Chromatography on Immobilized Artificial Membrane

Principles of measuring compounds affinity to phospholipids using immobilized artificial membrane chromatography
Chromatography on Immobilized Artificial Membrane (IAM)

Principles of measuring compounds affinity to phospholipids using immobilized artificial membrane chromatography

Pidgeon and Venkataran1 patented a method for immobilizing phospholipids on HPLC-grade silica stationary phases. The immobilized phospholipids mimic the lipid environment of a fluid cell membrane on a solid matrix. Regis Technologies offers various IAM HPLC stationary phases that are prepared by covalently bonding the phospholipids to amino-propyl silica phases. The remaining amino-propyl groups and the free silanol groups are treated to reduce their secondary interactions with the compounds. The IAM stationary phase contains covalently bonded phosphatidyl choline covering the silica particles at monolayer density.

The retention factor (k) of compounds obtained on the IAM stationary phase is proportional to its affinity (partition coefficient) (K) to phospholipids according to the equation (1):

\[
\log k_{(IAM)} = \log K_{(IAM)} + \log \left( \frac{V_s}{V_m} \right) \tag{1}
\]

Where \( k \) is the retention factor obtained from the retention time \( t_R \) and the dead time \( t_0 \) according to equation (2):

\[
k = \frac{t_R - t_0}{t_0} \tag{2}
\]

\( V_s/V_m \) is the volume ratio of the stationary and mobile phases, respectively. Normally we use physiological mobile phase pH (pH7 or pH 7.4) to mimic the physiological environment for the phospholipid binding. However, many drug molecules bind strongly to phospholipids as it is a pre-requisite for membrane permeability, therefore to reduce the retention times various concentrations of acetonitrile can be used in the mobile phase. Similarly to reversed-phase retention, there is the linear relationship between the log \( k \) values and the percentage of acetonitrile concentration in the mobile phase that allows the extrapolation of the retention factor to the zero percentage of acetonitrile, i.e. the pure aqueous buffer (log \( k_0 \)) as is shown by equation 3.

\[
\log k = \text{slope} \times \text{ACN\%} + \log k_0 \tag{3}
\]

Table 1 Calibration standards and typical gradient retention times (Conditions: Column: IAM.PC.DD2 100x4.6 mm, Flow rate: 1.8 ml/min, Mobile phase A; 50 mM ammonium acetate at pH 7.4; B: 100% acetonitrile; gradient: 0 to 5.0 0 to 80% B, 4 to 5 min 80% B; 5-5.25 min 80 to 0% B; 6 min run time, Detection wavelength 254 nm.)

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>TR 7.4</th>
<th>CHI IAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octanophenone</td>
<td>3.110</td>
<td>49.40</td>
</tr>
<tr>
<td>Heptanophenone</td>
<td>2.995</td>
<td>45.70</td>
</tr>
<tr>
<td>hexanophenone</td>
<td>2.855</td>
<td>41.80</td>
</tr>
<tr>
<td>Valerophenone</td>
<td>2.716</td>
<td>37.30</td>
</tr>
<tr>
<td>Butirophenone</td>
<td>2.534</td>
<td>32.00</td>
</tr>
<tr>
<td>Propiophenone</td>
<td>2.314</td>
<td>25.90</td>
</tr>
<tr>
<td>Acetophenone</td>
<td>1.999</td>
<td>17.20</td>
</tr>
<tr>
<td>Acetanilide</td>
<td>1.773</td>
<td>11.50</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>1.455</td>
<td>2.90</td>
</tr>
</tbody>
</table>
Equation 3 enables us to calculate the acetonitrile concentration that is necessary to achieve log $k=0$, when the retention time is exactly double of the dead time, which means equal distribution of the compound in the mobile and the stationary phase. This acetonitrile concentration expressed in volume percentage is called Chromatographic Hydrophobicity Index (CHI IAM). It has been also shown that the CHI IAM values show linear relationships to the gradient retention times of the compounds obtained on the IAM stationary phases. When using acetonitrile gradient 0 to max 80% the obtained gradient retention times can be calibrated by the CHI IAM values using a set of standards, so users do not need to carry out isocratic measurements. The so obtained CHI IAM values showed good correlation to the log $k_0$ (IAM) values, that are the extrapolated logarithmic retention factors to 0 acetonitrile concentration in isocratic mode. Table 1 shows typical retention times for the calibration set of compounds and the fixed CHI IAM values that should not be changed to get data suitable for inter-laboratory comparison. A typical chromatogram is shown in Figure 1.

By plotting the calibration standard’s retention times in the function of the pre-determined and fixed CHI IAM values the obtained straight line shown in Figure 2 can be used to convert any gradient retention times to CHI IAM values that are obtained under the same conditions on the same instrument. Therefore, the calibration has to be done before the measurements of each 96 well plate of discovery compounds. In this way, we ensure that the obtained CHI IAM values are suitable for inter-laboratory comparison.

In conclusion, the chromatographic determination of phospholipid binding is based on measuring gradient retention times. It is independent of the amount of compound injected on the column, so there is no need for quantitative analysis. The time measurement is very reproducible, and the calibrated (relative) gradient retention data are suitable for inter-laboratory comparison and applying them in published models of in vivo drug distribution. The so obtained data can be compiled in databases and suitable to establish quantitative structure-retention relationships, thus enabling chemists to design a compound with the appropriate IAM binding.

**Figure 1** A typical chromatogram for the calibration set of compounds. (Conditions: Column: IAM.PC.DD° 100 mm x 4.6 mm, Flow rate: 2.0 ml/min, Mobile phase A; 50 mM ammonium acetate at pH 7.4; B:100% acetonitrile; gradient: 0 to 3.5 0 to 80% B, 3.5 to 4 min 80% B; 4.0 to 4.5 min 80 to 0% B; 6 min run time, Detection wavelength 254 nm.)

**CHI IAM calibration**

$y=14.895x - 22.411$

$R^2=0.9998$

**Figure 2** Typical calibration plot obtained on IAM stationary phase with the calibration standards.
How can the IAM affinity data be used in early drug discovery?

Phospholipids, especially phosphatidylcholine are major components of cells, tissues and living organisms. The phospholipid bilayer covers every cell and even the cell organelles inside the cells. Various tissues contain various amounts of phospholipids. Compounds affinity to phospholipids is an important property to assess cell permeability and drug distribution between the plasma and various tissue compartments in the body.

A recent review summarizes the major applications of the IAM technology for compound characterization.³

Models with IAM Retention Data

Some of the models utilize the albumin binding data as well. For example volume of distribution can be modeled by the IAM and the Human Serum Albumin (HSA) binding. The two types of binding can be very different especially for positively and negatively charged compounds and these differences are very important in the models. Albumin binding can be easily determined by HPLC in a similar fashion to IAM chromatography. We need to use isopropanol gradient (up to 40%) to elute strongly bound compounds and the retention times have to be calibrated to get data suitable for the model. Detailed procedure is described in reference 5. The HSA HPLC columns are available from Chiral Technologies USA or Europe (http://chiraltech.com).

1. Intestinal Absorption

Intestinal absorption and drug distribution depend on compound’s partitioning into phospholipids. Compounds have to possess a certain degree of affinity to membranes in order to permeate through the biological phospholipid bi-layers. Therefore, IAM chromatography can provide insight of potential intestinal absorption of the compounds.⁴

2. Volume of Distribution

As tissues represent a more non-polar lipid environment relative to the plasma, the human steady state volume of distribution (Vdss) could be modeled (see Figure 2) by the binding differences between IAM and human serum albumin (HSA)⁵.

\[
\log Vdss = 0.44 \times \log K(IAM) - 0.22 \times \log K(HSA) - 0.66
\]

3. Unbound Fraction in Tissues (fut)

The sum of the albumin and phospholipid binding inversely correlates to the unbound fraction of the compounds in tissues according to equation (5). The agreement between the measured and estimated \( \log f_{ut} \) values are shown in Figure 3.

\[
\log f_{ut} = -0.66 \times \log K(HSA) - 0.52 \times \log K(IAM) + 0.55
\]

Figure 2 The plot of the human clinical volume of distribution data of 130 marketed drugs in the function of the estimated values from the difference of IAM partition and albumin binding of the compounds.

Figure 3 The plot of the measured and estimated unbound fraction of known drugs in tissue using equation 5.
4. Drug Efficiency—Dose Estimation

The in vivo drug efficiency could be modeled by the sum of the IAM and HSA binding of compounds\(^5\) according to equation 6. The in vitro DRUG\(_{eff}\) showed an excellent trend with the in vivo DRUG\(_{eff}\) (see Figure 4). Drug efficiency and potency enables early dose estimation as drug efficiency approximates the proportion of the free drug concentration and dose.

\[
\log \text{DRUG}_{eff} = 2 - (0.23 \log K(HSA) + 0.43 \log K(IAM) - 0.72) \tag{6}
\]

Figure 4 The good trend between the in vivo Drug\(_{eff_{\text{max}}}\) and the estimated Drug\(_{eff_{\text{max}}}\) from IAM and HSA binding data for 130 marketed drugs.

5. Phospholipidosis Potential—Hepatotoxicity

It was found that basic compounds that have higher than 50 CHI IAM values have phospholipidosis potential\(^7\). Phospholipidosis is an accumulation of lamellar phospholipids in the cell often caused by drugs. When compound binds strongly to phospholipids it can block the metabolizing enzymes responsible for the elimination of the excessive amount of phospholipids from the cells. Hepatotoxicity was measured in cells by Nile Red fluorescence emission. Good correlation was found between hepatotoxicity and CHI IAM values as is shown in Figure 5.

Figure 5 Good correlation between CHI IAM and hepatotoxicity detected by Nile Red cell assay.

6. Solubility Enhancement in Simulated Intestinal Fluid

The intestine contains phosphatidylcholine micelles that enhance the solubility and absorption of nutrients. It was found that the solubility enhancement showed good correlation to the IAM binding of compounds. Compounds that have CHI IAM values higher than 35, had significant enhancement of the buffer solubility in the intestinal fluid due to their partitioning to phosphatidylcholine micelles as is shown in Figure 6.

Figure 6 Solubility enhancement correlates with the CHI IAM values of the compounds.
**Structure - IAM Binding Relationships, Relation to Octanol/Water or C-18 Partition**

*a. Effect of charge*

Although both IAM and HSA binding depend on compound's lipophilicity, the presence of positive or negative charges drives compounds to bind more strongly to IAM and partition to tissues. The plot in Figure 7 shows the IAM binding as a function of HSA binding for 150 known drug molecules.

![Figure 7](image)

**Figure 7** The plot of IAM and HSA binding of neutral, acidic, and basic compounds

It can be seen that positively charged compounds have longer retention on IAM while negatively charged compounds have longer retention on the albumin columns, respectively.

When comparing acidic, basic and neutral molecules lipophilicity obtained on C-18 and IAM stationary phases it is clearly visible that basic compounds bind to the IAM surface much stronger than to the C-18 surface as is shown in Figure 8.

![Figure 8](image)

**Figure 8** The plot of CHI logD (obtained on C18 stationary phase) and log k (IAM) obtained on immobilized artificial membrane phase of basic, neutral and acidic compounds.
b. Comparison with reversed-phase retention and octanol/water partition

The IAM stationary phase with aqueous mobile phase can be considered as another type of reversed phase column. However, when plotting the CHI IAM values in the function of CHI C-18, it was found that H-bond donor compounds have longer retention (higher CHI IAM values) on the IAM columns as is shown in Figure 9.

Setting up the Abraham solvation equations it was found that the IAM lipophilicity is very similar to octanol/water lipophilicity for neutral compounds. The table below shows the relative coefficients of the Abraham descriptors for the most commonly used lipophilicity parameters in drug discovery. The IAM lipophilicity shows the closest similarity to the octanol/water partition system together with the albumin binding. e/v, s/v, a/v and b/v are the normalized coefficient of the excess molar refraction, the dipolarity/polarizability, the H-bond acidity and the H-bond basicity, respectively to the v size coefficient.

c. Effect of shape

There are several in silico approaches to calculating compound’s lipophilicity, phospholipid binding and protein binding. However, all these methods use 2D descriptors of the molecules that do not incorporate the shape. The shape of the molecules has a significant impact on their ability to partition into the phospholipid bi-layer. IAM retention can detect shape differences, too.6

Two molecules that are structural isomers, only the ortho- and meta- substitutions are different, had significantly different IAM retention (log K IAM), which is equivalent to half a log unit octanol/water lipophilicity. It shows that long and narrower shaped molecules can partition into the membrane more see Figure 10.

In conclusion, octanol/water partition and reversed phase (C-18) partition cannot describe appropriately the compound’s binding to phospholipids. IAM chromatography provided the best phospholipid binding parameter that described in vivo distribution behavior of compounds much better than other lipophilicity measures. IAM chromatography provides an invaluable tool for early lead optimization when the best compounds should be selected for further in vivo studies in order to reduce later stage attrition and reduce the number of animal experiments. The excellent structure-phospholipid binding relationship helps chemist to design compounds with the appropriate tissue binding properties to reduce potential safety issues and problems with the PK profile in pre-clinical and clinical studies.

Table 2 The relative coefficients of the molecular descriptors in various lipophilicity measures obtained by the Abraham solvation equation. e/v, s/v, a/v and b/v are the normalized coefficient of the excess molar refraction, the dipolarity/polarizability, the H-bond acidity and the H-bond basicity, respectively to the v size coefficient.

<table>
<thead>
<tr>
<th></th>
<th>e/v</th>
<th>s/v</th>
<th>a/v</th>
<th>b/v</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log K (HSA)</td>
<td>0.02</td>
<td>-0.07</td>
<td>0.16</td>
<td>-1.21</td>
</tr>
<tr>
<td>Log K (AGP)</td>
<td>0.46</td>
<td>-0.38</td>
<td>-0.33</td>
<td>-0.85</td>
</tr>
<tr>
<td>CHI&lt;sub&gt;RP,AcN&lt;/sub&gt;</td>
<td>0.09</td>
<td>-0.24</td>
<td>-0.30</td>
<td>-0.98</td>
</tr>
<tr>
<td>logP&lt;sub&gt;octanol&lt;/sub&gt;</td>
<td>0.15</td>
<td>-0.28</td>
<td>0.01</td>
<td>-0.91</td>
</tr>
<tr>
<td>Log K (IAM)</td>
<td>0.11</td>
<td>-0.03</td>
<td>0.01</td>
<td>-1.05</td>
</tr>
<tr>
<td>Blood/brain</td>
<td>0.59</td>
<td>-1.03</td>
<td>-0.84</td>
<td>-0.78</td>
</tr>
</tbody>
</table>

Figure 9 The plot of CHI IAM values in the function of the CHI C-18 values obtained on Inertsil stationary phase for neutral compounds. Compounds (marked red) that have H-bond donor groups had longer retention on IAM stationary phase.

Figure 10 The differences of IAM binding of structural isomers that have different shapes.
IAM

Rapid biomimetic screening of drug-membrane affinity
An advanced tool for drug discovery

<table>
<thead>
<tr>
<th>PRODUCT</th>
<th>DIMENSIONS</th>
<th>PARTICLE SIZE</th>
<th>IAM.PC.DD2 CATALOG #</th>
<th>IAM.PC CATALOG #</th>
<th>IAM.PC.MG CATALOG #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Columns</td>
<td>15 cm x 3 mm</td>
<td>10 µm</td>
<td>1-774004-300</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>10 cm x 3 mm</td>
<td>10 µm</td>
<td>1-774003-300</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>3 cm x 4.6 mm</td>
<td>10 µm</td>
<td>1-774010-300</td>
<td>1-770007-300</td>
<td>1-772007-300</td>
</tr>
<tr>
<td></td>
<td>10 cm x 4.6 mm</td>
<td>10 µm</td>
<td>1-774011-300</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>15 cm x 4.6 mm</td>
<td>10 µm</td>
<td>1-774014-300</td>
<td>1-770001-300</td>
<td>1-772001-300</td>
</tr>
<tr>
<td>Guard Kit</td>
<td>1 cm x 3 mm</td>
<td>10 µm</td>
<td>1-774012-300</td>
<td>1-771001-300</td>
<td>1-773001-300</td>
</tr>
<tr>
<td>Guard Cartridges</td>
<td>1 cm x 3 mm</td>
<td>10 µm</td>
<td>1-774013-300</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>IAM Fast Screen Mini Column Kit*</td>
<td>1 cm x 3 mm</td>
<td>10 µm</td>
<td>1-775014-300*</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Drug Screening Calibration Mixture</td>
<td>10 x 1 mL</td>
<td>N/A</td>
<td>1-774015-300</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

* Not associated with one type of IAM phase. Inquire for more details.

Useful References for Drug Membrane Affinity Screening with IAM

3. Tsopelas et al., The potential of immobilized artificial membrane chromatography to predict human oral absorption, European Journal of Pharmaceutical Sciences 2016, 81, 82-93.

Add Regis’ IAM columns to your drug discovery tool box today!