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Experimental and Virtual Physicochemical and Pharmacokinetic Profiling of New Chemical Entities

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Abstract: Physicochemical and pharmacokinetic profiling of new chemical entities (NCEs) allows the rapid identification and elimination of compounds with properties not suitable for further development as drug candidates. Among the complex panel of theoretical and experimental methods available to predict or measure physicochemical or pharmacokinetic properties, some key techniques developed or tested in the pharmacochemistry group at EPGL are presented. This paper focuses on virtual and experimental approaches dealing with key properties such as ionization, solubility, lipophilicity, and membrane permeation. In addition, the effect of the third dimension on intramolecular interactions is exemplified by lipophilicity variations in the conformational space of cyclosporin A and with a 3D solvatochromic model able to accurately predict the BBB permeation.

Keywords: *In vitro* intestinal perfusion model · PAMPA technique · Pharmacokinetic profiling · Physicochemical profiling · 3D Solvatochromic model

Introduction

Successful drug development requires not only optimization of specific and potent pharmacodynamic activity, but also efficient delivery to the target site. Following advances in rational drug design and combinatorial chemistry, the number of newly discovered and promising active compounds has increased dramatically in recent years, often making poor pharmacokinetic properties the rate-limiting step in drug research. Over the last decade, several approaches were introduced to incorporate estimation of ADMET behavior in the early steps of drug design. The pharmacochemistry group at the Ecole de Pharmacie Genève-Lausanne continues previous efforts to develop and validate experimental and theoretical methods to allow a better characterization of lead-like or drug-like profiles of new chemical entities (NCEs). These projects (in close collaboration with several partners) explore two directions, namely the physicochemical space of NCEs and their pharmacokinetic space above all the permeation of several biological barriers.

Theoretical models (*in silico* methods) allow a rapid exclusion of compounds presenting poor ADMET properties and thus reduce a chemically diverse collection of NCEs to a pre-focused library. These models have to be validated with experimental methods which are used as filters in preliminary *in vitro* screening during the generation of a hit collection.

Physicochemical Filters

Over the last decade, drug research has identified precisely the need for an early determination of pharmacokinetic properties of new chemical entities by computational estimation and/or fast experimental measurements in order to obtain better drug candidates in terms of bioavailability and safety. It must be emphasized that efficient pharmacokinetic estimation depends largely on the detailed knowledge of complex molecular interactions between NCEs and different surrounding media which can be approximated by their physicochemical profile.

Moreover, computational pharmacokinetic estimations are largely performed using quantitative structure-pharmacokinetics (QSPkR) with an enhanced quality when a detailed knowledge of the physicochemical profile of compounds is available.

Over the past years, a large number of different techniques (Fig. 1) has been developed to predict various physicochemical properties directly from the molecular structure of solutes (virtual physicochemical profiling) or to measure them (experimental physicochemical profiling). Extensive work was performed in the pharmacochemistry group to develop, test, and validate some of these methods with a particular attention given to the convergence between theoretical and experimental approaches. This paper will focus on the most recent developments.

Ionization Constants

Ionizable chemical functional groups, present in about 70% of pharmaceutical compounds, offer a better control of the NCE physicochemical properties such as solubility or partitioning in aqueous and lipidic media. Modulating the ionization

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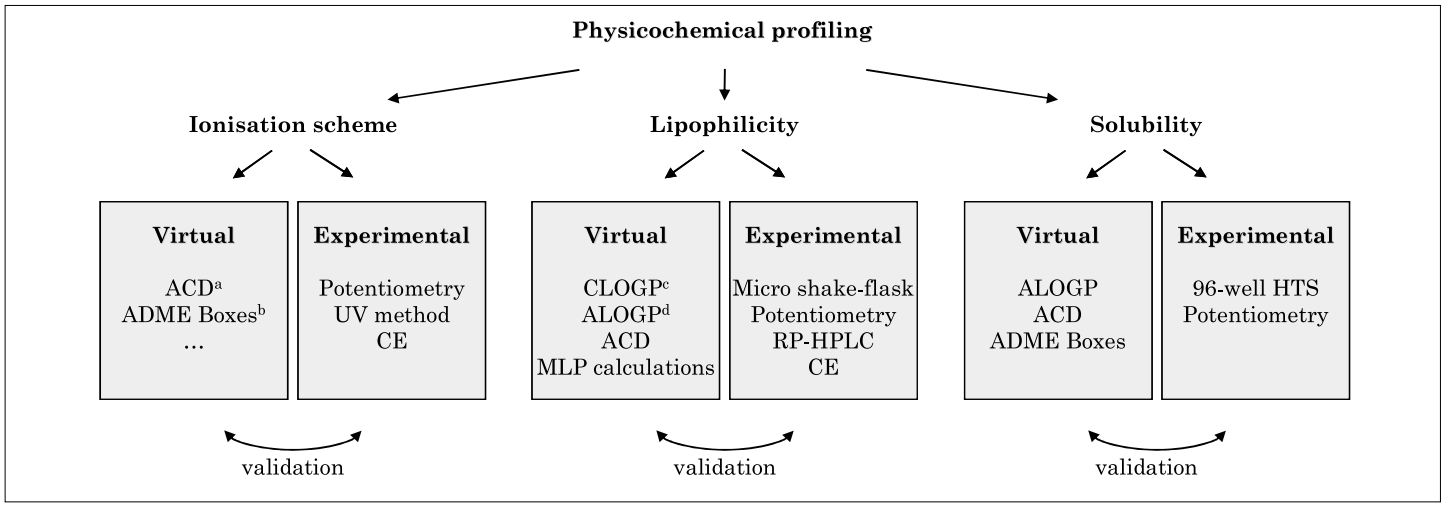

Fig. 1. Physicochemical profiling as applied in the pharmacochemistry group. (Abbreviations used: CE = Capillary Electrophoresis; RP-HPLC = Reversed Phase High Performance Liquid Chromatography). [a]ACD/Labs Software V.8.0; [b]ADME boxes software V.2.2; [c]CLOGP software V.4.83; [d]ALOGP software V.2.1.

power of ionizable groups is one of the key approaches to control the biodisponibility of drug candidates. Several methods already provide pK_a values with a high accuracy. For instance, the most commonly used are potentiometric and spectrophotometric methods, with their inherent limitations such as a compound's purity or the necessity of chromophore variations associated with acid–base equilibria. Computation of pK_a values using appropriate software is sometimes far from reality due to the small series of compounds available to validate their accuracy (see Table).

More recently, capillary electrophoresis (CE) has provided pK_a values of compounds presenting low solubility and modest purity. In addition this method requires low quantity of solvent and sample [1–3]. pK_a determinations by CE are based on the migration time or mobilities (μ) of an ionic species over a range of pH values. The effective mobilities μ_{eff} measured for a studied compound reported as a function of pH results in an S-shaped curve allowing the pK_a determination. Several studies have already shown the efficiency of CE to determine the ionization constants of molecules in good agreement with values obtained with different standard methods [4–7]. Even if this method is unable to deal precisely with compounds having two close pK_a values, the μ_{eff} variation presents a two-fold increase when the total charge increases by a factor of two. This can be illustrated with the zwitterionic antihistamine cetirizine: the two lowest pK_a are not fully separable due to their proximity (less than 1.0 pH unit) but a global charge variation of +2 is identified on the titration curve (gain of +1 corresponding to the second piperazine protonation and loss of –1 due to the carboxylic acid protonation) (Fig. 2) [7].

Table. Ionisation constant (pK_a), lipophilicity of the neutral form in n-octanol/water system (log P_{oct}) and solubility values of the neutral form obtained for the zwitterionic antihistamine cetirizine (Zyrtec®) using predictive and experimental methods

Methods	pK_{a1}	pK_{a2}	pK_{a3}	Log P_{oct}	S [mg/l]
Virtual methods					
ACD[a]	2.10	3.46	6.22	-0.33	580
ADME Boxes[b]	–	3.80	7.70	0.44	356
CLOGP[c]	–	–	–	2.08	–
ALOGP[d]	–	–	–	2.80	64.2
Experimental methods					
Potentiometric method	2.2[e]	2.9[e]	8.0[e]	1.50	–
CE	–	–	8.21[f]	–	–
RP-HPLC	–	–	–	2.15	–
96-well HTS method	–	–	–	–	>800[g]

Abbreviations used: CE = Capillary Electrophoresis; RP-HPLC = Reversed Phase High Performance Liquid Chromatography. [a] ACD/Labs Software V.8.0; [b] ADME boxes software V.2.2; [c] CLOGP software V.4.83; [d] ALOGP software V.2.1; [e] values obtained with a ionic strength adjusted water solution containing 0.15 M of KCl; [f] pK_a value corrected with activity coefficient to obtain a value independent from procedure [7]; [g] value obtained with 1% DMSO in phosphate buffer 0.01M.

Lipophilicity

Lipophilicity is a key parameter in the study of the pharmacokinetic behavior of NCEs because it represents the relative affinity of a solute for aqueous or lipidic media. In order to deal with lipophilicity very early in drug design, a large number of virtual methods were developed. However most methods have well-known limitations [8], such as in particular the effect of the molecular 3D structure so often neglected in the prediction of lipophilicity parameters. Useful tools were developed in the pharmacochemistry group in order to better clarify lipophilicity variation over a conformational space and, thus, to take into account better the effects of hydrophobic or hydrophilic collapses on partitioning of flexible solutes. This approach, based on the Molecular Lipophilicity Potential (MLP), is illustrated by the difference between the estimated partitioning of the two main conformers of cyclosporin A (3.5 log P units) due to a hydrophilic collapse by intramolecular H-bonds stabilizing the conformation present in non-polar media. As a result of this conformation change, the lipophilicity distribution around the mole-

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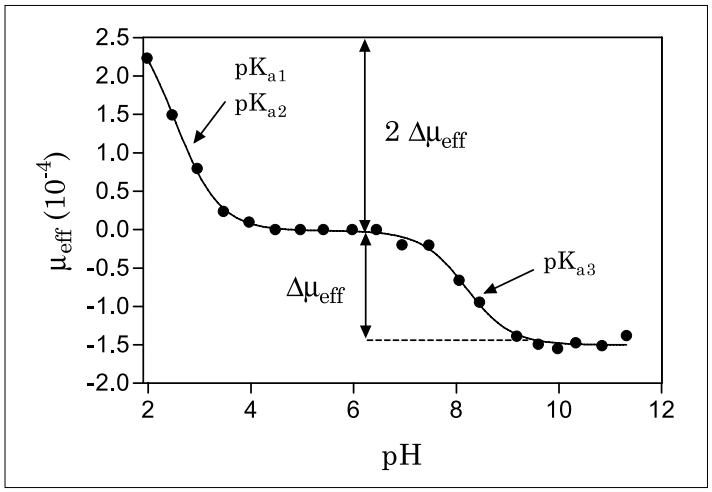

Fig. 2. Effective mobility (μ_{eff}) as a function of pH for the zwitterionic antihistamine cetirizine [7]. $\Delta\mu_{eff}$ represents the increment in effective mobility due to the global charge variation of +1 (or –1).

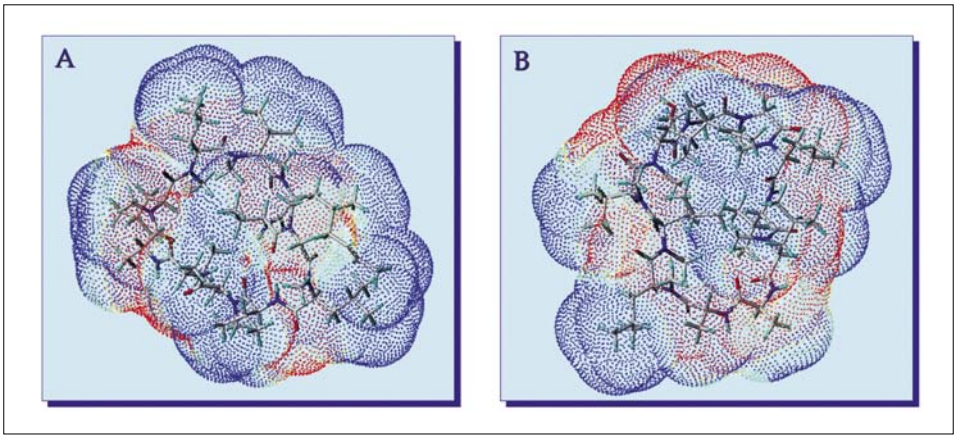

Fig. 3. Molecular lipophilicity potential for two representative conformers of cyclosporin A. Hydrophobic collapse in polar media stabilizes the conformation B comparatively to the conformer A which is more stable in apolar media. The calculated lipophilicity difference between these two conformers is $\Delta\log P_{MLP} = 3.5$.

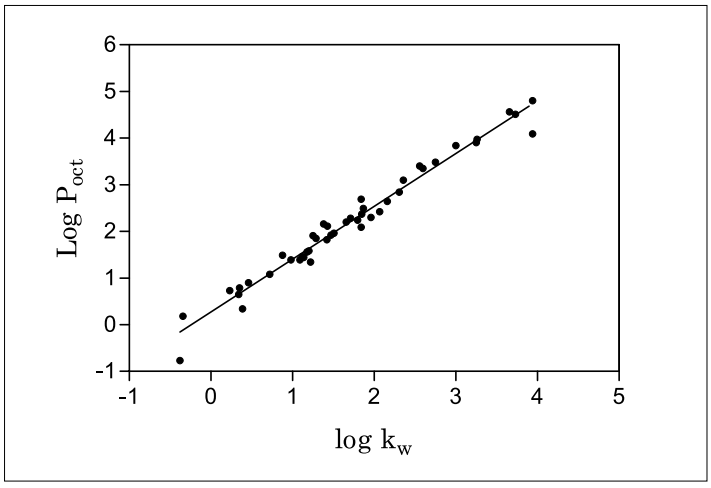

Fig. 4. Relationships between log k_w (extrapolated from log k obtained with different percentages of methanol as organic modifier) and log P_{oct} on discovery® RP Amide C16 stationary phase for acidic, basic and neutral compounds [11]

cule is very different from the distribution around the conformer present in polar media (Fig. 3) and is responsible for different intermolecular interactions between these two representative conformations and their biological partners.

The request for high-throughput methods to determine lipophilicity renewed recently interest in indirect reversed-phase HPLC methods [9] that allow the rapid evaluation of the relative affinity of a NCE for an apolar stationary and a polar mobile phase. This research was built on the correlation between the retention data and lipophilicity values in the n-octanol/water system for congeneric compounds. This method has been criticized in the past because the molecular interactions which occur were not considered similar to those occurring in the classical shake flask method. However, Lombardo *et al.* [10] demonstrated, using a linear free energy relationship (LSER) analysis on series of non-congeneric drugs, that this method encodes the same information obtained from a shake-flask log P_{oct}. We recently showed that Supelcosil ABZ$^+$Plus and Discovery® RP Amide C16 phases offer highly significant correlations between log k_w and log P_{oct} (Fig. 4) [11]. Acidic, basic, and neutral compounds were successfully studied with these two stationary phases. Moreover, preliminary studies of zwitterionic compounds on the Discovery® RP Amide C16 phase, revealed that the log P_{oct} obtained under the same conditions were higher than expected (Table) suggesting an influence of the tautomeric equilibrium between neutral unionized and neutral zwitterionic forms on retention mechanisms. Work is currently in progress to validate this hypothesis and to develop a novel HPLC method allowing, for the first time, the experimental determination of the lipophilicity of the neutral unionized tautomer of zwitterions.

Solubility

Drug candidates are often poorly soluble in water, which results in unacceptably low drug absorption. Indeed, several experimental methods and computational models were developed with variable accuracy and complexity for the prediction of aqueous solubility in the early stage of drug development. Despite numerous efforts, the computational methods remain currently too limited since they either require complex calculations [12] and thus are too slow, or they use simple models but give unreliable evaluation of aqueous solubility (Table). Thus, experimental methods are still preferable. We then adapted a 96-well UV detection approach already described by Millipore, where a DMSO stock solution is diluted in aqueous buffer in a 96-well filter plate to obtain a nominal compound concentration of 1000 μM. The filter plate is then gently shaken for 90 min and the precipitate is removed by application of weak vacuum. Because DMSO generally increases solubility of organic compounds compared to water solubility, evaluation of the co-solvent effect on the compound's solubility is currently tested using different percentages of DMSO varying from 0.33 to 5%.

As a general result, the decrease of the percentage of DMSO to 0.33% enhances the correlation between values obtained by the potentiometric method [13] and by the 96-well UV method (compared to 5% DMSO) but, even with only 0.33% of DMSO, the HTS method overestimates some solubility values (Fig. 5). Moreover, large variations between the solubility without and in presence of DMSO were observed only for some compounds suggesting a high compound-dependent solubility behavior in presence of DMSO. For example, the

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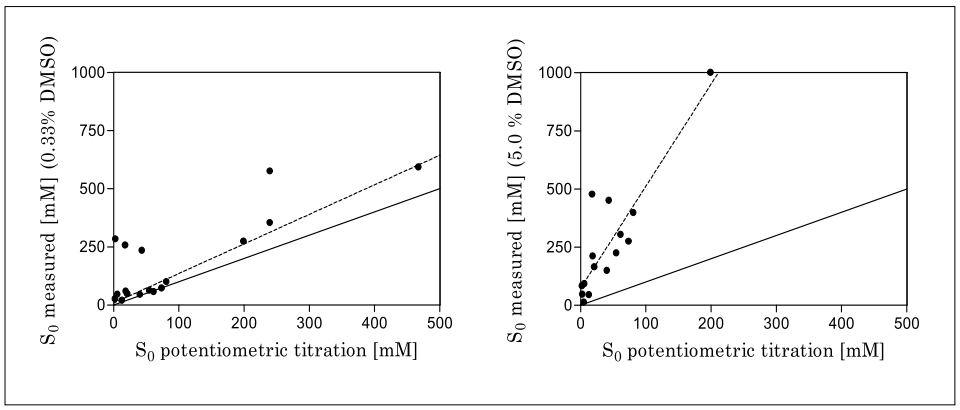

Fig. 5 Correlation (---) obtained between S_0 (solubility of the uncharged form) obtained by potentiometric method [13] (without DMSO) and by the 96-well UV method with 0.33% DMSO (right) and with 5% DMSO (left). The plain line represents the identity.

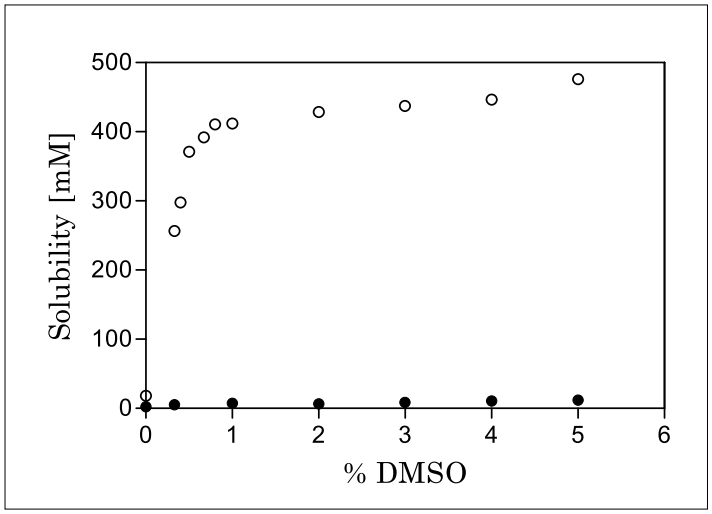

Fig. 6. Solubility values S_0 (solubility of the uncharged form) measured by 96-well UV method for chlorpromazine (●) and warfarine (○) as a function of percentage of DMSO

solubility of chlorpromazine is poorly enhanced by increasing the co-solvent fraction whereas, in contrast, the presence of 0.33% of DMSO increases dramatically the solubility of warfarine (Fig. 6). In order to identify some structural parameters which could explain these differences in solubility behavior, a systematic study is currently in progress using a large series of NCE.

Pharmacokinetic Filters

Various *in silico*, *in vitro*, and *in situ* methods have been developed to assess drug permeation across various barriers. As methods increase in complexity, a large number of factors influencing drug absorption and distribution is revealed. Unfortunately, the more closely the method approaches the *in vivo* situation, the more labor-intensive, time and material-consuming it becomes. Thus a large amount of research in drug design and development is devoted to obtain simple pharmacokinetic models which can be used as filters at different stages according mainly to the number of compounds to be tested.

To illustrate some activities in pharmacokinetic profiling of the pharmacochemistry group at Geneva, this article focuses on three models of different barriers, namely an *in silico* model of BBB permeation, a PAMPA method to predict skin permeation and an *in vitro* rat intestine segmental perfusion model to predict oral absorption. These models are linked to three main physiological barriers due to their large influence on drug delivery to the target site:

- Because about 90% of marketed drugs are administrated orally, permeation across the gastro-intestinal barrier has been widely investigated. Besides virtual, PAMPA, and Caco-2 cell models devoted to intestinal absorption, a performant *in vitro* rat intestine segmental perfusion model will help to optimize better the oral absorption of drug candidates.
- The main role of skin is to protect an organism from its environment. However, the skin can also be used as an alternative way for drug delivery. Even if some virtual and *in vitro* models already exist to predict skin permeation [14], simple HTS filters are still needed.
- The blood–brain barrier (BBB), separating the blood from the central nervous system (CNS), is important not only for development of CNS-active agents, but also for drugs which have to be excluded from the CNS to diminish unwanted side-effects. Having a valuable virtual filter based on molecular properties will help the selection of better hits.

A 3D Solvatochromic Model for the Prediction of BBB Permeation

QSAR analyses were applied in order to replace tedious *in vivo* evaluations of drug brain uptake with *in silico* models especially in the early screening of drug candidates. Indeed, for the past few years, 2D solvatochromic analyses were used to relate BBB permeation to physicochemical properties encoded by the solvatochromic parameters [15–17] describing the main intermolecular interaction forces by steric terms (molecular volume) and polar descriptors (polarisability/dipolarisability and H-bond capacity terms). Recently, a 3D-QSAR model based on four molecular interaction fields (MIFs), namely the Molecular Lipophilicity Potential (MLP), the acceptor and donor Molecular Hydrogen Bond Potential (MHBP) and the polarisability/dipolarisability GRID "DRY" field was developed to predict skin permeation [18]. This novel 3D solvatochromic approach was applied to a data set containing 30 heterogeneous compounds using the kinetic parameter PS (permeability-surface area product) measured by *in situ* rat brain perfusion for the quantification of their passive blood–brain barrier (BBB) permeation [19].

This novel methodology is composed of three parts, namely the calculation of spe-

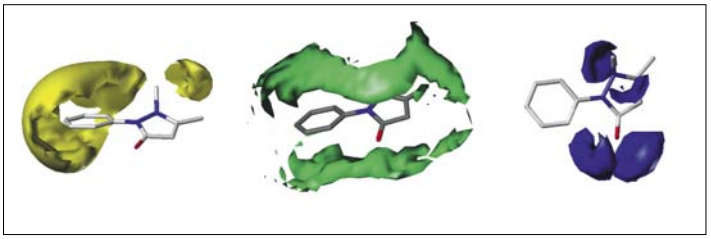

Fig. 7. MLP_{ho} (yellow), $MHBP_{ac}$ (blue) and "DRY" GRID field (green) represented on antipyrine

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cific molecular interaction fields (MIFs), their transformation into simpler descriptors by the Volsurf procedure and the generation of a PLS-based QSAR model. The molecular interaction fields proposed were the following and represented for antipyrine in Fig. 7:

- MLP_{ho} (hydrophobic part of the Molecular Lipophilicity Potential) computed using the Broto and Moreau atomic lipophilicity fragmental system and a Fermi distance function [20];
- The acceptor and donor molecular hydrogen bond potentials (MHBPac and MHBPdo) computed using Systahl 2.0 fragmental values of H-bond acidity and basicity weighed by a distance and angled function [21];
- The polarisability/dipolarisability ("DRY"), a Grid field based on the total energy interaction between the "DRY" probe and the compound.

The 3D solvatochromic descriptors were extracted by a VolSurf procedure from eight 3D isopotential contours for each MIF. Every contour was characterized by three main parameters, namely its volume (V), its integy moment (I) and its capacity factor (CF).

After a careful variable selection, a PLS analysis was used to relate the retained descriptors of MIFs to the BBB permeation values (kinetic parameters PS). This 3D PLS model has a good predictive power (q^2 = 0.77) (Fig. 8). In terms of molecular interactions, a high log PS is correlated to an important and localized hydrophobic field (positive coefficients of MLP_{ho} descriptors) and to low and localized hydrogen bond potentials (negative coefficients of MHBP volume and positive coefficients of MHBP integy I_w) (Fig. 9). The coefficient profiles of I_w "DRY" field and I_w MLP_{ho} are different (Fig. 9) because the DRY field represents more polarizability than hydrophobicity as shown for antipyrine (Fig. 7).

The proposed 3D solvatochromic model based on well-defined intermolecular forces involved in passive BBB uptake is able to discriminate BBB permeation of compounds and demonstrates that the effect of intermolecular interactions on permeation (*e.g.* hydrophobicity) is not only related to their power (intensity) but also to their radiance (localized or delocalized field). The training set will be extended to assess the statistical quality of the 3D solvatochromic model. Moreover this new approach will be explored for other *in vivo* or *in vitro* pharmacokinetic parameters in order to increase their interest in early ADMET predictions.

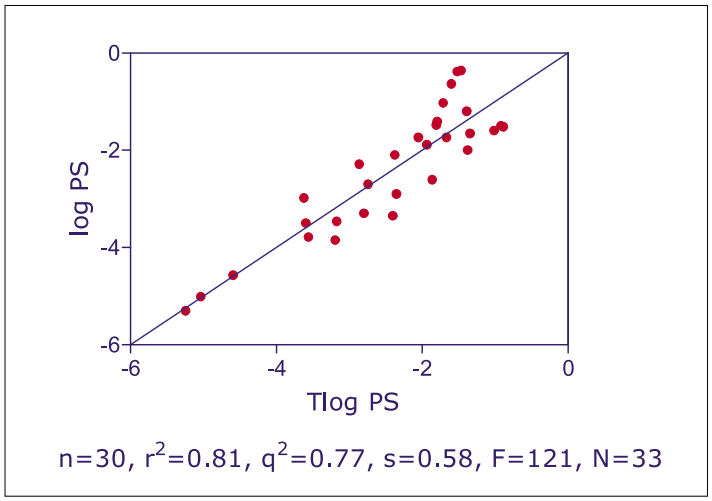

Fig. 8. Correlation plot of the experimental log PS versus the predicted log PS

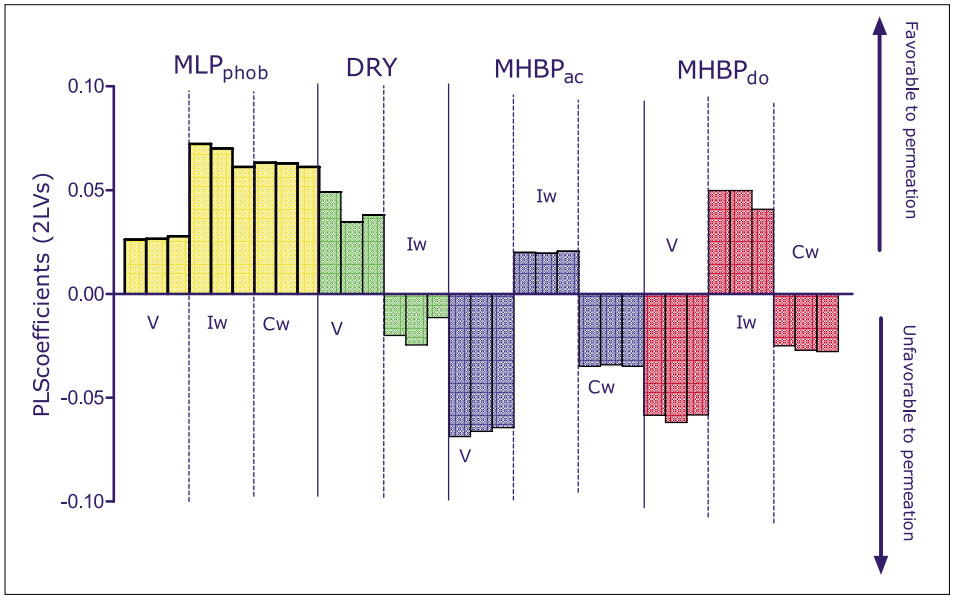

Fig. 9. PLS coefficients of the descriptors included in the model

Towards a PAMPA Method to Predict Skin Permeation

The prediction of chemical transport across the skin is important for the optimization of topical and transdermal drug delivery [22][23]. Among several *in vitro* models developed, the most commonly used method to estimate percutaneous absorption is based on isolated skin and diffusion cells (system containing a donor and an acceptor compartment) [24]. One drawback of this method is the impossibility to test a large number of compounds in a limited amount of time. In order to speed up this process, experimental conditions of an *in vitro* technique used to predict intestinal absorption, namely the PAMPA assay, were modified in order to model skin permeation as follows:

- In a typical PAMPA assay, a 'sandwich' is formed assembling a donor-filter plate supporting a liquid membrane and an acceptor compartment. The 'sandwich' is incubated under constant shaking.
- The rate of appearance of a solute in the acceptor compartment (or its disappearance from the donor compartment) is used to quantify the permeation across this artificial membrane according to the iso-pH permeability equation derived by Avdeef [13].

It was revealed that the partition coefficient measured in a water/1,2-dichloroethane system (log P_{dce}) described solute permeation across skin better than the partition coefficient in a water/n-octanol system (log P_{oct}). In this context, 1,2-dichloroethane (DCE) would be a promising candidate to assess percutaneous absorption based on the PAMPA technique. Thus 27 compounds (18 acids, 5 bases, 3 neutrals and 1 zwitterion) with known percutaneous permeability coefficients (K_p) corrected for ionization were selected from a validated database [25] and tested in PAMPA approach using 1,2-DCE to create the artificial membrane. The pH was carefully adjusted in order to measure all compounds in their neutral form.

Since permeability (log Pe) includes also membrane retention (R), these two parameters determined for the selected compounds

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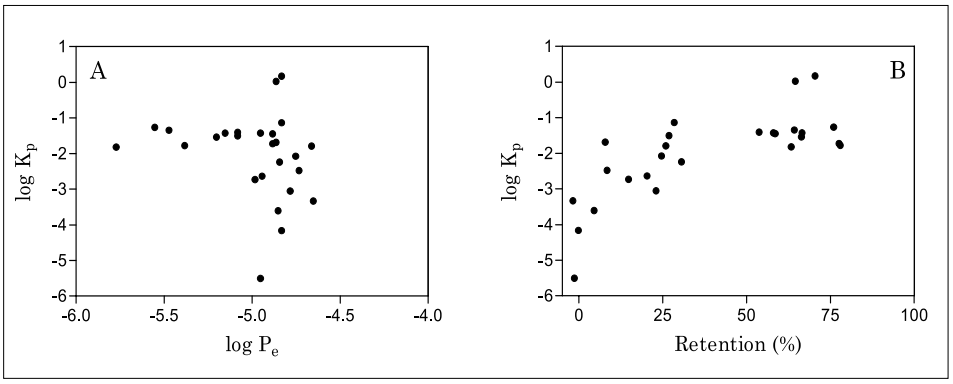

Fig. 10. Relation between log K_p [25] (from a validated database) and log P_e (**A**) or membrane retention R (**B**) for a series of 26 compounds in their neutral form

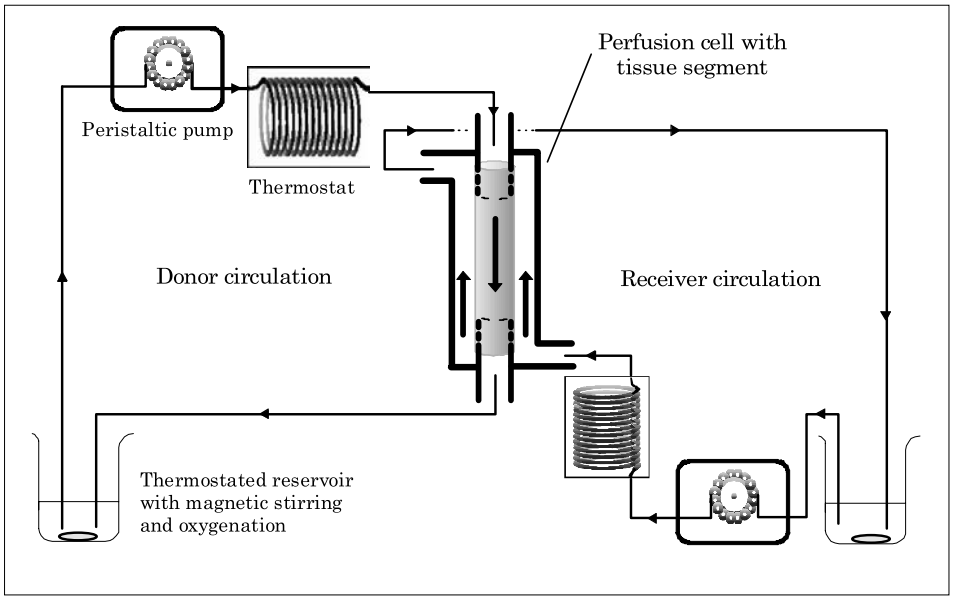

Fig. 11. Design of the *in vitro* segmental perfusion system

were compared to their log K_p (Fig. 10). Log K_p of neutral compounds were poorly correlated with log P_e (Fig. 10A) while a better correlation was obtained between log K_p and membrane retention (Fig. 10B). This unexpected result has to be clarified in order to better characterize the usefulness of the 1,2-DCE PAMPA model as a predictor of skin permeation.

An *in vitro* Rat Intestine Segmental Perfusion Model to Predict Oral Fraction Absorbed

Developing new chemical entities administered orally and improving their bioavailability are key objectives in drug research. Approaches that allow the evaluation of the extent, characteristics and mechanisms of absorption without having to study bioavailability *in vivo* in whole animals are essential to rationally select and optimize lead candidates. Ideally, an absorption model suitable for the lead finding and optimization phase should respect the relevant properties of the functional intestinal barrier as closely as possible, be reproducible and easy to use. Low animal requirement and prospect for automation would be advantageous. Considering these requirements, an *in vitro* rat intestine segmental perfusion model was designed and developed. This approach was based on a perfusion cell where a donor circulation passed inside a non-everted rat jejunal segment while a receiver circulation flowed outside the segment in the opposite direction as depicted in Fig. 11. Both circulations included a thermostated reservoir with oxygenation and magnetic stirring, a peristaltic pump and a thermostat to maintain perfusion solutions at 37 °C. Research with animals adhered to the 'Principles of Laboratory Animal Care' (NIH publication #85-23) and the protocol of this study was approved by the Cantonal Veterinary Service (VD, Switzerland).

Preliminary experiments were performed to investigate potential adsorption phenomena onto the perfusion material, which may lead to erroneous estimation of permeations. Testosterone, a compound with known adsorption propensity [26], was strongly bound onto silicone tubing. In contrast, no binding was observed (after 2 d at r.t.) onto Teflon® and Plexiglas®. Therefore, Teflon® tubing was chosen for the whole system including in the peristaltic pumps and the perfusion cell was made of Plexiglas®.

Polyethylene glycol 4000 (PEG 4000), atenolol and naproxen, with oral fractions absorbed (F_a) of 0, 50 and 100% respectively [27]) were selected to investigate whether the model would discriminate

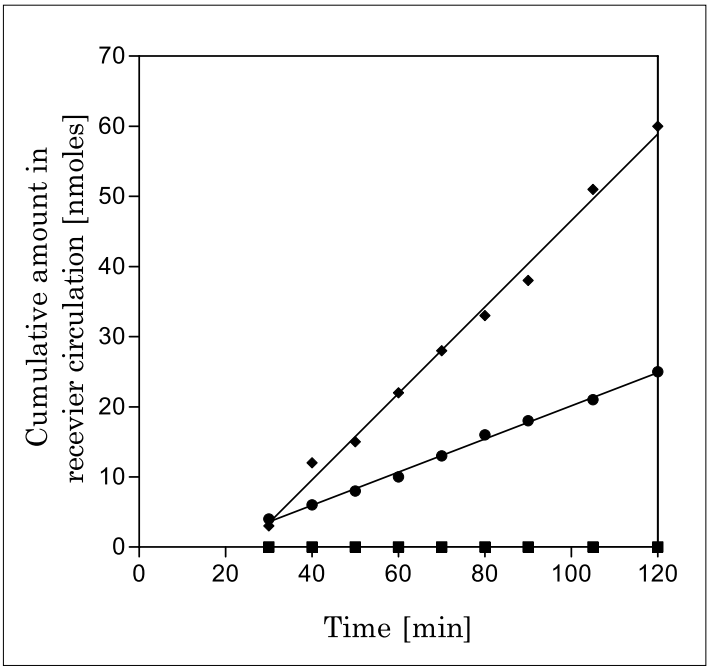

Fig. 12. Permeation kinetics of PEG 4000 (■), atenolol (●) and naproxen (♦) inserted together in a same experiment at an initial donor concentration of 100 μM

their permeation. None of the compounds displayed adsorption onto the material of the circulations. In the donor reservoir, 15.0 ml of tissue culture medium 199 containing the three test compounds, each at a concentration of 100 μM, were inserted. The reservoir of the receiver circulation was filled with 15 ml of medium 199. Then, the jejunal tissue was removed from a rat and ligatured on built elements of the perfusion cell using silk suture, exposing a defined length of tissue to permeation. Donor circulation was allowed to reach the perfusion cell. Then, its flow rate was set to 0.2 ml/min, a value frequently met during *in situ* perfusion studies [27]. On the other side, the receiver circulation's flow rate was set to 3 ml/min, which allowed a sufficient homogenization of the solution in this circulation. The permeation kinetics of the tested compounds were followed over 120 min in the receiver circulation (Fig. 11). Atenolol and naproxen were assayed by HPLC with fluorescence detection, whereas PEG was quantified using radiolabeled compound.

The pH in both reservoirs remained between 7.2 and 7.4 during the time course of the experiment. Since the appearance kinetics were linear after 30 min. (Fig. 12), the coefficients of permeation of PEG 4000, atenolol and naproxen could be calculated and were 0, 15 and 37 [$\cdot 10^{-6}$ cm/s] respectively. Hence, the developed model ranked correctly the three compounds according to their oral absorption potential and allowed discrimination between their permeations kinetics.

The permeation of further compounds will be investigated with this method in order to challenge its ability to predict their oral fraction absorbed more quantitatively and with more robustness. Other perspectives include the study of metabolism and the introduction of a dissolution chamber in place of the donor reservoir.

Conclusions

An early access to the estimation of NCE biodisponibility in order to generate more promising lead compounds has become a challenge in drug discovery. The development of highly specific theoretical and experimental methods to predict ADMET properties of hits will be accelerated with a better knowledge of the interactions between xenobiotics (*e.g.* drug candidates) and biological systems at a molecular and mechanistic level. Physicochemical profiling is without doubt an important source of detailed information on the interactions between a solute and its complex chemical or biological environment.

Novel technologies currently developed or tested in the pharmacochemistry group at EPGL offer promising rapid methods to better characterize molecular properties such as ionization, lipophilicity or solubility. These new methods allow a better understanding and description of molecular structure as a preliminary key step allowing progress towards structure–pharmacokinetic relationships with greater robustness, improved predictive and broader interpretative capacity as exemplified by the BBB permeation 3D solvatochromic model. Detailed physicochemical profiling is also helpful to refine and/or interpret pharmacokinetic filters used to predict NCE absorption such as PAMPA or *in vitro* intestinal perfusion experiments.

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