above equation as $\beta_{A \to I}$, which is equivalent to the ratio of K'_{AL}/K_{AL}. If the analysed interaction fulfill the assumption described by above equation (7), the plot of $k_0/(k_0-k)$ against $1 / [I]$ should be linear. Intercept in this case is equivalent to $1/(\beta_{A \to I} - 1)$, and the slope is $1/[(\beta_{A \to I} - 1)K_{IL}]$. Based on these values it is possible to calculate the $\beta_{A \to I}$ and K_{IL}. The value of $\beta_{A \to I} > 1$ indicates a positive allosteric effect of compound I on binding of the analyte A, while $\beta_{A \to I} < 1$ indicates a negative allosteric interaction between these two substances. The value of $\beta_{A \to I}$ equal to zero suggests competitive interaction between I and A on the immobilized ligand, while the value of $\beta_{A \to I}$ equal to unity indicates the absence of any effect of compound I on the binding of the substance A (Chen & Hage, 2004). Therefore, it is important that the retention of injected analyte resulted solely from its interaction with the immobilized ligand, and not from the column overload. In order to confirm this, the retention time should remain unchanged at different initial concentration of injected sample. If not, the amount should be decreased until the retention time remains constant and/or increase the volume of the column. Also number of injections at different flow rates should be done to confirm that the processes of association/dissociation are fast enough (compared to how much time analyte spends in the column) to create inside the column local state of a binding equilibrium (Loun & Hage, 1995). Considerations on the factors that need to be pointed out using the zonal QAC are described in publications by prof. D. S. Hage (2002).

Competition and displacement technique allows to study drug-protein interactions occurring on a single binding site, which interacts with injected compound even though the drug is bound to several different sites on the protein. In this case, the analysed compound (drug) should be used as a competitive addition to the mobile phase, and as injected compound analyte with known, specific binding site on the protein. In addition, this approach allows to directly calculate the binding equilibrium constant of analysed drug

from the ratio of slope to intercept in plot $1/k$ against *[I]* (Loun & Hage, 1995; Chen et. al., 2004; Yoo et al., 2009, Mallik et al., 2008).

Knowing the location of specific binding site for a substance on the target protein and applying an analyte on the column in presence of it, becomes possible to define binding site of analysed drug (Yoo et al., 2009; Mallik et al., 2008). Carrying out series of this type of experiments we can draw a map of allosteric protein binding sites (Chen et al., 2004).

It is also possible to use the same compound as an injected analyte and a competitive agent (self-competition studies). In this case the Equation 8 describing single site competitive interaction will take the form (Hage, 2002):

$$\frac{1}{k} = \frac{V_M[A]}{m_L} + \frac{V_m}{K_a m_L} \tag{8}$$

Plotting the dependence of $1/k$ on *[A]* we can obtain information about the number of binding sites for the analyte. In case of a single site interactions it should be a linear relationship with a slope equal to m_L/V_M, and the ratio of the slope to intercept will determine K_a (Xuan & Hage, 2005).

As the zonal elution method, frontal chromatography can also be used in combination with competition-displacement technique. Increasing concentrations of the competitive ligand in the presence of constant concentration of a marker are added to the mobile phase and the effect on the breakthrough volumes of marker is measured. Decreasing breakthrough time of marker with increasing concentration of competing agent suggests direct competition between them. If positive or negative allosteric effect of displacement agent on binding reaction is occurring, shift to lower or higher marker breakthrough times should be observed. Using Equation 9 the relationship between displacer concentration *[D]* and marker retention volume can be used to determine the K_d value of the displacer as well as the number of active binding sites (B_t).

$$[D](V - V_0) = \frac{B_t[D]}{(K_d + [D])} \tag{9}$$

In above equation V is the retention volume of marker and V_0 is the retention volume of marker when the specific interaction is completely suppressed. From the plot of $[D](V\text{-}V_0)$ versus *[D]*, dissociation constant values for displacer ligand can be obtained (Moaddel & Wainer, 2006).

Chan and co-workers (2003) reported an alternative ''competitive'' assay format for K_d determination of tested ligands. FAC column is equilibrated with increasing concentrations of test ligand, with no washing between infusions. Each equilibration is bracketed by an infusion of the indicator (marker ligand detectable by MS) and void marker, and the adjusted breakthrough volume ($V\text{-}V_0$) of the indicator is monitored by mass spectrometry. At high indicator dilution relative to K_d of the particular interaction ($[A]_0$ is negligible compared with K_d), the breakthrough value is insensitive to slight changes in its concentration and achieves its maximum value (V_{limit}). In this mode the indicator is not competing with test ligand, but merely quantitating uncomplexed immobilized protein

(column capacity). This operational mode is very practical because a weak ligand (i.e., with high K_d) can be applied at modest concentrations and still function in the linear region of its binding isotherm. Weak ligands break through the column quickly and are easily washed out, thus providing a rapid probe of column capacity. This method is referred to as the indirect staircase approach for K_d determination (Chan te al., 2003).

The competition displacement methods are insensitive to non-specific binding since they are measuring a retention changes only due to allosteric or competitive interactions between drugs on a specific binding surface of target protein.

Aside from the utility of the FAC technique to provide accurate dissociation constants measurements for individual ligands, combination of frontal QAC with mass spectrometry allows rapid screening of mixtures of substances for their pharmacological activity, and the results show a high correlation with those obtained by traditional methods. FAC-MS screenings can be done with and without an indicator. In the second case detection is performed at selected *m/z* values to detect individual ligands and void marker. This allows to evaluate set of ligands in a single experiment (Ng et al., 2007).

In the indicator method, analysed compound is applied on the affinity column simultaneously with the indicator and the extent (or percentage) to which they shifts an indicator is determined. To compare the reductions in the breakthrough times for indicator in the presence of the ligands, the %shift is quantified from Equation 10. This FAC–MS readout can be used to rank the binding of a series of ligand or ligand mixtures: the greater the percentage shifts, the greater the degree of competition with the indicator. Mixtures in which a significant displacement (or shift) of the indicator is observed merit further investigation and deconvolution (Slon-Usakiewicz et al., 2004, as cited in Calleri et al., 2010).

$$\%shift = \frac{t_I - t}{t_I - t_{NSB}} \times 100 \tag{10}$$

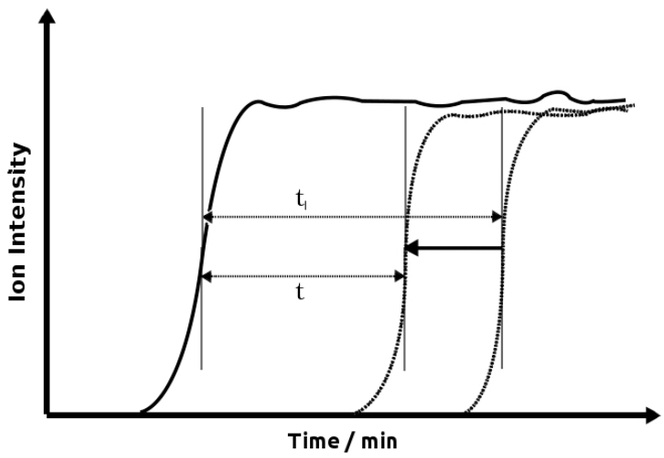


Fig. 6. Typical FAC curves obtained using the "indicator" screen method.

In above equation (10) t is the breakthrough time difference, measured at the inflection point, of the sigmoidal fronts between the indicator and a void marker in the presence of any competing ligand(s); t_{NSB} is the non-specific binding breakthrough time difference in the

absence of immobilized protein (and is a constant for the indicator used); and t_I is the breakthrough time difference in the absence of any competing ligands. Typical FAC curves obtained using the indicator screen method is shown in Figure 6.

For more detailed information about FAC-MS technique the readers are referenced to reviews (Chan et al., 2003; Slon-Usakiewicz et al., 2005; Calleri et al., 2009).

3.4 Nonlinear chromatography for determination of kinetics parameters

Peaks obtained in zonal AC differ from Gaussian shape observed frequently in classical chromatographic analyses. Because we have to deal with column overload and slow kinetics of adsorption/desorption during assays, peaks exhibit a strong tailing, which increases with increased concentration of an analyte. Observed asymmetry can arise from a variety of other factors-including extra column effects, heterogeneity of the stationary phase, heterogeneous mass transfer or a non-linear isotherm (Wade et al., 1987, as cited in Moaddel & Wainer, 2006). The degree of deviation from a Gaussian distribution is a function of applied ligand concentration and the kinetics of ligand–receptor interactions occurring during the chromatographic process. An example of the effect of solute concentration on peak shape is presented in Figure 7, which shows the example of usage of nonlinear chromatography (NLC) for determination of kinetic parameters for the α3β4 nAChR allosteric inhibitors (Jozwiak et al., 2002).

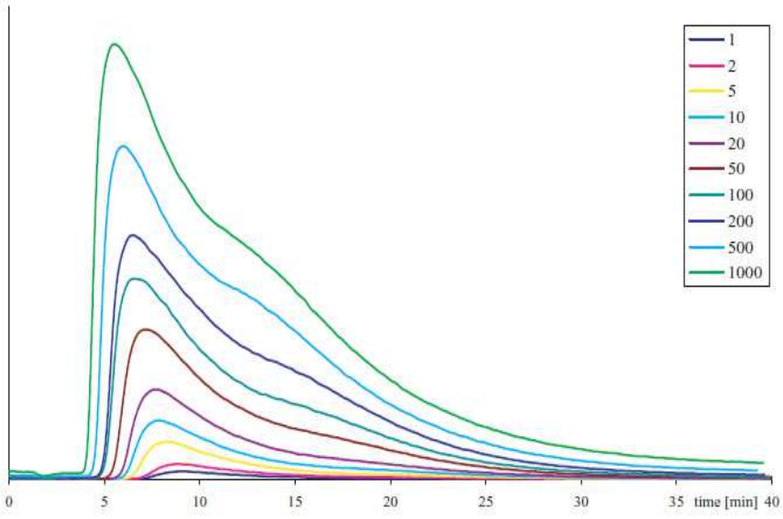


Fig. 7. The effect of increasing concentraions of mecamylamine, from 1 to 1,000 mM, on the chromatographic profiles of mecamylamine. For experimental details, see reference (Jozwiak et al., 2002).

While peak tailing (or fronting) is a problem in analytical separations, concentration-dependent asymmetry can be used with NLC techniques to characterize the separation processes occurring on the column. When the chromatographic process includes binding interactions between a ligand and an immobilized membrane bound receptor, the NLC

approach can be used to calculate the association rate constant (k_{on}), dissociation rate constant (k_{off}) for the ligand–receptor complex and the equilibrium constant for complex formation (K). One approach to the analysis of NLC data is the Impulse Input Solution for the mass balance equation developed in 1987 by Wade and collaborators (Wade et al., 1987, as cited in Moaddel & Wainer, 2006). This approach is based upon the observation that when adsorption/desorption rates are slow, band broadening is insensitive to a moderate degree of column overload. In contrast to numerical integration methods this approach uses the analytical solution, which can be applied directly to fit experimental peak profiles. The Impulse Input Equation has been included in commercially available deconvolution software, and can be easily applied to NLC studies. The peak area parameter (a_0), peak center parameter (a_1), its width (a_2) and distortion (a_3) are the parameters used to describe the chromatographic traces. Thermodynamic and kinetics parameters of a drug-receptor complex formation are then calculated on the basis of the relationship:

$k' = a_1$ the real thermodynamic capacity factor

$k_d = 1/a_2 t_0$ desorption reaction rate constant

$K_a = a_3/C_0$ adsorption equilibrium constants, where C_0 is the concentration of solute injected multiplied by the width of the injection pulse (as a fraction of column dead volume)

$k_a = k_d K_a$ reaction rate constant of adsorption

4. Examples of application

QAC was successfully used to describe drug interactions with multiple different system proteins. In case of soluble proteins this approach was applied to determine binding of different active substances (drugs, hormones) with e.g. serum albumins (HSA), α1-acid glycoprotein (AGP) or nucleic estrogen receptors (hER).

Xuan & Hage (2005) conducted research on immobilized α1-acid glycoprotein (AGP) demonstrating good correlation (0.954) of observed retention factors of several compounds with their equilibrium binding constants to AGP designated by other methods. In the same work authors, using the self-competition technique, confirmed the literature data on existence of one binding site for propranolol enantiomers on this protein. Determined values of K_a were also included in the range of values known from other experiments. Performing the analysis at different temperatures, changes in enthalpy and entropy of propranolol enantiomers binding to α1-acid glycoprotein, and their contribution in the total energy change in the process of binding were determined. It was found, that this reaction depends mainly on the enthalpy, but entropy change also significantly affects the binding of propranolol. Linearity of the plot confirmed the single-side character of binding of this compound by the AGP.

Combination of zonal QAC with competition-displacement studies confirmed the negative allosteric nature of the interaction between verapamil and tamoxifen. It was determined that verapamil causes 41% decrease in binding constant of tamoxifen by immobilized HSA (Malik et al., 2008). As mentioned earlier, using compound of interest as a competitive addition to the mobile phase it is possible to define its interactions with target protein on a single binding site even though the drug is bound to several different sites on the protein.

Such solution has been used in the study of binding of both; hormones (Loun & Hage, 1995) and drugs (Chen et. al., 2004; Yoo et al., 2009; Mallik et al., 2008) to different places in the HSA molecule. Simultaneous injection of imipramine with L-tryptophan on the column with immobilized HSA, showed a competitive interaction between those two drugs, confirming that imipramine specifically connects to the indole-benzodiazepine binding site on the molecule of albumin (Sudlow site II). This analysis also allowed to determine the equilibrium constant of this interaction (Yoo et al., 2009). Analogously was defined binding site for the verapamil on this protein, demonstrating in this case, competitive interaction with warfarin, specifically connecting to Sudlow site I. This was confirmed by elution in the presence of tamoxifen. Nonlinear dependence of retention of this compound on verapamil concentration showed a high correlation to the equation describing the allosteric interaction, characteristic for interaction that is observed between the tamoxifen binding site and the warfarin binding site (Sudlow I) (Mallik et al., 2008). Team of Chen and colleagues (2004) determined the nature of the interaction of phenytoin by drawing a map of interactions of the drug with the major binding sites on the surface of HSA on the basis of the relationship between $1/k$ of the compounds with confirmed specific binding site on albumin molecule, and various concentrations of phenytoin as a mobile phase additive during elution. Thus suggested the potential impact of other concomitant medications on the efficacy of therapy with phenytoin.

The effect of different assay conditions on drug-protein interaction was used, for example to define the influence of long-chain fatty acids concentration in the plasma and glycation of plasma transport proteins on the binding of sulfonylurea drugs in diabetes. Experiments were carried out using columns with immobilized HSA (Basiaga & Hage, 2010).

Recently Sanghvi and co-workers (2010) successfully immobilized the ligand binding domains of the estrogen related receptors ERRα and ERRγ onto the aminopropyl silica liquid chromatography stationary phase, as well as the surface of the open tubular capillaries, creating the ERR-silica and ERR-OT columns. Both types were characterized using frontal chromatographic techniques with diethylstibesterol and the binding affinities, expressed as K_d values, to the immobilized receptors were consistent with the literature data. Biochanin A, the ERRα agonist, was also used to further characterization of properties of the ERRα-silica column, and obtained K_d value was consistent with the previously reported data. The ERRγ-silica column was characterized using nonlinear chromatographic techniques using a series of tamoxifen derivatives (tamoxifen, 4-hydroxy tamoxifen and N-desmethyl 4-hydroxytamoxifen, Endoxifen). The relative K_d values obtained for the derivatives were consistent with relative ability of the compounds to inhibit the cellular proliferation of the human-derived T98G glioma cell line, expressed as IC_{50} values. The results indicate that the relative retention of compounds on these columns reflects the magnitude of their inhibitory activity. Therefore columns containing immobilized ERRs can be used for a preliminary screen for anti-glioma agents, such as tamoxifen, which work as selective estrogen receptor modulator and that this method may replace current laborious and time consuming cellular uptake studies.

QAC has been applied to study drug-receptor interactions in the α1 adrenergic receptor system. The results showed a positive correlation of chromatographic data (k') with literature data defining the affinity of ligands on the basis of radio ligand studies with cell membrane homogenates containing α1 adrenergic receptor (Yu et al., 2005).

QAC was also used in determination of binding site structure of the α3β4 nAChR. Comparing the retention time of tritium labelled epibatidine ([^{3}H] EB) on four different columns: with immobilized either receptor subunit α3 or β4 subunit, both subunits immobilized on the same column and complete pentameric α3β4 receptor, it was found that the binding site for this compound is formed by several subunits of the complete receptor, and not on the individual subunits. This was confirmed by assays with nicotine as a competitive additive to the mobile phase. No impact of nicotine on the retention of [^{3}H] EB on columns with immobilized subunits was observed, while the epibatidine retention on the column with complete receptor decreased from k = 8.4 to k = 1.5, which indicates the interaction of nicotine on [^{3}H] EB binding site (Wainer et al., 1999). In the same work the effect of pH and ionic strength of mobile phase on the retention of [^{3}H] epibatidine in the α3β4 nAChR column was also examined using zonal elution technique. Analyses showed an increase in epibatidine retention with increasing mobile phase pH from pH 4 to pH 7 and a significant decrease in the retention of this compound with increasing ionic strength of mobile phase (5-200 mM ammonium acetate concentration). These results showed that the binding of competitive agonists is mainly electrostatic interaction between drug molecule and the binding site on the nAChR.

QAC in combination with displacement studies were used in case of α3β4 nAChR to confirm the location of binding sites of allosteric inhibitors of this receptor. Elution of bupropion, ketamine and dextromethorphan was conducted in the presence of mecamylamine (nAChR blocker) as an addition to the mobile phase. The linear dependence of eluted compounds $1/k'$ on the displacer concentration indicates that all tested inhibitors compete for the same binding site on the receptor molecule. Additional studies also showed no effect of mecamylamine in the mobile phase on epibatidine retention and no effect of nicotine in the mobile phase on the retention of ketamine and bupropion. It indicates that ketamine, bupropion, mecamylamine bind to other sites on the receptor surface than nicotine and epibatidine and do not compete for the binding site (Jozwiak et al., 2002). In the same work nonlinear chromatography (NLC) model was used to analyse interactions between the immobilized α3β4 nAChR, and its allosteric inhibitors. Peak profiles from zonal elution of allosteric inhibitors (ketamine, bupropion, mecamylamine, and dextrometorfan), recorded by the mass spectrometry were numerically fitted to Impulse Input Solution model using PeakFit v4.11 software (SPSS Inc., Chicago, IL). The results (k_{on}, k_{off}, K) were consistent with available literature data, thus confirming the effectiveness of the NLC in receptor drug binding kinetics study, in this case allosteric nAChR inhibitors. In further work enantioselectivity of the interaction of dextromethorphan (DM) and levomethorphan (LM) with an immobilized α3β4 nAChR subtype liquid chromatographic stationary phase has been compared to DM- and LM-induced non-competitive blockade of nicotine-stimulated ^{86}Rb+ efflux from cells expressing the α3β4 nAChR. Since DM and LM are enantiomers and have the same physicochemical properties, any chromatographic and pharmacological differences must be due to specific interactions with nAChR. Asymmetrical peaks were observed for both compounds and DM had significantly longer retention time than LM (Figure 8). Determined by NLC k_{on} values of both compounds did not significantly differ while k_{off} of DM was significantly lower than k_{off} value of LM. That was in good agreement with results of the functional inhibition studies which showed that DM and LM had equivalent potencies, i.e. the same IC_{50} values, but that DM inhibition lasted longer than the effect produced by LM. The effect of temperature on the chromatographic retention of DM

and LM on the nAChR column was also determined using a sequence of temperature experiments ranging from 5 to 30°C. Respective *k'*s values of independently injected compound were determined and van't Hoff plots were constructed by plotting ln *k* versus 1/*T*. Results showed that the binding reaction is enthalpy driven. In addition, for DM and LM there was no significant difference in the $\Delta S°$ values, while the $\Delta H°$ value for the DM-nAChR interaction was significantly lower than respective value for LM-nAChR interaction showing that the enantioselectivity of α3β4 nAChR is mostly enthalpy-based (Jozwiak et al., 2003).The results of those studies demonstrated that non-linear chromatography approach of investigations of immobilized nAChRs can be useful in characterizing ligand–receptor binding interactions and predicting of properties of drugs and drug candidates. Additional NLC studies with this system were used to construct QSAR models of non-competitive inhibitors of the nAChR (Jozwiak et al., 2004), to develop a molecular model of these interactions (Jozwiak et al., 2004) and to predict IC_{50} values (Jozwiak et al., 2005)

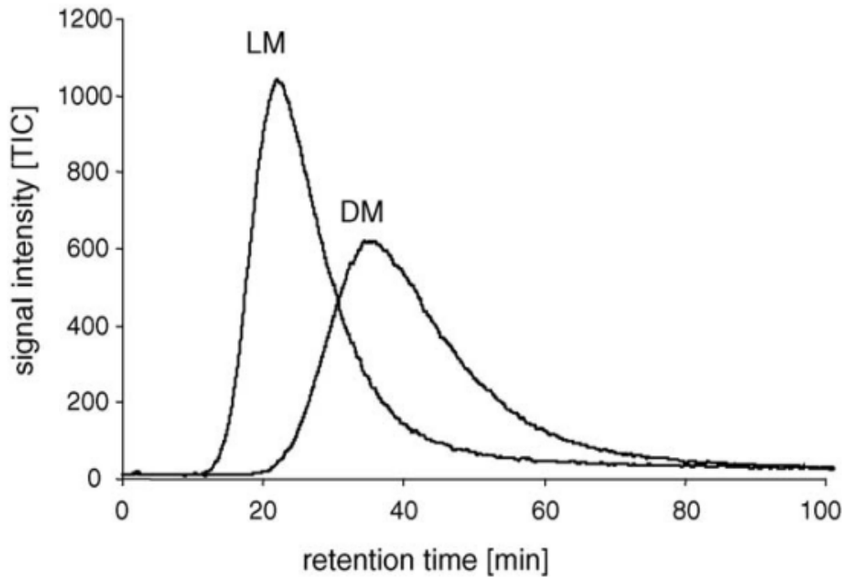


Fig. 8. The comparison of peak profiles of DM and LM obtained in independent experiments of consequent injections. Reprinted from *Journal of Chromatography B*, Vol. 797, No. 1-2, Jozwiak, K., Hernandez, S. C., Kellar, K. J., & Wainer, I. W. (2003). Enantioselective interactions of dextromethorphan and levomethorphan with the α3β4-nicotinic acetylcholine receptor: comparison of chromatographic and functional data, pp. 373-379, Copyright 2011, with permission from Elsevier

Competition displacement studies with [^{3}H]epibatidine and [^{125}I]α-bungarotoxin as marker radioligands specific for α3β4 and α7 nAChRs subtypes respectively were applied to determine K_i values and check subtype selectivity of newly synthesized derivatives of epiboxidine, synthetic epibatidine-related compounds (Rizzi et al., 2008).

Another interesting application of QAC is use of immobilized-enzyme reactors (IMERs). In medicinal chemistry research IMERs are applied to drug metabolism studies, enantioselective analyses and for the identification of substrates and inhibitors as potential drugs. Interestingly, the investigated enzymatic reaction took place directly on the column.

Attractive features of immobilized enzyme reactors are the increased enzyme stability and the reusability coupled to accuracy, automation and potential high throughput when they are inserted in a HPLC system (Bartolini et al., 2005). The approach requires neither highly purified enzyme nor use of labelled substrates (radio- or color labelled). In immobilized enzymes, inhibitors efficacies can be measured either as IC_{50} values or K_i values using numerical transformation. Enzyme kinetic parameters are determined by successive injection of a substrate at increasing concentrations and measuring the rate of enzymatic reaction (V) expressed as peak area of product formed after each injection. Fitting the data to Lineweaver–Burk double-reciprocal plot of $1/(V)$ against the substrate concentration *[S]* (what is a linear transformation of the Michaelis–Menten plot), K_m and V_{max} values can be obtained. The y-intercept of such graph is equivalent to the inverse of $1/V_{max}$, the x-intercept of the graph represents $-1/K_m$. In order to obtain correct results the concentration of substrate should be normalized according to the formula 11.

$$[S] = \frac{C_{inj} \times V_{inj}}{BV} \tag{11}$$

where C_{inj} is the injected substrate concentration, V_{inj} is the injected volume and BV is the bed volume of the IMER.

To determine the inhibition constant (K_i) for a test drug a set of inhibitor injections in several different concentrations ($[I]$) at two or more concentration of a substrate should be performed. As noted by Dixon in 1953, if $1/V$ is plotted against inhibitor concentration *[I]*, a straight line plot is obtained for each substrate concentration *[S]*. The *[I]* value of a intersect of those lines is equal to $-K_i$. If curves obtained for several different *[S]* converge in the left upper quadrant of a chart, the inhibitor is competitive. If curves converge on the *[I]* axis, the inhibitor is non-competitive. For not competitive inhibition, the lines are parallel.

Simultaneous injections of both a substrate at a fixed saturating concentration and increasing concentration of an inhibitor, result in increasing reduction of the peak area (A_i) in comparison to area obtained for a substrate alone (A_0). The percent inhibition ($100 - (A_i/A_0 \times 100)$) is then plotted against the inhibitor concentration to obtain the inhibition curves (Girelli & Mattei, 2005; Nie & Wang, 2009). Recently this technique has been used for the kinetic characterization of inhibitors specific to brain-targeted butyrylcholinesterase (BuChE) (Bartolini et al., 2009), acetylocholinesterase (AchE) (Bartolini et al., 2004; Bartolini et al., 2005; He et al., 2010) and β-secretase (human recombinant β-amyloid precursor protein cleaving enzyme, hrBACE1)(Mancini & Andrisano, 2010) as potential therapeutics for Alzheimer's disease. IMER was also used for rapid and cost-effective on-line chromatographic screening of matrix metalloproteinases (MMP-9 (Ma & Chun Yong Chan, 2010) and MMP-8 (Mazzini et al., 2011)) inhibitors that may be useful in cancer therapy and for determining the role of some derived plant products for treating NO-dependent smooth disorders using monolithic micro-IMER with covalently bounded arginase (André et al., 2011).

5. Conclusions

The drive to bring innovative drugs to market faster, without compromising quality and safety, induced need for new experimental techniques and methodologies. It is crucial to

determine biological activity, drug-target interactions and physico-chemical properties of drug candidates as predictors of administration, distribution, metabolism, excretion (ADME) characteristics. In fact, development of rapid methodologies enabling to obtain those information is a key aspects of the drug discovery process. QAC as rapid, relatively simple technique with the possibility of automation proved to be useful alternative to conventional methods in the field of drug discovery and analysis. This method is facilitated by multiple use of the same column with the immobilized target and as a consequence the reproducibility of assays is increased. Thanks to the above features QAC becomes popular method of measuring binding affinity of the drug-protein interactions. Variety of data that we can obtain via this technique allows characterization of binding reactions as well as description of the binding site. The development of techniques of high-yielding synthesis increases demand for technology that would allow pharmaceutical companies for efficient and rapid biological screening of thousands of synthesized compounds (Renaud & Delsuc, 2009). Taking into account that approximately one drug is produced after 8000-10000 compounds were subjected to primary and secondary drug screens, the classical approach, one compound-one assay, becomes unsatisfactory (Caldwell, 2000; as cited in Nie & Wang, 2009) . Studies conducted by Ng and colleagues have shown that use of an automated high performance chromatographic system consisting of two affinity columns and a mass spectrometer as the detector, allow to analyse and rank activity of 10 000 compounds in just 24 hours (Ng et al., 2007). Thus QAC techniques seem to be an promising method of preliminary verification of drug candidates, which is an alternative to expensive and tedious in vitro assays.

6. References

Andre, C., Herlem, G., Gharbi, T., & Guillaume, Y. C. (2011). A new arginase enzymatic reactor: development and application for the research of plant-derived inhibitors. *J Pharm Biomed Anal,* Vol. 55, No. 1, pp. 48-53, ISSN 1873-264X (Electronic)0731-7085 (Linking)

Bartolini, M., Cavrini, V., & Andrisano, V. (2004). Monolithic micro-immobilized-enzyme reactor with human recombinant acetylcholinesterase for on-line inhibition studies. *J Chromatogr A,* Vol. 1031, No. 1-2, pp. 27-34, ISSN 0021-9673 (Print)0021-9673 (Linking)

Bartolini, M., Cavrini, V., & Andrisano, V. (2005). Choosing the right chromatographic support in making a new acetylcholinesterase-micro-immobilised enzyme reactor for drug discovery. *J Chromatogr A,* Vol. 1065, No. 1, pp. 135-144, ISSN 0021-9673 (Print)0021-9673 (Linking)

Bartolini, M., Greig, N. H., Yu, Q. S., & Andrisano, V. (2009). Immobilized butyrylcholinesterase in the characterization of new inhibitors that could ease Alzheimer's disease. *J Chromatogr A,* Vol. 1216, No. 13, pp. 2730-2738, ISSN 1873-3778 (Electronic)0021-9673 (Linking)

Basiaga, S. B., & Hage, D. S. (2010). Chromatographic studies of changes in binding of sulfonylurea drugs to human serum albumin due to glycation and fatty acids. *J Chromatogr B Analyt Technol Biomed Life Sci,* Vol. 878, No. 30, pp. 3193-3197, ISSN 1873-376X (Electronic)1570-0232 (Linking)

Beigi, F., & Wainer, I. W. (2003). Syntheses of immobilized G protein-coupled receptor chromatographic stationary phases: characterization of immobilized mu and kappa opioid receptors. *Anal Chem,* Vol. 75, No. 17, pp. 4480-4485, ISSN 0003-2700 (Print)0003-2700 (Linking)

Beigi, F., Chakir, K., Xiao, R. P., & Wainer, I. W. (2004). G-protein-coupled receptor chromatographic stationary phases. 2. Ligand-induced conformational mobility in an immobilized beta2-adrenergic receptor. *Anal Chem,* Vol. 76, No. 24, pp. 7187-7193, ISSN 0003-2700 (Print)0003-2700 (Linking)

Calleri, E., Temporini, C., & Massolini, G. (2011). Frontal affinity chromatography in characterizing immobilized receptors. *J Pharm Biomed Anal,* Vol. 54, No. 5, pp. 911-925, ISSN 1873-264X (Electronic)0731-7085 (Linking)

Calleri, E., Temporini, C., Caccialanza, G., & Massolini, G. (2009). Target-based drug discovery: the emerging success of frontal affinity chromatography coupled to mass spectrometry. *ChemMedChem,* Vol. 4, No. 6, pp. 905-916, ISSN 1860-7187 (Electronic)1860-7179 (Linking)

Chan, N. W., Lewis, D. F., Rosner, P. J., Kelly, M. A., & Schriemer, D. C. (2003). Frontal affinity chromatography-mass spectrometry assay technology for multiple stages of drug discovery: applications of a chromatographic biosensor. *Anal Biochem,* Vol. 319, No. 1, pp. 1-12, ISSN 0003-2697 (Print)0003-2697 (Linking)

Chen, J., & Hage, D. S. (2004). Quantitative analysis of allosteric drug-protein binding by biointeraction chromatography. *Nat Biotechnol,* Vol. 22, No. 11, pp. 1445-1448, ISSN 1087-0156 (Print)1087-0156 (Linking)

Chen, J., Ohnmacht, C., & Hage, D. S. (2004). Studies of phenytoin binding to human serum albumin by high-performance affinity chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci,* Vol. 809, No. 1, pp. 137-145, ISSN 1570-0232 (Print)1570-0232 (Linking)

Cheng, Y., Ho, E., Subramanyam, B., & Tseng, J. L. (2004). Measurements of drug-protein binding by using immobilized human serum albumin liquid chromatography-mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci,* Vol. 809, No. 1, pp. 67-73, ISSN 1570-0232 (Print)1570-0232 (Linking)

Dixon, M. (1953). The determination of enzyme inhibitor constants. *Biochem J,* Vol. 55, No. 1, pp. 170-171, ISSN 0264-6021 (Print)0264-6021 (Linking)

Girelli, A. M., & Mattei, E. (2005). Application of immobilized enzyme reactor in on-line high performance liquid chromatography: a review. *J Chromatogr B Analyt Technol Biomed Life Sci,* Vol. 819, No. 1, pp. 3-16, ISSN 1570-0232 (Print)1570-0232 (Linking)

Gottschalk, I., Li, Y. M., & Lundahl, P. (2000). Chromatography on cells: analyses of solute interactions with the glucose transporter Glut1 in human red cells adsorbed on lectin-gel beads. *J Chromatogr B Biomed Sci Appl,* Vol. 739, No. 1, pp. 55-62, ISSN 1387-2273 (Print)1387-2273 (Linking)

Gottschalk, I., Lundqvist, A., Zeng, C. M., Hagglund, C. L., Zuo, S. S., Brekkan, E., Eaker, D., & Lundahl, P. (2000). Conversion between two cytochalasin B-binding states of the human GLUT1 glucose transporter. *Eur J Biochem,* Vol. 267, No. 23, pp. 6875-6882, ISSN 0014-2956 (Print)0014-2956 (Linking)

Gritti, F., & Guiochon, G. (2005). Critical contribution of nonlinear chromatography to the understanding of retention mechanism in reversed-phase liquid chromatography. *J Chromatogr A,* Vol. 1099, No. 1-2, pp. 1-42, ISSN 0021-9673 (Print)0021-9673 (Linking)

Guiffant, D., Tribouillard, D., Gug, F., Galons, H., Meijer, L., Blondel, M., & Bach, S. (2007). Identification of intracellular targets of small molecular weight chemical compounds using affinity chromatography. *Biotechnol J,* Vol. 2, No. 1, pp. 68-75, ISSN 1860-7314 (Electronic)1860-6768 (Linking)

Hage, D. S. (2002). High-performance affinity chromatography: a powerful tool for studying serum protein binding. *J Chromatogr B Analyt Technol Biomed Life Sci,* Vol. 768, No. 1, pp. 3-30, ISSN 1570-0232 (Print)1570-0232 (Linking)

Hage, D. S., Jackson, A., Sobansky, M. R., Schiel, J. E., Yoo, M. J., & Joseph, K. S. (2009). Characterization of drug-protein interactions in blood using high-performance affinity chromatography. *J Sep Sci,* Vol. 32, No. 5-6, pp. 835-853, ISSN 1615-9314 (Electronic)1615-9306 (Linking)

Hage, D. S., Ruhn, P. F. (2006). An Introduction to Affinity Chromatography, In: *Handbook of Affinity Chromatography,* Hage, D.S., pp. 3-15, CRC Press/Taylor&Francis, ISBN 978-0-8247-4057-3, Boca Raton

Hage, D.S. (Ed.). (2006). *Handbook of Affinity Chromatography,* CRC Press/Taylor&Francis, ISBN 978-0-8247-4057-3, Boca Raton, FL

Hage, D.S., Chen, J. (2006). Quantitative Affinity Chromatography: Practical Aspect, In: *Handbook of Affinity Chromatography,* Hage, D.S., pp. 595-628, CRC Press/Taylor&Francis, ISBN 978-0-8247-4057-3, Boca Raton

He, P., Davies, J., Greenway, G., & Haswell, S. J. (2010). Measurement of acetylcholinesterase inhibition using bienzymes immobilized monolith micro-reactor with integrated electrochemical detection. *Anal Chim Acta,* Vol. 659, No. 1-2, pp. 9-14, ISSN 1873-4324 (Electronic)0003-2670 (Linking)

Joseph, K. S., & Hage, D. S. (2010). Characterization of the binding of sulfonylurea drugs to HSA by high-performance affinity chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci,* Vol. 878, No. 19, pp. 1590-1598, ISSN 1873-376X (Electronic)1570-0232 (Linking)

Josic, D., & Buchacher, A. (2001). Application of monoliths as supports for affinity chromatography and fast enzymatic conversion. *J Biochem Biophys Methods,* Vol. 49, No. 1-3, pp. 153-174, ISSN 0165-022X (Print)0165-022X (Linking)

Jozwiak, K., Haginaka, J., Moaddel, R., & Wainer, I. W. (2002). Displacement and nonlinear chromatographic techniques in the investigation of interaction of noncompetitive inhibitors with an immobilized alpha3beta4 nicotinic acetylcholine receptor liquid chromatographic stationary phase. *Anal Chem,* Vol. 74, No. 18, pp. 4618-4624, ISSN 0003-2700 (Print)0003-2700 (Linking)

Jozwiak, K., Hernandez, S. C., Kellar, K. J., & Wainer, I. W. (2003). Enantioselective interactions of dextromethorphan and levomethorphan with the alpha 3 beta 4-nicotinic acetylcholine receptor: comparison of chromatographic and functional data. *J Chromatogr B Analyt Technol Biomed Life Sci,* Vol. 797, No. 1-2, pp. 373-379, ISSN 1570-0232 (Print)1570-0232 (Linking)

Jozwiak, K., Moaddel, R., Yamaguchi, R., Ravichandran, S., Collins, J. R., & Wainer, I. W. (2005). Qualitative assessment of IC50 values of inhibitors of the neuronal nicotinic acetylcholine receptor using a single chromatographic experiment and multivariate cluster analysis. *J Chromatogr B Analyt Technol Biomed Life Sci,* Vol. 819, No. 1, pp. 169-174, ISSN 1570-0232 (Print)1570-0232 (Linking)

Jozwiak, K., Ravichandran, S., Collins, J. R., & Wainer, I. W. (2004). Interaction of noncompetitive inhibitors with an immobilized alpha3beta4 nicotinic acetylcholine receptor investigated by affinity chromatography, quantitative-structure activity relationship analysis, and molecular docking. *J Med Chem,* Vol. 47, No. 16, pp. 4008-4021, ISSN 0022-2623 (Print)0022-2623 (Linking)

Kasai, K., & Ishii, S. (1975). Quantitative analysis of affinity chromatography of trypsin. A new technique for investigation of protein-ligand interaction. *J Biochem,* Vol. 77, No. 1?, pp. 261-264, ISSN 0021-924X (Print)0021-924X (Linking)

Kim, H. S., & Wainer, I. W. (2008). Rapid analysis of the interactions between drugs and human serum albumin (HSA) using high-performance affinity chromatography (HPAC). *J Chromatogr B Analyt Technol Biomed Life Sci,* Vol. 870, No. 1, pp. 22-26, ISSN 1570-0232 (Print)1570-0232 (Linking)

Kim, H.S., Hage, D.S., Immobilization methods for affinity chromatography, In: *Handbook of Affinity Chromatography,* Hage, D.S., pp. 35-79, CRC Press/Taylor&Francis, ISBN 978-0-8247-4057-3, Boca Raton

Kimura, T., Perry, J., Anzai, N., Pritchard, J. B., & Moaddel, R. (2007). Development and characterization of immobilized human organic anion transporter-based liquid chromatographic stationary phase: hOAT1 and hOAT2. *J Chromatogr B Analyt Technol Biomed Life Sci,* Vol. 859, No. 2, pp. 267-271, ISSN 1570-0232 (Print)1570-0232 (Linking)

Kitabatake, T., Moaddel, R., Cole, R., Gandhari, M., Frazier, C., Hartenstein, J., Rosenberg, A., Bernier, M., & Wainer, I. W. (2008). Characterization of a multiple ligand-gated ion channel cellular membrane affinity chromatography column and identification of endogenously expressed receptors in astrocytoma cell lines. *Anal Chem,* Vol. 80, No. 22, pp. 8673-8680, ISSN 1520-6882 (Electronic)0003-2700 (Linking)

Landry, Y., & Gies, J. P. (2008). Drugs and their molecular targets: an updated overview. *Fundam Clin Pharmacol,* Vol. 22, No. 1, pp. 1-18, ISSN 1472-8206 (Electronic)0767-3981 (Linking)

Lebert, J. M., Forsberg, E. M., & Brennan, J. D. (2008). Solid-phase assays for small molecule screening using sol-gel entrapped proteins. *Biochem Cell Biol,* Vol. 86, No. 2, pp. 100-110, ISSN 0829-8211 (Print)0829-8211 (Linking)

Loun, B., & Hage, D. S. (1992). Characterization of thyroxine-albumin binding using high-performance affinity chromatography. I. Interactions at the warfarin and indole sites of albumin. *J Chromatogr,* Vol. 579, No. 2, pp. 225-235, ISSN 0021-9673 (Print)0021-9673 (Linking)

Loun, B., & Hage, D. S. (1994). Chiral separation mechanisms in protein-based HPLC columns. 1. Thermodynamic studies of (R)- and (S)-warfarin binding to immobilized human serum albumin. *Anal Chem,* Vol. 66, No. 21, pp. 3814-3822, ISSN 0003-2700 (Print)0003-2700 (Linking)

Loun, B., & Hage, D. S. (1995). Characterization of thyroxine-albumin binding using high-performance affinity chromatography. II. Comparison of the binding of thyroxine, triiodothyronines and related compounds at the warfarin and indole sites of human serum albumin. *J Chromatogr B Biomed Appl,* Vol. 665, No. 2, pp. 303-314, ISSN 1572-6495 (Print)1572-6495 (Linking)

Lundqvist, A., Ocklind, G., Haneskog, L., & Lundahl, P. (1998). Freeze-thaw immobilization of liposomes in chromatographic gel beads: evaluation by confocal microscopy and effects of freezing rate. *J Mol Recognit,* Vol. 11, No. 1-6, pp. 52-57, ISSN 0952-3499 (Print)0952-3499 (Linking)

Ma, X., & Chan, E. C. (2010). On-line chromatographic screening of matrix metalloproteinase inhibitors using immobilized MMP-9 enzyme reactor. *J Chromatogr B Analyt Technol Biomed Life Sci,* Vol. 878, No. 21, pp. 1777-1783, ISSN 1873-376X (Electronic)1570-0232 (Linking)

Mallik, R., Yoo, M. J., Chen, S., & Hage, D. S. (2008). Studies of verapamil binding to human serum albumin by high-performance affinity chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci,* Vol. 876, No. 1, pp. 69-75, ISSN 1570-0232 (Print)1570-0232 (Linking)

Mancini, F., & Andrisano, V. (2010). Development of a liquid chromatographic system with fluorescent detection for beta-secretase immobilized enzyme reactor on-line enzymatic studies. *J Pharm Biomed Anal,* Vol. 52, No. 3, pp. 355-361, ISSN 1873-264X (Electronic)0731-7085 (Linking)

Markuszewski, M., & Kaliszan, R. (2002). Quantitative structure-retention relationships in affinity high-performance liquid chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci,* Vol. 768, No. 1, pp. 55-66, ISSN 1570-0232 (Print)1570-0232 (Linking)

Mateo, C., Fernandez-Lorente, G., Abian, O., Fernandez-Lafuente, R., & Guisan, J. M. (2000). Multifunctional epoxy supports: a new tool to improve the covalent immobilization of proteins. The promotion of physical adsorptions of proteins on the supports before their covalent linkage. *Biomacromolecules,* Vol. 1, No. 4, pp. 739-745, ISSN 1525-7797 (Print)1525-7797 (Linking)

Mazzini, F., Nuti, E., Petri, A., & Rossello, A. (2011). Immobilization of matrix metalloproteinase 8 (MMP-8) for online drug screening. *J Chromatogr B Analyt Technol Biomed Life Sci,* Vol. 879, No. 11-12, pp. 756-762, ISSN 1873-376X (Electronic)1570-0232 (Linking)

Moaddel, R., & Wainer, I. W. (2006). Development of immobilized membrane-based affinity columns for use in the online characterization of membrane bound proteins and for targeted affinity isolations. *Anal Chim Acta,* Vol. 564, No. 1, pp. 97-105, ISSN 1873-4324 (Electronic)0003-2670 (Linking)

Moaddel, R., Bullock, P. L., & Wainer, I. W. (2004). Development and characterization of an open tubular column containing immobilized P-glycoprotein for rapid on-line screening for P-glycoprotein substrates. *J Chromatogr B Analyt Technol Biomed Life Sci,* Vol. 799, No. 2, pp. 255-263, ISSN 1570-0232 (Print)1570-0232 (Linking)

Moaddel, R., Calleri, E., Massolini, G., Frazier, C. R., & Wainer, I. W. (2007). The synthesis and initial characterization of an immobilized purinergic receptor (P2Y1) liquid

chromatography stationary phase for online screening. *Anal Biochem,* Vol. 364, No. 2, pp. 216-218, ISSN 0003-2697 (Print)0003-2697 (Linking)

Moaddel, R., Cloix, J. F., Ertem, G., & Wainer, I. W. (2002). Multiple receptor liquid chromatographic stationary phases: the co-immobilization of nicotinic receptors, gamma-amino-butyric acid receptors, and N-methyl D-aspartate receptors. *Pharm Res,* Vol. 19, No. 1, pp. 104-107, ISSN 0724-8741 (Print)0724-8741 (Linking)

Moaddel, R., Jozwiak, K., Whittington, K., & Wainer, I. W. (2005). Conformational mobility of immobilized alpha3beta2, alpha3beta4, alpha4beta2, and alpha4beta4 nicotinic acetylcholine receptors. *Anal Chem,* Vol. 77, No. 3, pp. 895-901, ISSN 0003-2700 (Print)0003-2700 (Linking)

Moaddel, R., Lu, L., Baynham, M., & Wainer, I. W. (2002). Immobilized receptor- and transporter-based liquid chromatographic phases for on-line pharmacological and biochemical studies: a mini-review. *J Chromatogr B Analyt Technol Biomed Life Sci,* Vol. 768, No. 1, pp. 41-53, ISSN 1570-0232 (Print)1570-0232 (Linking)

Moaddel, R., Musyimi, H. K., Sanghvi, M., Bashore, C., Frazier, C. R., Khadeer, M., Bhatia, P., & Wainer, I. W. (2010). Synthesis and characterization of a cellular membrane affinity chromatography column containing histamine 1 and P2Y(1) receptors: a multiple G-protein coupled receptor column. *J Pharm Biomed Anal,* Vol. 52, No. 3, pp. 416-419, ISSN 1873-264X (Electronic)0731-7085 (Linking)

Moaddel, R., Oliveira, R. V., Kimura, T., Hyppolite, P., Juhaszova, M., Xiao, Y., Kellar, K. J., Bernier, M., & Wainer, I. W. (2008). Initial synthesis and characterization of an alpha7 nicotinic receptor cellular membrane affinity chromatography column: effect of receptor subtype and cell type. *Anal Chem,* Vol. 80, No. 1, pp. 48-54, ISSN 0003-2700 (Print)0003-2700 (Linking)

Moaddel, R., Rosenberg, A., Spelman, K., Frazier, J., Frazier, C., Nocerino, S., Brizzi, A., Mugnaini, C., & Wainer, I. W. (2011). Development and characterization of immobilized cannabinoid receptor (CB1/CB2) open tubular column for on-line screening. *Anal Biochem,* Vol. 412, No. 1, pp. 85-91, ISSN 1096-0309 (Electronic)0003-2697 (Linking)

Moaddel, R., Yamaguchi, R., Ho, P. C., Patel, S., Hsu, C. P., Subrahmanyam, V., & Wainer, I. W. (2005). Development and characterization of an immobilized human organic cation transporter based liquid chromatographic stationary phase. *J Chromatogr B Analyt Technol Biomed Life Sci,* Vol. 818, No. 2, pp. 263-268, ISSN 1570-0232 (Print)1570-0232 (Linking)

Narayanan, S. R., Kakodkar, S. V., & Crane, L. J. (1990). "Glutaraldehyde-P", a stable, reactive aldehyde matrix for affinity chromatography. *Anal Biochem,* Vol. 188, No. 2, pp. 278-284, ISSN 0003-2697 (Print)0003-2697 (Linking)

Ng, W., Dai, J. R., Slon-Usakiewicz, J. J., Redden, P. R., Pasternak, A., & Reid, N. (2007). Automated multiple ligand screening by frontal affinity chromatography-mass spectrometry (FAC-MS). *J Biomol Screen,* Vol. 12, No. 2, pp. 167-174, ISSN 1087-0571 (Print)1087-0571 (Linking)

Nie, Y.-l., Wang, W.-h. (2009) .Immobilized Enzyme Reactor in On-line LC and Its Application in Drug Screening. *Chromatographia,* Vol. 69, pp. S5–S12

Pidgeon, C., & Venkataram, U. V. (1989). Immobilized artificial membrane chromatography: supports composed of membrane lipids. *Anal Biochem,* Vol. 176, No. 1, pp. 36-47, ISSN 0003-2697 (Print)0003-2697 (Linking)

Renaud, J. P., & Delsuc, M. A. (2009). Biophysical techniques for ligand screening and drug design. *Curr Opin Pharmacol,* Vol. 9, No. 5, pp. 622-628, ISSN 1471-4973 (Electronic)1471-4892 (Linking)

Rizzi, L., Dallanoce, C., Matera, C., Magrone, P., Pucci, L., Gotti, C., Clementi, F., & De Amici, M. (2008). Epiboxidine and novel-related analogues: a convenient synthetic approach and estimation of their affinity at neuronal nicotinic acetylcholine receptor subtypes. *Bioorg Med Chem Lett,* Vol. 18, No. 16, pp. 4651-4654, ISSN 1464-3405 (Electronic)0960-894X (Linking)

Ruhn, P. F., Garver, S., & Hage, D. S. (1994). Development of dihydrazide-activated silica supports for high-performance affinity chromatography. *J Chromatogr A,* Vol. 669, No. 1-2, pp. 9-19, ISSN 0021-9673 (Print)0021-9673 (Linking)

Sanghvi, M., Moaddel, R., Frazier, C., & Wainer, I. W. (2010). Synthesis and characterization of liquid chromatographic columns containing the immobilized ligand binding domain of the estrogen related receptor alpha and estrogen related receptor gamma. *J Pharm Biomed Anal,* Vol. 53, No. 3, pp. 777-780, ISSN 1873-264X (Electronic)0731-7085 (Linking)

Slon-Usakiewicz, J. J., Ng, W., Dai, J. R., Pasternak, A., & Redden, P. R. (2005). Frontal affinity chromatography with MS detection (FAC-MS) in drug discovery. *Drug Discov Today,* Vol. 10, No. 6, pp. 409-416, ISSN 1359-6446 (Print)1359-6446 (Linking)

Soczewinski, E., Bieganowska, M. (1969). Investigations on the relationship between molecular structure and chromatogenic parameters: I. Two homologous series of pyridine derivatives suitable as reference compounds in liquid-liquid partition chromatography. *J Chromatogr A*, Vol. 40, pp. 431-439

Taylor, R.F. (Ed.). (1991). *Protein Immobilization: Fundamentals and Applications*, Marcel Dekker, ISBN10 0824782712, New York

Temporini, C., Ceruti, S., Calleri, E., Ferrario, S., Moaddel, R., Abbracchio, M. P., & Massolini, G. (2009). Development of an immobilized GPR17 receptor stationary phase for binding determination using frontal affinity chromatography coupled to mass spectrometry. *Anal Biochem,* Vol. 384, No. 1, pp. 123-129, ISSN 1096-0309 (Electronic)0003-2697 (Linking)

Trujillo, C. A., Majumder, P., Gonzalez, F. A., Moaddel, R., & Ulrich, H. (2007). Immobilized P2X2 purinergic receptor stationary phase for chromatographic determination of pharmacological properties and drug screening. *J Pharm Biomed Anal,* Vol. 44, No. 3, pp. 701-710, ISSN 0731-7085 (Print)0731-7085 (Linking)

Turkova, J. (1999). Oriented immobilization of biologically active proteins as a tool for revealing protein interactions and function. *J Chromatogr B Biomed Sci Appl,* Vol. 722, No. 1-2, pp. 11-31, ISSN 1387-2273 (Print)1387-2273 (Linking)

Tweed, S. A., Loun, B., & Hage, D. S. (1997). Effects of ligand heterogeneity in the characterization of affinity columns by frontal analysis. *Anal Chem,* Vol. 69, No. 23, pp. 4790-4798, ISSN 0003-2700 (Print)0003-2700 (Linking)

Wade, J.L., Bergold, A.F., Carr, P.W. (1987). Theoretical description of nonlinear chromatography, with applications to physicochemical measurements in affinity chromatography and implications for preparative-scale separations. *Anal. Chem.*, Vol. 59, No. 9, pp. 1286–1295

Wainer, I. W., Zhang, Y., Xiao, Y., & Kellar, K. J. (1999). Liquid chromatographic studies with immobilized neuronal nicotinic acetylcholine receptor stationary phases: effects of receptor subtypes, pH and ionic strength on drug-receptor interactions. *J Chromatogr B Biomed Sci Appl,* Vol. 724, No. 1, pp. 65-72, ISSN 1387-2273 (Print)1387-2273 (Linking)

Winzor, D. J. (2004). Determination of binding constants by affinity chromatography. *J Chromatogr A,* Vol. 1037, No. 1-2, pp. 351-367, ISSN 0021-9673 (Print)0021-9673 (Linking)

Xuan, H., & Hage, D. S. (2005). Immobilization of alpha(1)-acid glycoprotein for chromatographic studies of drug-protein binding. *Anal Biochem,* Vol. 346, No. 2, pp. 300-310, ISSN 0003-2697 (Print)0003-2697 (Linking)

Yang, J., & Hage, D. S. (1993). Characterization of the binding and chiral separation of D- and L-tryptophan on a high-performance immobilized human serum albumin column. *J Chromatogr,* Vol. 645, No. 2, pp. 241-250, ISSN 0021-9673 (Print)0021-9673 (Linking)

Yang, Q., & Lundahl, P. (1995). Immobilized proteoliposome affinity chromatography for quantitative analysis of specific interactions between solutes and membrane proteins. Interaction of cytochalasin B and D-glucose with the glucose transporter Glut1. *Biochemistry,* Vol. 34, No. 22, pp. 7289-7294, ISSN 0006-2960 (Print)0006-2960 (Linking)

Yoo, M. J., Smith, Q. R., & Hage, D. S. (2009). Studies of imipramine binding to human serum albumin by high-performance affinity chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci,* Vol. 877, No. 11-12, pp. 1149-1154, ISSN 1873-376X (Electronic)1570-0232 (Linking)

Yu, W., Yuan, B., Deng, X., He, L., Youyi, Z., & Qide, H. (2005). The preparation of HEK293 alpha1A or HEK293 alpha1B cell membrane stationary phase and the chromatographic affinity study of ligands of alpha1 adrenoceptor. *Anal Biochem,* Vol. 339, No. 2, pp. 198-205, ISSN 0003-2697 (Print)0003-2697 (Linking)

Yuan, B. X., Hou, J., He, L. C., & Yang, G. D. (2005). Evaluation of drug-muscarinic receptor affinities using cell membrane chromatography and radioligand binding assay in guinea pig jejunum membrane. *Acta Pharmacol Sin,* Vol. 26, No. 1, pp. 113-116, ISSN 1671-4083 (Print)1671-4083 (Linking)

Zeng, C. M., Zhang, Y., Lu, L., Brekkan, E., Lundqvist, A., & Lundahl, P. (1997). Immobilization of human red cells in gel particles for chromatographic activity studies of the glucose transporter Glut1. *Biochim Biophys Acta,* Vol. 1325, No. 1, pp. 91-98, ISSN 0006-3002 (Print)0006-3002 (Linking)

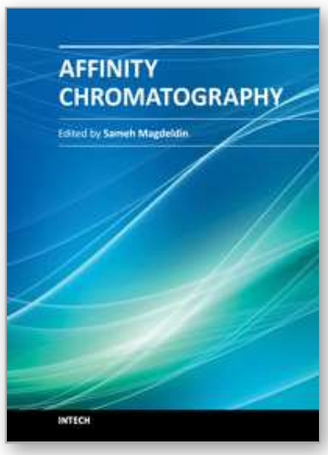
Zhang, Y., Leonessa, F., Clarke, R., & Wainer, I. W. (2000). Development of an immobilized P-glycoprotein stationary phase for on-line liquid chromatographic determination of drug-binding affinities. *J Chromatogr B Biomed Sci Appl,* Vol. 739, No. 1, pp. 33-37, ISSN 1387-2273 (Print)1387-2273 (Linking)

Zhang, Y., Xiao, Y., Kellar, K. J., & Wainer, I. W. (1998). Immobilized nicotinic receptor stationary phase for on-line liquid chromatographic determination of drug-receptor affinities. *Anal Biochem,* Vol. 264, No. 1, pp. 22-25, ISSN 0003-2697 (Print)0003-2697 (Linking)

Zhu, L., Chen, L., Luo, H., & Xu, X. (2003). Frontal affinity chromatography combined on-line with mass spectrometry: a tool for the binding study of different epidermal growth factor receptor inhibitors. *Anal Chem,* Vol. 75, No. 23, pp. 6388-6393, ISSN 0003-2700 (Print)0003-2700 (Linking)

IntechOpen

IntechOpen



Affinity Chromatography

Edited by Dr. Sameh Magdeldin

ISBN 978-953-51-0325-7
Hard cover, 368 pages
Publisher InTech
Published online 21, March, 2012
Published in print edition March, 2012

Most will agree that one major achievement in the bio-separation techniques is affinity chromatography. This coined terminology covers a myriad of separation approaches that relies mainly on reversible adsorption of biomolecules through biospecific interactions on the ligand. Within this book, the authors tried to deliver for you simplified fundamentals of affinity chromatography together with exemplarily applications of this versatile technique. We have always been endeavor to keep the contents of the book crisp and easily comprehensive, hoping that this book will receive an overwhelming interest, deliver benefits and valuable information to the readers.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Piotr Draczkowski, Dariusz Matosiuk and Krzysztof Jozwiak (2012). Affinity Chromatography as a Tool for Quantification of Interactions Between Drug Molecules and Their Protein Targets, Affinity Chromatography, Dr. Sameh Magdeldin (Ed.), ISBN: 978-953-51-0325-7, InTech, Available from: http://www.intechopen.com/books/affinity-chromatography/affinity-chromatography-as-a-tool-for-quantification-of-interactions-between-drug-molecules-and-thei

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

© 2012 The Author(s). Licensee IntechOpen. This is an open access article distributed under the terms of the Creative Commons Attribution 3.0 License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

IntechOpen

IntechOpen