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Modeling Caco-2 permeability of drugs using immobilized artificial membrane chromatography and physicochemical descriptors

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Abstract

This study evaluates the potential of immobilized artificial membrane (IAM) chromatography, in combination with other physicochemical descriptors for high-throughput absorption profiling during lead optimization. An IAM chromatographic method was developed and validated. Absorption profiles of 32 structurally diverse compounds (acidic, basic, neutral and amphoteric) were then evaluated based on their IAM retention factor ($\log k'_{IAM}$), molecular weight (MW), calculated $\log P$ ($C\log P$), polar surface area (PSA), hydrogen bonding capacity (HBD and HBA) and calculated Caco-2 permeability (QPCaco). Using regression and stepwise regression analysis, experimental Caco-2 permeability was correlated against $\log k'_{IAM}$ and a combination of various physicochemical variables for quantitative structural-permeability relationship (QSPR) study. For the 32 structurally diverse compounds, $\log k'_{IAM}$ correlated poorly with Caco-2 permeability values ($R^2 = 0.227$). Stepwise regression analysis confirmed that $C\log$, PSA, HBD and HBA parameters are not statistically significant and can be eliminated. Correlation between Caco-2 cell uptake and $\log k'_{IAM}$ was enhanced when molecular size factor (MW) was included ($R^2 = 0.555$). The exclusion of 11 compounds (paracellularly and actively transported, Pgp substrates and blocker, and molecules with MW lesser than 200 and greater than 800) improved the correlation between Caco-2 permeability, IAM and MW factors to R^2 value of 0.84. The results showed that IAM chromatography can only profile the passive absorption of drug molecules. Finally, it was confirmed in this study that the IAM model can accurately identify the Caco-2 permeability of nontransported Pgp substrates, such as verapamil and ketoconazole, through passive permeation because of their high permeability. IAM chromatography, combined with molecular size factor (MW), is useful for elucidating biopartitioning mechanism of drugs.

Keywords: IAM chromatography; Physicochemical descriptors

1. Introduction

Most commercially available drugs are administered orally as this noninvasive method is the preferred route of drug administration. To elicit their pharmacological and therapeutic effects, drug molecules first have to enter the systemic blood circulation, which requires the passage through the barrier membranes of the gastrointestinal tract. There are two possible pathways for permeation, the transcellular route (including passive diffusion, carrier-mediated uptake and receptor-mediated endocytosis) and paracellular route (passive diffusion via tight junctions). Most of these drugs are absorbed across the intestinal mucosa by passive diffusion via transcellular route [1].

The prediction of drug-membrane permeability is important during the lead optimization stage of drug discovery. The experimental difficulty, high cost and low-throughput involved in screening lead compounds in animals for oral drug absorption have led to the development of several in vitro prediction models. These models generally measure lipophilicity, as it directly influences the degree of membrane parti-

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tioning and hence passive transcellular transport of solutes [2].

Permeation (P_m) of a drug through membrane is directly proportional to D_m and K_m:

$$P_m = \frac{D_m K_m}{L} \quad (1)$$

where D_m is the membrane diffusion coefficient of the solute, K_m the membrane partition coefficient of the solute and L is the membrane thickness (a constant). Drug permeability is difficult to measure, thus it is simpler to estimate P_m based on K_m. Drug permeability varies less with D_m that is in turn influenced by the drug molecular size.

Caco-2 model has been previously reported to be predictive of oral absorption in humans [1,3]. The membranes of Caco-2 cells have useful properties for correlation with in vivo data and are used to study active transport, efflux mechanisms, paracellular and transcellular diffusion and metabolic activity during absorption [4,5]. However, the validation of the integrity and proper functionality of the Caco-2 cells monolayer is often tedious and not straightforward. Another commonly used in vitro model is the *n*-octanol/water partitioning system. Solute partitioning using *n*-octanol/water system (i.e., the logarithm of partition coefficient, $\log P$) is however only useful, when polar group interactions between the solute and the phospholipid bilayers are minimal or absent [6]. It lacks structural similarities to cell membranes, reflecting only the hydrophobicity of a compound and is not suitable for highly polar and ionic compounds [7]. Chromatography with octadecylsilica (ODS) as stationary phase models the *n*-octanol/water partitioning system [8]; and the measurement of its partition coefficients (K_{oct}) is comparatively simple. However, ODS chromatography inherits the same limitation as the *n*-octanol/water system. In contrast, fluid liposome system exhibits structural similarities to biological membrane. Many quantitative structure-activity relationship (QSAR) studies have successfully correlated drug activities with drug liposome partition coefficients [9–12]. Useful retention data for prediction of drug absorption can be obtained by chromatography of drugs on immobilized liposomes or biomembranes of heterogenous compositions [13]. However, the use of liposomal system is experimentally laborious and low-throughput, thus prohibiting large-scale screening.

Immobilized artificial membrane (IAM) as another approach of measuring partition coefficients consists of ordered phospholipid surface that supports drug-membrane partitioning based on lipophilicity and electrostatic interactions. It consists of a monolayer of phospholipids covalently bound to an inert propylamine-silica support, thus this surface contains one-half of the membrane bilayer [14]. Since IAM adequately models drug-membrane partitioning, this chromatographic method has been suggested to be an accepted model for predicting the drug transport across the intestinal membranes. The correlation between IAM and other lipophilicity parameters determined by *n*-octanol/water partitioning, ODS chromatography and liposomal partitioning had been investigated [7,15,16]. Solute partitioning into liposomes (K'_m) and IAMs (k'_{IAM}) is virtually identical for the 23 compounds tested [7]. Both K'_m and k'_{IAM} were correlated with solute partitioning into *n*-octanol/water system only when nonpolar interactions between solutes and membranes dominate the membrane binding energy. Performance of $\log k'_{IAM}$ was also shown to be as good as or even better than that of the reference hydrophobicity parameters ($\log P$ and $\log D$) in predicting biological activity (IC_{50} binding) [17,18].

Besides correlating very well with drug pharmacokinetic parameters, IAM chromatography has shown good correlation with transport of drugs across Caco-2 cells [15,19], T cells [19], and intestinal tissue [15,20]. One important finding from Ong et al. was that IAM chromatography always gives better correlations than ODS chromatography or *n*-octanol/water partitioning systems on modeling the interactions of drugs with fluid membranes [15]. Although a diverse set of 11 compounds was used, Ong et al. did not consider any other physicochemical descriptors during the correlation study. Stewart et al. demonstrated that correlation between Caco-2 uptake and $\log k'_{IAM}$ was improved, when molecular weight and hydrogen-bonding potential were included in multivariable regression analysis [19]. The results indicated the importance of physicochemical properties in data correlation. However, the homologous series of drugs selected in that study was limited in their number and chemical diversity. In another study by Genty et al., correlation between passive permeabilities of drugs in rat intestines and $\log k'_{IAM}$ was enhanced when molar volume was considered as an additional descriptor [20]. The findings underscored the importance of physicochemical parameter consideration during absorption data correlation. The data were not derived from correlation with Caco-2 uptake that is in turn accepted as the primary absorption-screening tool during pharmaceutical lead selection and optimization.

As an alternative to Caco-2 model, the availability of a high-throughput complementary drug absorption screening method may be desirable for early candidate profiling in drug discovery. This study aims to develop and validate an IAM chromatographic technique using a HPLC/UV system and evaluate its potential as a predictive tool for passive intestinal absorption of drugs when used in combination with physicochemical descriptors, such as molecular size, hydrogen bonding and polar surface area (PSA). Among computational methods, molecular polar surface area is a useful parameter for prediction of drug transport properties [21]. Polar surface area of a molecule is defined as the area of its van der Waals surface that arises from oxygen or nitrogen atoms or hydrogen atoms attached to oxygen or nitrogen atoms. PSA has been shown to correlate very well with the human intestinal absorption, Caco-2 monolayers permeability, and blood–brain barrier penetration [21–23]. A set of 32 drugs from about 10 therapeutic areas, consisting of neutral, acidic, basic and amphoteric compounds, is selected to cover a fair range in molecular size and chemical diversity. $\log k'_{IAM}$ values of these 32 compounds gen-

erated using the established IAM chromatographic technique were subsequently correlated with Caco-2 absorption values (literature and experimental), with and without the inclusion of physicochemical descriptors, using linear and multiple stepwise regression analysis. The findings of the study and their implications are elaborated in the subsequent sections.

2. Experimental

2.1. Chromatographic system

The stainless-steel column is an IAM.PC.DD.2 (150 mm × 4.6 mm, particle diameter 12 μm and pore diameter 300 Å) purchased from Regis Technology (Morton Grove, IL, USA). The retention measurements were performed by HPLC using a Waters Delta 600 pumping system and a Waters Controller equipped with a Waters 2487 dual λ absorbance detector (Waters, Milford, MA, USA).

2.2. Chemicals

The following chemicals were of analytical grade and used without further purification: acetaminophen, acetylsalicylic acid, ampicillin, atenolol, caffeine, captopril, carbamazepine, cephalexin, chloramphenicol, cimetidine, diclofenac, diltiazem, etoposide, hydrocortisone, ibuprofen, indomethacin, ketoconazole, labetalol, lucifer yellow, pindolol, prednisolone, procaine, propranolol, quinidine, ranitidine, rifampicin, salicylic acid, tamoxifen, terfenadine, testosterone, verapamil, vinblastine, phenol, 3-nitrobenzoic acid, benzoic acid, 3-nitroaniline, potassium chloride, sodium chloride, potassium phosphate, sodium dihydrogen phosphate, *ortho*-phosphoric acid and methanol. High-purity water was produced using a Millipore Milli-Q system (Bedford, MA, USA).

2.3. Chromatographic conditions

The eluents were either 0.01 M phosphate buffer saline (PBS) at pH 7.0 (2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4 and 8.1 mM Na_2HPO_4) or mixtures of methanol and PBS at various compositions. The flow rate at 1.0 ml/min was used throughout the study. The organic modifier and the aqueous phase were degassed by sparging inert helium at a rate of 60–100 ml/min. Capacity factors on the IAM column (k'_{IAM}) were determined with eluents at pH 7.0 to mimic the physiological pH and be compatible with the stability of IAM stationary phase. The chromatography was carried out isocratically at room temperature (about 23 °C). Stock solutions (0.5 mg/ml) of compounds were prepared in PBS or methanol. The solutes were diluted suitably before analysis with PBS and the dilution factors were between 5 and 10 times. The injection volume was 10–30 μl. UV detection was monitored at 210 and 254 nm. Data were acquired, processed and reported using the Waters Empower software.

2.4. IAM capacity factor (k'_{IAM}) profiling

Using IAM chromatography to measure the partition coefficient between the aqueous mobile phase and the IAM bonded phase only requires measurements (in min) of the solute retention time (t_r) and the column dead time (t_0). A nonretained compound, such as citric acid, is used to determine t_0. From the retention time of the analyte and the column dead time, the solute's capacity factor k'_{IAM} can be calculated using the following equation:

$$k'_{IAM} = \frac{t_r - t_0}{t_0} \quad (2)$$

The capacity factor, k'_{IAM} is linearly related to the equilibrium partition coefficient, K'_{IAM}:

$$K'_{IAM} = \left(\frac{V_m}{V_s}\right) k'_{IAM} = \Phi k'_{IAM} \quad (3)$$

where V_m is the total volume of the solvent within the IAM-HPLC column, V_s the volume of the IAM interphase created by the immobilized phospholipids, and Φ ($=V_m/V_s$), the phase ratio is a constant for a given column.

The HPLC system was conditioned by passing the mobile phase for 5 min at a flow rate of 10 ml/min. The flow rate was changed to 1 ml/min to equilibrate the column and a stable UV baseline was observed before injecting the samples. Retention times (t_r) and $\log k'_{IAM}$ values of 3-nitrobenzoic acid, benzoic acid, phenol and 3-nitroaniline were obtained using 0.01 M PBS as eluent on 3 separate days. Triplicate values were obtained for each compound on each day. The intra-day variations and inter-day variations of these data were determined for method validation.

Using the established IAM chromatographic technique, the $\log k'_{IAM}$ values for 32 compounds were determined. Intra-day variation of solute retention times was calculated for each compound. For the elution of the more lipophilic compounds, mixtures of methanol/PBS at compositions ranging from 20 to 80% organic modifiers were used. For each compound, the $\log k'_{IAM}$ value at a particular composition of methanol/PBS mixture was determined under that condition. The $\log k'_{IAM}$ values at 100% aqueous phase were then extrapolated by linearly plotting the $\log k'_{IAM}$ values (averages of triplicate measurements) against the percentage (v/v) of organic modifier in the eluent mixtures.

2.5. Physicochemical descriptors profiling

Molecular weight (MW), number of hydrogen bond donor (HBD), number of hydrogen bond acceptor (HBA) and hydrophobicity parameter or calculated octanol–water partition coefficient ($C\log P$) were calculated using Daylight Toolkit from Daylight Chemical Information Systems (Mission Viejo, CA, USA). Polar surface area (PSA) was cal-

culated using topological PSA or TPSA from Cheminformatics at Novartis Pharma (Basel, Switzerland). Calculated Caco-2 cell permeability or QPCaco was measured using QikProp from Schrödinger (Portland, OR, USA).

2.6. Caco-2 permeability assay

Cell culture media and buffer components were purchased from GibcoBRL (Invitrogen, Paisley, UK). HTS Transwell-24 plates (insert with 6.5 mm diameter, 0.4 μm pore size, polycarbonate membrane, cell growth area 0.33 cm^2) were purchased from Corning Costar (Corning Incorporated Life Sciences, Acton, MA, USA). Caco-2 cells were purchased from ATCC and maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 1% nonessential amino acids, and 2 mM fresh L-glutamine at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. Cells were passaged at 80–90% confluence (every 3–4 days) using Trypsin–EDTA solution. Caco-2 cells were used at passage numbers 31–42 and seeded with 1×10^5 cells/cm^2. Cells on the inserts were incubated in the same culture conditions as above with the change of growth medium every 48 h.

Absorption was measured as the apparent permeability coefficient (Papp) of transport in the apical to basolateral direction. Assay was performed with cell monolayers cultured for 13–25 days after seeding. Cells were fed with fresh growth medium 1 day before experiment. On the experiment day, cells were allowed to adapt for the assay buffers for at least 30 min at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. The assay was performed in a 2 h transport incubation period with 0.2 ml/insert of the apical buffer (Hank's Balanced Salt Solution HBSS Gibco 14025-092, 0.35 g/l sodium bicarbonate, 5 mM Mes, pH 6.5) containing 50 μM testing compound and lucifer yellow, and 1 ml/well the basolateral buffer (HBSS, 0.35 g/l sodium bicarbonate, 5 mM Hepes, pH 7.4). All compounds were prepared at 10 mM in DMOS or H_2O and tested in six replicate monolayers. Monolayers were incubated at 37 °C in an atmosphere of 5% CO_2 and 95% relative humidity with 50 rpm shaking during 2 h transport. Samples were collected in a 96-well plate, 50 μl from donors collected at time 0 and 2 h, 300 μl from receivers collected after 2 h incubation. Samples were read at 485 nm excitation and 530 nm emission for lucifer yellow and then quantified by LC/MS (Waters Alliance 2795 and Micromass ZQ single-quadrupole mass spectrometer).

Lucifer yellow, a fluorescent marker for the paracellular pathway, was used as an internal control in every test to verify tight junction integrity of the monolayer during the assay. Caco-2 monolayers with lucifer yellow Papp $>1 \times 10^{-6}$ cm/s were not used in compound Papp calculations. A set of reference compounds, propranolol, labetalol, ranitidine, and vinblastine, were used to quality control the assay. The Papp (in 10^{-6} cm/s) was calculated according to the formula $\text{Papp} = (\Delta Q/\Delta t)/(AC_0)$ where $\Delta Q/\Delta t$ is expressed in mmol/s, A the surface area of the cell monolayers (in cm^2), and C_0 is the initial concentration of the compound on the donor side (in mmol/cm^3).

2.7. Statistical methods

$\log k'_{IAM}$ values of the 32 drugs were correlated with their respective Caco-2 absorption values. Linear and multiple stepwise regression (MLR) of Caco-2 absorption values with $\log k'_{IAM}$ values were performed and examined with the inclusion of the physicochemical descriptors using Minitab 14 statistical software from Minitab (State College, PA, USA).

3. Results and discussion

3.1. Validation of IAM chromatographic technique

Reference compounds, benzoic acid, 3-nitrobenzoic acid, phenol and 3-nitroaniline, were used for validating the IAM chromatographic technique with respect to retention time reproducibility. These compounds were selected as they elute within a reasonable short period of time (less than 16 min) under 100% aqueous mobile phase condition and they are stable and readily available. As computed from the retention values (mean ± S.D.) in Table 1, solute retention times (t_r) exhibited an intra-day coefficient of variation (C.V.) of less than 2% and inter-day C.V. of less than 6% for all the four reference compounds. Subsequently, the calculated $\log k'_{IAM}$ values exhibited an inter-day C.V. of less than 8%, indicating a good reproducibility and robustness of the method. It was noted that thorough degassing of the mobile phase using helium sparging was paramount in generating reproducible chromatographic profiles. A test-mixture of these four reference compounds was prepared and analyzed using the HPLC/UV system. The mean retention times of benzoic acid, 3-nitrobenzoic acid, phenol and 3-nitroaniline were 2.12, 2.68, 9.84 and 15.73 min, respectively. This test-mixture

Table 1
Intra- and inter-day variations of retention times (t_r) and inter-day variations of $\log k'_{IAM}$ values of 3-nitrobenzoic acid, benzoic acid, phenol and 3-nitroaniline

Compounds ($n = 3$)	t_r (min) [intra-day (mean ± S.D.)]	t_r (min) [inter-day (mean ± S.D.)]	$\log k'_{IAM}$ [inter-day (mean ± S.D.)]
3-Nitrobenzoic acid	2.65 ± 0.003	2.64 ± 0.05	−0.30 ± 0.03
Benzoic acid	2.11 ± 0.004	2.12 ± 0.004	−0.67 ± 0.03
Phenol	9.60 ± 0.002	9.91 ± 0.4	0.67 ± 0.03
3-Nitroaniline	15.24 ± 0.2	15.76 ± 0.8	0.90 ± 0.02

was used for system validation during future IAM applications.

3.2. $\log k'_{IAM}$ profiling

Fig. 1 illustrates the structures of the 32 drugs being examined. The set comprises acidic (acetylsalicylic acid, captopril, diclofenac, ibuprofen, indomethacin and salicylic acid), basic (atenolol, carbamazepine, cimetidine, diltiazem, ketoconazole, labetalol, lucifer yellow, pindolol, procaine, propranolol, quinidine, ranitidine, tamoxifen, terfenadine, verapamil and vinblastine), amphoteric (ampicillin, cephalexin and rifampicin) and neutral (acetaminophen, caffeine, chloramphenicol, etoposide, hydrocortisone, prednisolone and testosterone) compounds.

Compounds like carbamazepine, chloramphenicol, diclofenac, diltiazem, etoposide, hydrocortisone, ibuprofen, indomethacin, ketoconazole, labetalol, pindolol, prednisolone, procaine, propranolol, quinidine, ranitidine, rifampicin, tamoxifen, terfenadine, testosterone, verapamil and vinblastine did not elute within a reasonable time (~10 min) with 100% aqueous mobile phase and the addition of various methanol percentages (%) to the eluent was needed. This approach of extrapolating $\log k'_{\mathrm{IAM}}$ was well established and reported [19,20]. In our study, a linear relationship between $\log k'_{\mathrm{IAM}}$ and methanol percentages was found for all drugs over the range of the eluent composition examined. The r^2 values for all linear regression plots were larger than 0.93 (3-methanol/PBS mixtures). As an illustration, Table 2 shows the retention times (mean ± S.D.) of ampicillin at different compositions of methanol, namely 20, 40 and 60%. The $\log k'_{\mathrm{IAM}}$ values of ampicillin at different percentages of methanol were calculated using the respective mean retention times. The final equation used to extrapolate the $\log k'_{\mathrm{IAM}}$ of ampicillin at 100% aqueous mobile phase is:

$$\log k'_{\mathrm{IAM}} = -0.0182y - 0.0908 \quad (4)$$

In this equation, y represents the percentage of methanol. This extrapolation method proved to be suitable as $\log k'_{\mathrm{IAM}}$ value of ampicillin measured at 100% aqueous phase (−0.068) was close to the $\log k'_{\mathrm{IAM}}$ value determined by the extrapolation method (−0.091). Other compounds that are retained longer than ampicillin provided similar correlation between actual measurement and extrapolation. Table 3 shows the logarithms of the capacity factors ($\log k'_{\mathrm{IAM}}$) extrapolated to or measured at 100% aqueous phase. Solute retention times exhibited an intra-day C.V. of less than 3% for all 32 compounds. This indicated the high reproducibility of the IAM profiling method. Furthermore, $\log k'_{\mathrm{IAM}}$ values generated in this study for testosterone, procaine, caffeine, hydrocortisone and propranolol were found to be comparable to the values obtained by Valko et al. [24]. This highly comparable interlaboratory IAM data further underscores the ruggedness and reproducibility of IAM profiling technique and demonstrates that it may be adopted by different laboratories for consistent absorption profiling.

3.3. Quantitative structure-permeability relationship (QSPR) of 32 diverse compounds

All experimental Caco-2 absorption values, summarized in Table 3, are obtained from literatures [3,25–30], except for diclofenac, procaine and vinblastine, in which their Caco-2 values were determined in-house. Although evaluating calculated Caco-2 permeability is not an objective in this project, we were interested to determine if these values were near to the experimentally determined ones. Correlation of calculated Caco-2 permeabilities (QPCaco) to experimental Caco-2 values was relatively poor ($R^2 < 0.387$) and it indicated that the prediction of experimental Caco-2 permeabilities using QikProp approach might not be accurate. As such, QPCaco value, that ought to be interpreted prudently, was not used for regression analysis against $\log k'_{\mathrm{IAM}}$ or $C\log P$ in our study.

Regression analysis of log Caco-2 versus $\log k'_{\mathrm{IAM}}$ for the diverse set of 32 compounds generated the following equation:

$$\log \text{Caco-2} = 0.375(0.213;\ 0.088) + 0.386(0.130;\ 0.006)\log k'_{\mathrm{IAM}},$$
$$n = 32,\ R^2 = 0.227,\ s = 0.745,\ F = 8.79 \quad (5)$$

In this equation, n is the number of compounds, s the standard deviation, R^2 the squared correlation coefficient and F is the Fisher F-statistic. The figures in parentheses are the standard deviation and P-values of coefficients, respectively. Regression analysis of log Caco-2 against the respective $C\log P$, MW, HBD, HBA and PSA generated similarly poor squared correlation coefficients (R^2) of 0.214, 0.061, 0.160, 0.207 and 0.256. These results differ from the findings of Stewart et al. in that log Caco-2 was better correlated with the respective hydrophobicity measures ($C\log P$, $\log D$, $\log k'_{\mathrm{IAM}}$ and $\log k'_{\mathrm{C18}}$) in their permeability regression analysis [19]. This difference could be due to the homologous series of drugs used in their study, that were limited in their number and chemical diversity [19]. The poor statistics of the regression analyses in our study showed that $\log k'_{\mathrm{IAM}}$ and the other physicochemical parameters could not be used singly to predict the Caco-2 permeabilities of drugs. This is not unexpected as the transport of drug molecules across the biomembranes is a complex process influenced by many factors.

In the regression analysis of log Caco-2 against $\log k'_{\mathrm{IAM}}$, the experimental log Caco-2 values of three of the four compounds with molecular weights less than 200 (salicylic acid (138.1), acetaminophen (151.2) and caffeine (194.2)) were found to lie above the linearly fitted line. This may be related to the concurrent paracellular diffusion of these compounds through the tight junctions of the biomembranes,

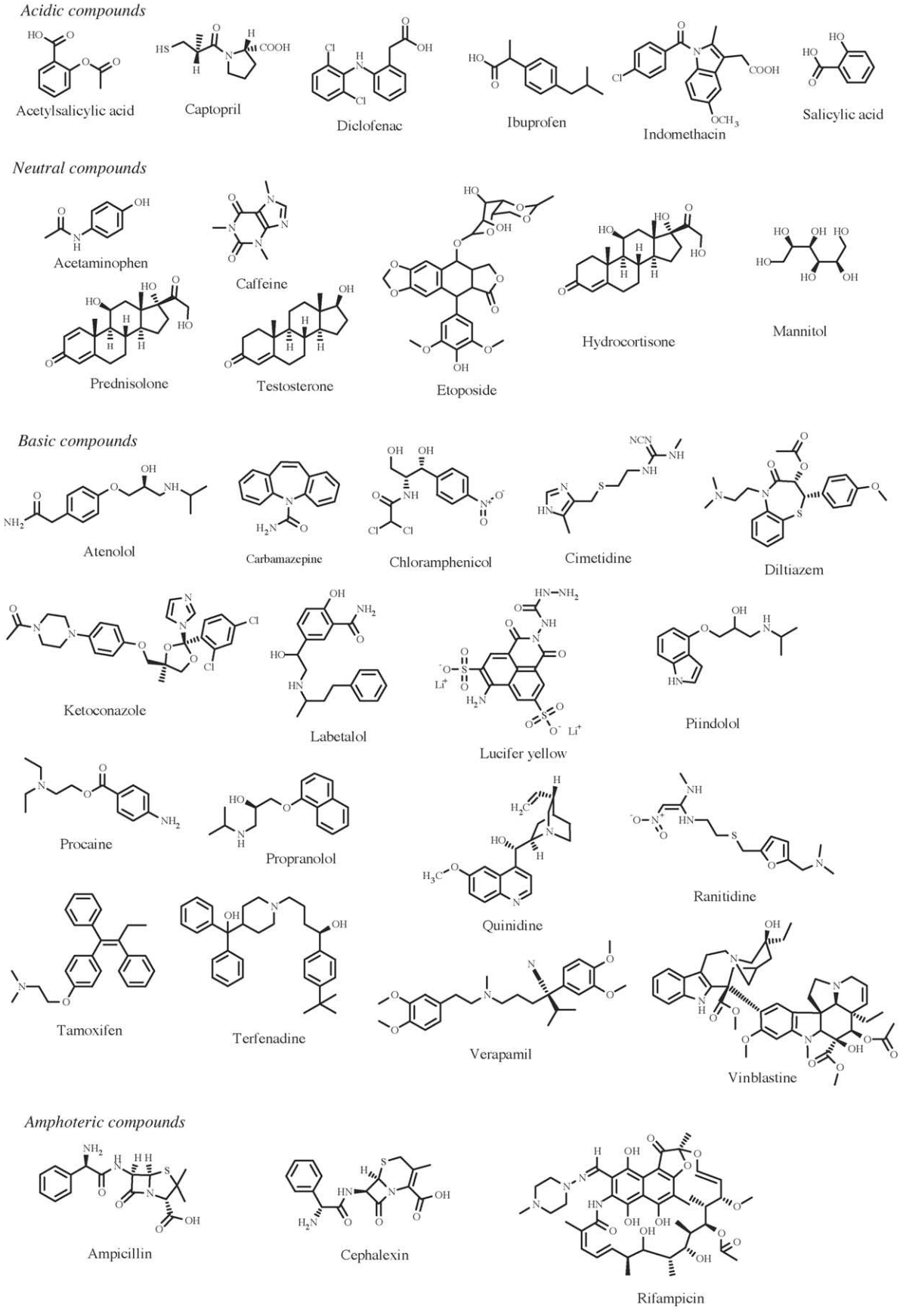

Fig. 1. Chemical structures of 32 test compounds.

thus accounting for the higher experimental log Caco-2 values. These lower molecular weight compounds are known to tend to cross the membrane via the paracellular pathway. On the other hand, the experimental $\log k'_{IAM}$ values of compounds with relatively larger molecular size (etoposide (588.6), rifampicin (823.0) and vinblastine (811.0)) were found to lie below the linearly fitted line. This reflected a stronger binding of these molecules to the phosphatidylcholine groups of the artificial membranes leading to "longer than expected" in-column retention. It may be induced from these results that IAM profiling may not be sufficient to account for the partition of these molecules across the biomembranes.

In the stepwise regression of log Caco-2 against $\log k'_{IAM}$ and the other physicochemical parameters ($C\log P$, MW, HBD, HBA and PSA), The following regression equation

Table 2
Retention times (t_r) of ampicillin at different methanol composition

Percentage of methanol composition (%)	t_r (min) [mean ± S.D.]
20	2.36 ± 0.0125
40	2.06 ± 0.00625
60	1.87 ± 0.00665

with the best statistics was obtained:

$$\log \text{Caco-2} = 1.220(0.245; 0) + 0.666(0.117; 0)\log k'_{IAM} - 0.00344(0.000744; 0)\text{MW},$$
$$n = 32,\ R^2 = 0.555,\ s = 0.575,\ F = 18.11 \quad (6)$$

These results showed that $C\log P$, HBD, HBA and PSA variables are not statistically significant, and there-

Table 3
IAM capacity factors and physicochemical variables of 32 test compounds

Compounds	$C\log P$	$\log k'_{IAM}$	log Caco-2	QPCaco	MW	HBD	HBA	PSA
Acetaminophen[a]	0.494	0.346	1.83059[b]	1046.66	151.2	2	3	49.33
Acetylsalicylic acid[a]	1.023	−0.0442	0.380211[c]	35.50	180.2	1	4	63.6
Ampicillin[a]	−1.204	−0.0684	−0.377675[d]	1.409	349.4	3	7	112.73
Atenolol[a]	−0.109	0.628	−0.10791[d]	50.02	266.3	3	5	84.58
Caffeine[a]	−0.040	0.230	1.88195[d]	3007.03	194.2	0	6	61.82
Captopril[a]	0.890	−0.834	−0.815308[e]	71.79	217.3	1	4	57.61
Carbamazepine	1.980	1.634	1.84572[d]	2328.33	236.3	1	3	46.33
Cephalexin[a]	−1.640	−0.125	−0.301029[c]	1.19	347.4	3	7	112.73
Chloramphenicol	1.283	1.212	1.20952[d]	130.24	323.1	3	6	112.7
Cimetidine[a]	0.351	0.875	−0.130768[c]	353.68	252.3	3	6	88.89
Diclofenac	4.726	2.282	1.81425[f]	106.40	296.2	2	3	49.33
Diltiazem	3.647	2.449	1.43297[d]	765.61	414.5	0	6	59.08
Etoposide	−0.34	1.555	−0.181774[g]	74.833	588.6	3	13	160.83
Hydrocortisone	1.697	1.562	1.20683[d]	157.82	362.5	3	5	94.83
Ibuprofen	3.679	1.281	1.72016[c]	143.46	206.3	1	2	37.3
Indomethacin	4.180	2.019	1.30963[c]	58.09	357.8	1	5	68.53
Ketoconazole	3.102	2.288	1.51851[d]	3816.89	531.4	0	8	69.06
Labetalol	2.500	1.900	1.06819[d]	54.43	328.4	4	5	95.58
Lucifer yellow[a]	−6.846	−0.680	−0.522878[d]	0.10	443.4	4	14	244.95
Mannitol[a]	−2.046	−1.872	0.301030[h]	6.01	182.2	6	6	121.38
Pindolol	1.671	1.074	1.22272[c]	312.91	248.3	3	4	57.28
Prednisolone	1.423	1.648	1.32634[g]	123.25	360.5	3	5	94.83
Procaine	2.538	0.986	1.24055[f]	208.61	236.3	1	4	55.56
Propranolol	2.753	2.404	1.47129[d]	612.30	259.3	2	3	41.49
Quinidine	2.785	2.461	1.30963[d]	2722.83	324.4	1	4	45.59
Ranitidine	0.630	0.984	−0.229147[d]	186.75	314.4	2	6	83.58
Rifampicin	3.770	2.881	0.294466[d]	4.55	823	6	16	220.15
Salicylic acid[a]	2.187	−0.0417	1.62221[c]	58.40	138.1	2	3	57.53
Tamoxifen	6.818	1.651	0.1673173[d]	2494.28	371.5	0	2	12.47
Terfenadine	6.073	1.110	0.737987[d]	689.61	471.7	2	3	43.7
Testosterone	3.219	1.990	1.85896[i]	1980.96	288.4	1	2	37.3
Verapamil	4.466	2.726	1.29447[d]	1490.71	454.6	0	6	63.95
Vinblastine	5.227	2.555	0.705008[f]	20.25	811	3	13	154.1

[a] Compounds had their retention times measured at 100% aqueous phase.
[b] Literature Caco-2 P_{app} ($cm\,s^{-1}$, $\times 10^{-6}$) were cited from ref. [25].
[c] Literature Caco-2 P_{app} ($cm\,s^{-1}$, $\times 10^{-6}$) were cited from ref. [26].
[d] Literature Caco-2 P_{app} ($cm\,s^{-1}$, $\times 10^{-6}$) were cited from ref. [27].
[e] Literature Caco-2 P_{app} ($cm\,s^{-1}$, $\times 10^{-6}$) were cited from ref. [28].
[f] Caco-2 P_{app} ($cm\,s^{-1}$, $\times 10^{-6}$) were determined in-house experimentally.
[g] Literature Caco-2 P_{app} ($cm\,s^{-1}$, $\times 10^{-6}$) were cited from ref. [29].
[h] Literature Caco-2 P_{app} ($cm\,s^{-1}$, $\times 10^{-6}$) were cited from ref. [30].
[i] Literature Caco-2 P_{app} ($cm\,s^{-1}$, $\times 10^{-6}$) were cited from ref. [3].

fore they can be excluded. The $\log k'_{IAM}$ parameter, together with the size parameter (MW) gave an enhanced correlation ($R^2 = 0.555$) as compared to using $\log k'_{IAM}$ alone ($R^2 = 0.227$). The negative dependence of log Caco-2 on the molecular size, as reflected by the MW coefficient, can be rationalized based on the inverse relation that exists between the diffusion coefficient of a solute and its molecular weight. The statistical quality of Eq. (5) is not optimal and accounts for 55.5% of the variance in log Caco-2. This result shows that the use of $\log k'_{IAM}$ and size parameter is not sufficient to predict the partition of a chemically diverse set of drug molecules that cross the biomembranes either by active or passive transported process.

3.4. Modeling biopartitioning of passively transported compounds

Due to the nature of the system, absorption profiling of drug candidates using IAM technique only generates information on their passive absorption. It would not be applicable to compounds that are actively transported and effluxed (e.g. by the ATP-dependent P-glycoprotein (Pgp) transporter-mediated process). Despite this, IAM would still be useful to the pharmaceutical industry for passive absorption profiling, as many pharmaceutical drugs are absorbed across the intestinal mucosa by passive diffusion via transcellular route [1].

In the second part of the stepwise regression analysis, compounds that have established transport mechanisms such as paracellular transport and active Pgp efflux and compounds with extreme molecular size (MW lesser than 200 and greater than 800), are excluded from regression analysis sequentially (Table 4). The objective is to test the relevance of $\log k'_{IAM}$ and/or other physicochemical parameters in the prediction of passive absorption of drug. Molecular size was also considered here as it was established in our earlier regression analysis that molecular size is of prime importance in Caco-2 permeability determination. Molecules with low (below MW 200) and high (above MW 800) molecular weights tend to fall outside the linearly fitted curve (Table 4). These compounds consist of actively transported drug (salicylic acid), paracellularly transported drugs (acetaminophen, atenolol [31], caffeine, cimetidine [31] and ranitidine [31]) and Pgp-mediated drugs (cimetidine [32], terfenadine [33], vinblastine [34,35], tamoxifen [35], ketoconazole [36] and verapamil [37]). Tamoxifen is a known Pgp blocker. Using the monolayer efflux, ATPase and calcein-AM assays, Polli et al. [37] further classified the Pgp substrates into unambiguous substrates (terfenadine and vinblastine), transported substrates (cimetidine) and nontransported substrates (ketoconazole and verapamil). The unambiguous substrates demonstrated positive outcomes for the three assays while the transported substrates were positive for monolayer efflux assay and negative for either ATPase or calcein-AM assays. The nontransported substrates were positive for both ATPase and calcein-AM assays and negative for the efflux assay; these substrates also demonstrated high passive permeability ($P_{app\,A-B} > 300$ nm/s).

For the first stepwise regression analysis, the actively transported (salicylic acid), paracellularly transported (acetaminophen, atenolol, caffeine, cimetidine and ranitidine), low and high molecular weight compounds (acetaminophen, acetylsalicylic acid, caffeine, rifampicin, salicylic acid and vinblastine), transported and unambiguous Pgp substrates (cimetidine, terfenadine and vinblastine) and Pgp blocker (tamoxifen) were excluded. It may be noted that tamoxifen acts as a non-competitive inhibitor [37]). Its transport may be deviated from passive permeation in Caco-2 experiments, especially if the compound is tested at low concentration.

After deletion of the abovementioned compounds, the following regression equation with the best statistics was obtained:

$$\begin{aligned}\log \text{Caco-2} &= 1.133(0.289; 0.001) \\ &\quad + 0.732(0.0781;\ 0)\log k'_{IAM} \\ &\quad - 0.00348(0.000808;\ 0)\text{MW}, \\ n &= 21,\ R^2 = 0.840,\ s = 0.359,\ F = 47.35 \end{aligned} \quad (7)$$

Table 4
Compounds excluded during stepwise regression analysis and the associated factors that impact their biopartitioning

Compounds	Transport mechanism	Molecular size	P-glycoprotein class[a]
Acetaminophen	Paracellular	MW < 200	
Acetylsalicylic acid		MW < 200	
Atenolol	Paracellular		
Caffeine	Paracellular	MW < 200	
Cimetidine	Paracellular		Transported substrate
Ketoconazole			Nontransported substrate
Ranitidine	Paracellular		
Rifampicin		MW > 800	
Salicylic acid	Active transport	MW < 200	
Tamoxifen			PGP blocker
Terfenadine			Unambiguous substrate
Verapamil			Nontransported substrate
Vinblastine		MW > 800	Unambiguous substrate

[a] P-glycoprotein classification is based on ref. [37].

These results showed consistently that $C\log P$, HBD, HBA and PSA variables are not statistically significant. The negative dependence of log Caco-2 on the molecular size was also reflected by the MW coefficient. The statistical quality of Eq. (6) has significantly improved (reduced standard deviations of coefficients and better correlation factor) and accounts for 84% of the variance in log Caco-2. The results underscore the importance of $\log k'_{\mathrm{IAM}}$ and molecular size parameters in describing Caco-2 permeability for passively transported compounds. A recent report discussed that hydrogen bonding, an important factor in liposome or biomembrane partitioning, played only a minor role in IAM retention [16]. This is, however, not consistent with our results as HBD and HBA are not significant in our regression analysis and our data indicated that electron pair donor-acceptor interactions such as hydrogen bonding may be accounted by IAM binding.

Verapamil is a Pgp substrate and is the standard commonly used in Caco-2 Pgp transport studies [27]). While ketoconazole has a low affinity for Pgp (~2 μM), it does not show up as Pgp substrate in Caco-2 cell transport studies [27]). It has been identified as Pgp substrate in other membrane and cell-based assays [37]). Nonetheless, both verapamil and ketoconazole were found to fit the regression line relatively well in this study. Stepwise regression analysis with additional exclusion of these two drugs generated the following equation:

$$\begin{aligned}\log \text{Caco-2} &= 1.306(0.314; 0.001) \\ &\quad + 0.723(0.0786; 0)\log k'_{\mathrm{IAM}} \\ &\quad - 0.00402(0.000887; 0)\mathrm{MW}, \\ n = 19,\ R^2 &= 0.866,\ s = 0.344,\ F = 51.61 \end{aligned} \quad (8)$$

As shown in Eq. (8), although there was a slight increase in the squared correlation coefficient with the exclusion of verapamil and ketoconozole, the enhancement is not marked. Polli et al. [37] categorized compounds like verapamil and ketoconazole as nontransported Pgp substrates (positive in the ATPase and calcein-AM assays). As both compounds have high passive permeabilities, the monolayer efflux effect becomes insignificant. Since these compounds show high passive permeability, IAM technique would provide a fairly accurate prediction on their biopartitioning activity. This explains the good fit of these compounds in the regression analysis and the consistency between our observation and the findings of Polli et al.

4. Conclusions

The validated IAM chromatographic method is simple, rapid and reproducible for measuring membrane partition coefficients. The retention data on IAM for 32 structurally diverse compounds were found to be poorly correlated to the Caco-2 absorption values. The correlation was however significantly improved after stepwise regression analysis using IAM and physicochemical parameters. The results indicated the importance of IAM and molecular size variables in measurement of biopartitioning. Other physicochemical variables such as HBD, HBA and PSA, were found to have less influence in describing the Caco-2 absorption. An optimal squared correlation coefficient ($R^2 = 0.840$) was obtained after stepwise regression analysis with the exclusion of compounds that are transported actively/paracellularly, affected by Pgp efflux mechanism and compounds that have molecular weight lower than 200 and higher than 800. It was also determined that molecules that are nontransported Pgp substrates with high passive permeability demonstrate a good fit with our IAM model. IAM chromatographic technique is useful in the early profiling of drug candidates, thus reducing the number of actual Caco-2 cell experiments needed. The correlation model described by Eq. (8) may be applied to the prediction of Caco-2 permeability of compounds that cross cell membranes mainly by passive diffusion. However, it was demonstrated in this study that several factors could influence the prediction of passive absorption by IAM chromatography. Hence, IAM retention factors have to be interpreted and correlated carefully as some drug candidates that have certain physicochemical properties may be potential outliers.

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