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Article

Orthogonal Chromatographic Descriptors for Modelling Caco-2 Drug Permeability

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The use of chromatographic descriptors as alternative for Caco-2 permeability in drug absorption screening was evaluated. Therefore, retentions were measured on 17 Reversed-Phase Liquid Chromatographic systems, considered to be orthogonal or dissimilar, and an Immobilized Artificial Membrane (IAM) system. Retentions on a Micellar Liquid Chromatography system were taken from the literature. From this set of systems, those found dissimilar for the used data set were selected. The retention factors on these systems were then used as descriptors in QSAR modelling. Modelling was performed using Stepwise Multiple Linear Regression. This resulted in a model using only two chromatographic systems with good descriptive and acceptable predictive properties.

A high qualitative model was obtained by combining both chromatographic systems selected in the previous model with a lipophilicity parameter (the squared Moriguchi n-octanol/water partition coefficient) and the molecular volume.

Introduction

Assessment of ADME-Tox (Absorption, Distribution, Metabolisation, Elimination, and Toxicity) properties of potential drug molecules constitutes one of the major bottlenecks of modern drug development. When a molecule, found to be potentially useful since it interacts with a target molecule, fails in the preclinical phase where the ADME-Tox properties are investigated *in vivo*, it causes a loss of time and resources for the industry. Therefore the pharmaceutical industry is interested in the development of cheap and fast screening approaches for ADME-Tox properties. This paper will focus on the problem of screening for the absorption properties of molecules.

To replace the complex, time-consuming and labour-intensive *in vivo* experiments, several in vitro techniques were developed. The latter can be divided in animal tissue-based, cell-based and artificial membrane-based methods. In animal tissue-based methods, excised intestinal tissue is used to study intestinal drug absorption. A solution, containing the drug, is applied to one side of the tissue and the rate of drug absorption is measured by the disappearance of the drug at one side and/or the appearance at the other. Examples of tissue-based methods are the perfused intestinal segments, the everted sac technique and the side-by-side Ussing chambers. One of the major drawbacks is that the viability of the isolated tissue is difficult to maintain, since the tissue is cut off from the blood circulation and therefore needs continuous oxygenation (1, 2).

A high variety of different cell monolayers also has been used to study gastro-intestinal absorption of drugs (1–4). Mostly human tumor cells are used since they grow rapidly in confluent monolayers followed by a spontaneous differentiation. The most popular cell line is Caco-2. Caco-2 cells originate from a human adenocarcinoma that undergoes spontaneous differentiation to enterocytes in culture. To obtain a confluent monolayer, these cells are grown on a semi-permeable porous filter. The solution containing the investigated substance is applied at the apical side of the monolayer. After incubation samples are taken in the compartments at both sides of the monolayer (1–5). Different studies showed good correlation between the Caco-2 permeability and the human intestinal absorption (3–8). One of the major drawbacks of the technique is the high interlaboratory variability between the cell-lines and thus of the measured Caco-2 permeability. Even though this method is very popular, the culture of the cell line still represents high costs and a high work load. Therefore methods based on artificial membranes were developed. The most popular in this category is the Parallel Artificial Membrane Permeability Assay (PAMPA) (1, 2). A PAMPA experiment is run on a 96-well plate that consists of two parts. The bottom part is filled with a buffer containing the investigated substance. On top of it an artificial phospholipid/dodecane membrane supported by a polycarbonate filter forms a barrier. The well plate with the acceptor wells is placed on top of the membrane. After incubation, concentrations of the investigated substance in donor and acceptor wells are measured using a 96-well UV spectrophotometric plate reader. Even though several authors (9–15) found good correlations between PAMPA results and human intestinal absorption, the method suffers from some serious drawbacks. The method is limited to molecules measurable with UV spectrophotmetry and requires long incubation times, which might be problematic for unstable molecules. The method also measures only passive diffusion (1, 2).

Since several years, attention has been paid to the use of chromatographic techniques in screening for absorption of molecules. The developed techniques use either classical reversed-phase conditions (16), special stationary phases or special mobile phases (1, 2, 17, 18). In IAM systems, columns are used that are essentially reversed-phase liquid chromatographic columns where the classic hydrocarbon groups bonded to the solid silica support are replaced by covalently-bonded membrane phospholipids. In this way a monolayer of phospholipids bonded to silica is obtained that should mimic

the lipophilic environment of the cell membrane (1, 2, 19). Good correlations were obtained between IAM retention factors and membrane permeability processes as Caco-2 permeability (3, 4) and gastro-intestinal absorption (1, 2, 19–23).

Micellar liquid chromatography typically uses a reversed-phase HPLC column combined with a mobile phase containing a surfactant above its critical micellar concentration, forming micelles in the mobile phase (24). These micelles show structural similarities with the structure of the cell membranes, making MLC retentions interesting for the prediction of membrane permeability. A few recent studies have shown the potential of MLC methods in screening for drug absorption (18, 25–27).

In this paper the use of dissimilar chromatographic systems in screening for absorption was evaluated. Therefore a data set (25) containing the Caco-2 permeability values for 28 structurally diverse drug molecules was used. Retention of the molecules was measured on a set of 17 dissimilar chromatographic systems. The data set was extended with the retentions measured on an IAM system and the MLC retentions, extracted from literature (25). In a first step, the system, with the highest correlation with the Caco-2 permeability values as well as the systems orthogonal to the latter were selected. The idea here is that orthogonal systems are based on different retention mechanisms and thus provide different information about the investigated molecules. In a second step, Multiple Linear Regression models were built using the selected systems. They were evaluated for their descriptive and predictive properties. In a third step, it was evaluated whether better models could be obtained by combining the chromatographic with some theoretical descriptors.

Theory

Orthogonal chromatographic systems

In statistics two parameters are orthogonal when their correlation coefficient is zero. In the context of chromatographic systems the term orthogonal is not used in its strict mathematical sense. Orthogonal systems are defined as systems that differ significantly in chromatographic selectivity. This means that two systems with a low correlation coefficient (e.g., $r < 0.4$) between their retention data can also be considered or called orthogonal. In this case the term dissimilar chromatographic systems could be preferred (28). A discussion on the selection of orthogonal, similar or dissimilar systems can be found in references (28–30). In this study seventeen orthogonal RPLC systems, identified by Van Gyseghem et al. (28), were used. They were selected from a set of 46 systems. In the selected set not all pairs of systems have a mutual correlation coefficient below 0.4. The systems are selected in such a way that they are the most dissimilar towards the other systems tested.

Chemometric Techniques

Multiple linear regression

Multiple linear regression (MLR) is the most widely applied multiple linear modelling technique. Often MLR can not be used to model complex QSAR data due to the fact that the number of descriptive variables exceeds the number of objects. Therefore variable selection prior to modelling is necessary. In this work, a stepwise selection procedure was used. In this technique a forward selection procedure iterates with a backward elimination procedure. The forward selection procedure starts with the variable that has the highest correlation with the response variable, for example the Caco-2 permeability. If this variable results in a significant regression, evaluated with an overall F-test, the variable is retained and selection continues. In a next step the variable that gives the largest significant increase of the regression sum of squares, evaluated with a partial F-test, is added. After each step of the forward selection procedure, the backward elimination procedure is applied. In this procedure a partial F-test for the variables already in the model is performed. If a variable is found that does not longer contributes significantly to the regression it is removed from the model. The iterative process is repeated until the model can not be improved anymore by adding or removing variables (31).

Clustering

To cluster the different systems according to their similarity, the Weighted Pair Group Method using arithmetic Averages (WPGMA) was applied.

This is a hierarchical agglomerative clustering technique. Hierarchical means that smaller clusters are included in larger ones or vice versa, and agglomerative means that objects are sequentially merged (32). The goal of the method is to classify m objects in m-1 steps. In each consecutive step, the two most similar objects (clusters) are merged. The objects (clusters) to merge are derived from the dissimilarity matrix, representing the dissimilarity between each pair of objects (clusters). Dissimilarities or dissimilarity coefficients are positive numbers that are small if two objects are closely related and large if they differ (28–30). In this study $1-|r|$, with r the pearson correlation coefficient, was used as dissimilarity criterion (29, 30). The two least dissimilar objects (clusters) are merged and the dissimilarity matrix is recalculated for the new situation (32).

In WPGMA, also called weighted average linkage, the dissimilarity between two clusters is defined as the average of all dissimilarities calculated between any object in both clusters. This method considers all objects equally important and thus having the same weight. This means that clusters consisting of a larger number of objects carry a larger weight (33, 34). The results of the clustering are represented as a dendrogram.

Molecular descriptors

A molecular descriptor is either the final result of a logical and mathematical procedure, which converts chemical information from a symbolic representation of the molecule into a useful numerical value (theoretical descriptor), or is the result of a standardized experiment (experimental descriptor) (35).

Molecular descriptors can be classified in different ways, e.g. in experimental and theoretical descriptors, as indicated above. The latter are further subdivided depending on the molecular representation they are derived from. Descriptors derived from a molecular formula are called zero-dimensional (0D) (e.g., molecular weight, atom-counts . . .). One-dimensional

(1D) descriptors are derived from a substructure list representation of the molecule [(e.g. log P calculated with the method of Rekker (360)]. Two-dimensional (2D) [e.g. connectivity indices (35)] and three-dimensional (3D) [e.g., the molecular volume, different geometrical and steric descriptors (35)] descriptors are calculated from a topological and a geometrical molecular representation, respectively. Finally, the descriptors derived from a stereo-electronic or lattice representation are called four-dimensional (4D). For more information on theoretical descriptors we refer to (35).

Materials and Methods

Drugs and reagents

The 28 substances used are listed in Table I, together with their supplier, CAS-number and Caco-2 permeability value, given as log P_{app}. The latter values are taken from reference (25). For all substances, sample solutions were prepared by dissolving 1 mg substance in 10 mL. To inject on the orthogonal systems, solutions were prepared in 1:1 (v/v) organic modifier/Milli-Q water. The organic modifier used was either acetonitrile or methanol, both HPLC grade from Fisher Scientific (Loughborough, Leicestershire, UK).

For the IAM system, the solutions are prepared by dissolving the appropriate amount of test component in mobile phase.

Phosphoric acid solution min. 85% (Carlo Erba, Milan, Italy), acetic acid glacial 100%, anhydrous disodium tetraborate, boric acid, disodium hydrogenium phosphate dihydrate, sodium dihydrogenium phosphate monohydrate, sodium hydroxide pellets, all pro analysis (GR quality) (all from Merck, Darmstadt, Germany), sodium dodecyl sulfate (SDS) and polyoxyethylene lauryl ether (both from Sigma-Aldrich, Steinheim, Germany) were used in the mobile phases.

Chromatographic conditions

All measurements were carried out on an HPLC-instrument consisting of an L-7100 liquid chromatograph pump, an L-7612 solvent degasser, an L-7250 autosampler with a 100 μL loop, an L-7350 column oven, an L-7400 UV detector and a D-7000 interface (Merck-Hitachi, Tokyo, Japan). The chromatographic data were gathered and treated with the D-7000 HPLC System Manager software (Merck-Hitachi).

The chromatographic conditions for the set orthogonal systems are summarized in Table II. Nine stationary phases were used: (a) a Chromolith Performance, RP-18e column (100 x 4.6 mm i.d.) (Merck), a monolithic phase, (b) a Zorbax Extend-C18 column (150 x 4.6 mm i.d., 3.5 μm) (Agilent, Palo Alto, California, USA), a bidentate bonded and double-endcapped octadecylsilica column, (c) a ZirChrom-PS column (100 x 4.6 mm i.d., 3 μm) (ZirChrom Separations, Anoka, MN, USA), a zirconia-based phase coated with polystyrene, (d) a Zorbax Eclipse XDB-C_8 column (150 x 4.6 mm i.d., 5 μm) (Agilent), a densily bonded double-endcapped C8-silica column, (e) a Betasyl Phenyl Hexyl column (100 x 4.6 mm i.d., 5 μm) (Thermo Hypersyl Keystone, Cheshire, UK), a phenyl-hexyl-

Table I
The Set of 28 Substances and their log P_{app} Values

Substance (concentration in mg/10ml)	Distributor	CAS number	Log P_{app}
Acebutolol.HCl	Sigma-Aldrich (Steinheim, Germany)	37517-30-9	−6.35
Alprenolol.HCl	Sigma-Aldrich	13655-52-2	−4.54
Astemizole	J&J Pharmaceutical Research & Development (Beerse, Belgium)	68844-77-9	−5.86
Atenolol	Sigma-Aldrich	29122-68-7	−6.68
Carbamazepine	Sigma-Aldrich	298-46-4	−4.55
Chlorpheniramine maleate	Sigma-Aldrich	113-92-8	−4.43
Chlorpromazine.HCl	Fluka Chemie (Buchs, Switzerland)	69-09-0	−5.03
Cimetidine	SK&F (Herts, United Kingdom)	51481-61-9	−6.04
Clonidine.HCl	Sigma-Aldrich	4205-90-7	−4.52
Desipramine.HCl	Sigma-Aldrich	50-47-5	−4.76
Diphenhydramine.HCl	Sigma-Aldrich	147-24-0	−4.50
Enilconazole	J&J Pharmaceutical Research & Development	35554-44-0	−4.57
Esmolol.HCl	Du Pont De Nemours (Le Grand Saconnex, Switzerland)	103598-03-4	−5.52
Imipramine.HCl	Sigma-Aldrich	50-49-7	−4.53
Ketoconazole	J&J Pharmaceutical Research & Development	65277-42-1	−4.67
Labetalol.HCl	Sigma-Aldrich	36894-69-4	−4.89
Mebenazole	J&J Pharmaceutical Research & Development	31431-39-7	−4.60
Metoprolol	Astra Hassle AB (Lund, Sweden)	37350-58-6	−4.56
Oxprenolol.HCl	Cynamid Benelux n.v. (Brussels, Belgium)	6452-71-7	−4.55
Pindolol	Sigma-Aldrich	13523-86-9	−4.64
Promazine.HCl	Sigma-Aldrich	53-60-01	−4.54
Propranol.HCl	Fluka (Neu-Ulm, Switzerland)	525-66-6	−4.59
Ranitidine.HCl	Sigma-Aldrich	66357-35-5	−6.08
Terconazole	J&J Pharmaceutical Research & Development	67915-31-5	−4.73
Thioridazine	Merck (Darmstadt, Germany)	50-52-2	−5.24
Timolol maleate	Sigma-Aldrich	26839-75-8	−4.81
Trifluoperazine.HCl	Sigma-Aldrich	117-89-5	−5.39
Flubenazole	J&J Pharmaceutical Research & Development	31430-15-6	−4.71

Table II
Chromatographic Conditions for the 17 Orthogonal RPLC Systems [30]

No.	Stationary phase	Mobile phase conditions and column temperature
CS1	Chromolith Performance	methanol/0.08M sodium phosphate buffer pH 3.0 from 10:90 to 75:25% (v/v) in 4 min; flow rate 2.0 mL/min; 40°C
CS2	Chromolith Performance	methanol/0.08M sodium phosphate buffer pH 6.8 from 10:90 to 75:25% (v/v) in 3 min; flow rate 2.0 mL/min; 40°C
CS3	Zorbax Extend-C18	methanol/0.08M sodium borate buffer pH 10.0 from 10:90 to 75:25% (v/v) in 6 min; flow rate 1.0 mL/min; 40°C
CS4	ZirChrom-PS	methanol/0.08M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 6 min; flow rate 1.5 mL/min; 40°C
CS5	ZirChrom-PS	methanol/0.08M sodium phosphate buffer pH 6.8 from 10:90 to 70:30% (v/v) in 4 min; flow rate 1.5 mL/min; 40°C
CS6	ZirChrom-PS	methanol/0.08M sodium borate buffer pH 10.0 from 10:90 to 70:30% (v/v) in 4 min; flow rate 1.5 mL/min; 40°C
CS7	ZirChrom-PS	acetonitrile/0.04M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 mL/min; 40°C
CS8	ZirChrom-PS	acetonitrile/0.04M sodium phosphate buffer pH 6.8 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 mL/min; 40°C
CS9	Zorbax Eclipse XDB-C_8	methanol/0.04M sodium phosphate buffer pH 6.8 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 mL/min; 40°C
CS10	Zorbax Eclipse XDB-C_8	acetonitrile/0.04M sodium phosphate buffer pH 6.8 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 mL/min; 40°C
CS11	Betasil Phenyl Hexyl	methanol/0.04M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 mL/min; 40°C
CS12	Betasil Phenyl Hexyl	acetonitrile/0.04M sodium phosphate buffer pH 6.8 from 10:90 to 70:30% (v/v) in 8 min; flow rate 1.0 mL/min; 40°C
CS13	Suplex pKb-100	methanol/Britton-Robinson buffer pH 2.5 from 30:70 to 75:25% (v/v) in 20 min; flow rate 1.0 mL/min; 40°C
CS14	ZirChrom-PBD	methanol/Britton-Robinson buffer pH 2.5 from 30:70 to 75:25% (v/v) in 20 min; flow rate 1.0 mL/min; 40°C
CS15	ZirChrom-PBD	acetonitrile/0.04M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 5 min; flow rate 2.0 mL/min; 75 °C
CS16	Shodex Rspak DE-413	methanol/0.04M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 5 min; flow rate 1.2 ml/min; 40°C
CS17	Discovery RP-AmideC16	acetonitrile/0.04M sodium phosphate buffer pH 3.0 from 10:90 to 70:30% (v/v) in 5 min; flow rate 1.5 ml/min; 40°C

silica column, (f) a Suplex pK_b-100 column (150 x 4.6 mm i.d., 5 μm) (Supelco, Bellefonte, PA, USA), a base-deactivated hexadecylsilica column, (g) a ZirChrom-PBD column (100 x 4.6 mm i.d., 3 μm) (ZirChrom Separations), a zirconia-based phase coated with polybutadiene-polymer, (h) a Shodex RSpak DE-413 column (150 x 4.6 mm i.d., 4 μm) (Showa Denko, Tokyo, Japan), a polymethacrylate-packed column and (i) a Discovery RP-Amide C16 column (100 x 4.6 mm i.d., 5 μm) (Supelco), a high-purity hexadecylsilica with a polar-embedded amide function bonded to the silica surface with a propyl group (28, 29). Gradient elution was used and the gradient profiles are summarized in Table II.

The IAM system (CS18) was that described by Nasal et al. (16). The stationary phase was an IAM-PC-MG column (150 x 4.6 mm i.d., 12 μm) (Regis Technologies Inc, Mortom Grove), an IAM phase formed by 1-myristoyl-2((13-carboxyl)-tridecoyl)-sn-3-glycerofosfocholin, chemically bonded to silica propylamin. The mobile phase consisted of a mixture of 30:70 (v/v) acetonitrile/ 0.1 M phosphate buffer pH 7.0. Measurements were carried out isocratically.

All buffers were filtered through a 0.2 μm membrane filter (Schleicher & Schuell, Dassel, Germany).

The retentions values on a MLC system using SDS as surfactant (CS19) were taken from reference (25). A Discovery C8 silica column (50 x 4.6 mm i.d., 5 μm) (Supelco) was used as stationary phase. The mobile phase consisted of a mixture of 15:85 (v/v) *n*-propanol−0.01M phosphate buffer-0.15M SDS-pH 7.4. (25).

Molecular structure optimization

The 3-dimensional structures of the molecules were drawn and optimized using the Hyperchem 6.03 Professional software (Hypercube, Gainesville, Florida). After inputting the molecule as a topological structure, geometry optimisation was obtained by the Molecular Mechanics Force Field method (MM+) using the Polak-Ribière conjugate gradient algorithm with a RMS gradient of 0.1 kcal/(Å mol) as stop criterion. The optimisation of the structure results in a data matrix consisting of the Cartesian coordinates of the atoms, defining the structure. This data matrix was used to calculate molecular descriptors.

Descriptor calculation

The Hyperchem 6.03 Professional software (Hypercube, Gainesville, Florida, USA) was used to calculate solvent accessible surface area (SASA), molecular volume (HyVol), (Ghose-Crippen) octanol/water partition coefficient (HyLogP) (35), hydration energy, molar refractivity, molar polarisability and molar mass. The Mc Gowan volume, one of the descriptors of the linear free energy relationship (LFER), was calculated manually (37, 38).

The Dragon 5.0 Professional software (39) allows the calculation of 20 different classes of molecular descriptors. In this paper, Dragon was used to calculate some additional molecular properties like the number of H-acceptors (nHacc), the number of H-donors (nHdonn), the polar surface area (PSA), the Moriguchi n-octanol/water partition coefficient (MlogP), the squared MlogP values ($MlogP^2$), the hydrophilic factor (Hy) and the Ghose-Crippen molar refractivity (AMR).

Model building

All models were built using SPSS version 13.0 for Windows (SPSS inc, Chicago, IL). All data was autoscaled prior to modeling. Clustering and cross validation was performed using in house algorithms written for Matlab 5.3 (The Mathworks, Natick, MA).

Results and Discussion

Chromatographic measurements

The retention of the 28 drug molecules was measured on each system. The logarithms of the retention factors log k were used. Results are not shown here but are available as supplementary data with the online version of the paper. For the IAM-system (CS18) the dead volume was determined by injecting a mixture of 1:1 (v/v) methanol/Milli-Q water and for the other 17 systems (CS1-CS17) of 1:1 (v/v) organic modifier/Milli-Q water. The organic modifier was either methanol or acetonitrile. All dead volume measurements were repeated three times. The retentions of the MLC system (CS19) were taken from reference (25).

Retention times were corrected for column ageing (40). for this purpose, a reference substance (desipramine) was injected after each tenth sample injection. The retention of a substance was corrected relativily to the retention time change observed for the reference substance. Corrections were carried out as follows:

$$y_{i,corrected} = y_{i,measured} + y_{ref,begin} - \left(\frac{(p+1-i)y_{ref,before} + iy_{ref,after}}{p+1}\right) \quad (1)$$

where i = 1, 2, . . ., p and p is the number of injections between two consecutive injections of the reference substance. $y_{i,corrected}$ and $y_{i,measured}$ are the corrected and the measured retention times of a test substance, respectively, $y_{ref,begin}$ is the retention time of the reference substance, measured before the start of the test samples injections, $y_{ref,before}$ and $y_{ref,after}$ are the retention times of the reference substance before and after a series of ten injections in which the corrected test substance is measured [40].

Selection of the most dissimilar systems

The set of 17 systems was selected earlier using a data set consisting of 68 structurally diverse molecules (28). Since in this study another data set of 28 structurally diverse molecules was used and the fact that the seventeen systems were extended with two systems, i.e. the IAM- (CS18) and the MLC (CS19)-system, it was necessary to recheck the orthogonality of the systems. The orthogonality between the systems was determined from the Pearson's correlation coefficients between the log k values for the 28 substances. To visualise the dissimilarity of the systems weighted-avarage-linkage clustering (WPGMA) was applied. The WPGMA-dendrogram is shown in Figure 1. It shows the dissimilarity, defined as 1- |r|, with r the Pearson's correlation coefficient, as a function of the system number. When drawing an arbitrary line at 0.5, three groups of similar systems can be observed as well as three individual systems (CS4, CS5 and CS13) considered orthogonal

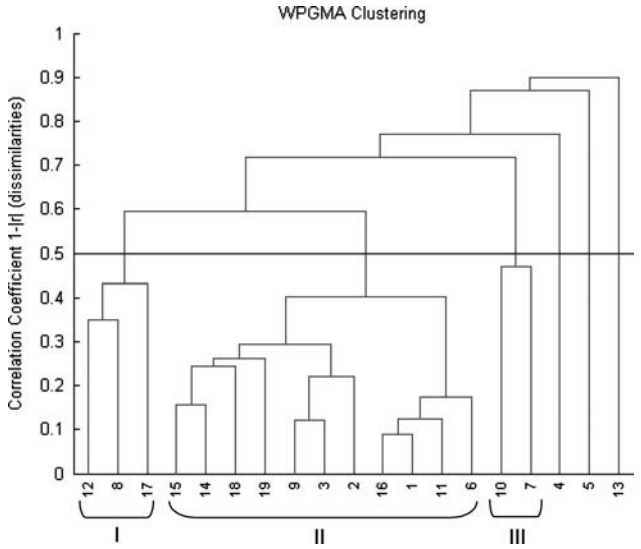

Figure 1. WPGMA-dendrogram for the 19 chromatographic systems.

with each other and the rest of the set. The IAM (CS18) and the MLC (CS19) are both in group II. The retentions on both systems are rather highly correlated (r = 0,75) and the correlations with the other systems in the group are above 0,60. This means that the IAM (CS18) and the MLC (CS19) systems can not be considered orthogonal with each other, nor with the other systems in the set and will not add supplementary information to the 17 systems of the orthogonal set.

Based on the results shown in Figure 1, the set of systems can be reduced to six systems: One system of each group of similar systems and the three isolated systems (CS4, CS5 and CS13), considered dissimilar.

The selection of the systems from the three similar groups was done according to the principle of the Stepwise MLR algorithm. The algorithm starts with selecting the variable with the highest correlation with the response variable, i.e. with log P_{app} (31). Therefore the system with highest correlation with log P_{app} was selected. Further, the systems most dissimilar to this system were also maintained in the set. System 11 shows the highest correlation ($r = 0.65$) with log P_{app}. Figure 2 shows the correlation between log P_{app} and the log k values on system 11. This system used a Betasil Phenyl Hexyl stationary phase. This column is a mixed mode column with both aromatic phenyl and aliphatic hexyl groups. The presence of aromatic groups provides the possibility of π-π electron interactions with the chromatographed compounds. This kind of interactions seems important in the process of Caco-2 permeation and more generally in the absorption of molecules. In previous work (41), we observed the importance of zirconium-based stationary phases in the prediction of gastro-intestinal absorption. These columns also show π-π electron interactions (42) with the chromatographed compounds. The IAM (CS18) and MLC (CS19) systems show very low correlation (r = 0.29 and r = 0.26, respectively) with log P_{app}, meaning that neither of the systems could be used individually in a regression model for Caco-2 permeability. This is not surprising. Detroyer et al. (25) showed for the same data set that the retentions on the MLC system are not linearly correlated with the log P_{app} values. They could mark cut-off values for the retentions, between

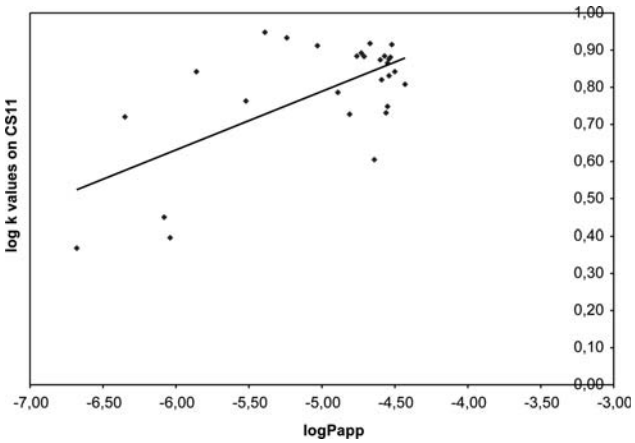

Figure 2. Correlation between $LogP_{app}$ and the log k values on system 11 (CS11).

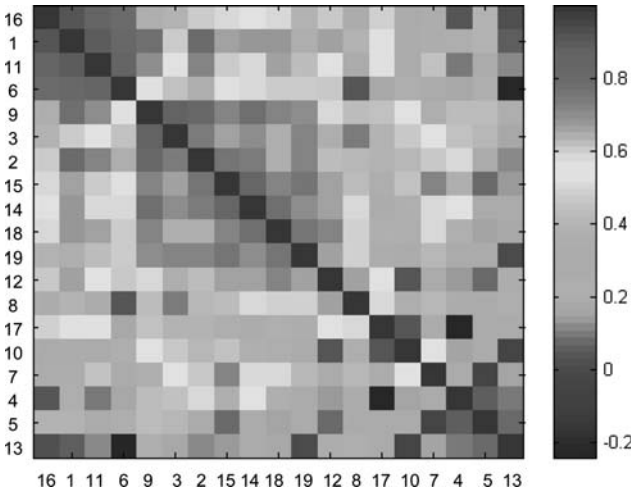

Figure 3. Color map representing the correlation matrix for the 19 chromatographic systems. The systems are arranged according to increasing dissimilarity.

which molecules show permeability through the Caco-2 layer. The retentions on the IAM system showed a similar trend when plotted against log P_{app}, even though no clear cut-off values could be defined.

The correlation matrix for all systems is represented by a color map (Figure 3) in which the systems were sorted by increasing dissimilarity based on the WPGMA-dendrogram (Figure 1).

Since MLR is not applicable with highly intercorrelated descriptive variables, only systems with a correlation coefficient with CS 11 below 0.4 were selected. Only 5 systems fulfill this criterion, i.e. CS13, CS5, CS4, CS10 and CS8 (Figure 3). The first three are those considered dissimilar to all other systems, CS 10 belongs to group III and CS 8 to group I (Figure 3). Since CS11 belongs to group II, all groups of the WPGMA-dendrogram (Figure 1) are represented in the set of 6, which will now be used to model log P_{app}.

Models using the selected chromatographic descriptors

In the previous section six dissimilar chromatographic systems were selected. The logarithms of the retention factors on the

selected systems were used as descriptive variables for modelling log P_{app}. Each time two different models were build: one using just the log k values for the six systems and one including also the quadratic term of the retention factors. In a first step classical MLR models, using all six systems, were built. Following models were obtained:

$$\hat{y} = -6.53 + 0.53\,CS4 + 0.03\,CS5 - 0.70\,CS8 - 0.02\,CS10 + 2.86\,CS11 - 0.92\,CS13 \quad (2)$$

$$\hat{y} = -5.48 + 0.81\,CS4 - 0.30\,CS5 + 0.54\,CS8 - 0.36\,CS10 + 0.93\,CS11 - 3.70\,CS13 - 0.33\,CS4^2 + 1.0\,CS5^2 - 2.24\,CS8^2 + 0.18\,CS10^2 + 1.31\,CS11^2 + 2.31\,CS13^2 \quad (3)$$

These models show correlation coefficients of 0.75 and 0.80 between measured and predicted values, standard errors of the estimate of 0.49 and 0.52, F values, obtained for lack of fit of 4.40 and 2.15, which indicate significant regressions ($p < 0.05$) and root mean squared errors of cross-validation (RMSECV) of 0.73 or 32,4% and 1.00 or 44.4%, obtained by leave-one-out cross-validation (LOOCV). The percentage was calculated over the range of log P_{app} values in the data set. Concerning the significance of the different coefficients in the equation only the constant function and the coefficient for CS11 are significant ($p < 0.05$) for model 2. In model 3 none of the regression coefficient could be considered significant at the 0.05 level. From the residual plots (results not shown) it could be seen that one molecule shows a high residual. This molecule is acebutolol. Investigating the data set showed that this molecule has a deviating retention behaviour. Acebutolol always had a lower retention factor than the other molecules with similar log P_{app} values on the systems most correlated with log P_{app} (CS11, CS6 and CS16). Therefore it was decided not to include acebutolol in further data analysis. New MLR models were built with the 27 remaining molecules. Following equations were obtained:

$$\hat{y} = -6.01 + 0.46\,CS4 + 0.05\,CS5 - 0.86\,CS8 - 0.11\,CS10 + 2.82\,CS11 - 1.22\,CS13 \quad (4)$$

$$\hat{y} = -6.34 + 0.74\,CS4 - 0.13\,CS5 + 2.04\,CS8 + 0.51\,CS10 + 1.92\,CS11 - 2.30\,CS13 - 0.26\,CS4^2 + 0.42\,CS5^2 - 3.00\,CS8^2 - 0.58\,CS10^2 + 0.36\,CS11^2 + 1.11\,CS13^2 \quad (5)$$

These models show correlation coefficients between measured and predicted values of 0.84 and 0.86 respectively, standard errors of the estimate of 0.37 and 0.41, F values, obtained for lack of fit of 7.76 and 3.34, which indicate a significant regression ($p < 0.05$) and RMSECV values of 0.62 or 27.5% (4) and 0.78 or 34.6% (5), obtained by LOOCV. For model 4 only three regression coefficients are significant at the 0.05 level, i.e. the constant function and the factors concerning CS11 and CS13. For model 5 none of the regression coefficients are significant ($p < 0.05$). From these results it can be seen that the introduction of quadratic terms of the retention factors does not improve the model, since it results in higher standard errors and cross-validation errors. From the investigation of the correlation and the residual plots (not shown) of both models it could be seen that the majority of the molecules are quite well described by the models, but that the models can be improved. Also the high cross validation errors and the relatively high correlation coefficients point at possible overfitting, especially for model 5.

Since in model 4 the majority of the coefficients and in model 5 all coefficients in the equations are insignificant, a lot of these factors introduce noise in the model. Therefore MLR models were built using a stepwise selection procedure. The stepwise procedure selects only the significant variables (systems) to model the response variable (log P_{app}). Following models were obtained:

$$\hat{y} = -6.06 + 2.71\,CS11 - 1.28\,CS13 \quad (6)$$

$$\hat{y} = -11.29 + 16.89\,CS11 - 10.79\,CS11^2 \quad (7)$$

The obtained models have correlation coefficients of respectively 0.79 and 0.80, standard errors of the estimate of 0.38 and 0.37, F values, obtained for lack of fit of 20.20 and 22.24, which indicate a significant regression ($p < 0.05$) and RMSECV values of 0.39 or 17.3% for model 6 and 0.37 or 16.3% for model 7, obtained by LOOCV. Even if the correlation coefficients are comparable to the models obtained without feature selection, the cross-validation errors are significantly lower, pointing at better predictive properties. This is an indication for the fact that the models shown in equation 4 and 5 were overfitting. From the results it can also be seen that the models with or without quadratic terms have comparable properties. Model 6 consists of three terms, using the retention on systems CS11 and CS13, which was expected based on the significance of the different factors in equation 4. Model 7 also consists of three terms but only uses the retentions on system CS11, which can be an advantage concerning workload, since the retention on only one system has to be measured. Not surprisingly the system is selected with the highest correlation with log P_{app}. Figure 4 shows the residual plots for both models.

The models obtained in equation 6 and 7 have acceptable descriptive and predictive properties, since the experimental error for permeability measurements is around 0.3 log units and the fact that only three molecules fall out of the residual range of -0,4 to 0,4 for model 6 and four for model 7. Still the residual plots do not show a complete random distribution of the residuals and quite high residuals are obtained for some of the molecules. Therefore it was considered that possibly better models could be obtained by adding some theoretically derived molecular properties to the data set and building a new stepwise MLR model, combining both theoretical and chromatographic descriptors.

Models combining chromatographic and theoretical descriptors

The molecular properties calculated in section 3.4. were added to the set of descriptive variables, resulting in a descriptor set combining theoretical molecular properties and chromatographic descriptors. Stepwise MLR was used to select the most

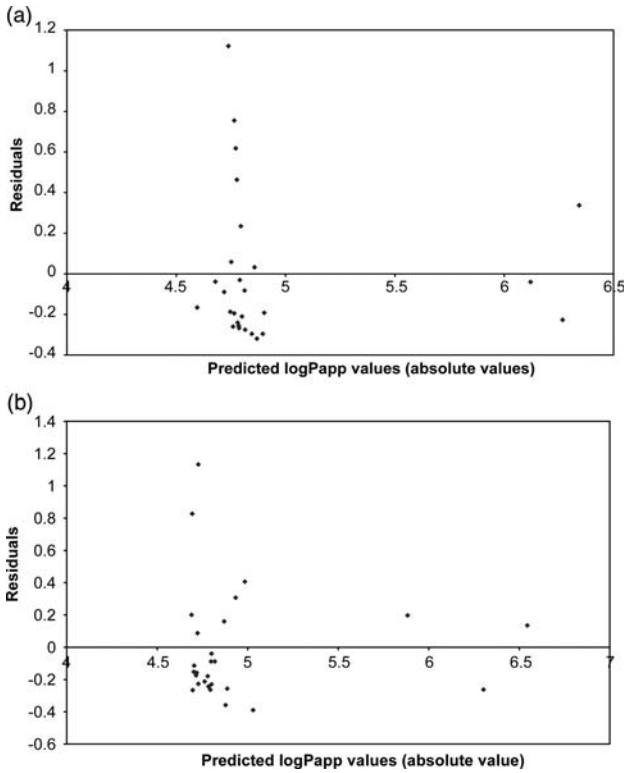

Figure 4. Stepwise MLR model using the selected chromatographic descriptors: (a) residual plot; (b) residual plot for the model including quadratic terms.

significant descriptors to model log P_{app}. Two models were built: one using just the theoretical descriptors and the log k values and one using the same set and adding the quadratic terms of the log k values. Both sets resulted in exactly the same model:

$$\hat{y} = -6.69 - 0.92\ \text{MlogP}^2 - 0.66\ \text{HyVol} + 3.90\ \text{CS11} - 0.91\ \text{CS13} \quad (8)$$

The obtained model has a correlation coefficient of 0.91, a standard error of the estimate of 0.27, an F value, obtained for lack of fit of 24.84, which indicate a significant regression ($p < 0.05$) and a RMSECV of 0.31 or 13.7%, obtained by LOOCV. The stepwise procedure selected the two chromatographic systems of the previous models (CS11 and CS13), together with MlogP^2 and HyVol as variables to describe the data set.

Adding the calculated properties, i.e. MlogP^2 and HyVol, measures for lipophilicity and molecular volume respectively, significantly improved the properties of the model, compared to the model obtained in equation 6. Both selected properties are known to play an important role in the process of membrane permeation. The lipophilicity is one of the key properties determining the passage through cell membranes and thus also a very important property for the passage through the Caco-2 cell monolayer (43–45). Also the molecular volume plays an important role in determining if a molecule will pass the cell membrane. This is for instance reflected by the use of the Mc Gowans volume in the Linear Free Energy Relationships built for different membrane permeability processes (37, 38, 46).

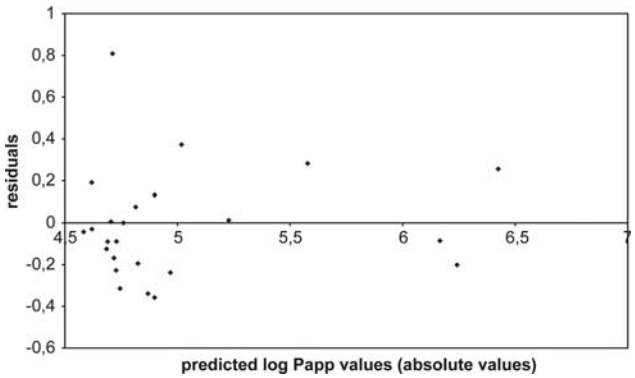

Figure 5. Stepwise MLR model combining the selected chromatographic descriptors and some theoretical molecular properties: residual plot.

Just for comparison a stepwise MLR model was built using only the calculated theoretical descriptors. Following model was obtained:

$$\hat{y} = -5.21 - 1.81\ \text{nHdonn} + 1.21\ \text{PSA} - 0.96\ \text{SASA} + 1.46\ \text{HylogP} \quad (9)$$

The obtained model has a correlation coefficient of 0.73, a standard error of the estimate of 0.44, an F value, obtained for lack of fit of 6.41, which indicate a significant regression ($p < 0.05$) and a RMSECV of 0.62 or 27.4%, obtained by LOOCV.

From the described results it is clear that the selection of two chromatographic descriptors, together with two theoretical descriptors by the stepwise procedure, designed to select the most significant variables out of the descriptor set, results in a better model. It should also be mentioned that the model based on only chromatographic descriptors has better properties than model 9.

In conclusion it can be stated that for this data set the combination of chromatographic with theoretical descriptors gives better results than those obtained with the two types of descriptors separately. It can also be seen that this model shows a cross-validation error (0.31) which is comparable with the experimental error for the permeability measurements, pointing at good predictive properties. Figure 5 shows the residual plot for the model presented in equation 8. From the plot it can clearly be seen that smaller residuals were obtained as well as a better distribution of these residuals compared to the model presented in equation 6.

Conclusions

This research showed that chromatographic descriptors can be useful in modelling membrane passage properties of drugs. It was shown that for the structural diverse data set used in this paper higher correlations could be obtained between the retention and the permeability parameters for a chromatographic system based on a Betasyl Phenyl Hexyl stationary phase, than for the IAM and the MLC systems. This is an advantage because this column is less sensitive for column ageing than the IAM column and no special mobile phase is necessary in contrast with MLC. This results in less labour intensive measurements and better reproducibility.

It was also shown that the combination of the two most orthogonal systems in the set, resulted in a model that gives a close description of the used data set. This shows that the use of a combination of orthogonal systems can result in better models compared to the use of a single chromatographic or chromatographic based method as IAM. Orthogonal systems are based on different retention mechanisms and so describe different properties of the chromatographed molecules.

From the results obtained with the different models it could also be seen that both the models based on chromatographic descriptors as the ones based on the combination of chromatographic descriptors and theoretical ones give better results than the model based on only *in silico* descriptors.

The final model presented, consists of four descriptors: two chromatographic and two theoretical descriptors. The two theoretical descriptors can easily be calculated with computer software, while the use of only two chromatographic systems, limits the work load of the experimental work. The model is easily applicable in high throughput screening and can be considered as a reasonable alternative for Caco-2 in early ADME-Tox screening. This final model shows a correlation coefficient between real and predicted values of 0.91 and a cross validation error of 0.31 log units, which is comparable to the experimental error for permeability measurements of 0.3 log units. Chan et al. [47] presented a linear model for Caco-2 permeability, based on IAM retention and the molecular weight. This model showed a correlation of only 0.86 and no evaluation of the predictive abilities was performed. The advantage of our model is that higher correlation is obtained and that no retentions on the very sensitive and expensive IAM columns are used.

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