Assessment of drug permeation:
Theory, methods and applications to skin and bacteria.

Thèse de doctorat

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# Table of contents

## TABLE OF CONTENTS

I

## LIST OF ABBREVIATIONS

VII

## PART I: SKIN PERMEATION: A REVIEW

1

### 1 SKIN BARRIER FUNCTION

3

1.1 Introduction

3

1.2 The stratum corneum and routes of passive permeation

4

1.3 Characterization of the passive transdermal route of polar/ionic permeants

8

1.3.1 A dual-pathway model

8

1.3.2 A domain mosaic model

11

1.4 Factors influencing percutaneous absorption

15

1.4.1 Physiological factors

15

1.4.2 Extrinsic factors

18

1.5 References

20

## 2 BIOLOGICAL AND ARTIFICIAL MODELS TO STUDY SKIN PERMEATION

25

2.1 In vivo models

25

2.1.1 A pharmacokinetic approach to in vivo studies

25

2.1.2 Skin permeation in humans

26

2.1.3 Animal skin permeation

29

2.1.4 “Hybrid” models

30

2.2 In vitro models

31
2.2.1 Diffusion cells for measuring *in vitro* permeation 31
2.2.2 Skin models 32
2.2.3 Cell-culture techniques 35
2.2.4 Artificial membranes 36
2.3 Aims of the thesis 38
2.4 References 39

3 QUANTITATIVE STRUCTURE-PERMEATION RELATIONSHIPS (QSPERS) TO PREDICT SKIN PERMEATION: A CRITICAL REVIEW 47

3.1 Introduction 47
3.2 Statistical analysis 48
3.3 Common molecular parameters used in QSPeRs 48
  3.3.1 Molecular weight and size 48
  3.3.2 Solvatochromic parameters 51
  3.3.3 Lipophilicity and related parameters 51
3.4 Quantitative structure-permeation relationships: pre-1990 period 53
3.5 Analyses based on Flynn’s dataset 54
3.6 Other recent QSPeR analyses 63
3.7 Conclusion 66
3.8 References 67

PART II: EXPERIMENTAL STUDIES ON SKIN PERMEATION 73

4 QUANTIFICATION OF LIPOPHILICITY 75

4.1 Experimental measurements of lipophilicity 75
  4.1.1 Introduction 75
  4.1.2 The shake-flask method 76
  4.1.3 The potentiometric method 76
  4.1.4 Cyclic voltammetry at the interface between two immiscible electrolyte solutions (ITIES) 78
4.2 Computational estimation of lipophilicity
4.3 References

5 SILICONE MEMBRANE: A MODEL FOR THE BARRIER FUNCTION OF SKIN

5.1 Introduction
5.2 Mathematical model
5.3 Materials and methods
  5.3.1 Materials
  5.3.2 pK_a measurements
  5.3.3 Partition coefficients measurements
  5.3.4 Assessment of hydrogen-bonding capacity
  5.3.5 Permeation experiments
  5.3.6 Adjustment for ionization
5.4 Results and discussion
  5.4.1 Permeability of phenolic compounds across silicone membranes
  5.4.2 Permeability of selected drugs across silicone membranes
  5.4.3 Comparison with human skin permeation
5.5 Conclusion
5.6 Appendix: Development of the mathematical model
5.7 References

6 MOLECULAR FIELDS IN QUANTITATIVE STRUCTURE-PERMEATION RELATIONSHIPS: THE VOLSURF APPROACH

6.1 Introduction
6.2 TLSER: a 2D-QSAR theoretical tool
6.3 Molecular fields: 3D-QSAR theoretical tools
  6.3.1 The GRID force field
  6.3.2 The Molecular Lipophilicity Potential (MLP)
  6.3.3 The Molecular Hydrogen-Bonding Potential (MHBP)
6.4 The VolSurf procedure
6.4.1 The water probe of GRID 120  
6.4.2 The amide and carbonyl probe of GRID 120  
6.4.3 The DRY probe of GRID 120  
6.4.4 VolSurf and the 1D descriptors 122

6.5 Chemometrics tools 128  
6.5.1 Principal Component Analysis (PCA) 128  
6.5.2 Principal Component Regression (PCR) 130  
6.5.3 Partial Least Squares (PLS) 130  
6.5.4 Cross-validation 132

6.6 Application of 2D and 3D-QSAR analysis to skin permeation 133  
6.6.1 Data validation criteria 134  
6.6.2 Material and methods 141  
6.6.3 Results and discussion 142  
6.6.4 Conclusion 163

6.7 References 165

PART III: PARTITIONING OF IONS: INFLUENCE ON THE  
PERMEATION OF A SERIES OF SULFONAMIDES INTO BACTERIA 171

7 SULFONAMIDES AND BACTERIA PERMEATION 173

7.1 Classification and identification of bacteria 173  
7.1.1 Bacteria: a definition 173  
7.1.2 Taxonomy 173  
7.2 Antibacterial sulfonamides 175  
7.2.1 Historical background 175  
7.2.2 Chemotherapeutic agents 176  
7.2.3 Mechanism of action 177  
7.2.4 Therapeutic use 179  
7.2.5 Nomenclature and Classification 180  
7.2.6 Structure and biological activity of sulfonamides 181  
7.3 Materials and methods 183
7.3.1 Chemical compounds and reagents 183
7.3.2 Bacterial strain 184
7.3.3 Determination of the minimal inhibitory concentration (MIC) 185
7.3.4 pKₐ measurements 187
7.3.5 Partition coefficients measurements 189
7.3.6 Cyclic voltammetry measurements 189

7.4 Results and discussion 191
7.4.1 Viability of the microorganisms 191
7.4.2 Antibacterial activity of sulfonamides 196
7.4.3 Experimental versus theoretical lipophilicity 201

7.5 Conclusion 203

7.6 References 204

CONCLUSION 209

8 GENERAL CONCLUSION AND PERSPECTIVES 211

SUMMARY 215

RÉSUMÉ 216
**List of abbreviations**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha, \beta$</td>
<td>Scaled values of H-bond donor and acceptor capacity</td>
</tr>
<tr>
<td>$\pi^*$</td>
<td>Solvatochromic parameter standing for the solute's dipolarity/polarizability</td>
</tr>
<tr>
<td>$\Sigma f_{ac-\text{(ihb)}}$</td>
<td>Sum of fragmental values ($f_{\beta}$) for H-bond acceptor capacity (taking into account intramolecular interactions)</td>
</tr>
<tr>
<td>$\Sigma f_{do-\text{(ihb)}}$</td>
<td>Sum of fragmental values ($f_{\alpha}$) for H-bond donor capacity (taking into account intramolecular interactions)</td>
</tr>
<tr>
<td>$\Sigma \pi_i$</td>
<td>Sum of fragmental $\pi^*$ values</td>
</tr>
<tr>
<td>alk</td>
<td>$n$-alkane</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection, catalog of reference strains</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units, number of bacterial colonies counted on a solid medium.</td>
</tr>
<tr>
<td>dce</td>
<td>1,2-dichloroethane</td>
</tr>
<tr>
<td>$D_M$</td>
<td>Diffusion coefficient in membrane M</td>
</tr>
<tr>
<td>F</td>
<td>Fischer's test</td>
</tr>
<tr>
<td>GOLPE</td>
<td>Generating optimal linear PLS estimations</td>
</tr>
<tr>
<td>$h$</td>
<td>Diffusional path length</td>
</tr>
<tr>
<td>$K_p$</td>
<td>Permeability coefficient</td>
</tr>
<tr>
<td>L</td>
<td>Membrane thickness</td>
</tr>
<tr>
<td>$\text{log } P_I$</td>
<td>Logarithm of the partition coefficient of the ionic form</td>
</tr>
<tr>
<td>$\text{log } P_N$</td>
<td>Logarithm of the partition coefficient of the neutral form</td>
</tr>
<tr>
<td>$\text{log } P_{dce}$</td>
<td>Logarithm of the partition coefficient measured in the 1,2-dichloroethane/water system</td>
</tr>
<tr>
<td>$\text{log } P_{oct}$</td>
<td>Logarithm of the partition coefficient measured in the octanol/water system</td>
</tr>
<tr>
<td>$\text{log } P_{dce}^{0,I}$</td>
<td>Logarithm of the standard partition coefficient of the ionic form of an ionizable solute in the 1,2-dichloroethane/water system</td>
</tr>
</tbody>
</table>
\( \Delta \log P \) Difference between the log P values of a solute present in a single electric state, measured in two distinct solvent systems

\( \text{diff}(\log P^{\text{N-I}}) \) Difference between the log P values of the neutral and the ionized form of a given solute, measured in the same solvent system

LSERs Linear solvation energy relationships

LV Latent variable

MHB Mueller-Hinton broth used as culture medium for bacterial growth

MHBP\textsubscript{ac(do)} Molecular hydrogen-bonding potentials

MHBP\textsubscript{ac(do)}\textsubscript{ac} Acceptor (donor) MHBP

MIC Minimal inhibitory concentration of an antibacterial agent to stop bacterial growth

MIFs Molecular interaction fields

MLP Molecular lipophilicity potential

MLP\textsubscript{hi(ho)} Hydrophilic (hydrophobic) part of the MLP

MxPs MHBP\textsubscript{s} or MLP fields

oct \( n\)-octanol

PCA Principal component analysis

PC Principal component

PLS Partial least squares

\( q^2 \) Predictive correlation coefficient

QSARs Quantitative structure-activity relationships

QSPeRs Quantitative structure-permeation relationships

\( r^2 \) Square correlation coefficient

s Standard deviation

SC Stratum corneum

SDEP Standard deviation of error of predictions

TLSERs Theoretical linear solvation energy relationships

\( V_D \) Volume of the diffusion cell donor compartment

\( V_R \) Volume of the diffusion cell receptor compartment

\( V_w \) Van der Waals volume
Part I:

Skin permeation: a review

3 blood vessels, 10 hairs, 12 nerves, 15 sebaceous glands, 100 sweat glands, 92 cm of blood vessels, 360 cm of nerves, 3'000'000 cells
1 Skin barrier function

1.1 Introduction

Skin, the major function of which is to compartmentalize and protect an animal organism from its environment, was originally considered an essentially impermeable barrier. However, sensitive techniques of blood and urine analysis showed that the skin represents a potential portal of entry through which many chemicals can gain access to the systemic circulation. Consequently, much effort is currently directed towards utilizing the skin as a non-invasive route for drug administration [1]. One of the main advantages of transdermal drug delivery compared to oral administration is the avoidance of variability associated with the gastrointestinal tract (effects of pH, motility, transit time and food intake) and first-pass metabolism. Although these advantages are similar to those associated with intravenous infusion, transdermal drug delivery is a safer and more convenient method of drug administration [2].

During skin absorption, drugs have to pass a complex multilayer structure before reaching the blood [3]. Structurally, the skin can be viewed as a series of layers, the three major divisions being epidermis, dermis and hypodermis or subcutis (Fig. 1.1). The hypodermis lies below the dermis and functions as a fat-storage layer; being below the vascular system, this layer is not relevant to percutaneous penetration. The dermis (2 mm) is essentially an acellular collagen-based connective tissue that supports the many blood vessels, lymphatic channels and nerves of the skin. The extensive microvasculature network found in the dermis represents the site of resorption for drugs absorbed across the epidermis; at this point, transdermally absorbed molecules gain entry into the systemic circulation and access to their central targets. Hair follicles with their associated sebaceous glands, eccrine and apocrine sweat glands are skin appendages that originate in this layer. The avascular epidermis consists of stacked layers of cells whose thickness is about 0.2 mm but
varies between 0.1 mm (eyelids) and 0.8 mm (palms, soles) [4,5]. Cells, which provide the epidermal tissue, differ from those of all other organs in that they ascend from the proliferative layer of basal cells. They change in an ordered fashion from metabolically active and dividing cells to become dense, dead and keratinized [6]. The outermost layer of the epidermis, namely the stratum corneum, is composed of 10-15 layers (10-20 µm) of flat keratin-filled cells or corneocytes, closely packed in a nonpolar lipid matrix. As this horny layer and its components are continuously exposed to physical and chemical degradation, it is a requirement that the barrier is constantly renewed to maintain the homeostasis of the body.

![Diagram of skin structure](Image)

**Fig. 1.1.** Structure of the skin and routes of passive permeation: (1) across the transcellular pathway, (2) via the intercellular pathway, (3) through the hair follicles and (4) their associated sebaceous glands, (5) via the sweat ducts (adapted from Ref. [7]).

1.2 The stratum corneum and routes of passive permeation

The skin is the largest organ of the body (1.5-2.0 m² in man) and offers a very accessible surface for drug delivery [8]. To bypass this
biological barrier, two main routes of permeation are possible. The first involves diffusion through the intact epidermis and the other through the skin appendages, i.e. hair follicles and sweat glands, which form shunt pathways through the intact epidermis (Fig. 1.1). However, these skin appendages occupy only 0.1% of the total human skin surface and the contribution of this pathway is usually considered to be small, with only a few exceptions [9,10]. It is actually well established that drug permeation through the skin is limited by the stratum corneum [11,12]. Two pathways through the intact barrier may be identified (Fig. 1.2): the intercellular lipid route between the corneocytes and the transcellular route across the corneocytes and the intervening lipids. In both cases the permeant must diffuse at some point through the intercellular lipid matrix, which is now recognized as the major determinant of percutaneous transport rate [13].

Fig. 1.2. Permeation routes through the stratum corneum: (i) across the corneocytes and the intercellular lipid matrix (transcellular route) and (ii) via the lipid matrix between the corneocytes (intercellular route) (adapted from Ref. [14]).
As stated above, the barrier function of mammalian skin is principally attributed to the stratum corneum (SC). The barrier properties of this unique biomembrane are based on the specific content and composition of the intercellular lipid matrix which accounts for ~10% of the dry weight of this layer, 90% of which are intracellular proteins (mainly keratin) [15-17]. These lipids are distinctive in many respects: (1) they provide the only continuous phase from the skin surface to the base of the SC; (2) their composition (ceramides, free fatty acids and cholesterol, summarized in Table 1.1), is unique among biomembranes (particularly noteworthy is the absence of phospholipids); (3) despite this deficit of polar bilayer-forming lipids, the SC lipids exist as multilamellar sheets; and (4) the predominantly saturated, long-chain hydrocarbon tails facilitate a highly ordered, interdigitated configuration and the formation of gel-phase membrane domains as opposed to the more usual liquid crystalline membrane systems [18].

Table 1.1. Composition of mammalian epidermal lipids [19].

<table>
<thead>
<tr>
<th>Lipid type</th>
<th>Living layers (weight %)</th>
<th>Stratum corneum (weight %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipids</td>
<td>40</td>
<td>Trace</td>
</tr>
<tr>
<td>Sphingolipids</td>
<td>10</td>
<td>35</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>25</td>
<td>Trace</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>Other</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>

To understand better the skin barrier function of the stratum corneum, structural characteristics of the intercellular lipids have to be considered closely. As indicated in Table 1.1, the major lipids are the sphingolipids (35%), which represent a structurally heterogeneous and complex group of at least six ceramides [20,21]. A ceramide is a
combination of a fatty acid and a sphingoid base, joined by an amide bond between the carboxyl group of the fatty acid and the amino group of the base. The fatty acyl moiety is either non-hydroxylated or $\alpha$-hydroxylated, while the sphingoid moiety is sphingosine or phytosphingosine. These compounds are optically active and are numbered from 1 to 6 in increasing order of polarity as determined by thin-layer chromatography [22,23]. Two examples of ceramides are depicted in Fig. 1.3.

Being the least abundant of the ceramides, ceramide 1 or acylceramide is thought to play a crucial role as a “molecular rivet” stabilizing the multilamellar lipid array in the stratum corneum. Interdigitated long-chain $\omega$-hydroxy-ceramides provide cohesion between corneocytes by forming tight lipid envelopes around the corneocyte protein component [24-26]. Moreover, the barrier function of the skin appears to depend on the specific ratio of various lipid; studies in which non-polar and relatively polar lipids were selectively extracted with petroleum ether and acetone, respectively, indicate that the relative polar lipids are more crucial to skin barrier integrity [17].

It seems that the unusual lipid matrix alone cannot entirely explain the outstanding resistivity of the membrane, and the SC architecture as a whole has been proposed to play an instrumental role in the barrier function of the membrane. Hence, the staggered corneocyte arrangement
in a lipid continuum (similar to a brick and mortar assembly) [27,28] is suggested to bestow a highly tortuous lipoidal diffusion pathway rendering the membrane 1000-times less permeable to water relative to most other biomembranes [29]. The transport role of this sinuous pathway is further supported by visualization studies localizing several permeants in the intercellular channels [30,31], by kinetic analysis of the in vivo skin penetration rates of model compounds [32], and by evidence from thermotropic biophysical studies of lipid domains [33].

1.3 Characterization of the passive transdermal route of polar/ionic permeants

The barrier properties of human skin, localized primarily in the stratum corneum, have been an area of focused research since at least three decades. This research has originated from diverse scientific fields, including pharmaceutics, dermatology, physiology, toxicology, and biochemistry. The pharmaceutical community has recently shown particular interest in this area. A fundamental understanding of the microenvironments encountered by drugs diffusing through the skin should help in the rational design and optimization of transdermal drug delivery systems. Despite substantial interest in this field, however, the complex heterogeneous structural properties of the skin have prevented the development of a universally accepted model describing transdermal diffusion of molecules.

1.3.1 A dual-pathway model

The concept of an alternative pathway in addition to the lipoidal pathway, existed for several years. The diffusional transport data for a series of steroid compounds revealed that much of the observed presteady-state flux was due to diffusion through “shunt” routes [34]. In discussing the transdermal permeation of polar permeants such as polyfunctional alcohols, it was noted that a finite limiting permeability may be reached [35]. A “limiting permeability” is expected, as the polarity
of the permeants increases, for a membrane with a porous pathway in parallel with a lipoidal pathway [36,37]. One of the primary sources of evidence cited in support of a porous pathway has been data of hairless mouse skin permeation for several permeants with a wide range of polarity and molecular weight (MW) [38]. The permeation characteristics of the moderately polar permeants within a homologous series of compounds (e.g., \( n \)-alkanols) were unquestionably consistent with transport through a lipoidal membrane. However, the more polar compounds showed a positive deviation from expectations based upon transport through a lipoidal membrane and reached a plateau at a permeability coefficient range of approximately \( 10^{-8} \text{ cm/s} \). This positive deviation from what is expected for permeability based strictly upon a lipid membrane partition/diffusion model has been viewed as evidence for a porous/polar pathway [3] (Fig. 1.4).

![Diagram of dual-pathway model](image)

**Stratum corneum**  **Epidermis-Dermis**

---

Fig. 1.4. Schematic diagram of the dual-pathway model. The stratum corneum consists of alternating parallel lipoidal and aqueous pores and is in series with the porous epidermis-dermis layer.

However, the validity of this interpretation has been questioned since the analysis did not consider the influence of permeant molecular volume (MV) upon permeation [13].

The strong MV dependence of permeation through lipid membranes is well established [39,40]. The first model that included a molecular
weight dependence in describing skin permeation through the lipoidal regions was based on the following equation [41]:

\[
\log K_{p(lip)} = a \cdot \log P_{oct} - b \cdot MV + c
\]

Eq. 1.1

where \( K_{p(lip)} \) is the lipid pathway permeability coefficient, \( P_{oct} \) is the octanol/water partition coefficient, and \( a, b, \) and \( c \) are constants. This model proposed an exponential dependence upon MV of the solute with regard to skin permeation. Later, other investigators fitted several data sets to a correlation similar to Eq. 1.1 [13].

Beside the previous studies cited in support of a porous pathway in parallel with a lipoidal one, further investigations on chemical permeation enhancers have reinforced the concept of a dual-pathway model [42-45]. In an attempt to better understand the different viewpoints of transdermal diffusion, a close examination of the permeants and data included in the development of Eq. 1.1 seems appropriate.

Of the human skin permeation data that have been compiled [3] and analyzed [13], only six of the 92 compounds have \( \log P_{oct} \) values below zero, and only two of these six have \( \log P_{oct} \) values below \(-1\). Clearly, the performed regression for human skin data was weighted almost entirely by relatively lipophilic permeants. Hence, it seems worthwhile to focus on the polar permeants included in the analysis to see how well the proposed correlation actually predicts their permeability coefficients. Of the permeants included in the human skin analysis, sucrose and water are the most polar (\( \log P_{oct} = -3.7 \) and \(-1.4\), respectively). The correlation that results from the fit of the compiled data to the Eq. 1.1 predicts permeability coefficients for sucrose and water that are 150 and 3 times lower than the reported experimental values. The fit between predicted and experimental permeability of the compounds with \( \log P_{oct} \) values between \(-1\) and 0 is more satisfactory.

Raising these issues illustrates that there is experimental evidence that a single lipoidal pathway cannot sufficiently account for all permeation data. An objective assessment of the existing data, however,
also indicates that there is a definite need for additional systematic research focusing upon the porous/polar permeation pathway. Moreover, as previously noted, relatively little permeation data have been generated for polar permeants. The interest in performing permeation studies with polar and ionic solutes has been limited due to the inherently low permeability of skin to polar permeants. Also, factors such as skin sample-to-sample variability make it difficult to obtain data from passive permeation experiments with polar compounds that lead to quantitative conclusions \[46,47\]. Despite the difficulty of characterizing polar solute diffusion across the skin, the importance of a fundamental understanding of transdermal permeation pathways should not be overlooked.

### 1.3.2 A domain mosaic model

Lipids that can form biological membranes are characterized by a hydrophobic part, generally fatty acid hydrocarbon chains, and a more or less hydrophilic head. For thermodynamic reasons the hydrophobic parts of the lipid molecules are segregated to form a separate region, while the hydrophilic headgroups face the surrounding aqueous phase \[48\]. The cohesion of such aggregates, e.g. liposomes and bilamellar structures, depends on a number of factors such as the charge of the headgroup and its hydration sphere, the hydrocarbon chain length, the degree of unsaturation of the aliphatic chains, and temperature. The major forces involved in a bilamellar organization are hydrophobic interactions between the hydrocarbon tails which cause the molecules to associate, and also the hydrophilic character of the headgroups which requires that they remain in contact with the water phase. Lipids in lamellar bilayers of liposomes and membranes can be found to exist in either of two main states depending on the temperature of the system, a fluid crystalline state and a crystalline or a gel state (Fig 1.5). When the temperature is lowered, the lipids are forced into a crystalline state. When such crystalline bilayers have water on both sides they are termed a gel phase.
The Singer-Nicholson model of the cell membrane [50] allows a free, bi-directional diffusion of water over the membrane bilayer. The osmotic control of intracellular water content is related to an active and passive transport of ions over the membrane of a living cell [51]. This is fundamentally different from the function of the stratum corneum barrier, which is designed to enclose the entire body system in a water-tight envelope. Consequently, if the barrier properties of the skin are such as to effectively prevent water from leaving the organism, the bulk of the lipids is expected to be in a crystalline or gel phase. It has been shown that lipid extracts from human skin have several transition temperatures [52,53], a lower one around 37°C to 40°C, i.e. above the normal temperature range of the skin, but still at a physiologically acceptable temperature. In addition there are further indications of hydrocarbon chain melting at higher (unphysiological) temperatures. It was also demonstrated that membrane lipids of the epidermis show increased fluidity in the basal layers compared to the horny layer. This corresponds to a dominance of shorter
aliphatic chains (<C_{20}) in contrast to the much longer chains (C_{22} to > C_{30}) of ceramides in the stratum corneum.

Evidence for segregation of lipids in the skin barrier has accumulated in recent years [43,54-56]. Such a segregation may occur if there is a significant difference in the chain length of different lipid types in bilamellar structures. Undoubtedly influenced by the Singer-Nicholson model [50], dermatologists and scientists perceive the arrangement of the lipid units in the skin barrier as completely randomized (Fig. 1.6). If this were the correct model it would mean that diffusion in the plane of the lipid bilayers would be feasible, i.e. the bilayers would be in the fluid crystalline state. However, this is not compatible with the presence of long fatty acid chains in the barrier lipids; thus a crystalline or a gel phase would dominate the barrier.

Fig. 1.6. Traditional model of “stochastic” lipid arrangement in bilamellar structures. There is a completely random distribution of the lipid units in the plane of the lipid bilayers (adapted from Ref. [57]).
To account for both the barrier function and the current concept of hydrophilic and hydrophobic pathways through this barrier, the “domain mosaic model” of the skin barrier has been proposed [57]. This model depicts the bulk of the lipids as segregated into crystalline/gel domains bordered by “grain borders” where lipids are in the fluid crystalline state (Fig. 1.7). Such an arrangement provides for an effective water-tight barrier that allows a controlled loss of water to keep the corneocytes moistened. In addition, the model provides for the necessary mechanical properties permitting bending and stress imposed on the skin surface. Furthermore, the fluid character of the “grain borders” represents areas where lipid and hydrophobic molecules may diffuse through the system on down-hill gradients. It is suggested that in the border areas between the crystalline domains, structural transformations of the lipid organization due to permeation enhancers may take place without structural changes in the bulk organization of lipids in the crystalline or gel phase.

Fig. 1.7. The “domain mosaic model” including “grain borders”. Lipids with very long chain lengths are segregated into domains in the crystalline/gel phase separated by grain borders populated by lipids with relatively short chain lengths in the lipid crystalline state (adapted from Ref. [57]).
1.4 Factors influencing percutaneous absorption

1.4.1 Physiological factors

**Integrity of the barrier**

As discussed above, the stratum corneum (SC) is the primary barrier to drug absorption across the skin, with the intercellular lipids constituting the main milieu through which transport must occur. It is thus clear that transdermal bioavailability depends upon the barrier function of the SC and that strategies to change the composition or the organization of the intercellular lipids must be considered to improve delivery. Such enhancing technologies are feasible, but not without problems and limitations [14,58,59].

The integrity of this barrier may also be altered by skin diseases that generally result either from variation of the lipid/protein composition of the SC or from abnormal epidermal differentiation. In psoriasis, for example, hyperproliferation of the epidermis leads to a stratum corneum that has a modified lipid composition, a lower resistance to transepidermal water loss, and an anticipated higher permeability to topically applied drugs [60].

As far as transdermal bioavailability is concerned, however, patches intended for systemic therapy are labeled for application only at “normal” skin sites, free from dermatological pathology. In the same way, skin areas which are excessively dry, or which have been subjected to an abrasive trauma, should be avoided.

**Anatomic site**

Although not extensively studied, there is a belief that skin absorption varies widely from site to site on the body depending on the thickness and nature of the stratum corneum, and – to a degree overemphasized in some publications – on the density of skin appendages
More accurately, it can be said that there is variability, but that over most of the surface this does not exceed by much the normal inter-individual variability observed at a specific site. It is true that certain regions are significantly more permeable – the genitalia (especially the scrotum), axilla, face, scalp, and post-auricular skin. In some cases, these high-permeability sites have been used to optimize transdermal delivery of particular drugs: e.g. the first testosterone patch was designed to be worn on the scrotum; and scopolamine is delivered behind the ear. However, most transdermal systems will function comparably at many different sites, and the recommended location will most likely depend primarily upon convenience (e.g., choosing a discreet site for an estradiol system) and/or psychological factors (e.g., placing a nitroglycerin patch over the heart).

Age

Skin age is another factor believed to impact upon percutaneous absorption and transdermal bioavailability. The evidence behind this conclusion, though, is not particularly extensive. It is known that from soon after birth until early “old-age”, there is little change in the rate of transepidermal water loss across normal, intact skin [62,63]. At the older extreme of the age scale, there are data pointing to changes in barrier function, but these are not dramatic when viewed in the context of typical variability across the entire population. A more important information is that as the skin ages, it becomes progressively more fragile and requires a longer period of time for recovery after injury. Thus the chronic application of transdermal systems to elderly patients should be carefully monitored.

At the opposite end of the age spectrum, as mentioned above, neonates born normally at 40 weeks gestational age possess an essentially complete stratum corneum barrier. Premature neonates, on the other hand, particularly those born at less than 30 weeks gestational age have poorly developed barriers and must face various risks including
percutaneous intoxication due to inadvertent chemical absorption. However, this negative feature also offers a potentially beneficial application of transdermal delivery. As drug administration to very premature neonates is fraught with difficulties (e.g., finding a vein for injection can require considerable time and cause much discomfort to the infant), the skin offers a route of entry that is easily accessible. This possibility has already been exploited for theophylline [64]. The challenge now is to ascertain whether the approach is feasible and manageable during the early weeks following premature birth, when the skin barrier function is maturing and changing.

**Skin metabolism**

Presystemic metabolism in the skin can obviously modify transdermal drug bioavailability. The cutaneous first-pass effect for nitroglycerin, for example, has been estimated at 15-20% [65]. The viable epidermis is a biochemically active tissue with metabolic capability [66,67]. Indeed, a multitude of enzymes have been identified in the skin, including a cytochrome P450 system [68]. However, the capacity of the viable epidermis below a transdermal patch to metabolize a delivered drug is limited (it must be remembered that nitroglycerin is an exceptionally sensitive compound, with a systemic half-life of only a few minutes), and the role of biodegradation is likely to be minor. Indeed, one of the advantages of transdermal delivery is avoidance of presystemic metabolism and an excellent illustration of this attribute is found with estradiol.

Ultimately, the importance of metabolism in the skin depends upon the capacity of the enzyme system(s) involved and the relative rates of diffusion and bioconversion. As the metabolizing capacity is typically small, biodegradation is likely to be most significant only when transport fluxes are low. The use of enhancing technologies, therefore, with the specific aim to increase flux, will probably reduce any problems associated with metabolism.
1.4.2 Extrinsic factors

**Occlusion**

Of the numerous methods to enhance the percutaneous absorption of drugs, occlusion is the simplest and most common one. It is defined as the complete impairment of passive transepidermal water loss at the application site. The predominant effect of occlusion is increased hydration of the stratum corneum (SC), swelling of the corneocytes, and uptake of water into intercellular lipid domains. The normal water content of the SC is 5-15%; this figure can be increased up to 50% by occlusion. Moreover, skin temperature generally increases from 32°C to 37°C with occlusion and also contributes to penetration enhancement [69]. In some cases, however, raised hydration may not always increase drug permeation [70].

**Nature of the vehicle**

The flux \( J \) of a drug through the stratum corneum can be described most simply by Fick's first law [71]:

\[
J = \frac{D_M \cdot c_{s,M} \cdot \frac{c_v}{c_{s,v}}}{L}
\]

where \( D_M \) is the diffusion coefficient of the drug in the membrane, \( c_{s,M} \) its solubility in the membrane, \( L \) the diffusion path length across the membrane, \( c_v \) the concentration of the drug dissolved in the vehicle, and \( c_{s,v} \) the solubility of the drug in the vehicle. Three strategies of permeation enhancement may be postulated based on Eq. 1.2: (i) increased \( D_M \), (ii) increased \( c_{s,M} \), i.e. increased drug partitioning into the membrane, and (iii) increased \( c_v/c_{s,v} \), i.e. the degree of saturation of the drug in the vehicle (the supersaturation approach). The latter strategy is based on interactions between the drug and the vehicle, the first two approaches imply an effect of the vehicle on the barrier function of the stratum corneum. This can be exemplified by chemical penetration enhancers.
diffusing into the stratum corneum and subsequently disordering the intercellular stratum corneum lipids, or by the extraction of such lipids by a solvating component in the formulation [14,72].

Microbial metabolism

A topically applied compound will also encounter the micro-organisms found on both healthy and diseased skin. These organisms have been shown to be capable of metabolizing drugs such as glucocorticoid esters (bethamethasone-17-valerate) and glyceryl trinitrate. Although microbial degradation may act on compounds applied to the skin surface, this effect is limited and may become of more importance with the proliferation of transdermal delivery systems. Such devices may be left on the skin surface for periods of up to one week and could provide an environment in which the micro-organisms can flourish [73].
1.5 References


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2 Biological and artificial models to study skin permeation

The prediction of chemical transport across the skin is important for both the optimisation of topical and transdermal drug delivery, and the assessment of risk following dermal exposure to chemicals [1,2]. To facilitate the estimation of percutaneous absorption, a number of in vivo, in vitro and cell-culture models have been developed (Table 2.1) [3-6]. The predictive capacity of the different approaches as well as their relative utility will be discussed here in order of decreasing complexity; those most worthy of further study will be highlighted.

2.1 In vivo models

2.1.1 A pharmacokinetic approach to in vivo studies

A general approach to model skin absorption has been to build “pharmacokinetic” models in which various compartments represent the delivery device, the stratum corneum, the viable tissue and the blood. Normal elimination kinetics are assumed from the blood compartment (Fig. 2.1). The first-order rate constants connecting the compartments can be ascribed physical significance [7], e.g. the slowest step $k_1$ equals transfer across the stratum corneum equals $D_{sc}/h_{sc}$ where $h_{sc}$ is the diffusional path length and $D_{sc}$ the diffusional coefficient of the drug in the stratum corneum. The rate constant $k_2$ describes the diffusion of the penetrant through the viable epidermis. Thus, as for $k_1$, it may be evaluated from the relationship $k_2 = D_{epid}/h_{epid}^2$ where $D_{epid}$ is the diffusion coefficient of the drug through the viable epidermis of diffusional path length $h_{epid}$. The rate constant $k_3$ is related to back diffusion from the viable epidermis to the stratum corneum and thus reflects the affinity of a drug for the stratum corneum.
The ratio $k_3/k_2$ is related to the partition coefficient between the lipophilic stratum corneum and the aqueous viable epidermis, and has been shown to be linearly correlated with the octanol/water partition coefficient [8]. The model has been used successfully to predict the transdermal delivery of a number of drugs [9-14].

Fig. 2.1. Schematic representation of the pharmacokinetic model.

2.1.2 Skin permeation in humans

Percutaneous penetration studies performed in humans provide the most relevant information. These studies are considered as the “gold standard” against which all methods for measuring skin absorption should be evaluated [3,15]. However, such experiments are not always easy to carry out, they may be expensive, and they can involve unacceptable risks to the volunteers. Among the advantages, in addition to the relevance and realism provided by a human in vivo experiment, it is at least theoretically possible to obtain important pharmacokinetic information pertaining to skin absorption. On the other hand, however, there are certain disadvantages, not the least of which is the variability associated with human percutaneous absorption [16] and the fact that not all experiments are conducted in the same homogenous population of volunteers under identical conditions, nor on the same anatomic site, and so on.
Table 2.1. Different biological and artificial models to assess percutaneous absorption.

<table>
<thead>
<tr>
<th></th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vivo models</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>Gold standard of relevance</td>
<td>Ethical considerations</td>
</tr>
<tr>
<td></td>
<td>Pharmacokinetic information is obtainable</td>
<td>Time-consuming experiments</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inter-individual variability</td>
</tr>
<tr>
<td>Animal</td>
<td>Good alternative for toxicological studies</td>
<td>Skin barrier may be more permeable than in man</td>
</tr>
<tr>
<td></td>
<td>Good correlation with <em>in vivo</em> human data</td>
<td>Large interspecies variability</td>
</tr>
<tr>
<td>“Hybrid” models</td>
<td>Experiments performed with intact tissues</td>
<td>Low throughput</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Complicated model</td>
</tr>
<tr>
<td><strong>In vitro models</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin fragments</td>
<td>Good intermediate level of complexity</td>
<td>High variability within treatments</td>
</tr>
<tr>
<td></td>
<td>Tissue source does not need to be human</td>
<td>Time-limited experiments</td>
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<tr>
<td></td>
<td></td>
<td>due to short viability</td>
</tr>
<tr>
<td>Cell cultures</td>
<td>Used to investigate both cutaneous metabolism and dermal irritation</td>
<td>Poor prediction of skin permeation in man</td>
</tr>
<tr>
<td>Artificial membranes</td>
<td>Fast screening possible</td>
<td>Difficult <em>in vivo</em> extrapolation</td>
</tr>
<tr>
<td></td>
<td>Experiments at low cost</td>
<td>Poorly characterized systems</td>
</tr>
</tbody>
</table>

Historically, human experiments were performed using small amounts of radiolabelled permeant applied to the skin in a small volume of organic solvent which quickly evaporated [17,18]. Thereafter, the elimination of the administrated radioactivity in the urine and/or feces was determined and, from this information, the percentage dose absorbed was deduced. While this experiment is of interest from a practical standpoint in that it is conducted in man, it has certain limitations in that
the measurement compartment, i.e. the excreta, is far-removed from the application site on the skin. Any conclusion about the percutaneous absorption phenomenon, beyond the cumulative amount absorbed, is therefore difficult to reach. One also has to question the ethics of applying radioactive substances to human skin in vivo — a practice that is accepted in some countries, but absolutely forbidden elsewhere.

Beside the extremely rigorous methods (e.g. full pharmacokinetic evaluation) used to assess skin absorption, a range of different studies have been performed and the information obtained therefrom varies in both quality and quantity [19,20]. More recently, an alternative in vivo experiment has replaced the evaluation of drugs in a compartment distant from the application site with, instead, determination of the permeant in the compartment to which it is being administrated, i.e. in the stratum corneum [21]. In its simplest form, post-application of a drug-containing formulation, this method involves removal of the stratum corneum by repeated adhesive-tape-stripping and quantification of the amount of substance absorbed by extraction and analysis of the individual tapes. This technique holds much promise for the comparison of different formulations containing the same drug and the determination of bioequivalence, for example, and as a rather tedious way to screen formulations.

It should be noted that this current method was somewhat anticipated in a series of studies published several years ago [22]. In that work, the stratum corneum was also removed after a short period (30 minutes) of contact between the applied vehicle and the skin, and the amount taken up into the barrier layer was determined. It was shown that this amount was highly correlated with the percentage dose applied which would have ultimately permeated into the body (measured by urinary excretion over 4 days) following an identical exposure. While this relationship is hardly surprising given the brief period of skin exposure involved, the methodology provided an extremely simple approach to evaluate the body burden of a chemical following its brief contact with the skin. This is an invaluable aid, therefore, when assessing the potential for
toxicity after dermal exposure to cosmetic or personal-care products which typically remain on the skin for only relatively short periods of time.

2.1.3 Animal skin permeation

The use of in vivo animal models to study skin permeation represents another considerable body of work [4]. Many different species have been examined, not the least of which include the rhesus monkey, the pig (mini- and weanling pigs), the rabbit, and many different species of rodents (rats, mice, guinea pigs, both hairy and hairless). The advantage of using an animal model as opposed to man is that animals are generally more accessible, and the procedures used can be more invasive than those possible in human volunteers. The disadvantages of using animals, on the other hand, are mainly due to the relevance of such models to represent and predict permeation in man. Furthermore, animal experiments may be quite expensive (especially if one uses a rhesus monkey, for example), may be as time-consuming as human studies since the animals need to be acclimated, and may require specialized equipment.

Looking at the animal models used to date, some observations can be made. First of all, it is clear that there exist animal models which correlate well with human models. The most notable examples are the rhesus monkey and the pig, including the minipig and weaning pig [23]. Unfortunately, these animals are neither the easiest nor the most economic to use. With respect to the more classical laboratory animals such as the hairy rat, mouse, rabbit or guinea pig, the literature shows that percutaneous absorption in these models is much higher than in man. To understand why these models overestimate human skin permeation, one has to take into account the possibility of a significant role in skin uptake via follicular pathways, which are much more developed than in the generally “bald” human skin. Moreover, these rodents typically have a thinner stratum corneum than man [24]. These physiological characteristics could partially explain the use of rat as the standard animal of choice for toxicological testing. This argument, however, is far
from convincing given that the differences in percutaneous absorption between rat and man can be as great as 10-fold.

Similarly, for the hairless rodent species, the permeation of chemicals is again higher than through human skin. Again, this is presumably due to the thinner barrier layer and to a different lipid composition compared to humans. Differences range from 2-3 fold up to nearly one order of magnitude. Nevertheless, hairless species are qualitatively reasonable predictors of trends in absorption. There is, however, an important problem with hairless species when one examines the influence of formulation components such as penetration enhancers on drug transport across the skin. It has been demonstrated that the more fragile stratum corneum of a hairless animal exaggerates the effects of these excipients and may significantly over-predict their effects in man.

2.1.4 “Hybrid” models

There have been other in vivo models developed which are more sophisticated, more complicated and, in consequence, less practical to use. For example, it was shown several years ago that it was possible to graft human skin onto the backs of athymic mice [25]. The viability of the skin after the graft had taken was good, and the existence of a functional cutaneous microcirculation could be demonstrated. Rejection could be reduced by treating the animals with immunosuppressive agents. An in vivo model was therefore available which allowed permeation across human skin to be investigated in an easy to handle rodent. Furthermore and importantly the human skin retained its permeability characteristics. Nevertheless, this is a time-restricted and labor-intensive model.

Another similar model involves the so-called skin sandwich flap [26]. Following a complex surgical procedure, an external skin “sandwich” consisting of the host athymic rat skin on one side and grafted human skin on the other, was grown on the animal and perfused via single afferent and efferent blood vessels, much like an isolated perfused organ preparation. Quite detailed pharmacokinetic analyses of the absorption and distribution of topically applied drugs could be undertaken, at least
within the constraints of the period available for experimentation (necessarily short because anesthesia of the animal was required). Neither of these models has been much used, primarily because of their technical complexity and expense. While useful perhaps as research tools, these approaches are not practical for routine use, and literature citations describing their use are now very rare.

### 2.2 In vitro models

An *in vitro* experiment to measure the percutaneous absorption of a drug is a much simpler approach than an *in vivo* measurement [27]. However, with this simplicity comes a number of limitations, and the question of relevance to predict *in vivo* permeation must always be raised. The literature is replete with papers describing *in vitro* percutaneous-absorption studies. The range of compounds studied and the variety of skin membranes used is quite staggering. As for *in vivo* methods, the range of animal species (in addition to man) which have given their skin for such permeation experiments is very broad.

#### 2.2.1 Diffusion cells for measuring *in vitro* permeation

The most frequently used *in vitro* method is a simulated membrane permeation experiment. Its design involves various rate-limiting membranes (e.g., excised animal or human skin, artificial membranes), adjustable environments, numerous protocols and the use of diffusion cells, i.e. systems containing a donor and a receptor compartment [28]. The donor compartment can be closed or open, depending on the type of administration protocol. As for the receptor compartment, it can be either closed (static design) or open (flow-through design) [29-33]. An appropriate analytical method (UV-spectrophotometry, liquid-scintillation counting, etc.) is used to quantify and monitor the appearance of drug in the receptor solution and/or the decline from the donor compartment as a function of time [34].
The major advantage of *in vitro* investigations is that the experimental conditions can be controlled precisely, such that the only variables are the membrane and the test material. A potential disadvantage, however, is that little information on the metabolism, distribution, and effects of blood flow on permeation can be obtained.

### 2.2.2 Skin models

*Isolated perfused tissues*

Perfused tissue models use excised regions of skin, complete with their associated microvasculature, immediately after sacrifice and with continuous perfusion (e.g., with Krebs-Ringer buffer, glucose and albumine aerated with oxygen and carbon dioxide). Several variations involve both different species and different areas of skin. Isolated perfused porcine skin flaps [35], bovine udders [36], and rabbit ears [37,38] have also been used. The perfused pig ear model uses an isolated ear perfused with oxygenated blood from the same pig [39]. These techniques allow the investigation of the effect of local blood circulation on the accumulation and removal of topically applied materials. In addition, one of the major disadvantages of the usual *in vitro* method, namely the lack of a perfused network of skin capillaries, is nearly overcome. Nevertheless, these preparations are complex for routine use and animal skin is not spared.

*Human skin*

Among the range of models available for *in vitro* experiments, human skin has been described as the material of choice [3]. The principal sources of human skin are cosmetic-surgical procedures, cadavers, and tissue banks. Not surprisingly, tissue obtained immediately following plastic surgery is preferred since the skin can be maintained metabolically active for some time, and the source and condition of the tissue can be ascertained during surgery. On the other hand, with skin obtained from a cadaver or from a tissue bank, one does not always know the origin of the
tissue, nor what may have happened to the tissue between the time that it is harvested and the moment that it is used in an *in vitro* experiment. Equally, it is very unlikely that such tissue would retain much in the way of metabolic activity, and it cannot be used, therefore, in experiments which require full skin viability [40].

The preparation of skin tissue for an *in vitro* experiment depends, of course, on the investigation envisaged. Every conceivable slice of skin has probably been examined: full-thickness skin, skin dermatomed to a particular depth (from a few to several hundred µm), heat- or otherwise separated epidermis, dermis, skin from which the barrier has been removed (e.g., by tape-stripping) and, most simply, stratum corneum isolated by enzymatic treatment (trypsinization).

Based on the assumption that the stratum corneum represents the main barrier in skin penetration, solute permeability across the skin can thus be determined by using a diffusion cell where the isolated stratum corneum is embedded as a membrane [41]. Although permeability experiments through this horny layer have been carried out *in vitro*, they do not differ markedly from an *in vivo* situation since this dead layer is also present in the living organism. However, such studies are carried out in water, resulting in an artifactual hydration which promotes solute penetration [42]. Therefore, any deduction one can make will not pertain directly to an *in vivo* situation, but to an extensively hydrated skin. This drawback may be tolerable, given that under normal *in vivo* conditions the skin is easily hydrated, particularly when a drug is applied is an aqueous gel or under occlusion [43].

**Animal skin**

Like biological membranes, isolated skin fragments from animals (e.g., rat, mouse, rabbit, squirrel, guinea pig, snake and pig) are frequently used [44-49]. Depending on the number of hair follicles seen in such skins preparations, three different phenotypic hair densities have been defined as hairy, fuzzy and hairless [50].
Among the many animal skins used, those from snakes and domestic pigs have been proposed as relevant models. Thus, the response of shed snake skin to lipophilicity and molecular weight mimics that of the human stratum corneum more closely than that of hairless mouse skin [51]. In addition, the rate of water evaporation of shed snake skin is comparable to that of human skin. Nevertheless, the lack of an optimum correlation between snake and human skin is likely due to the differences of their lipids in structure and proportions [52]. A particular advantage of this model, however, is that snakes shed their skins on a regular basis, thereby providing a renewable source of tissue without the need to kill the animal. Otherwise, the domestic pig is perhaps the best skin model, as it has been demonstrated to have histological and physiological properties similar to those of human skin. The epidermal lipid biochemistry of the pig has also been shown to be similar to that of humans [53]. Although animal skin is more readily available than human skin, care must be taken when selecting a type of skin, because the method remains time-consuming and may show poor reproducibility [5]. There is no question, however, that \textit{in vitro} skin-permeation experiments have yielded a wealth of information about the percutaneous penetration process, in particular with respect to the key structure-penetration relationships involved [54].

As described by the European Centre for the Validation of Alternative Methods (ECVAM), the commonly used methods for measuring percutaneous absorption can be ranked in a hierarchy (Fig. 2.2). Although the ranking of animal skin types represented here is generally appropriate, one must take exception to the ranking of methodologies on the left side of the diagram. The choice of different \textit{in vivo} and \textit{in vitro} methodologies depends on the compounds being studied, and on the questions being asked. Thus, in predicting human dermal absorption, an \textit{in vivo} study may not be preferable to an appropriately designed \textit{in vitro} experiment.
Confidence level
High

In vivo
Perfused skin
Whole skin (viable)
Whole skin (non-viable)
Keratome slices
Stratum corneum
Model membranes
Mathematical models

Human
Primate
Swine
Guinea-pig
Rat
Rabbit
Mouse

Hair-less strains

Fig. 2.2. Hierarchy of physiological levels (on the left side) and skin types (on the right side) for measuring percutaneous absorption, as recommended by the European Centre for the Validation of Alternative Methods (ECVAM) [3].

2.2.3 Cell-culture techniques

In recent years, attention has been focused on the development of skin substitutes to provide biological materials for the treatment of acute and chronic wounds [55,56], and to provide models permitting toxicological and pharmacological studies in vitro [57-59]. The skin equivalents used for permeation testing are typically the epidermis or full-thickness skin. The full-thickness equivalents are composed of both dermal and epidermal tissues, with the dermis being constituted from a collagen matrix populated or not by living fibroblasts. The epidermal substitutes are composed of normal human keratinocytes that have been cultured at the air-liquid interface to insure proper development and terminal differentiation [60,61]. These skin equivalents have many advantages, including the ability to eliminate animal experimentation. Also, they use human skin cells which exhibit morphological and biochemical features
similar to native skin. Beside such analogies, the barrier function of these substitutes is significantly deficient in comparison to human skin, and the epidermal lipids show abnormalities in composition [62]. Such cell cultures are more permeable than intact skin and their lower content in ceramide, the most important lipid class for the barrier function, may be the cause for this quantitative difference [63-65].

More recent comparisons of permeability have shown that these cutaneous models are discriminative in terms of absorption rates and yield the same ranking of permeants as normal human skin [66].

2.2.4 Artificial membranes

Several synthetic membranes can be used to model the transport of drugs across the skin [5,67]. They are exemplified by cellulose acetate membranes, which have been used in various diffusion studies [68-70]. Like all dialysis membranes, those made of cellulose acetate are porous barriers which model the skin seen as a passive diffusion barrier, with very little involvement of drug partitioning into the intercellular lipid pools. Consequently, in these experimental models, the rate of diffusion is determined by solute concentration and the length of the diffusion pathway. Although this mechanism is not representative of diffusion through intact skin, it may be relevant as a model for damaged skin, where the relatively aqueous dermis is the only remaining barrier [5].

In addition to hydrophilic acetate membranes, an array of synthetic polymer membranes containing hydrophobic groups have been evaluated as potential models for penetration of compounds through the skin. The hydrophobic groups are intended to provide functional mimics for drug partitioning into lipids. Among these mixed-polarity membranes, foils of dimethylpolysiloxane (silicone rubber or Silastic®) have been most extensively used [45,71-74]. These models, however, do not incorporate all of the structural features needed to evaluate interactions occurring during transport across lipoidal intercellular channels.

Besides such solid membranes, organic liquid-supported membranes containing hydrocarbons, long-chain alcohols or isopropyl myristate have
been investigated [75-78]. Also, skin lipids (ceramides, fatty acids, cholesterol, etc.) or liposomes composed of various skin lipids, have been tested [79,80]. Several synthetic (mixed cellulose ester, polyethylene-vinylacetate, polypropylene, etc) or natural materials (e.g. eggshell membrane) have been used to prepare artificial membranes. Such systems have been proposed as reasonable models for the epidermal barrier[81].

Other devices combine two or more different membranes into laminate or composite systems to mimic the hydrophilic and hydrophobic domains of the skin [5]. Such models, however, are not relevant because the desorption of solutes out of the stratum corneum is linear with the square root of time, whereas linear relationships have been obtained with laminates. More recent experiments have used synthetic graft copolymers to model the heterogeneity of skin domains [82-84]. These latter membranes have proved useful as relevant models for the quantitative evaluation of transdermal penetration.

Although skin-imitating membranes will continue to play a role in the future, artificial membranes should provide the most useful forecast of in vivo transdermal delivery processes when: (1) the passive diffusion barrier imposed by the stratum corneum is the major resistance to transport; (2) the drug of interest is known to be metabolically inert and not specifically bound in viable skin; (3) the formulation does not contain a permeability enhancer which can interact with the skin but not the membrane; and (4) in vivo experiments of similar design have been or can be performed and correlated with in vitro results.
2.3 Aims of the thesis

The aims of this thesis are to highlight the relevant physicochemical properties responsible for permeation across biological membranes, namely human skin and bacterial cells. This thesis is divided into three parts.

Part I is devoted to a critical review on skin permeation. First the predictive capacity of the different in vivo and in vitro models used to estimate skin permeation is examined in chapter 2. Then, quantitative structure-permeation relationships (QSPeRs) to predict percutaneous absorption are reviewed in chapter 3, and their statistical reliability is also discussed.

Part II illustrates the application of experimental and computational models in predicting skin permeation. In this context, chapter 5 is based on the use of silicone membranes, as artificial models for the barrier function of skin. A set of phenolic derivates in addition to heterogeneous compounds are investigated in terms of physicochemical parameters relevant in permeation. The application of a computational tool, the VolSurf procedure, to predict human skin permeation is shown in chapter 6. Specific considerations on the nature of molecular interaction fields used in this method are also discussed.

Part III attempts to correlate the antibacterial activity, measured in terms of minimal inhibitory concentration (MIC), of a set of sulfonamides with their physicochemical properties.

Finally, a general conclusion and perspectives for future work is presented in chapter 8.
2.4 References


3 Quantitative structure-permeation relationships (QSPeRs) to predict skin permeation: a critical review

3.1 Introduction

Because the determination of absorption of chemicals into and through the skin involves ethical difficulties with experiments on animals and human skin [1], the quantification of transdermal penetration raises great interest in industrial, governmental and academic circles.

Quantitative structure-permeation relationships (QSPeRs) relate variations in the permeation of series of compounds with variations in their physicochemical and/or structural properties. QSPeRs provide insights into permeation mechanisms, prediction of the permeation of novel compounds (even before their synthesis) and allow to limit in vivo experiments. In transdermal studies, QSPeR models are often based on multivariate regression analysis to encode an experimental index of permeation such as the permeability coefficient, \( K_p \). A key feature in the development of QSPeRs is that all available data must be consistent and reliable. Variations caused by heterogeneous experimental conditions will decrease the statistical validity of models. Furthermore, the physicochemical parameter space should be broadly explored to provide relevant information on the factors governing skin permeation.

The ability of a compound to diffuse passively across the skin can be variously assessed. Commonly, parameters from Fick's first law (Eq. 3.1), including flux and permeability coefficient, are used [2]:

\[
J = \frac{D_M \cdot P \cdot \Delta C_M}{h} = K_p \cdot \Delta C_M
\]

Eq. 3.1

where \( J \) is the flux, \( D_M \) the diffusion coefficient into the membrane, \( P \) the partition coefficient of the solute between the membrane and solvent, \( h \)
the diffusional path length, $\Delta C_M$ the difference in concentrations between both sides of the membrane, and $K_p$ the permeability coefficient. Typically such assessment is made following in vitro exposure of skin fragments to the permeant. Other assessments may include the amount, or percentage, of an exogenous chemical being absorbed in vivo.

The purpose of this review is to give an overview of some important models to evaluate skin permeation. Physicochemical determinants in passive drug absorption and the relevance of the various approaches are also discussed. A summary of the chemical classes and ranges covered in terms of molecular weight, lipophilicity and permeability is presented in Table 3.1.

### 3.2 Statistical analysis

Most published QSPeR models appeared without complete statistics and were recalculated here using a stepwise multiple regression procedure in Tsar 3.3 (Oxford Molecular) running on Windows 2000 PC. All calculations were performed in an identical manner, the statistical parameters highlighted being the number of compounds included in the analysis ($n$), the square correlation coefficient ($r^2$), the standard deviation ($s$), Fischer's test ($F$), and the predictive correlation coefficient ($q^2$). This latter parameter is obtained by cross-validation using a “leave-one-out” deletion pattern (see section 6.5.4) [3-5]. Moreover, 95% confidence limits are given in parentheses and the relative contributions of each variable to the model is calculated using Mager’s standardization procedure [6].

### 3.3 Common molecular parameters used in QSPeRs

#### 3.3.1 Molecular weight and size

Molecular size is believed to play a distinct role in permeation processes [7,8]. Drug diffusivity, in general, is size-dependent, i.e., large molecules diffuse more slowly than small ones.
Table 3.1. Summary of the chemical classes and range of molecular weight (MW), lipophilicity (log P<sub>oct</sub>) and permeability (log K<sub>p</sub>) upon which each model was developed.

<table>
<thead>
<tr>
<th>Equation</th>
<th>Reference</th>
<th>Chemical class</th>
<th>Ranges a)</th>
</tr>
</thead>
</table>
| Eq. 3.9 | Potts & Guy 1992 [9] | Flynn’s database (n = 93) | 18 < MW < 765  
-2.3 < log P<sub>oct</sub> < 5.5  
-6.1 < log K<sub>p</sub> < 0.1 |
| Eq. 3.12 | Lien & Gao 1995 [10] | Alkanols, phenols, steroids (n = 22) | 18 < MW < 362  
-1.4 < log P<sub>oct</sub> < 3.9  
-5.5 < log K<sub>p</sub> < 12 |
| Eq. 3.13 | Barratt 1995 [11] | Alkanols, phenols, steroids, barbiturates, other drugs (n = 60) | 18 < MW < 362  
-2.3 < log P<sub>oct</sub> < 5.5  
-5.5 < log K<sub>p</sub> < 0.1 |
| Eq. 3.15 | Potts & Guy 1995 [12] | Alkanols, phenols, miscellaneous compounds (n = 37) | 18 < MW < 158  
-1.4 < log P<sub>oct</sub> < 3.2  
-3.5 < log K<sub>p</sub> < 0.1 |
-0.8 < log P<sub>oct</sub> < 3.7  
-5.5 < log K<sub>p</sub> < 12 |
| Eq. 3.20 | Abraham et al. 1997 [14] | Alkanols, phenols, steroids, miscellaneous compounds (n = 53) | 32 < MW < 392  
-0.8 < log P<sub>oct</sub> < 4.0  
-4.2 < log K<sub>p</sub> < 0.1 |
| Eq. 3.21 | Pugh et al. 1996 [15] | Alkanols, phenols, drugs (n = 52) | 32 < MW < 303  
-0.8 < log P<sub>oct</sub> < 4.0  
-4.3 < log K<sub>p</sub> < 11 |
| Eq. 3.22 | Deardan et al. 2000 [16] | Flynn’s database (n = 91) | 18 < MW < 765  
-2.3 < log P<sub>oct</sub> < 5.5  
-6.1 < log K<sub>p</sub> < 0.1 |
| Eq. 3.23 | Cronin et al. 1999 [17] | Flynn’s database, miscellaneous compounds (n = 107) | 30 < MW < 391  
-1.5 < log P<sub>oct</sub> < 7.5  
-5.5 < log K<sub>p</sub> < 0.2 |

a) Permeability coefficients (K<sub>p</sub>) are expressed in [cm/s] (Eqs 3.9, 3.15, 3.17, 3.20, 3.21) and in [cm/h] (Eqs 3.12, 3.13, 3.22 and 3.23).
In simple liquids, this dependence is often expressed by the Stokes-Einstein equation [18]:

\[
D = \frac{R \cdot T}{6 \cdot \pi \cdot \eta \cdot r \cdot N}
\]

Eq. 3.2

where \(D\) is the diffusion coefficient obtained from Fick's law (Eq. 3.1), \(R\) the molar gas constant, \(T\) the absolute temperature, \(\eta\) the viscosity of the solvent, \(r\) the solvated radius of the diffusing solute, and \(N\) Avogadro's number. Assuming spherical molecules, Eq. 3.2 means that \(D\) is inversely proportional to the cube-root of the molecular volume (\(V\)).

In more organized media such as polymers and the stratum corneum, the dependence on size is more severe and has been empirically described by an exponential relationship [9]:

\[
D = D_0 \cdot e^{-\beta(V)}
\]

Eq. 3.3

where \(D_0\) is the diffusivity of a hypothetical molecule having zero molecular volume, and \(\beta\) is a constant. This means that drug diffusivity across the stratum corneum decreases as molecular weight (MW) increases since \(V\) and MW are interdependent.

In terms of permeability coefficient (\(K_p\)), the diffusion parameter (\(D\)) plays an important role in addition to lipophilicity (\(P\)), as described in the following equation:

\[
\log K_p = \log \frac{D \cdot P}{h} = \log D + \log P - \log h
\]

Eq. 3.4

The partition coefficient (\(P\)) of a solute between the skin and water has been frequently described in terms of octanol/water partition coefficient (\(P_{oct}\)) [19,20] which depends on molecular volume and polar interactions (see section 3.3.2). The combination of diffusion and partition coefficient in a single equation (Eq. 3.4) shows that the solute's volume
contributes both positively and negatively to its permeation, as illustrated later by Eqs 3.9 and 3.17. A practical result of this observation is that small polar compounds often have better permeabilities than expected from their lipophilicity alone, due to compensation by a higher diffusivity.

3.3.2 Solvatochromic parameters

Linear solvation free-energy relationships (LSERs) are based on the solvatochromic parameters developed by Taft, Kamlet, Abraham and co-workers [21-25]. LSER analyses serve to factorize some given molecular property (S_p) of neutral organic solutes, e.g. partitioning, retention or permeation [19,26-29], in terms of structural parameters such as the calculated van der Waals volume (V_w) and the so-called solvatochromic parameters (dipolarity/polarizability π*, hydrogen-bond donor acidity α, and hydrogen-bond acceptor basicity β). The linear equation 3.5 reflects a differential solvation model constructed with an endergonic factor (i.e. the volume term accounting for solvophobic/hydrophobic and dispersive forces) and an exergonic factor (i.e. the polar interactions represented by π*, α and β terms).

\[ \log S_p = v \cdot V_w + p \cdot \pi^* + a \cdot \alpha + b \cdot \beta + c \]  
\[ \text{Eq. 3.5} \]

where v, p, a, and b are the regression coefficients which reflect the relative contribution of each solute parameter to S_p. The constant term c includes information on the analyzed system.

3.3.3 Lipophilicity and related parameters

Lipophilicity is classically recognized as a meaningful parameter in structure-activity relationships (QSARs). It is also the single most informative and successful physicochemical property in medicinal chemistry [30,31]. Not only has lipophilicity found innumerable applications in QSARs, but its study has revealed a wealth of information on intermolecular forces, intramolecular interactions, and molecular structure in the broadest sense [32-34].
Lipophilicity is determined experimentally as partition coefficients (log P, valid only for a single ionic state) or as distribution coefficients (log D, referring to a pH-dependent mixture of ionic forms, see chapter 4) [35]. As a ratio of two concentrations at equilibrium, the partition coefficient is the net result of all intermolecular forces linking a solute and the two phases between which it partitions. When a given type of interaction elicited by the solute is of similar energy in the two solvents, the two contributions compensate each other and log P will contain no information about this type of interaction.

It is now common to factorize lipophilicity into two sets of terms (Eq. 3.6), namely a cavity term positively related to lipophilicity, and a negatively related polarity term [36]:

\[
\log P = a \cdot V - \Lambda
\]

where \( V \) is the molar volume and \( \Lambda \) the global polarity of a given solute in a given solvent system. The slope \( a \) depends on the solvent system and on the method used to calculate molecular volumes, whereas the \( \Lambda \) term is mostly related to the solute's H-bonding capacity. This latter parameter, however, also contains information on dipolarity/polarizability.

The contributions of the volume term in octanol and alkanes are known to be similar [27,37] so that the difference \( \log P_{\text{oct}} \) minus \( \log P_{\text{hep}} \) (noted \( \Delta \log P_{\text{oct-hep}} \)) is a function of the polar terms only, and in fact mostly of the H-bonding donor acidity (\( \alpha \)) (Eqs 3.7 and 3.8), as demonstrated by a factorization into the solvatochromic parameters:

\[
\Delta \log P_{\text{oct-hep}} = 0.12 (\pm 0.30) \cdot \pi^a + 1.96 (\pm 0.42) \cdot \beta \\
+ 3.40 (\pm 0.25) \cdot \alpha - 0.43 (\pm 0.27)
\]

\( n = 75; \ r^2 = 0.92; \ s = 0.31; \ F = 288 \)  

\[
\Delta \log P_{\text{oct-hep}} = 3.54 (\pm 0.36) \cdot \alpha - 0.37 (\pm 0.15)
\]

\( n = 75; \ r^2 = 0.84; \ s = 0.45; \ F = 325 \)
The $\Delta\text{log} P$ parameter has found valuable applications in QSPeR studies, e.g. in percutaneous absorption or crossing of the blood-brain barrier [19,38-40].

Lipophilicity is also markedly influenced by intramolecular interactions which can be classified into three main classes [34]: electronic conjugations; interactions involving polar groups; and steric and hydrophobic effects. Several structural factors have been found to be responsible for intramolecular interactions, rendering them too complex to be factorized into simple molecular descriptors such as log P and log D.

Two experimental methods have been proposed to deal with intramolecular forces affecting lipophilicity. The first method is the calculation and application of a number of $\text{diff}$ parameters, deduced from lipophilicity descriptors obtained in the same solvent system for two states (e.g. ionic) of a given solute. Recently, the various $\text{diff}$ parameters and their significance has been reviewed [37]. The second method refers to a pair of solvent systems and is based on the graph obtained by plotting $\text{log Poct}$ vs $\text{log P xxx}$ (being xxx a second system solvent) for a balanced training set of compounds [27]. The addition to this graph of the solutes under study can reveal the existence of intramolecular effects rendering these solutes deviant. Alternatively, the same information can be obtained by calculating and analyzing $\Delta\text{log} P$ parameters, but the results may not be as clear-cut as those obtained with the graphical approach.

### 3.4 Quantitative structure-permeation relationships: pre-1990 period

Over the last thirty years, there have been significant attempts to relate the skin permeability of compounds to their physicochemical properties. Quantitative models (i.e. QSPeRs) were seldom presented, particularly in the earlier studies. Most of these were based on the analysis of homologous or closely related series comprising relatively few compounds. Many of these studies revealed a linear relationship with lipophilicity, i.e. increasing lipophilicity was associated with increasing
skin permeation [41,42]. Some studies also reported a parabolic relationship with lipophilicity, particularly when highly lipophilic compounds were included [42]. Whilst these studies showed that it was possible to derive QSPeR models for skin penetration, each model was valid only for a particular chemical class or a series of compounds. A further problem of analyzing homologous or closely related series is that there is little physicochemical variety in the data. This results in co-linearity between descriptors, especially for congeneric series, so that it is not possible to separate the effects of lipophilicity and molecular size. A number of excellent reviews dealing with the modeling of skin permeability of homologous or closely related series of compounds have been published [43-45].

3.5 Analyses based on Flynn’s dataset

Attempts to develop a more global approach to the QSPeR prediction of skin permeability were greatly facilitated by the large database published by Flynn in 1990 [46]. A collection of 97 permeability coefficients (K_p) for 94 compounds (3 triplicates) with a relatively broad range of properties (18 < MW < 765 and -2.3 < log P_oct < 5.5) were gathered in this publication. Until recently, this provided the largest heterogeneous database of in vitro skin permeabilities through human skin. It should be noted, however, that these data were a compilation from 15 different literature sources and that they necessarily contain a high degree of experimental error due to inter-laboratory variability. The predictive models presented below will be based on Flynn’s database and on related studies, published during the last decade.

In his original paper, Flynn proposed a number of algorithms to predict skin permeability. His results showed that lipophilicity (octanol/water partition coefficient) and molecular size were relevant parameters. A simple approach with algorithms for low and high molecular weight compounds has been described, which can be presented
as a decision tree (Fig. 3.1). No statistical measure of fit was provided at this time.

![Decision Tree Diagram]

**Fig. 3.1.** Decision tree for predicting approximate skin permeability coefficients ($K_p$), based on Flynn’s algorithm [46].

The data published by Flynn were subsequently analyzed by many workers. Potts and Guy [9] demonstrated the use of log $P_{oct}$ in combination with either molecular weight (MW) or molecular volume to predict the permeability coefficients collected by Flynn. For 93 of the compounds they reported the following relationship:

$$\log K_p = 0.72 (\pm 0.13) \cdot \log P_{oct} - 0.0059 (\pm 0.0014) \cdot MW - 6.36 (\pm 0.28)$$

Eq. 3.9

$$n = 93; \quad r^2 = 0.67; \quad q^2 = 0.65; \quad s = 0.74; \quad F = 92$$

relative contributions: log $P_{oct}$ (53%); MW (47%)

It should be noted that this equation is used for predicting the permeability coefficient from an aqueous solution of the diffusant. The physical significance of this empirical equation is clear. As the permeants become more lipophilic, their permeability increases due to better
partitioning into the skin but as they become larger, their diffusion into
the skin is reduced. Our statistical analysis shows that the model is
significant in terms of the descriptors used. Although the statistical fit to
Eq. 3.9 is comparatively poor, the authors did observe that up to 30%
variability in the experimental data was to be expected [47], so that an r²
of approximatively 70% was considered to be significant. Moreover, the
authors did not discuss the problem of outliers.

In a recent study, five algorithms based on log P_{oct} and molecular
weight were compared in terms of predictive reliability for skin
permeation [48-52]. A large database of 99 solutes was used, and two
particular algorithms stood out as better predictors of the skin permeation
of highly hydrophilic and highly lipophilic chemicals compared to the
model of Potts and Guy.

At a similar time, El Tayar and co-workers [19] examined various
subsets from Flynn's dataset. They confirmed the role of lipophilicity in
controlling skin permeability for a variety of these subsets. They also
described a correlation with the parameter ∆log P_{oct-alk} (the partition
coefficient in octanol/water minus the partition coefficient in
alkane/water).

\[
\log K_p \ [\text{cm/s}] = -1.36 (\pm 0.40) \cdot \Delta \log P_{oct-alk} - 3.39 (\pm 0.99)
\]

\[
n = 21; \ r^2 = 0.81; \ q^2 = 0.76; \ s = 0.50; \ F = 80
\]

Eq. 3.10

This latter parameter is considered to be a measure of the hydrogen
bond donor acidity of solutes. El Tayar et al. further suggested that
molecular size was not relevant in the data set analyzed. Statistical bias
due to two clustered congenic series and/or differences in partition
coefficient measurement are responsible for this erroneous conclusion.
Whilst the fact that hydrogen bonding is important in controlling skin
permeability has been taken up by many other workers, the induction that
molecular weight was not important was strongly and rightly refuted by
Potts and Guy [9], as underlined by the improved equation.
log $K_p$ [cm/s] = 0.80 (±0.16)·log $P_{oct}$ - 0.0108 (±0.0015)·MW - 5.91 (±0.28)

Eq. 3.11

$n = 21; \ r^2 = 0.89; \ q^2 = 0.86; \ S = 0.40; \ F = 70$

relative contributions: log $P_{oct}$ (42%); MW (58%)

This fact suggests that the complex mechanism of skin permeation cannot be described by a single physicochemical parameter. In addition to hydrogen bond donor acidity implicitly contained in lipophilicity, an additional term (MW) is necessary to better predict the dataset.

The Flynn dataset was also analyzed by Pugh and Hadgraft using a novel approach [53], namely a so-called ab initio approach based on indicator variables for various molecular sub-structures and features. The results using a 17- or 11-descriptor model were comparable, but not significantly better, than those of Potts and Guy [9]. This was a fundamentally empirical approach to the prediction of skin permeability, but lacked the uniform approach and mechanistic simplicity of methods based on physicochemical properties. Using this approach, however, Pugh and Hadgraft did identify a number of compounds as significant outliers (notably atropine, estriol, naproxen, nicotine, nitroglycerine, sucrose, and toluene). There is considerable agreement between these outliers and those identified in later studies.

Lien and Gao [10] analyzed a subset from Flynn’s dataset which included non-electrolytes and steroids. They demonstrated that the number of hydrogen bonds formed by a compound ($H_b$), plus molecular weight and a biphasic response to lipophilicity, could model skin permeability in a satisfactory manner:

$$
\log K_p \ [\text{cm/h}] = -0.08 (±0.13) \cdot (\log P_{oct})^2 + 0.84 (±0.59) \cdot \log P_{oct} - 0.25 (±0.12) \cdot H_b - 1.8 (±2.0) \cdot \log MW + 4.2 (±3.3)
$$

Eq. 3.12

$n = 22; \ r^2 = 0.95; \ q^2 = 0.91; \ s = 0.30; \ F = 88$

relative contributions: log $P_{oct}$ (12%); log $P_{oct}$ (43%); $H_b$ (26%); log MW (19%)
This model is clearly at the limit of statistical validity in terms of the number of variables included to the observations, although it emphasizes the possible importance of hydrogen bonding. The authors, however, did not pinpoint the strong correlation between the squared $\log P_{oct}$ value and $\log P_{oct}$ ($r^2 = 0.77$) and between $\log P_{oct}$ and $\log MW$ ($r^2 = 0.53$) for the set of compounds. Moreover, two parameters ($\log P_{oct})^2$ and $\log MW$) are non significant due to large confidence intervals. Such statistical anomalies due to an insufficient number of compounds leads to an over-interpretation of the data.

Barratt [11] subsequently analyzed the complete Flynn's dataset and developed a QSPEr model after subdividing the original set into three distinct groups (steroids, other pharmacologically active compounds and small compounds). He noted that a subset of hydrocortisone derivates within the steroid group were very poorly predicted. To model the data, Barratt extended Potts and Guy's approach [9] by using molecular volume as a measure of molecular size and adding melting point (mpt). The usefulness of this latter descriptor has never been fully addressed. The author proposed that, combined with $\log P_{oct}$, it offers a measure of aqueous solubility. From the dataset, it is not possible to prove or disprove this suggestion. However, it should be noted that melting point is a physicochemical property highly dependent on hydrogen bonding, hence this descriptor may emphasize the importance of hydrogen bonding to model skin permeability. After removing the hydrocortisone derivates, the most significant equation reported for a subset of 60 “small molecules and steroids” was:

$$\log K_p \ [cm/h] = 0.820 (\pm 0.091) \cdot \log P_{oct} - 0.0093 (\pm 0.0021) \cdot MV$$
$$- 0.004 (\pm 0.032) \cdot mpt - 2.36 (\pm 0.22)$$

Eq. 3.13

$$n = 60; \ r^2 = 0.90; \ q^2 = 0.89; \ s = 0.39; \ F = 176$$

relative contributions: $\log P_{oct}$ (49%); MV (38%); mpt (13%)
The statistical results clearly indicate that the melting point parameter is non significant. Indeed, its removal does not decrease the predictivity of the model as described by Eq. 3.14:

$$\log K_p \, [\text{cm/h}] = 0.830 \, (\pm 0.085) \cdot \log P_{oct} \, - \, 0.0119 \, (\pm 0.0015) \cdot MV$$

$$-2.31 \, (\pm 0.22)$$

$$n = 60; \, r^2 = 0.89; \, q^2 = 0.87; \, s = 0.42; \, F = 219$$

relative contributions: $\log P_{oct}$ (51%); $MV$ (49%)

The role of hydrogen bonding was further investigated by Potts and Guy [12]. A QSPeR model based on 37 “non-electrolyte” compounds selected from Flynn’s compilation was developed. The algorithm did not contain a lipophilic term, but included molecular volume and descriptors for hydrogen bond donor ($H_d$) and acceptor ($H_a$) capacity:

$$\log K_p \, [\text{cm/s}] = 2.56 \, (\pm 0.50) \cdot MV/100 \, - \, 1.74 \, (\pm 0.46) \cdot H_d$$

$$- 3.85 \, (\pm 2.44) \cdot H_a \, - \, 4.89 \, (\pm 0.50)$$

$$n = 37; \, r^2 = 0.94; \, q^2 = 0.92; \, s = 0.24; \, F = 169$$

relative contributions: $MV/100$ (38%); $H_d$ (30%); $H_a$ (32%)

Whilst Eq. 3.15 has some utility in shedding mechanistic light onto the prediction of skin permeability (i.e. in that hydrogen bonding capacity is inversely related to skin permeability, and hydrogen bond acceptor ability is more important than hydrogen bond donor capacity) the reduced size of the dataset limits its predictability, especially for large compounds (see range in Table 3.1). In order to compare the relevance of H-bonding to lipophilicity, a new model (Eq. 3.16) has been calculated using only two parameters. The high correlation between molecular volume (MV) and molecular weight (MW) for the set of compounds ($r^2 = 0.81$) allows one parameter to be replaced by the other.
\[
\log K_p \ [\text{cm/s}] = 0.93 (\pm 0.37) \cdot \log P_{\text{oct}} - 0.013 (\pm 0.013) \cdot MW - 5.67 (\pm 0.80)
\]

\[\text{Eq. 3.16}\]

\[
n = 37; \quad r^2 = 0.78; \quad q^2 = 0.72; \quad s = 0.44; \quad F = 61
\]

relative contributions: \(\log P_{\text{oct}} (71\%); MW (29\%)\)

It must be noted that Eq. 3.16 is very similar to Eq. 3.9. The difference in intercepts is related to the specific set of compounds. Due to the smaller dataset, however, molecular weight appears statistically non significant.

Other similar results indicating the importance of molecular size and hydrogen bonding, were also published by Abraham et al. [13]. For a set of 46 compounds (mainly the “non-electrolytes” selected by Potts and Guy [12]) in Flynn's dataset, it was demonstrated that:

\[
\log K_p \ [\text{cm/s}] = -0.59 (\pm 0.23) \cdot \pi_2^H - 0.62 (\pm 0.43) \cdot \Sigma\alpha_2^H - 3.43 (\pm 0.41) \cdot \Sigma\beta_2^H + 1.80 (\pm 0.30) \cdot V_x - 5.07 (\pm 0.21)
\]

\[\text{Eq. 3.17}\]

\[
n = 46; \quad r^2 = 0.96; \quad q^2 = 0.95; \quad s = 0.25; \quad F = 242
\]

relative contributions: \(\pi_2^H (17\%); \Sigma\alpha_2^H (4\%); \Sigma\beta_2^H (43\%); V_x (36\%)

where \(\pi_2^H\) is dipolarity/polarisability, \(\Sigma\alpha_2^H\) hydrogen-bond donor acidity, \(\Sigma\beta_2^H\) hydrogen-bond acceptor basicity and \(V_x\) McGowan volume [54].

The positive coefficient associated with molecular size (\(V_x\)) reflects an unbalance between the opposite influences of molecular size on lipophilicity (Eq. 3.18) and diffusion (Eq. 3.3).

\[
\log P_{\text{oct}} = -0.98 (\pm 0.25) \cdot \pi_2^H + 0.68 (\pm 0.47) \cdot \Sigma\alpha_2^H - 3.72 (\pm 0.44) \cdot \Sigma\beta_2^H + 4.12 (\pm 0.33) \cdot V_x - 0.08 (\pm 0.27)
\]

\[\text{Eq. 3.18}\]

\[
n = 46; \quad r^2 = 0.96; \quad q^2 = 0.94; \quad s = 0.25; \quad F = 222
\]

relative contributions: \(\pi_2^H (18\%); \Sigma\alpha_2^H (3\%); \Sigma\beta_2^H (29\%); V_x (50\%)\)
Based on the relationship described by Potts and Guy [9], Eq 3.17 is rewritten as follows:

\[
\log K_p \ [\text{cm/s}] = 0.788 \ (\pm 0.096) \cdot \log P_{\text{oct}} - 1.44 \ (\pm 0.18) \cdot V_x \\
- 5.64 \ (\pm 0.26)
\]

\[\text{Eq. 3.19}\]

\[n = 46; \ r^2 = 0.90; \ q^2 = 0.89; \ s = 0.37; \ F = 196\]
relative contributions: \(\log P_{\text{oct}} \ (43\%); \ V_x \ (57\%)\)

A clearly negative influence of molecular size is highlighted, reflecting the effect of size on the diffusion component of permeation (Eqs 3.3 and 3.4).

Abraham et al. [14,55] later considered the issue of outliers and the possible erroneous values of some steroids. In their review based on the prediction of transport properties of solutes [55], a slightly expanded equation was proposed:

\[
\log K_p \ [\text{cm/s}] = 0.45 \ (\pm 0.24) \cdot R_2 - 0.49 \ (\pm 0.23) \cdot \pi_H^2 \\
- 1.49 \ (\pm 0.27) \cdot \Sigma \alpha_H^2 - 3.43 \ (\pm 0.31) \cdot \Sigma \beta_H^2 \\
+ 1.93 \ (\pm 0.25) \cdot V_x - 5.13 \ (\pm 0.21)
\]

\[\text{Eq. 20}\]

\[n = 53; \ r^2 = 0.96; \ q^2 = 0.95; \ s = 0.21; \ F = 216\]
relative contributions: \(R_2 \ (7\%); \ \pi_H^2 \ (12\%); \ \Sigma \alpha_H^2 \ (11\%); \ \Sigma \beta_H^2 \ (38\%); \ V_x \ (32\%)\)

where \(R_2\) represents the excess molar refraction. The work of Abraham and co-workers confirms that there may be problems with the permeability coefficients of some of the compounds, especially steroids. It also confirms the importance of hydrogen bonding when an explicit lipophilicity descriptor is absent from the QSPeR equation.

Pugh et al. [15] also addressed the issue of hydrogen bonding and molecular size in skin permeation. Their findings demonstrated that hydrogen bond donor acidity is dominant over acceptor basicity. The relative effect of the donor versus acceptor capacity was quantified as being 0.6:0.4. A later study by Pugh et al. [56] demonstrated the usefulness of calculated molecular charges (i.e. the sum of partial charges
on atoms) as descriptors of hydrogen bonding, as opposed to the solvatochromic parameters. The relationship obtained for a set of 52 values was:

\[
\log \left( \frac{D}{h} \right) = -2.69 (\pm 0.13) \cdot 0.00268 (\pm 0.00032) \cdot MW \cdot \text{charge} \\
\text{n} = 52; \quad r^2 = 0.83; \quad q^2 = 0.82; \quad s = 0.33; \quad F = 242
\]

\[\text{Eq. 3.21}\]

where \(D [\text{cm}^2/\text{s}]\) is the diffusion coefficient and \(h [\text{cm}]\) is the diffusional path length through the skin.

The complexity in modeling the Flynn’s dataset was exemplified by Magee [57]. In order to test the hypothesis that drug absorption changes with its lipophilicity, the dataset was factorized into three overlapping regions of log \(P_{oct}\) (-1.38 to 1.96; 1.53 to 2.97; 2.50 to 5.49). A difference in the QSPeRs based on log \(P_{oct}\) and molecular weight was reported, and an increased importance of hydrogen bonding for the most lipophilic group of chemicals was highlighted. Individual chemical classes were also analyzed within the heterogeneous dataset. Predictably, correlations for individual chemical classes (probably measured in the same laboratory) were better than for the complete dataset. These results are not necessarily relevant in terms of being predictive models, but they do highlight the mechanistic possibilities of QSPeR analysis.

The complete Flynn’s dataset was also analyzed by Dearden et al. [16] who utilized a total of 81 physicochemical descriptors including those for hydrophobicity, molecular size and hydrogen bonding. A highly predictive but mechanistically complex QSPeR model was reported based on six parameters:

\[
\log K_p [\text{cm/h}] = 0.834 - 0.626 \cdot \Sigma \text{Ca} - 23.8 \cdot \Sigma (Q+) / \alpha \\
- 0.289 \cdot \text{SsssCH} - 0.0357 \cdot \text{SsOH} - 0.482 \cdot I_B \\
+ 0.405 \cdot B_R
\]

\[\text{n} = 91; \quad r^2 = 0.83; \quad s = 0.56; \quad F = 69\]

\[\text{Eq. 3.22}\]

where \(\Sigma \text{Ca}\) is the HYBOT-PLUS H-bond acceptor free energy factor, \(\Sigma (Q+) / \alpha\) the HYBOT-PLUS positive charge per unit volume, SsssCH and
SsOH are electrotopological atom-type indices for single bonded CH and OH, IB is the Balaban index, and Bř the number of rotatable bonds. Although atropine, naproxen and nicotine were poorly predicted, the results clearly indicate that H-bond acceptor capacity and polarity lower skin permeability. Moreover, SsssCH and IB, both reflecting molecular size, are better descriptors than molecular weight itself. The inclusion of the Bř term (a measure of conformational flexibility) is most interesting, and suggests that this property enhances permeability. However, due to the lack of data in the publication it is difficult to evaluate the pertinence of all these terms and/or their possible intercorrelation.

3.6 Other recent QSPeR analyses

A larger database of 114 skin permeability values established by Kirchner et al. [58] incorporates many data from the Flynn’s dataset, with considerable additional data from regulatory reports from Health Canada. In an attempt to model these data, Kirchner et al. subdivided the dataset into 5 groups according to calculated molar volume. Correlations for each group were obtained with log Poct, indicating the dependence on lipophilicity. The dependence of the complete dataset on lipophilicity was, however, rather poor (r^2 = 0.32).

All these data were subsequently reanalyzed by Cronin et al. [17], who demonstrated that seven significant outliers occurred in the set. These included compounds that had more than ten sites to accept or donate a hydrogen bond (i.e., sucrose and digitoxin), and well as large compounds such as estriol, atropine, hydrocortisone and etorphine. Propylene chloride was also excluded from the list as having a spurious permeation value. Removal of these outliers and reanalysis against a wide variety of QSPeR parameters, including those for hydrogen bonding and other molecular properties, revealed the following, highly significant, equation:
\[
\log K_p [\text{cm/h}] = 0.773 \pm 0.082 \cdot \log P_{oct} - 0.0103 \pm 0.0014 \cdot MW - 2.33 \pm 0.15
\]

\[n = 107; \quad r^2 = 0.86; \quad q^2 = 0.85; \quad s = 0.39; \quad F = 317\]

relative contributions: \(\log P_{oct}\) (57%); MW (43%)

Eq. 3.23

It was noted that despite the larger data set, Eq. 3.23 is very similar to Eq. 3.9 proposed by Potts and Guy [9] (the difference in intercepts is due to the difference in units in the permeability coefficients). The statistical improvement over the original Potts and Guy model \((r^2 = 0.86\) vs \(r^2 = 0.67\)) was closer investigated in the recent study of Frasch et al. [59]. The results indicated that of the 107 compounds studied, 63 were taken from the Occupational Safety and Health Association (OSHA) and had calculated \(\log K_p\) values based on a method which is a trivial modification of the original Potts and Guy equation. Thus, the apparent statistical improvement reported by Cronin et al. [17] is an artifact because the permeation values are not experimentally determined. Therefore, continued use of these data would lead to a biased selection of the statistical models, an underestimation of the experimental variability, and an overestimation of the predictivity of the models.

The problem of identifying and dealing with outliers is a controversial issue that has seldom been addressed sufficiently in QSPeR analyses. The reasons for the removal of outliers, and the consequence of so-doing (or indeed not doing) are poorly understood. It is clear from a number of studies described above [11,17,53] that some compounds are consistently found to be outliers. To shed some light on these outliers, it is pertinent to consider observations by Johnson et al. [60] and Degim et al. [61]. Johnson et al. [60] reinvestigated the steroid permeability values taken from Scheuplein et al. [62] and recorded in Flynn’s dataset. Large discrepancies were found between the values from Scheuplein et al. [62] and other, more recently, published data. Degim et al. [61] re-measured a number of permeability coefficients, including three compounds (naproxen, atropine and nicotine) that had been found to be outliers. For these later compounds, measured values were found to be significantly different from
those compiled by Flynn. Thus, there is considerable evidence that some of the permeability coefficients in Flynn’s dataset may have considerable error associated with them. The removal of such outliers was thus justified to produce a statistically valid model. For future predictions of skin permeability based on Flynn’s dataset, the reported values should be treated with caution to avoid affecting the validity of resulting models.

Whilst a number of QSPeR approaches have been attempted, few have considered the possible effect of differences in skin conditions. An exception to this was the work of Hostynek and Magee [63]. These authors indicated that for the human in vivo absorption of miscellaneous chemicals both the vehicle in which the material was delivered and the degree of occlusion of the device were important. For the former property, a significant relationship was developed for the in vivo absorption data (penetration constant at the point of maximum flux ($K_{p_{\text{Max}}}$)) of 20 miscellaneous compounds with an indicator variable (VEH) for the different vehicles used (i.e. a value of 1 for acetone and 2 for ethanol):

$$\log K_{p_{\text{Max}}} \text{ [cm/h]} = -0.60 \cdot \text{VEH} - 0.014 \cdot \text{MR} - 0.16 \cdot \text{HBA}$$
$$+ 0.29 \cdot \text{HBD} + 2.1$$
\[\text{Eq. 3.24}\]

where $n = 20$; $r^2 = 0.80$; $s = 0.35$; $F = 15$

This model appears very limited due to the large number of parameters with respect to the number of compounds. Moreover, no data was available to assess statistical pertinence.

A larger dataset was available to assess the effect of the degree of occlusion on the maximum flux of compounds through human skin in vivo. Here, an indicator variable (OCCL) for occlusion was used (i.e. a value of 1 for open, 2 for protected, 3 for occluded):

$$\log J_{\text{Max}} \text{ [(\mu g/cm^2)/h]} = 0.15 \cdot \log P_{\text{oct}} - 0.013 \cdot \text{MR} - 0.29 \cdot \text{HBD}$$
$$+ 0.28 \cdot \text{OCCL} + 0.11$$
\[\text{Eq. 3.25}\]

where $n = 48$; $r^2 = 0.66$; $s = 0.40$; $F = 21$
As well as the importance of such features as the vehicle and degree of occlusion, the same parameters for molecular size, lipophilicity and hydrogen bonding were seen to be important. The relatively low correlation coefficient is another indication of the errors in the dataset, undoubtedly due to their compilation from a variety of different sources.

Other more recent studies have again illustrated the importance of partitioning in the structure-based prediction of abdominal skin permeability coefficients [64]. Also, Ghafourian and Fooladi [65] demonstrated the use of theoretical parameters derived from structural chemistry to replace solvatochromic descriptors in QSPeRs.

3.7 Conclusion

The aim of this review was to survey the most significant models for the prediction of skin permeation. The large number of models that have been developed highlights their usefulness as predictive tools in both the drug delivery and toxicology fields. Such quantitative structure-permeation relationships do not resort to expensive and time-consuming human studies or animal models. Moreover, they are most accurate for compounds within similar classes and useful for predicting drug absorption at the conceptual stage, i.e., prior to their synthesis and further development or formulation spending. Certain limitations of these models, however, are to be pinpointed. An inherent problem lies in the fact that their applicability is restricted by the limited range of polarity and size of the permeants on which they are based. In addition, most of the models use large compilations of data from various investigators and laboratories employing different experimental protocols. Thus, these models show fair explanation of variance in the lower molecular size and the middle range of lipophilicity, while the divergences for both very hydrophilic and very lipophilic compounds become conspicuous. Furthermore, rigorous statistics show that some models are based on non-significant descriptors. As such, they do not afford mechanistic insights and may lead to an over-interpretation of the data.
3.8 References


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Part II:

Experimental studies on skin permeation
4 Quantification of lipophilicity

4.1 Experimental measurements of lipophilicity

4.1.1 Introduction

Lipophilicity is a molecular property influencing the pharmacokinetic behavior and pharmacodynamic properties of many compounds [1]. Since most drugs are partly or largely ionized at physiological pH, an understanding of the lipophilicity of both their neutral and ionic form is important.

Conventionally, lipophilicity is expressed as the logarithm of the partition coefficient (P) of a solute present in a single electrical state and is given by Eq. 4.1:

$$\log P = \log \left( \frac{C_o}{C_w} \right)$$  
Eq. 4.1

where $C_o$ and $C_w$ are the concentrations at equilibrium of the solute in the organic and aqueous phase, respectively. $\log P$ depends on the temperature and the solvent system used [2].

When more than one electrical species are present in solution, as can be the case with ionizable solutes, the observed ratio of concentrations is the distribution coefficient (D), which takes into account the intrinsic lipophilicity of the different electrical species present and their relative concentrations. At a given pH, the lipophilicity of an ionizable solute is thus described by Eq. 4.2:

$$\log D^{pH} = \log \left[ f_N \cdot P_N^{N} + \Sigma (f_I \cdot P_I^{N}) \right]$$  
Eq. 4.2

where $f_N$ and $f_I$ are the molar fractions of the neutral and ionized form(s) and $P_N^{N}$ and $P_I^{N}$ are the partition coefficients of the neutral and ionized
species, respectively. Sigmoidal curves called lipophilicity profiles are obtained when plotting the log D values against pH. At pH values where the neutral form predominates, the log D values are identical to log $P_N$ values, whereas with increasing ionization the log D values decrease linearly, until again a constant log D value is reached and equals the log $P_I$ value.

4.1.2 The shake-flask method

Probably the most widely used technique to measure partition coefficient is the so-called “shake-flask method”, which involves shaking the two solvents together until the partition equilibrium of the solute is reached, and then measuring the concentration in each phase. By controlling the pH in the aqueous phase, the log P of neutral solutes can be measured by this method. Because of its simplicity and clear relationship to the partitioning phenomenon, the shake-flask technique is regarded as a benchmark method against which other methods are validated. However, it is tedious and suffers from a number of practical limitations, such as the precision of phase volume ratio, solute stability or volatility, solute impurities, formation of microemulsions and time consumption [3].

A lipophilicity profile can be obtained by measuring log D at several different pH values, but it has to be highlighted that the log P values measured for ionized solutes are apparent values (see Eq. 4.13) and thus strongly depend on experimental conditions such as phase volume, nature of the buffer and ionic strength [4].

4.1.3 The potentiometric method

The potentiometric technique allows measurement of the ionization constants and the log P values of ionizable solutes exclusively [5-8]. This method is based on the observation that when an ionizable compound in aqueous solution is titrated in the presence of an organic phase, the titration curve shifts to the right for acids and to the left for bases. This
shift is related to the phase ratio and to the lipophilicity of both electrical species of the solute [9-11]. In the simple case of a monobasic drug B, the apparent pKₐ (pKₐ^{app}) of the substance measured in the presence of the organic phase is given by Eq. 4.3:

$$\text{pK}ₐ^{\text{app}} = \text{pK}ₐ \cdot \log \left( \frac{r \cdot P^B + 1}{r \cdot P^{BH^+} + 1} \right)$$

Eq. 4.3

where P^B and P^{BH^+} are the partition coefficients of the neutral and protonated drug, respectively, and r is the phase volume ratio. Thus the determination of the log P values of a monobasic (or monoacid) compound requires knowledge of its pKₐ and a minimum of two titrations in the presence of two different phase volume ratios, yielding two pKₐ^{app} values. By assuming that log P^{BH^+} is a constant, the resolution of the system given by Eq. 4.4 (2 equations, 2 unknown parameters) then yields log P^B and log P^{BH^+} values.

$$\begin{bmatrix}
\text{pK}ₐ^{\text{app}1} = \text{pK}ₐ \cdot \log \left( \frac{r_1 \cdot P^B + 1}{r_1 \cdot P^{BH^+} + 1} \right) \\
\text{pK}ₐ^{\text{app}2} = \text{pK}ₐ \cdot \log \left( \frac{r_2 \cdot P^B + 1}{r_2 \cdot P^{BH^+} + 1} \right)
\end{bmatrix}$$

Eq. 4.4

The advantage of the potentiometry method is the possibility to use a large set of solvents and to measure a large range of log P values [12]. Nevertheless its main limitation comes from the assumption that log P^{BH^+} is a constant since Eq. 4.12 (see later) shows clearly the dependence of log P^{BH^+} on experimental conditions, and particularly phase volumes. Some general considerations have thus to be taken into account when analyzing the results:
When $P^B >> P^{BH^+}$, $pK_a$ shifts obtained by Eq. 4.3 do not depend on $\log P^{BH^+}$ and the potentiometric method yields reliable $\log P^B$ values.

When $P^{BH^+}$ cannot be neglected relative to $P^B$, the results given by potentiometric titrations must be considered with caution.

### 4.1.4 Cyclic voltammetry at the interface between two immiscible electrolyte solutions (ITIES)

Cyclic voltammetry is an electrochemical method to determine the lipophilicity of ions and to study their mechanism of transfer at the liquid-liquid interface [4,13,14]. This technique requires the use of a polarizable interface which is available with a solvent system such as 1,2-dichloroethane/water [15,16]. The main advantage of cyclic voltammetry is to provide standard $\log P$ values for ions, independent on the experimental conditions (except on temperature and solvents) [17]. At this stage, some general thermodynamic aspects of the partitioning of ions and ionizable compounds need to be shortly recalled.

The partition of salts can be described by a system consisting of two immiscible liquid phases $\alpha$ and $\beta$ (for example water and 1,2-dichloroethane) and containing $n$ ions $X_i$:

\[
\begin{align*}
\alpha \text{ phase} & \quad \sum_{i=1}^{n} X_i^\alpha \\
(V_\alpha) & \quad \sum_{i=1}^{n} X_i^\beta \\
\beta \text{ phase} & \quad \sum_{i=1}^{n} X_i^\beta
\end{align*}
\]

where $V_\alpha$ and $V_\beta$ are the volumes of phases $\alpha$ and $\beta$.

At equilibrium, the electrochemical potential of each ion $X_i$ is the same in both phases $\alpha$ and $\beta$: 

\[
\frac{\sum_{i=1}^{n} X_i^\alpha}{V_\alpha} = \frac{\sum_{i=1}^{n} X_i^\beta}{V_\beta}
\]
\[ \bar{\mu}_i^\alpha = \mu_i^\beta \]  

Eq. 4.5

The electrochemical potential of the ion \( X_i \) is the sum of a chemical and an electrical term:

\[ \bar{\mu}_i^\alpha = \mu_i^\alpha + z_i \cdot F \cdot \phi^\alpha \]  

Eq. 4.6

where \( \mu_i^\alpha \) is the chemical potential of ion \( X_i \) in phase \( \alpha \), \( z_i \) is the charge of ion \( X_i \), \( F \) is the Faraday constant \( (9.65 \cdot 10^4 \text{ C mol}^{-1}) \) and \( \phi^\alpha \) the Galvani potential of phase \( \alpha \) in Volt (i.e. the bulk potential of phase \( \alpha \)).

The chemical potential is defined by:

\[ \mu_i^\alpha = \mu_i^{0,\alpha} + R \cdot T \cdot \ln a_i^\alpha \]  

Eq. 4.7

where \( \mu_i^{0,\alpha} \) and \( a_i^\alpha \) are respectively the standard chemical potential and the activity of \( X_i \) in phase \( \alpha \), \( R \) is the gas constant \( (8.31 \text{ J K}^{-1} \text{ mol}^{-1}) \) and \( T \) is the temperature in Kelvin.

From Eq. 4.5, we obtain:

\[ \Delta_{\beta}^\alpha \phi = \phi^\alpha - \phi^\beta = \frac{\mu_i^{0,\beta} - \mu_i^{0,\alpha}}{z_i \cdot F} + \left( \frac{R \cdot T}{z_i \cdot F} \right) \cdot \ln \left( \frac{a_i^\beta}{a_i^\alpha} \right) \]  

Eq. 4.8

where \( \Delta_{\beta}^\alpha \phi \) is the Galvani potential difference between phases \( \alpha \) and \( \beta \) and represents the potential difference across the interface between two immiscible electrolyte solutions (noted ITIES) \( \alpha / \beta \).

Eq. 4.8 can be written as:

\[ \Delta_{\beta}^\alpha \phi = \Delta_{\beta}^\alpha \phi_i^0 + \left( \frac{R \cdot T}{z_i \cdot F} \right) \cdot \ln \left( \frac{a_i^\beta}{a_i^\alpha} \right) \]  

Eq. 4.9

where \( \Delta_{\beta}^\alpha \phi_i^0 \) is the standard transfer potential of ion \( X_i \) between phases \( \alpha \) and \( \beta \). Supposing that the ion transfers without formation of any ion-pair and without any chemical or redox reaction, \( \Delta_{\beta}^\alpha \phi_i^0 \) can be expressed by:
\[ \Delta \mu_i^0 = \frac{\mu_i^0,\beta - \mu_i^0,\alpha}{z_i \cdot F} = \frac{\Delta G_{\text{tr},i}^{0,\alpha \rightarrow \beta}}{z_i \cdot F} \]  

Eq. 4.10

where \( \Delta G_{\text{tr},i}^{0,\alpha \rightarrow \beta} \) is the standard Gibbs energy of transfer of ion \( X_i \) from \( \alpha \) to \( \beta \).

The 1,2-dichloroethane/water partition coefficient of ion \( X_i \) (noted \( P_{\text{dce}}^{i,0} \)) between phases \( \alpha \) and \( \beta \) is potential-dependent and is given by Eq. 4.11:

\[
\log P_{\text{dce}}^{i} = \log \left( \frac{a_{\beta}^i}{a_{\alpha}^i} \right) = \frac{z_i \cdot F}{R \cdot T \cdot \ln 10} \cdot \Delta \phi_i^0 + \frac{z_i \cdot F}{R \cdot T \cdot \ln 10} \cdot \Delta \phi \] 

Eq. 4.11

Log \( P_{\text{dce}}^{0,i} \) represents the partition coefficient of \( X_i \) when the interface is not polarized and depends only on the chemical structure of \( X_i \) and on the temperature. The standard partition coefficient is given by:

\[
\log P_{\text{dce}}^{0,i} = - \frac{z_i \cdot F}{R \cdot T \cdot \ln 10} \cdot \Delta \phi_i^0 
\]

Eq. 4.12

Thus Eq. 4.11 can be rewritten as:

\[
\log P_{\text{dce}}^{i} = \log P_{\text{dce}}^{0,i} + \frac{z_i \cdot F}{R \cdot T \cdot \ln 10} \cdot \Delta \phi 
\]

Eq. 4.13

Cyclic voltammetry at the ITIES is a potential-controlled electrochemical method so that the standard partition coefficient obtained by this method is independent on the Galvani potential difference between the two phases (\( \Delta \phi \)) and corresponds to \( \log P_{\text{dce}}^{0,i} \). At the opposite, the shake-flask method is not a potential-controlled method. Each experiment corresponds to a particular value of \( \Delta \phi \), according to particular experimental conditions (nature and concentration of all ions, phase volumes). The partition coefficient measured by the shake-flask method is thus an apparent partition coefficient (noted \( \log P_{\text{dce}}^{i} \)).
The experimental set-up used on cyclic voltammetry experiments is illustrated in Fig. 4.1. The ITIES are polarized by using a 4-electrode potentiostat with two reference electrodes to control the Galvani potential difference and two counter-electrodes allowing the passage of the current.

![Fig. 4.1. Schematic representation of the electrochemical cell used for cyclic voltammetry measurements. (1) reference electrode (Ag/AgCl or Ag/Ag$_2$SO$_4$); (2) counter-electrodes (platinum wire sealed in glass); (3) reference electrode (Ag/AgCl); (4) aqueous phase (LiCl or Li$_2$SO$_4$); (5) organic phase (BTPATPBCl); (6) aqueous reference (BTPPACl + LiCl).](image)

The Galvani potential difference ($\Delta \phi$) is varied from an initial value, where the ion of interest is in one phase only, to a final value at which the ion has migrated into the other phase, and vice versa. This migration of ions results in a current which increases as the flux of ions across the interface increases [4].

Analysis of the current response can give information about the thermodynamics, kinetics and mechanisms of ion transfer reactions across the liquid-liquid interface [18]. The current response is visualized in a voltammogram (Fig. 4.2), from which the half-wave potential of the transferring ion $X_i$ can be measured ($\Delta \phi^{1/2}$). Using this half-wave
potential, the standard transfer potential of ion $X_i$ ($\Delta_{\beta}^{\alpha}_{\Phi_i}^0$) can be calculated by Eq. 4.14:

$$\Delta_{\beta}^{\alpha}_{\Phi_i}^{1/2} - \Delta_{\beta}^{\alpha}_{\Phi_i}^0 = \Delta_{\beta}^{\alpha}_{\Phi_{\text{ref}}}^{1/2} - \Delta_{\beta}^{\alpha}_{\Phi_{\text{ref}}}^0$$  \hspace{1cm} \text{Eq. 4.14}$$

where $\Delta_{\beta}^{\alpha}_{\Phi_{\text{ref}}}^{1/2}$ and $\Delta_{\beta}^{\alpha}_{\Phi_{\text{ref}}}^0$ are the half-wave potential and the standard transfer potential of a reference ion, respectively.

Fig. 4.2. Typical cyclic voltammogram obtained at the water/1,2-dichloro-ethane interface for the transfer of the cation tetramethyl-ammonium. The potential scan rate $\nu$ is 20, 40, 60, 80 and 100 mV/s; the forward scan (FWD) is from left to right and the reverse scan (REV) from right to left. The half-wave potential of the cation ($\Delta_{\beta}^{\alpha}_{\Phi_{i}}^{1/2}$) is localized between the two pics of current.
It is important to note that the applied potential difference between two reference electrodes depends on the nature of the electrodes used, and it is thus necessary to convert the scale of applied potential to the Galvani potential scale. This can be achieved by internally referencing each measurement with a transferring ion for which the standard transfer potential is already known in most solvent systems.

Using Eqs 4.10 and 4.12, the standard Gibbs energy of transfer of the ion \( X_i \) \( (\Delta G^{0\rightarrow\alpha\rightarrow\beta}_{tr,i}) \) and its standard partition coefficient \( (\log P_i^0) \) can be calculated.

### 4.2 Computational estimation of lipophilicity

The computational methods to calculate \( \log P \) values have been reviewed [19,20]. They are based on a one-, two-, three- or four-dimensional description of molecular structure [21].

The two-dimensional approach of Rekker [22,23] is based on the concepts of fragments and correction factors. The main advantage of this method is its simplicity, which makes it well-suited to identify the discrepancies between experimental and calculated \( \log P \) values. Moreover in contrast with most computational methods, Rekker's fragmental method allows the calculation of \( \log P \) not only in \( n \)-octanol/water system but also in alkane/water.

Among two-dimensional approaches, the method of Leo and Hansch (CLOGP) is probably the most frequently used [19,20,24]. The CLOGP algorithm is based on the additivity of molecular fragments and the use of correction factors. Whereas the large and increasing number of correction factors continues to improve the accuracy and usefulness of CLOGP for calculating partition coefficient of complex compounds, the large number of factor makes it difficult to unravel the origin of discrepancies between experimental and calculated \( \log P \) values.

The main disadvantage of two-dimensional methods remains the loss of information induced by a two-dimensional consideration of molecular structure. The introduction of tri-dimensional methods such as the
Molecular Lipophilicity Potential (MLP) \([25,26]\) to calculate a log P value (obtained by back-calculation from the solvent-accessible surface area) allows to take into account the influence of conformational changes. This computational method and its application to the prediction of skin permeation will be detailed in chapter 6.
4.3 References


5 Silicone membrane: a model for the barrier function of skin

5.1 Introduction

Transdermal drug delivery is an important area of pharmaceutical and toxicological research. The skin acts as a highly efficient barrier preventing the ingress of xenobiotics and reducing water loss from the body. Notwithstanding this barrier property, the topical application of drugs is a promising route of administration whose potential advantages are well documented [1].

Understanding the physicochemical factors that control passive percutaneous absorption is a topic of current interest [2-4]. Knowledge of these factors may allow one to predict the absorption characteristics of candidates for transdermal delivery. In this respect, many in vivo and in vitro experimental techniques have been used to elucidate the underlying diffusion mechanisms [5]. However, the ultimate outcome of any model system is obviously its ability to yield observations in agreement with the more complex process it is meant to mimic. For percutaneous penetration, this means in vivo experiments in humans, which are often ethically unacceptable (e.g., during early-stage drug development), expensive and time-consuming. Among the various models used to mimic in vivo experiments in humans, a wide variety of synthetic membranes may be identified [6].

The skin is a heterogeneous membrane, and it is recognized that the superficial stratum corneum most frequently controls percutaneous absorption, i.e. this layer represents the rate-determining step for diffusion [7]. The lipoidal nature of the stratum corneum diffusion pathway suggests that artificial lipophilic membranes may provide useful in vitro models for permeation studies [8]. Silicone membranes are of particular interest in this context [3,9,10].
The purpose of the present study was to characterize the mechanisms of permeation across silicone (polydimethylsiloxane) membranes by emphasizing the most distinctive structural parameters in a series of permeants. As previously described, the hydrogen-bonding capacity of compounds restricts their skin permeation [11]. An initial set of substituted phenols was therefore chosen to evaluate how H-bonding influences permeation across such membranes, since in such compounds simple substitutions produce significant variations in H-bonding properties with only little change in other molecular parameters such as size and shape. In addition to these phenols, a small additional set of four drugs was also examined.

Experimental and computational parameters were used to quantify the H-bonding capacity. The experimental parameter was the difference between solute partition coefficients measured in two solvent systems ($\Delta \log P_{oct-dce}$) [12]. The computational parameter was calculated by the Molecular Hydrogen-Bonding Potentials (MHBPs) recently developed in our laboratory [13]. Permeation experiments were performed in a simple diffusion cell, and a mathematical model of pseudosteady-state diffusion was developed to determine the permeability coefficients.

5.2 Mathematical model

The most commonly used method to analyze in vitro permeation data obtained by an infinite dose technique is the lag time method of the steady-state results [14]. Whereas this approach is readily applicable, it is often difficult to determine when steady-state is achieved, a serious shortcoming since a misleading interpretation at this stage can lead to large errors in permeation values [15]. Furthermore, for this analysis to apply, concentrations in the receptor solution must be kept constant (usually at nearly zero) throughout the experiment. The pseudosteady-state method described here bypasses such a problem. Taking into account drug concentration buildup in the receiving chamber,
the mathematical model removes the experimental constraint of maintaining sink conditions.

The experimental permeation method (Fig. 5.1) involved placing a dilute solution of the test compound in the donor chamber (D) and monitoring the accumulation of that compound in the initially solute-free solution of the receiving chamber (R). A membrane of thickness L and area A separated the two well-stirred compartments (of volumes V_D and V_R, respectively) containing the same solvent. Assuming a one-directional flux, the solute diffused from the donor chamber (C_D), shown on the left-hand side of the membrane, into the less concentrated solution (C_R), shown on the right.

![Fig. 5.1. Schematic diagram of the in vitro permeation experiment.](image)

M = membrane, D = donor chamber, R = receptor chamber.

This in vitro permeation system is described mathematically with a pseudosteady-state model as outlined in the Appendix [16] with the result:

$$\ln \left( \frac{C_D \cdot C_R}{C_D^0} \right) = -\gamma \cdot t$$  \hspace{1cm} \text{Eq. 5.1}
in which

\[ \gamma = \frac{A \cdot K_p}{V_D \cdot V_R} \cdot (V_D + V_R) \]

Eq. 5.2

and \( K_p \), the permeability coefficient, is defined as:

\[ K_p = \frac{D_M \cdot P}{L} \]

Eq. 5.3

where \( D_M \) is the diffusion coefficient in the membrane and \( P \) is the partition coefficient of the solute between the membrane and solvent.

Plotting the negative natural logarithm of the concentration ratio against time, a straight line with a slope equal to \( \gamma \) is observed from which \( K_p \) can be determined as

\[ K_p = \frac{\gamma \cdot V_D}{2 \cdot A} \]

Eq. 5.4

in which it was assumed that \( V_D = V_R \).

5.3 Materials and methods

5.3.1 Materials

2,6-Difluorophenol, 2,6-dichlorophenol, 2,6-dibromophenol, 2,5-dinitrophenol and orphenadrine were purchased from Aldrich (Buchs, Switzerland). Phenol, 2-bromophenol, 4-bromophenol, 2-nitrophenol, 3-nitrophenol, 4-nitrophenol, 2,4-dinitrophenol and nitrobenzene were obtained from Fluka (Buchs, Switzerland). Lidocaine and (S)-nicotine were provided by Sigma (Buchs, Switzerland) and diazepam by Lipomed (Arlesheim, Switzerland). The Silatos™ silicone sheeting (150 x 200 x 0.12 mm, \( d = 1.33 \text{ g/cm}^3 \)), a medical grade dimethylsiloxane polymer, was purchased from Atos Medical (Hörby, Sweden). Analytical grade 1,2-dichloroethane (DCE) and \( n \)-octanol were obtained from Fluka (Buchs,
Switzerland). All other reagents were of analytical grade and were employed as received. Distilled water was used throughout.

5.3.2 pKₐ measurements

The protonation constants were determined by potentiometric titrations using the GLpKₐ apparatus (Sirius Analytical Instruments Ltd, Forrest Row, East Sussex, UK) as previously described [17]. The low aqueous solubility of compounds 3, 4, 5, 6 and 13 (Table 5.1) required pKₐ measurements in the presence of methanol as co-solvent. For acidic compounds, at least five separate 20-mL semi-aqueous solutions of ca. 1 mM, in 20-40% (w/w) methanol, were initially alkalinized to an appropriately high pH with standardized KOH. The solutions were then titrated with HCl 0.5 M to low pH (minimum 2.0). The same procedure was applied for basic compounds but the analysis ran from low to high pH (maximum 12.0) with standardized KOH. The titrations were conducted under an inert gas atmosphere (Ar) at 25.0 ± 0.1°C. The initial estimates of pₐₙₐ values (the apparent ionization constants in the H₂O/co-solvent mixture) were obtained from Bjerrum plots. These values were refined by a weighted non-linear least-squares procedure. The refined values were then extrapolated to zero percent of co-solvent by the Yasuda-Shedlovsky procedure [18].

5.3.3 Partition coefficients measurements

The partition coefficients in octanol/H₂O and DCE/H₂O were determined by the pH-metric method with the GLpKₐ apparatus. The principle of the pH-metric method for pKₐ and log P measurements has been explained in detail elsewhere [17,18]. At least three separate titrations of compounds 1-15 (ca. 1 mM) were carried out in the pH range 1.8 to 12.2, using various volumes of octanol or DCE (volume ratios of organic solvent/H₂O ranging from 0.3 to 0.8). All experiments were performed under Ar at 25.0 ± 0.1°C.
5.3.4 Assessment of hydrogen-bonding capacity

\( \Delta \log P_{oct-alk} \) has been shown to express essentially the capacity of solutes to donate hydrogen bonds [19]. However, the determination of partition coefficients in alkane/water systems is often difficult because of the low solubility of many compounds. The DCE/water system appears to be a promising alternative by which to overcome these experimental constraints [12]. Hence, the H-bond donor acidity of each drug was assessed by the difference (\( \log P_{oct} - \log P_{dce} \)).

In addition to experimental approaches to quantify a molecule’s capacity to form hydrogen bonds, a computational tool — Molecular Hydrogen-Bonding Potentials (MHBPs) — has been recently described [13] comprising a H-bonding donor potential (MHBP_{do}) and a H-bonding acceptor potential (MHBP_{ac}), which are calculated in a stepwise procedure. First, a H-bondingfragmental system containing literature donor (\( \alpha \)) and acceptor (\( \beta \)) values [20,21] was developed, as well as geometric functions relating the variations in potential with distance and angle. The fragmental system and the geometric functions were then combined to generate the H-bonding potentials. These are calculated at each point of the molecular surface and the sums of the donor and acceptor potentials are the two parameters which characterize the molecular H-bonding capacity (\( \Sigma \)MHBPs).

5.3.5 Permeation experiments

A wide variety of diffusion cell systems have been developed for use with rate-limiting membranes, but many show relatively poor mixing hydrodynamics and lack the possibility of automation [22]. In this study, all \textit{in vitro} experiments were performed with a specially designed diffusion cell consisting of two half-compartments of 9 mL volume and an effective diffusion area of 2.0 cm\(^2\) (Fig 5.2). Silicone membranes were cut into round pieces of 24 mm diameter and clamped between the two glass chambers using a Teflon joint. The membranes were immersed in distilled water for 1h before use. The donor chamber was filled with a dilute drug
solution (ca. 1 mM), buffered to pH 4.0 (50 mM citrate-phosphate salts) for phenols or pH 7.4 (50 mM phosphate salts) for basic compounds. All buffers contained 5% EtOH [23,24] although the presence of the solvent was necessary only for the less soluble compounds. Both compartments were stirred with Teflon-coated magnetic bars at 150 rpm; two other stirrers, connected to a motor, were positioned below the cell system to produce synchronous stirring in both cells. This entire device was fastened in a Plexiglas cage and temperature-controlled by immersion in a water bath at 37°C. Finally, the solution in each compartment was separately circulated through a flow-through cell (Hellma, type 176.700-QS, Müllheim, Germany) mounted in a spectrophotometer (Perkin Elmer, Lambda 11, Ueberlingen, Germany) using Teflon tubing (1/30 in. internal diameter, Zeus Industrial Products, Raritan, N.J., USA). A peristaltic pump set at 9 mL/min (Ismatec, Reglo FMI 005, Glattbrugg, Switzerland) allowed on-line measurements in the chambers and data collection by a computer connected to the spectrophotometer.

Fig. 5.2. System used for permeation studies allowing on-line measurements, see details in text.
It must be noted, however, that the low permeation of relatively hydrophilic solutes \((\log P_{oct} < 1)\) precluded the accurate determination of their \(K_p\).

### 5.3.6 Adjustment for ionization

Permeability coefficients were measured for chemicals with different ionization behavior. Some of the analyzed compounds were essentially neutral at the experimental pH, whereas others were partly or mostly ionized. As a first approximation, penetration can be attributed to the neutral species alone, particularly when the fraction unionized is not too low (i.e., \(f_{ui} > 0.1\)). Consequently, the permeation coefficients of unionized species were calculated by dividing the observed permeability coefficient, based on the total concentration, by the unionized fraction \((f_{ui})\). For compounds with a single acid-base reaction, this parameter is correlated to the dissociation constant \((pK_a)\) and the pH of the donor chamber by the following equation:

\[
\begin{align*}
  f_{ui} &= \frac{1}{(1+10^g)} \\
  \text{Eq. 5.5}
\end{align*}
\]

where the exponent \(g = (pH - pK_a)\) for acids and \((pK_a - pH)\) for bases.

### 5.4 Results and discussion

The experimental and computational physicochemical parameters for the two sets of compounds are summarized in Table 5.1. All experiments were performed at a fixed pH value of 4.0 or 7.4 for the initial or additional set, respectively, and the calculated permeation coefficients were adjusted for ionization according to Eq. 5.5.
Table 5.1. Experimental and computational physicochemical parameters for the compounds studied.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Permeation</th>
<th>Physicochemical properties</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_p a) [cm/h]</td>
<td>log K_p</td>
</tr>
<tr>
<td>1 phenol</td>
<td>0.129</td>
<td>-0.889</td>
</tr>
<tr>
<td>2 2,6-difluorophenol</td>
<td>0.333</td>
<td>-0.478</td>
</tr>
<tr>
<td>3 2,6-dichlorophenol</td>
<td>0.912</td>
<td>-0.040</td>
</tr>
<tr>
<td>4 2,6-dibromophenol</td>
<td>1.521</td>
<td>0.182</td>
</tr>
<tr>
<td>5 2-bromophenol</td>
<td>0.701</td>
<td>-0.154</td>
</tr>
<tr>
<td>6 4-bromophenol</td>
<td>0.360</td>
<td>-0.444</td>
</tr>
<tr>
<td>7 2-nitrophenol</td>
<td>1.233</td>
<td>0.091</td>
</tr>
<tr>
<td>8 4-nitrophenol</td>
<td>0.030</td>
<td>-1.527</td>
</tr>
<tr>
<td>9 3-nitrophenol</td>
<td>0.045</td>
<td>-1.347</td>
</tr>
<tr>
<td>10 2,4-dinitrophenol</td>
<td>0.300</td>
<td>-0.523</td>
</tr>
<tr>
<td>11 2,5-dinitrophenol</td>
<td>0.505</td>
<td>-0.297</td>
</tr>
<tr>
<td>12 nitrobenzene</td>
<td>2.009</td>
<td>0.303</td>
</tr>
<tr>
<td>13 diazepam</td>
<td>0.450</td>
<td>-0.347</td>
</tr>
<tr>
<td>14 lidocaine</td>
<td>0.322</td>
<td>-0.493</td>
</tr>
<tr>
<td>15 nicotine</td>
<td>0.184</td>
<td>-0.735</td>
</tr>
<tr>
<td>16 orphenadrine</td>
<td>22.289</td>
<td>1.348</td>
</tr>
</tbody>
</table>
Footnotes for Table 5.1:

a) Calculated permeation coefficient using the non-steady-state diffusion model and adjustment for ionization (see Eq. 5.5); n = 3; SD < 0.02.

b) Determinated by potentiometry; n = 3; SD < 0.1.

c) Logarithm of n-octanol/water partition coefficient determined by potentiometry; n = 3; SD < 0.02.

d) Logarithm of 1,2-dichloroethane/water partition coefficient determined by potentiometry; n = 3; SD < 0.02.

e) Difference between the log P_{oct} and log P_{dce} of a solute.

f) Molecular Hydrogen-Bonding Potential, calculated on the molecular surface for an acceptor (ac) or a donor (do) solute.

g) Sum of the fragmental values of polar atoms (ac) or polar hydrogen atoms (do) in a solute.

* Measured by centrifugal partition chromatography, with pH 4.6 buffer as the stationary phase [12].

5.4.1 Permeability of phenolic compounds across silicone membranes

Effect of lipophilicity

The actual partition coefficient between a membrane and the bathing solution is a key determinant of permeability. In this study, the amphiprotic n-octanol and the inert 1,2-dichloroethane (DCE) were chosen as surrogate models. As revealed by Fig. 5.3A and 5.3B, log P_{dce} describes solute permeation across silicone membranes better than log P_{oct}. The reason may be that the polydimethylsiloxane membrane, like DCE but unlike octanol, has little ability to form hydrogen bonds (see below). Moreover, although there is a general trend of increasing permeability with lipophilicity in 1,2-dichloroethane as depicted in Fig. 5.3B, two distinct groups of compound are apparent in the graph. The first represents the halophenols, the second the nitro-derivates.
Fig. 5.3. Permeability of the initial set of compounds across silicone membranes as a function of lipophilicity measured in terms of $n$-octanol/water partitioning (A), 1,2-dichloroethane/water distribution (B), and by the difference between these two solvent systems (C). ● halophenols, dihalophenols and phenol, ○ nitrophenols, * di-nitrophenols, □ nitrobenzene. Compound numbering follows that in Table 5.1.
As is readily appreciated, lipophilicity is strongly influenced by the nature of the substituents and by their position around the aromatic ring.

**Effect of hydrogen-bonding capacity**

This property has often been used in structure-permeation relationships and is related to skin permeation [11,25]. Different experimental approaches can be used to assess H-bonding capacity; e.g., the solvatochromic parameters $\alpha$ (H-bond donor acidity) and $\beta$ (H-bond acceptor basicity) [26-28]. The $\Delta \log P_{\text{oct-dce}}$ ($\log P_{\text{oct}} - \log P_{\text{dce}}$) parameter expresses mainly the hydrogen-bond donor acidity of a solute [12]. Indeed, $\log P_{\text{oct}}$ contains no contribution from the solute H-bond donor capacity, unlike $\log P_{\text{dce}}$, of which it is a major component.

The separation between the two groups of compounds seen in Fig. 5.3B (the halophenols and the nitro-derivates) is clearer in Fig. 5.3C. For the halophenols, the $\Delta \log P_{\text{oct-dce}}$ parameter decreases when the substituent is located close to the hydroxy group; compare, for example, 2-bromophenol (5) and 4-bromophenol (6). The presence of a halo-atom in the ortho- rather than para-position weakens the H-bond donor acidity of the -OH moiety due to proximity effects, and facilitates permeability across the polymer membrane (see Table 5.1).

For the nitrophenols, the influence of the substitution pattern on membrane permeability can be summarized as follows. First, the presence of a single phenolic group produces a positive $\Delta \log P$ value relative to non-H-bond forming solutes. The lower $\log P_{\text{dce}}$ relative to $\log P_{\text{oct}}$ is consistent with the fact that 1,2-DCE does not form H-bonds with solutes. The presence of nitro substituents in the para- or meta-position means that the phenolic group predominates over the nitro group in terms of $\Delta \log P_{\text{oct-dce}}$ value. The hydrogen-bond donor acidity of para-nitrophenol is slightly reinforced compared to meta-nitrophenol due to an inductive effect, thereby decreasing its permeability. Finally, the $\Delta \log P_{\text{oct-dce}}$ values of ortho-substituted phenols are significantly reduced due to their high $\log P_{\text{dce}}$ values, indicating that this H-bond donor capacity is not
expressed in the DCE/water system. A strong intramolecular hydrogen bond between the nitro group and the phenolic function accounts for this behavior.

The strong stabilization of this intramolecular H-bond in non-polar solvents [29] renders these compounds much more lipophilic than other nitrophenols in the DCE/water system. In the octanol/water system, the large amount of water in the organic phase offers additional possibilities for the formation of intermolecular H-bonds which compete with intramolecular H-bonds and affect the lipophilicity ranking of these compounds (Table 5.1).

In addition to experimental approaches to quantify a molecule's capacity to form hydrogen bonds, a computational tool — Molecular Hydrogen-Bonding Potentials (MHBPs) — has been developed [13]. Examples of such calculated potentials are illustrated in Fig. 5.5 for ortho and para-nitrophenol. The H-bonding acceptor potential on the molecular surface of 4-nitrophenol (A) and 2-nitrophenol (C) are similar, in stark contrast to their H-bonding donor potential (B) and (D), respectively. Indeed, the presence of an intramolecular interaction greatly decreases or abolishes the H-bonding donor capacity of a solute. As depicted in Fig. 5.4B and the corresponding parameters of the linear regression (n = 12, $r^2 = 0.60$, $q^2 = 0.40$), a major determinant of diffusion across silicone membranes is clearly the H-bond donor acidity but not the H-bond acceptor basicity (Fig. 5.4A). This observation agrees with the permeation results from human skin experiments [3]. Moreover, Fig. 5.4B illustrates the influence of an ortho-nitro group on the MHBPs. As can be seen, the dinitrophenols (10, 11) deviate from the linear relationships between H-bonding capacity and permeation through silicone membranes, suggesting that other factors beside H-bonding influence permeation. Several studies based on silicone membrane permeation support this observation [30,31].
Fig. 5.4. Permeability of the initial set of compounds across silicone membranes as a function of their molecular H-bonding acceptor (A) and donor (B) potential. ● halophenols, dihalophenols and phenol, ○ nitrophenols, * di-nitrophenols, □ nitrobenzene.

5.4.2 Permeability of selected drugs across silicone membranes

A set of four additional drugs was investigated to assess the generality of the previous observations. The influence of physicochemical and structural variability was therefore analyzed over an extended range of lipophilicity and molecular weight compared to the initial set.
As seen in Fig. 5.6, log \( P_{dce} \) is a significant parameter for the prediction of solute permeability across silicone membranes. As mentioned above, the major contributions to log \( P_{dce} \) are hydrophobicity and H-bond donor acidity [12]. Eq. 5.6 illustrates the linear relationship between permeability coefficient and partition coefficient in DCE/water; that is, a single parameter allows a fair prediction of permeability:

\[
\log K_p = 0.49 \pm 0.26 \cdot \log P_{dce} - 1.36 \pm 0.55
\]

\( n = 16; \quad r^2 = 0.70; \quad q^2 = 0.56; \quad s = 0.38; \quad F = 32 \)  \hspace{1cm} \text{Eq. 5.6}

In this and the following equations, 95% confidence limits are given in parentheses; \( n \) is the number of compounds, \( r^2 \) the square correlation
coefficient, $q^2$ the cross-validated correlation coefficient, $s$ the standard deviation, and $F$ the Fischer's test.

![Graph showing permeability of the initial set and additional set of compounds through silicone membranes as a function of lipophilicity](image)

**Fig. 5.6.** Permeability of the initial set (●) and additional set of compounds (○) through silicone membranes as a function of lipophilicity measured in terms of 1,2-dichloroethane/water partitioning (Eq. 5.6).

The hydrogen-bonding donor acidity is a relevant parameter which greatly influences permeation across polydimethylsiloxane membranes. Adding the four drugs to the linear regression between log $K_p$ and MHBP$_{do}$ yields a poorer correlation ($n = 16$, $r^2 = 0.42$, $q^2 = 0.22$) since diazepam, nicotine and orphenadrine have no H-bond donor capacity. However, a better relationship is recovered by adding log $P_{oct}$ in the multilinear equation 5.7.

\[
\log K_p = 0.56 \pm 0.37 \cdot \log P_{oct} - 0.0108 \pm 0.0064 \cdot \Sigma \text{MHBP}_{do} - 1.16 \pm 0.72
\]

\[\text{Eq. 5.7}\]

$n = 16; \; r^2 = 0.77; \; q^2 = 0.61; \; s = 0.35; \; F = 21$

Statistically, Eq. 5.7 is comparable to Eq. 5.6. This shows that predictions of silicone membrane permeation can be based on the single log $P_{dce}$ parameter (Eq. 5.6) which expresses the same intermolecular
forces as the combined \( \log P_{\text{oct}} \) and a H-bond donor parameter (e.g. \( \Sigma \text{MHBP}_{\text{do}} \) in Eq. 5.7, or \( \Delta \log P \)) [12,28].

5.4.3 Comparison with human skin permeation

As silicone membranes are artificial barriers used to model skin lipids [3,6], the membrane permeability coefficients (\( K_{p(\text{sil})} \)) of seven compounds were compared to their corresponding \textit{in vitro} values across human epidermis (\( K_{p(\text{epid})} \)) (Fig. 5.7). The correlation was good (Eq. 5.8):

\[
\log K_{p(\text{sil})} = 1.15 (±0.36) \cdot \log K_{p(\text{epid})} + 1.29 (±0.58)
\]

Eq. 5.8

\( n = 7; \; r^2 = 0.90; \; q^2 = 0.83; \; s = 0.19; \; F = 46 \)

Fig. 5.7. Correlation of solute permeabilities through human epidermis and silicone membranes. See Table 5.2 for data and Eq. 5.8.

As indicated by the positive intercept in Eq 5.8, the permeability through silicone membrane is about ten times higher than that through excised human skin (Table 5.2). The relationship indicates that silicone membranes may be a useful trend-predictive model for skin permeation.
Table 5.2. Experimental permeability coefficients of selected compounds through silicone membranes and across human epidermis.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Silicone membrane</th>
<th>Human epidermis$^{a)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_p$ [cm/h]</td>
<td>log $K_p$</td>
</tr>
<tr>
<td>1 phenol</td>
<td>0.129</td>
<td>-0.889</td>
</tr>
<tr>
<td>6 4-bromophenol</td>
<td>0.360</td>
<td>-0.444</td>
</tr>
<tr>
<td>7 2-nitrophenol</td>
<td>1.233</td>
<td>0.091</td>
</tr>
<tr>
<td>8 4-nitrophenol</td>
<td>0.030</td>
<td>-1.527</td>
</tr>
<tr>
<td>9 3-nitrophenol</td>
<td>0.045</td>
<td>-1.347</td>
</tr>
<tr>
<td>14 lidocaine</td>
<td>0.322</td>
<td>-0.493</td>
</tr>
<tr>
<td>15 nicotine</td>
<td>0.184</td>
<td>-0.735</td>
</tr>
</tbody>
</table>

a) Data from Flynn et al. [32].

b) Data from Johnson et al. [33].

5.5 Conclusion

This study demonstrates that the permeation of xenobiotics across polydimethylsiloxane membranes is controlled primarily by their H-bond donor capacity, in turn strongly influenced by intramolecular interactions. Lipophilicity also plays a role. Thus, a single H-bond donor parameter is shown to correlate with silicone membrane permeability for a congeneric set of phenols, whereas a lipophilicity term must be added when heterogeneous drugs are included in the regression (Eq. 5.8). Interestingly, the permeation of the extended set is also well described by lipophilicity in the 1,2-dichloroethane/water system ($\log P_{\text{dce}}$, Eq. 5.6), which encodes a strong contribution from H-bond donor capacity [12], in contrast to $\log P_{\text{oct}}$ which does not.
5.6 Appendix: Development of the mathematical model

The key assumptions are: (i) depletion of the donor chamber; (ii) accumulation in the receiving chamber; (iii) instantaneous equilibrium at the skin-solution interfaces; (iv) a homogeneous membrane as the barrier to diffusion; (v) a constant diffusion coefficient; (vi) no binding or metabolism within the membrane; (vii) no solvent diffusion.

To determine the solute concentration profile and the flux across the membrane, it is necessary to write three unsteady-state solute mass balances:

membrane M: \[
\frac{\partial C_M}{\partial t} = D_M \frac{\partial^2 C_M}{\partial x^2}
\]

Eq. A1

donor chamber D: \[
\frac{V_D}{A} \frac{dC_D}{dt} = D_M \frac{\partial C_M}{\partial x} \bigg|_{x=0}
\]

Eq. A2

receptor chamber R: \[
\frac{V_R}{A} \frac{dC_R}{dt} = - D_M \frac{\partial C_M}{\partial x} \bigg|_{x=L}
\]

Eq. A3

where \( C \) is the concentration of the permeant expressed in g/cm\(^3\), \( V \) is the volume in cm\(^3\) of each chamber, \( D_M \) is the diffusion coefficient in the membrane in cm\(^2\)/sec, \( A \) is the area of the membrane in cm\(^2\), and \( x \) is the depth in membrane M.

These differential equations are subject to specific conditions:

at \( t = 0 \), \[
C_D = C_D^0, \ C_R = 0, \ C_M = 0
\]

Eq. A4

at \( x = 0 \), \[
C_M = P_{M/D} \cdot C_D
\]

Eq. A5

at \( x = L \), \[
C_M = P_{M/R} \cdot C_R
\]

Eq. A6
If the two compartments contain the same solvent conditions, the following simplification is allowed: \( P_{M/D} = P_{M/R} = P \). Described as the partition coefficient of the solute between the membrane and solvent, this latter parameter is assumed not to vary with solute concentration.

Moreover, if \( P \cdot L \cdot A \ll V \), the amount of solute in the membrane will be always negligible compared to the amount of solute in the two compartments. As a result, the variation in concentration in the donor and receptor solutions will be slow compared to the diffusion rate across the membrane. In other words, the concentration profile across the membrane will always be close to its steady-state value, even though the compartment concentrations are time-dependent. This suggests that a pseudosteady-state solution strategy is appropriate.

Assuming pseudosteady-state, where the rate of change of concentration, \( \partial C_M / \partial t \), will be zero, Eq. A1 is first integrated twice and combined with Eqs A5 and A6, to obtain the following expression for the concentration profile

\[
C_M = P \cdot C_D - \frac{P}{L} \cdot x \cdot (C_D - C_R) \quad \text{Eq. A7}
\]

Eq. A7 is differentiated and evaluated at \( x = 0 \) and \( x = L \) to obtain:

\[
\frac{\partial C_M}{\partial x} \bigg|_{x=0} = \frac{\partial C_M}{\partial x} \bigg|_{x=L} = - \frac{P}{L} (C_D - C_R) \quad \text{Eq. A8}
\]

It has been shown that it is experimentally more robust to analyze the data as the difference \([C_D(t) - C_R(t)]\) rather than either \( C_D(t) \) or \( C_R(t) \) individually (14). Subtracting Eq. A3 from Eq. A2 and substituting for Eq. A8 yields:

\[
\frac{d}{dt} (C_D - C_R) = -\gamma (C_D - C_R) \quad \text{Eq. A9}
\]

in which \( \gamma \) is defined by Eq. 5.2.
Eq. A9 is solved subject to the initial condition Eq. A4 to give:

\[ \ln \left( \frac{C_D \cdot C_R}{C_D^0} \right) = -\gamma \cdot t \]  \hspace{1cm} \text{Eq. A10}

This pseudosteady-state analysis holds as long as \( P \cdot L \cdot A / V_D < \sim 0.1 \), assuming that \( V_D = V_R \) [34].
5.7 References


6 Molecular fields in quantitative structure-permeation relationships: 
the VolSurf approach

6.1 Introduction

In traditional 2D-QSARs (Quantitative Structure-Activity Relationships), also known as the Hansch approach, descriptors for hydrophobic, steric, and electronic effects are generally used to correlate and predict biological activities [1-3]. These descriptors are taken as substituent constants assumed to be transferable from one series of compounds to another. They do not involve 3D molecular properties and one often encounters the problem of missing parameter values. However, numerous successful applications are described in the literature [4,5] despite the fact that the biological activities and physicochemical properties of a compound are strongly dependent on its 3D shape. By limiting the analysis to the 2D aspects of molecular structure, 2D-QSAR models can usually be applied only to congeneric datasets. By taking into account tri-dimensional aspects of molecular structure, 3D-QSARs and related approaches can directly address the fundamental principles of intermolecular recognition which determine many biological properties [6].

3D-QSARs often apply to a more diverse set of compounds than traditional 2D-QSARs, since molecules are described by properties calculated directly from their tri-dimensional structures. 3D-QSAR methods define molecular properties in terms of 3D molecular interaction fields (MIFs).

6.2 TLSER: a 2D-QSAR theoretical tool

As described in chapter 3, linear solvation free-energy relationships (LSERs) are used to factorize some given molecular properties (S_p) of neutral organic compounds, e.g. partitioning, retention or permeation
[7-9], in terms of structural parameters such as the calculated van der Waals volume ($V_w$) and the so-called solvatochromic parameters (dipolarity/polarizability $\pi^*$, hydrogen-bond donor acidity $\alpha$, and hydrogen-bond acceptor basicity $\beta$) [10]. The coefficients of the descriptors in these multivariate equations reflect the nature of solute-solvent interactions.

Although there are tables of LSER parameters and predictive relations to help estimate them, LSER values of complex molecules are not easy to determine. Thus, other alternative methods to LSERs called Theoretical Linear Solvation Energy Relationships (TLSERs) have been developed [11-13].

In this study, the theoretical parameters were estimated as follows. The molecular volume was easily calculated by a standard computational software, and the hydrogen-bond capacity parameters were available from the Molecular Hydrogen-Bonding Potentials (MHBPs, see section 6.3.3). As for the $\pi^*$ parameter, a new fragmental system called Pistal 1.0 was used [14]. This latter is associated with a research algorithm to compute automatically the $\pi^*$ values for any compound. Pistal 1.0 is available on the Web:

http://www-ict.unil.ch/ict/pistal/

The TLSER models so established can be used in a predictive way for new compounds whose descriptors can be easily calculated.

6.3 Molecular fields: 3D-QSAR theoretical tools

One method to investigate interactions between a molecule and its environment is the generation of Molecular Interaction Fields (MIFs). These fields describe the variation of interaction energy between a target molecule and a chemical probe moved in a 3D grid constructed around the target (see Figs 6.1A and 6.1B). The total interaction energy is calculated at each point of the 3D grid. The regions of interest around the target molecule are highlighted by color coding (see Figs 6.1C and 6.1D). Regions of large positive energies indicate the zones from which the probe would be
repelled, while those of large negative energies correspond to energetically favorable binding regions [15].

Since the information contained in 3D molecular fields is related to the interacting partners, the amount of information in MIFs is clearly greater than that in one- or two-dimensional computed molecular descriptors.

6.3.1 The GRID force field

The GRID force field [16-18] is one of the most widely used computational tools to map the molecular surfaces of molecules and macromolecules. It uses a potential based on the total energy of interaction (E_{tot}) between a target molecule and a probe. The GRID energy function is given by Eq. 6.1:

$$E_{tot} = \sum E_{LJ} + \sum E_{el} + \sum E_{Hb}$$  \hspace{1cm} Eq. 6.1

where $E_{LJ}$ (Lennard-Jones potential) measures the induction and dispersion interactions, $E_{el}$ (electrostatic potential) measures the charged interactions and $E_{Hb}$ the hydrogen-bond (acceptor and donor capacity) interactions.

A probe is a small molecule or chemical fragment, e.g. a water molecule, a methyl group, a carboxylate group or a carbonyl oxygen atom. By moving the probe over all nodes of the grid, GRID yields the distribution of attractive and repulsive forces between the probe and the target.

The water probe is used to simulate the enthalpy part of the solvation-desolvation processes. The hydrogen-bonding carbonyl and amide probes and the hydrophobic probe (called DRY in the GRID force field) are used to simulate interactions with the polar headgroups and the hydrophobic core of biological membranes, respectively, or with polar or hydrophobic regions in proteins. As GRID probes are selective, their use allows to collect data on molecular property fields which can be used in QSAR, 3D-QSAR and selectivity studies [19-22].
Fig. 6.1. Graphical definition of a typical molecular interaction field (MIF), using indomethacin. A. 3D grid surrounding the compound. B. Interaction between the probe (e.g. a water molecule) and the target compound. C. The MIF represented by colored dots. D. The MIF represented by volumes.

6.3.2 The Molecular Lipophilicity Potential (MLP)

The Molecular Lipophilicity Potential (MLP) is a transformation of log $P_{oct}$ values (conceptually one-dimensional representations) into three-dimensional representations [23,24]. The MLP describes the combined lipophilic influence of all fragments in a molecule on its
environment and can be calculated at any given point in space around a molecule. Two components are needed to calculate a lipophilicity potential: a fragmental system of lipophilicity and a distance function, as expressed by the following general equation:

\[
\text{MLP}_k = \sum_{i=1}^{N} f_i \cdot \text{fct}(d_{ik})
\]

where \( k \) indicates a given point in space (a node in the grid), \( i \) indicates a given molecular fragment, \( N \) is the total number of fragments in the molecule, \( f_i \) the lipophilic increment of fragment \( i \), \( \text{fct} \) a distance function \( (e^{-d/2} \text{ in the present MLP}) \), and \( d_{ik} \) the distance between fragment \( i \) and point \( k \).

In sharp contrast to the GRID force field, the MLP is not obtained by calculating the interactions between a probe and the molecule. Rather, all interactions with the molecular environment are implicitly contained in the lipophilic atomic fragmental values. The system of Broto et al. [25] with new fragments and adapted values was used as fragmental system.

The MLP can spread out the molecular lipophilicity on the Solvent-Accessible Surface (SAS) of a molecule, allowing a quantitative visualization by color-coding (see Fig. 6.2) of the hydrophilic (polar) regions (\( \text{MLP}_{\text{h}} \)) and the hydrophobic ones (\( \text{MLP}_{\text{h}} \)).

The MLP can be used for the back-calculation of log P values [26], to explore conformational effects on lipophilicity, as a docking tool, and as an additional field in 3D-QSARs [27,28].

6.3.3 The Molecular Hydrogen-Bonding Potential (MHBP)

Hydrogen bonds are major forces of recognition in biochemistry and molecular pharmacology, and as such they are an essential component of intermolecular interactions. The conformational stabilization of proteins by internal H-bonds and the influence of H-bonds in oral and percutaneous absorption are telling examples [29-33].
Fig. 6.2. MLP 3D molecular fields of ibuprofen calculated at three potential levels (no unit). Hydrophilic (polar) regions ($\text{MLP}_{\text{hi}}$) are represented in magenta and hydrophobic regions ($\text{MLP}_{\text{ho}}$) in yellow.
To explore three-dimensional H-bonding properties, a computational tool — the Molecular Hydrogen-Bonding Potentials (MHBPs) — has recently been created [34], comprising a H-bonding donor potential (MHBP\textsubscript{do}) and a H-bonding acceptor potential (MHBP\textsubscript{ac}). The development of this tool is based on a stepwise procedure similar to the one used to generate the MLP. First, a H-bonding fragmental system, called Systahl 1.0, was developed starting from published, solvatochromic parameters [35,36]. An atomic H-bonding donor fragmental value (α) was associated to each hydrogen atom in a polar moiety. Similarly, an atomic H-bonding acceptor fragmental value (β) was associated to each polar atom. An angular function taking into account the directionality of H-bonds, and a distance function, were defined to calculate variations of the MHBPs in space. The fragmental system and the geometric functions were then combined to generate the MHBPs. These are calculated for each point \( k \) on a molecular surface (located 1.8 Å away from the center of each atom in the molecule) or at each node of a grid (to be imported into VolSurf) according to Eq. 6.3:

\[
\text{MHBP}_{k} = \sum_{i=1}^{N} \sum_{j=1}^{n} f_{ij} \cdot f(d_{jk}) \cdot g(U_{jk})
\]

Eq. 6.3

where \( k \) is a given point in space, \( N \) the number of molecular fragments identified in the compound, \( n \) the number of polar atoms in the molecular fragment \( i \), \( f_{ij} \) the \( \alpha \) and/or \( \beta \) value of atom \( j \) in the fragment \( i \), \( d_{jk} \) the distance between the polar atom \( j \) and the point \( k \), \( f(d_{jk}) \) the distance function, \( U_{jk} \) the angle defined by the point \( k \), the polar atom \( j \) and the polar hydrogen or the lone pair belonging to the polar atom \( j \), and \( g(U_{jk}) \) the angular function. Fig. 6.3 shows the MHBPs regions (MHBP\textsubscript{ac} and MHBP\textsubscript{do}) of ibuprofen computed at different potential levels.
Fig. 6.3. MHBP 3D molecular fields of ibuprofen calculated at three potential levels (no unit). Acceptor H-bonds (MHBP_{ac}) regions are represented in red and donor H-bonds (MHBP_{do}) regions in blue.
The distance function $f(d_{jk})$ is given by Eq. 6.4:

$$f(d_{jk}) = e^{-\pi(d \cdot a)^2}$$  \hspace{1cm} \text{Eq. 6.4}

where $a$ is the optimal distance between the polar hydrogen or polar atom and the calculated point, set at 1.8 Å. A cutoff of 2.6 Å was added to the distance function to avoid unrealistic long-distance effects.

The angular function used to describe how the potential decreases when moving away from the axis of the H-bond is given by Eq. 6.5:

$$g(U_{jk}) = \cos\left(U_{jk} \cdot \frac{90}{\text{max}}\right)$$  \hspace{1cm} \text{Eq. 6.5}

where max is set at 30° for a donor H-bond and 60° for an acceptor H-bond. Moreover, the procedure allows major intramolecular H-bonds to be taken into account [37].

The MHBPs are specifically devoted to the computation of donor and acceptor hydrogen-bond capacity of solutes and can be used as additional fields in 3D-QSARs.

### 6.4 The VolSurf procedure

Molecular interaction fields (MIFs) contain a large amount of data some of which are redundant or not relevant for a given problem. Recently a new method called VolSurf has been developed [38] in order to transform the information present in 3D molecular interactions fields into a limited number of quantitative 1D numerical descriptors. It is important to note that VolSurf descriptors can be obtained for small, medium, and large molecules, as well as for biopolymers such as DNA sequences and proteins.

Initially the standard procedure of VolSurf involves the calculation of GRID force field energies with the water and the DRY probes (see below). Specific 1D descriptors were developed to extract a maximum of information from the standard GRID probes. However, other MIFs
produced by different methods (e.g. molecular mechanics or semi-empirical) can also be used.

In our laboratory the MHBP and the MLP (symbolized as MxPs) have been interfaced with VolSurf, and new descriptors taking into account the specificity of both fields have been developed.

6.4.1 The water probe of GRID

The molecular envelope produced by the water probe of GRID defines hydrophilic regions which are accessible to and attract water molecules. This probe is used to simulate solvation-desolvation processes and the overall energy of interaction is computed at each grid node by Eq. 6.1.

Fig. 6.4 shows an example of calculation where the hydrophilic regions (GRID\textsubscript{water}) of ibuprofen are computed at three energy levels.

6.4.2 The amide and carbonyl probe of GRID

The amide NH group and the carbonyl oxygen atom are two GRID probes more specific than the water probe. Although the total interaction energy is the sum of the Lennard-Jones, electrostatic and hydrogen-bond terms, they allow the donor and acceptor hydrogen-bond capacity of a target molecule to be distinguished. The amide probe can donate one hydrogen-bond, while the carbonyl probe can accept two hydrogen-bonds.

6.4.3 The DRY probe of GRID

When a hydrophobic probe (e.g. a methyl or phenyl group) interacts with a target molecule, hydrophobic interactions are generated and reported in 3D molecular fields. The GRID force field uses a special probe called DRY to generate a 3D lipophilic field (see Fig. 6.4).
Fig 6.4. GRID 3D molecular fields of ibuprofen calculated at three energy levels (expressed in kcal/mol) with a water probe (GRID$_{\text{water}}$) and a DRY probe (GRID$_{\text{DRY}}$). Hydrophilic regions are represented in magenta and hydrophobic regions in yellow.
The DRY probe specifically generates a “hydrophobic” interaction energy, which is computed at each grid point as:

\[ E_{\text{tot}} = E_{\text{entropy}} + E_{\text{LJ}} \cdot E_{\text{Hb}} \]  \hspace{1cm} \text{Eq. 6.6}

where \( E_{\text{entropy}} \) is the ideal entropic component of the hydrophobic effect in an aqueous environment, \( E_{\text{LJ}} \) measures the induction and dispersion interactions occurring between any pair of molecules, and \( E_{\text{Hb}} \) measures the hydrogen-bond interactions between water molecules and polar groups on the target surface.

6.4.4 VolSurf and the 1D descriptors

From points in the MIFs containing the same information, VolSurf builds a framework (a volume or a surface) related to specific molecular properties. Different images can be obtained for the regions by contouring the MIFs points at different energies or potential levels. Afterwards a simple summation over the selected points yields back the overall volume for the considered properties at the defined level of energy or potential defining a 1D descriptor. Consequently a change of energy level or potential modifies the size and shape of the related volume, and the information content is altered. In order to take into account this effect, VolSurf uses several ranges of energy or potential levels to compute molecular volumes and surfaces (see Table 6.1). So each level becomes a 1D descriptor.

As VolSurf was initially developed to process energy quantities, i.e. negative values, positive potential values produced by the MxPs fields are multiplied by a negative factor of -1.0.
Table 6.1. Definition of eight VolSurf levels for the GRID force field and the MxPs fields.

<table>
<thead>
<tr>
<th>Level</th>
<th>GRID probes</th>
<th>MHBPs b)</th>
<th>MLP b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water a)</td>
<td>DRY</td>
<td>Donor</td>
</tr>
<tr>
<td>1</td>
<td>-0.20</td>
<td>-0.20</td>
<td>-0.05</td>
</tr>
<tr>
<td>2</td>
<td>-0.50</td>
<td>-0.40</td>
<td>-0.15</td>
</tr>
<tr>
<td>3</td>
<td>-1.00</td>
<td>-0.60</td>
<td>-0.25</td>
</tr>
<tr>
<td>4</td>
<td>-2.00</td>
<td>-0.80</td>
<td>-0.35</td>
</tr>
<tr>
<td>5</td>
<td>-3.00</td>
<td>-1.00</td>
<td>-0.45</td>
</tr>
<tr>
<td>6</td>
<td>-4.00</td>
<td>-1.20</td>
<td>-0.55</td>
</tr>
<tr>
<td>7</td>
<td>-5.00</td>
<td>-1.40</td>
<td>-0.65</td>
</tr>
<tr>
<td>8</td>
<td>-6.00</td>
<td>-1.60</td>
<td>-0.75</td>
</tr>
</tbody>
</table>

a) Identical for GRID polar probes (e.g. amide or carbonyl).

b) The choice of levels is explained in the thesis of S. Rey [14].

Standard VolSurf descriptors are classified into three categories:

- The size and shape of the molecule, e.g. molecular volume or weight.
- The size and shape of the contoured regions of the MIFs.
- The calculation from existing 1D descriptors, e.g. the ratio between the total molecular volume and the total molecular surface.

**Size and shape descriptors**

The study of molecular surfaces and volumes has predictive value in various areas of chemistry. The concept of molecular shape is of fundamental importance to assess similarities, regularities and correlations in drug design and optimization, and in toxicity analysis. Moreover, the characterization of macromolecular surfaces is one of the
most important problems in molecular biophysics. VolSurf produces four descriptors of size and shape.

- **Molecular volume** ($V_{\text{tot}}$): This descriptor is represented by the water-excluded volume (in Å$^3$), or the volume enclosed by the repulsive interaction between the water probe and the target molecule computed at +0.25 kcal/mol.

- **Molecular surface** ($S_{\text{tot}}$): This descriptor represents the surface (in Å$^2$) traced out by a water probe interacting at +0.25 kcal/mol.

  The following descriptors are directly calculated from the molecular volume and surface:

- **Molecular rugosity** ($R_{\text{mol}}$): defined by the ratio volume/surface ($V_{\text{tot}}/S_{\text{tot}}$). This is a measure of molecular wrinkled surface (rugosity). For a sphere, the rugosity is the radius of the sphere divided by 3. The greater the difference from a sphere, the smaller the ratio and the smaller the rugosity.

- **Molecular globularity** ($G_{\text{mol}}$): This is defined as $S/S_{\text{equiv}}$, in which $S_{\text{equiv}}$ is the surface area of a sphere of equal volume to that one of the considered molecule ($V_{\text{tot}}$). Globularity is 1.0 for perfect spherical molecules. It assumes values greater than 1.0 when the shape of the molecule differs from a sphere. The greater the difference from a sphere, the greater the globularity.

**Descriptors of hydrophilic regions**

Hydrophilic regions are defined as the molecular envelope accessible by solvent water molecules. The volume of this envelope varies with the level of interaction energies between water and the solute molecule. In general, hydrophilic descriptors computed from molecular fields of -0.2 to -1.0 kcal/mol account for polarisability and dispersion forces, whereas descriptors computed from molecular fields of -1.0 to -6.0 kcal/mol account for polar and H-bond donor-acceptor regions (see Table 6.1).
Descriptors of hydrophobic regions

In analogy with the hydrophilic regions, “hydrophobic” regions are defined as the molecular envelope generating attractive “hydrophobic” interactions. VolSurf computes “hydrophobic” descriptors from the DRY probe at eight different energy levels adapted to the usual range of hydrophobic interactions (i.e. from 0.0 to -2.0 kcal/mol).

INTeraction Enery (Integy) moments

The spatial distribution of molecular interactions can be characterized by additional descriptors, namely the integy moments. Integy moments are vectors pointing from the centre of mass to the centre of the hydrophilic, respectively hydrophobic regions. When the integy moment is high, there is a clear concentration of interaction regions in only one part of the space. If the integy moment is small, the interaction areas are well-distributed around the molecule. All the integy moments can be visualised in the real 3D molecular space.

Integy moments are defined for each type of MIFs at eight different energy or potential levels used to generate the corresponding regions (see Table 6.2).

Capacity factors

Capacity factors are defined as the ratio of the considered hydrophilic surface \( (S_{\text{pol}}) \) over the total molecular surface \( (S_{\text{pol}}/S_{\text{tot}}) \), i.e. the polar interactions per surface unit. Capacity factors are calculated at eight different energy or potential levels (see Table 6.2), the same levels used to compute the corresponding hydrophilic descriptors (see Table 6.1).

Hydrogen-bonding parameters

These variables are obtained as the differences between the hydrophilic volumes between the water and another polar probe (e.g. amide or carbonyl) calculated for each energy level considered. They
encode the hydrogen-bonding capabilities of target molecules, which can be different depending upon the nature of the polar probe used.

**Hydrophilic-Lipophilic Balance (HLB)**

This is the ratio between the hydrophilic regions measured at -4 kcal/mol and the “hydrophobic” regions measured at -0.8 kcal/mol. The balance, namely HLB, describes which effect dominates in the molecule, or if they are roughly balanced. If the interactions energy of a probe with a target molecule is smaller than the reported levels, -3 and -0.6 kcal/mol levels are used respectively.

**Amphiphilic moment**

It is defined as a vector, noted A, pointing from the center of the “hydrophobic” domain to the center of the hydrophilic domain, its length is proportional to the strength of the amphiphilic moment.

**Critical packing parameters**

These descriptors define a ratio between the hydrophilic and “hydrophobic” part of a molecule. In contrast to the hydrophilic-lipophilic balance (HLB), critical packing refers just to molecular shape and is defined by Eq. 6.7:

\[
CP = \frac{\text{Volume } D_3 \text{ (water probe)}}{\text{Surface of } W_5 \text{ (DRY probe)} \cdot \text{Length of } D_3}
\]

Eq. 6.7

The “hydrophobic” and hydrophilic calculations are performed at -0.6 and -3.0 kcal/mol, respectively. Critical packing (CP) is a good parameter to predict molecular packing such as in micelle formation, and may be relevant in solubility studies.

The development of similar descriptors using the MxPs fields is currently in progress.
Table 6.2. Summary of 1D descriptors of VolSurf for the GRID probes and MxPs fields (MHBPs and MLP). n varies from 1 to 8, and m from 1 to 3.

<table>
<thead>
<tr>
<th>Interaction regions</th>
<th>GRID probes</th>
<th>MHBP</th>
<th>MLP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>DRY</td>
<td>Polar b)</td>
</tr>
<tr>
<td></td>
<td>W_n</td>
<td>D_n</td>
<td>PW_n</td>
</tr>
<tr>
<td>Integer moments</td>
<td>IW_n</td>
<td>ID_n</td>
<td>−</td>
</tr>
<tr>
<td>Capacity factors</td>
<td>CW_n</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Local interaction</td>
<td>E_m</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>energy or potential</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>minima</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Local interaction</td>
<td>D_m</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>minima distances</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrophilic-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lipophilic balance</td>
<td>−</td>
<td>HLB a)</td>
<td>−</td>
</tr>
<tr>
<td>Amphiphilic</td>
<td>−</td>
<td>A a)</td>
<td>−</td>
</tr>
<tr>
<td>moment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Critical packing</td>
<td>−</td>
<td>CP a)</td>
<td>−</td>
</tr>
<tr>
<td>Hydrogen bonding</td>
<td>−</td>
<td>−</td>
<td>HB_n</td>
</tr>
<tr>
<td>descriptors</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) Variables computed only when the water probe (GRID water) is present.
b) Polar probe (e.g. amide or carbonyl).
c) Descriptors under progress.

**Local interaction energy or potential minima**

These represent the energy of interaction (in kcal/mol) of the highest three local energy minima (E_1 to E_3) between the water probe and the target molecule. Alternatively, the minima refers to the three deepest local minima of the MxPs fields (PA_n for MHBP_{ac}, PD_n for MHBP_{do}, PH_{ohn} for MLP_{ho} and PH_{in} for MLP_{hi} regions).
**Energy or potential minima distances**

These descriptors represent the distances between the highest three local energy or potential minima (\(D_n\) refers to the GRID water probe, \(DA_n\) to \(MHBP_{ac}\), \(DD_n\) to \(MHBP_{do}\), \(DH_{on}\) to \(MLP_{ho}\) and \(DH_{in}\) to \(MLP_{hi}\) regions).

**Hydrogen-bonding descriptors**

These descriptors (\(HB_1\) to \(HB_8\)) are obtained as the differences between the hydrophilic negative volumes (\(W_1\) to \(W_8\)) calculated with the water and any other polar probe (e.g. amide or carbonyl).

### 6.5 Chemometrics tools

As mentioned above the information contained in 3D molecular fields is very large. Moreover, when various compounds are studied at the same time, a simple graphic analysis and visualization of the fields generated by each single compound is no longer sufficient to highlight the useful information. In this case, appropriate chemometric methods are needed to condense and extract the desired information. Chemometrics comprises the application of multivariate statistics, mathematics, and computational methods to chemical results. Principal component analysis (PCA), principal component regression (PCR) and partial least squares (PLS) are very useful techniques allowing to summarize the information provided by the numerous variables (PCA) and to correlate them with a biological activity (PCR and PLS) [39].

#### 6.5.1 Principal Component Analysis (PCA)

PCA is a statistical multivariate technique very useful to handle a data matrix (X-matrix) with \(n\) objects and \(p\) variables (\(X(n,p)\)). Even if the objects are less than one hundred and the variables are several thousands, PCA is a powerful tool to decompose the data and to unravel the hidden information.
The PCA works by decomposing the data matrix as the product of two smaller matrices (Eq. 6.8):

- The **loading matrix** \( (P(p,a) \text{ with } a < p) \), which contains information about the variables. It consists of a few vectors called Principal Components (PCs), obtained as linear combinations of the original X-variables.
- The **score matrix** \( (T(n,a)) \), which contains information about the objects. Each object is described in terms of its projection onto the PCs, instead of the original variables. The score of an object is its coordinates in the PC space.

\[
X(n,p) = T(n,a) \cdot P'(a,p) + E(n,p) \quad \text{Eq. 6.8}
\]

where \( n \) is the number of objects, \( p \) is the number of variables, \( a \) is the number of principal components, and the prime signifies a transposed matrix.

The information not encoded in the \( P \) and \( T \) matrices remains in the **residual matrix** \( (E(n,p)) \), which contains the unexplained part of the X-variance.

The principal components (PCs) have two fundamental properties. First, they are extracted by linear combinations of the original X-variables in decreasing order of importance. The first PC always lies along the direction of maximum variance and therefore contains the maximal information, followed by the second PC and so on. Usually the first four or five PCs explain more than 90% of the X-variance. Second, the PCs are orthogonal to each other, thus containing no correlated information.

Score plots (plots of the objects in the PC’s space) are used to reveal the presence of clusters of objects, thus indicating the structural uniformity of a set of molecules, or to highlight the peculiar behavior of outliers. Loading plots (plots of variables in the PC’s space) are useful to discover the relation between the original variables and the PCs, i.e. the
loading of a single variable indicates how much this variable participates in defining the PC.

### 6.5.2 Principal Component Regression (PCR)

Principal components extracted from a X-matrix of variables can be used as independent variables (X's) in a regression model, the Y-matrix containing the dependent variables (e.g. biological properties). PCR is a distinct two-step process: first a PCA is carried out on the X-matrix and then a Multiple Linear Regression (MLR) is performed on the T-matrix produced by the PCA. The goal is to explain a dependent variable (Y) in terms of a number of explanatory variables. In PCR, however, the information extracted comes only from the X-matrix without taking into account the target property [40]. To deal with two different blocks of variables (X's and Y's), use of the PLS procedure (see next section) is strongly recommended.

### 6.5.3 Partial Least Squares (PLS)

The classical regression technique (Multiple Linear Regression, MLR) is inadequate in 3D-QSAR studies because it assumes that the X-variables are uncorrelated and because it can be used only with a small number of descriptors relative to the number of compounds. The needed ratio of 5 between the number of compounds and the number of variables is totally overwhelmed in 3D-QSAR data matrices (large number of variables).

The X-variables derived from an interaction field do not fulfill these requirements. For example, in VolSurf the variables generated are numerous (VolSurf descriptors) in comparison to the number of objects (compounds). Moreover, these variables are highly correlated due to the continuity properties of the interaction field.

PLS is the method of choice to handle the kind of matrices present in 3D-QSAR studies because it relates structural descriptors (stored in the
X-matrix) to activities or properties (stored in the Y-matrix) by using the target properties along with the decomposition of the X-matrix [41].

PLS works by decomposing the X-matrix as the product of two smaller matrices (Eq. 6.9), like in the PCA procedure:

- The *loading matrix* \((P)\), which contains information about the variables. It contains a few vectors called Latent Variables (LVs) obtained as linear combinations of the original X-variables.

- The *score matrix* \((T)\), which contains information about the objects. Each object is described in terms of its projection onto the LVs.

The PLS model has to satisfy the following conditions:

\[
X(n,p) = T(n,a) \cdot W'(a,p) + E(n,p) \quad \text{Eq. 6.9}
\]

\(X\) is decomposed in X-scores \((T)\) and X-weights \((W)\)

\[
Y(n,k) = U(n,a) \cdot C'(a,k) + F(n,k) \quad \text{Eq. 6.10}
\]

\(Y\) is decomposed in Y-scores \((U)\) and Y-weights \((C)\)

\[
U(n,a) = T(n,a) \cdot B(a,a) + H(n,a) \quad \text{Eq. 6.11}
\]

\(X\)-scores \((T)\) correlates \(Y\)-scores \((U)\) (inner relation)

where \(n\) is the number of objects, \(p\) is the number of X-variables, \(k\) is the number of Y-descriptors (target properties), \(a\) is the number of LVs, \((B)\) is the diagonal matrix, \((E)\) is the X-residual matrix, \((F)\) is the Y-residual matrix, and \((H)\) is the residual matrix. The weights \((W)\) represent the coefficients that multiply the X-variables to best fit the Y-variables. Therefore, the loadings represent better the first constraint used to built the PLS model (the representation of the X-matrix) while the weights represent better the second constraint (the fitting of the Y-matrix).

The LVs have at least three fundamental properties. First, they must correlate as well as possible with the Y-variables. Second, they are extracted in decreasing order of importance. Third, they are orthogonal to
each other. Thus PLS uses the structure of the Y-data directly as a guide to decompose the X-matrix, so that the outcome constitutes an optimal regression. The statistical significance for each model dimension is determined by cross-validation methods (see next section).

6.5.4 Cross-validation

Cross-validation is an approach for selecting which model gives the best result in predictivity. It is particularly useful to determine the best level of complexity (number of principal components or latent variables) for a model in order to distinguish between information and noise. In this method, many models are derived each with one or several compounds excluded from the dataset and predicted by the corresponding model. This approach is very valuable because it performs an internal validation of the model. In the most common “leave-one-out” cross-validation, every compound is once eliminated. Thus N models (N = number of compounds) are derived and the N predictions are compared with the experimental values. The predictivity of the model is evaluated by $q^2$, the predictive correlation coefficient, which is defined as follows:

$$q^2 = 1 - \frac{\sum_{i=1}^{N} (Y - Y')^2}{\sum_{i=1}^{N} (Y - \bar{Y})^2}$$

Eq. 6.12

where $Y$ is the experimental value, $Y'$ is the predicted value, $\bar{Y}$ is the average of all predicted values, and $N$ is the number of objects. The SDEP (Standard Deviation of Errors of Prediction) is calculated by Eq. 6.13:

$$SDEP = \sqrt{\frac{\sum_{i=1}^{N} (Y - Y')^2}{N}} = \sqrt{\frac{PRESS}{N}}$$

Eq. 6.13

The PRESS (Predictive Error Sum of Squares) parameter measures the predictive power of the model. Actually, $q^2$ and SDEP are calculated from PRESS in the same manner as the squared correlation coefficient $r^2$. 


and the standard deviation $s$ are calculated when no cross-validation is performed. Most often, the highest $q^2$ or the lowest SDEP is taken as the criterion for the optimal number of principal components or latent variables [42,43]. For theoretical reasons, $q^2$ cannot be higher than $r^2$ and SDEP cannot be smaller than $s$. A value of $q^2$ equal to 1.0 corresponds to perfect predictions, while a negative value means that the predictions by the model are worse than taking the average value of all biological data as the predictions. A “good” value of $q^2$ ($q^2 > 0.4$) shows that a model is statistically significant [44,45].

There are other modes than the “leave-one-out” one to perform a cross-validation, i.e “leave-two-out”, “fixed groups” and “random groups”. For the two latter, the objects are assigned in a fixed or random way to $n$ groups, each one containing an equal number of objects. Then models are built keeping one of these groups out of the analysis until all objects were kept once. A main inconvenient of cross-validation is that there is no general agreement on how to built the reduced groups and how many objects to keep. Another way to evaluate the predictivity of the model is to use an external prediction set, namely a test set. The compounds belonging to the test set are included with the training set in the data table for the generation of the variables. The training set is used to built the model which will help to predict the activities or properties of the compounds belonging to the test set. These predicted values are then compared with the experimental ones.

6.6 Application of 2D and 3D-QSAR analysis to skin permeation

Skin permeation is a phenomenon of major significance not only in drug research, but also in a cosmetic and toxicological perspective. As reviewed in chapter 3, several 2D-QSAR models based on experimental solvatochromic parameters have been developed to predict skin permeation [33,46-49]. Although these models gave some mechanistic
insight onto skin permeation, their predictivity was limited due to the small datasets analyzed.

The use of TLSER parameters, based on the replacement of experimental by calculated solvatochromic parameters, allow increase of the number of compounds studied for which experimental data are not available. However, by considering only the 2D aspects of molecular structure, 2D QSARs are inherently empirical and limited. By considering the 3D aspects of molecular structure, 3D QSARs and related techniques pinpoint that the bioactivities and physicochemical properties of a compound are consequences of its 3D size, general 3D shape and precise geometry.

The usefulness of VolSurf descriptors is exemplified in this study by correlating molecular properties with pharmacokinetic data taken from the literature [50]. Three distinctive molecular interaction fields (GRID, MLP and MHBPs) were used to derive 3D quantitative structure-activity relationships.

A series of 86 chemically heterogeneous compounds, including alcohols, phenols, steroids, barbiturates and other drugs, was analyzed (Table 6.3). Skin permeability coefficients (K_p), expressed in cm/h, cover a range of permeation values from -5.52 (hydrocortisone) to 0.16 (flurbiprofen) log units, where -5.52 represents poor percutaneous penetration, and 0.16 a very good percutaneous penetration. All data were obtained with human skin either epidermis (71 compounds) or full-thickness of skin (15 compounds).

6.6.1 Data validation criteria

In order to optimize the prediction of skin permeation, all permeability coefficients (K_p) analyzed in this study were taken from a validated database [50]. The permeation data of five compounds (aldosterone, β-estradiol, corticosterone, progesterone, and testosterone) were modified according to recent studies on skin permeation of steroids [51] (see chapter 3). Each data point was measured in vitro through human skin from aqueous vehicles and had to meet five criteria:
(1) the temperature was known and ranged from 20 to 40°C,
(2) more than 10% of the penetrating compound was in the unionized form,
(3) a valid log $P_{oct}$ (either a log $P^*$ value [52] or else a value calculated using ClogP 4.41 [53] which was developed from these recommended log $P_{oct}$) was available,
(4) the measurement had been determined at steady-state,
(5) the donor and receptor fluids did not affect (more than water does) the barrier of the skin. Steady-state permeability coefficients require a constant vehicle concentration (or adjustment to account for changing vehicle concentration).

**Retained temperature range**

The effect of temperature on permeation has been explored experimentally by Blank et al. [54] who showed that permeability coefficients ($K_p$), measured for normal alcohols, increased approximately 2.9 fold when the temperature increased by 10°C. Other examples [55] highlight the influence of temperature on permeation so that the range of temperature retained was limited to 20-40°C.

**Adjustment for ionization**

The permeability coefficients were measured for chemicals which exhibit diverse ionization behavior. While many compounds are essentially unionized at the experimental pH, others exist in equilibrium with a charged species (frequently protonated amines or dissociated carboxylic acids, e.g. ibuprofen and nicotine). Still others coexist as a complex mixture of zwitterionic, charged, and unionized species (e.g., 2-amino-4-nitrophenol). Moreover, it has been found that permeability coefficients for unionized compounds are frequently one or two orders of magnitude larger than those of their ionized forms [56-59]. The relationship depends upon the compounds and in particular on the lipophilicity of the unionized species. Consequently, the unionized fraction
available for penetration can greatly influence the magnitude of the observed permeability coefficient.

As a first approximation, penetration can be attributed to the neutral species alone, particularly when the fraction unionized is not too low (i.e., $f_{ui} > 0.1$). Consequently, the permeation coefficients of unionized species were calculated by dividing the observed permeability coefficient, based on the total concentration, by the unionized fraction ($f_{ui}$). When more than 90% of the compound is ionized (i.e., $f_{ui} < 0.1$), the rate of penetration of the ionized species can not be neglected. Thus, the data in the validated database meet the criteria that $f_{ui} \geq 0.1$). Assuming that unionized compounds penetrate two orders of magnitude faster than their ionized forms, this limit will ensure that errors in estimating the permeability coefficient should not exceed 10%.

For compounds with a single acid-base reaction, the unionized fraction in the vehicle ($f_{ui}$) is correlated to the dissociation constant ($pK_a$) and the pH of the donor chamber by the following equation:

$$f_{ui} = \frac{1}{(1+10^{-g})}$$  \hspace{1cm} \text{Eq. 6.14}

where the exponent $g = (pH - pK_a)$ for acids and $(pK_a - pH)$ for bases. The vehicle pH rather than the skin pH (typically about pH = 4-5 at the outer surface of the stratum corneum) was used due to the low buffer capacity of the skin to balance a vehicle with large volume.
Table 6.3. Physicochemical and permeation parameters for the compounds of the database.

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<tr>
<th>No.</th>
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<th>log $P_{oct}$ b)</th>
<th>log $K_p$ c)</th>
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**Barbiturates**

| 72 | [Amobarbital]                                    | 226.3 | 2.07                   | -2.64       | 30     | [71] |
| 73 | [Barbital]                                       | 184.2 | 0.65                   | -3.96       | 30     | [71] |
| 74 | [Butobarbital]                                   | 212.3 | 1.73                   | -3.72       | 30     | [71] |
| 75 | Cyclobarbital                                    | 236.3 | 1.77                   | -3.09       | 37     | [72] |
| 76 | [Phenobarbital]                                  | 232.2 | 1.47                   | -3.35       | 30     | [71] |

**Others**

| 77 | Aminopyrine                                      | 231.3 | 1.00                   | -2.99       | 37     | [72] |
| 78 | Antipyrine                                       | 188.2 | 0.38                   | -4.18       | 37     | [72] |
| 79 | Flurbiprofen                                     | 244.3 | 4.16                   | 0.16        | 37     | [72] |
| 80 | Ibuprofen                                        | 206.3 | 3.50                   | 0.01        | 37     | [72] |
| 81 | [Isoquinoline]                                   | 129.2 | 2.08                   | -1.77       | 30     | [71] |
| 82 | Isosorbide dinitrate                             | 236.1 | 1.31                   | -1.79       | 37     | [72] |
| 83 | Ketoprofen                                       | 254.3 | 3.12                   | -1.15       | 37     | [72] |
| 84 | Nicorandil                                       | 211.2 | [0.65]                 | -3.58       | 37     | [73] |
| 85 | Nicotine                                         | 162.2 | 1.17                   | -1.70       | 30     | [71] |
| 86 | Ouabain                                          | 584.7 | -1.70                  | -5.40       | 30     | [74] |

a) Compounds contained within brackets also appeared in Flynn’s database [75] (see chapter 3). Those indicated with an asterisk to the right were used in the TLSER analysis.

b) Reported log $P_{\text{oct}}$ are taken from the Starlist [52], within brackets, calculated CLOGP values [53].

c) Logarithm of permeability coefficients adjusted for ionization.

d) Data modified with respect to the validated database [50] according to recent studies on skin permeation of steroids [51] (see chapter 3).
6.6.2 Material and methods

*Hardware and software*

All calculations were performed on Silicon Graphics Indy R4400 175 MHz, O2 R5000 180 MHz or Origin 2000 4·R10000 195 MHz workstations using the SYBYL 6.5 molecular modeling package (Tripos Associates, St. Louis, MO, USA), GRID 1.7 (Molecular Discovery Ltd, Oxford, UK) [76], Tsar 3.3 (Oxford Molecular Ltd, Oxford, UK) [77] including CORINA algorithm [78], VolSurf 2.0.6 and Golpe 4.5.12 (Multivariate Infometric Analysis, Perugia, Italy).

*TLSER computation*

The TLSER parameters were calculated by SPL (Sybyl program language) macro commands in the Sybyl software. Five descriptors were generated for each molecule, i.e. the fragmental sums of acceptor (respectively donor) MHBPs with and without intramolecular hydrogen-bonds, \(\Sigma f_{ac}\) and \(\Sigma f_{ac-ihb}\) (respectively \(\Sigma f_{do}\) and \(\Sigma f_{do-ihb}\)), the fragmental sum of \(\pi^*\) value, \(\Sigma pi\), and the molecular van der Waals volume, \(V_w\).

*Generation of molecular descriptors*

The starting geometries were built by the CORINA algorithm and energy-optimized using the Merck Molecular Force Field (MMFF94s) including MMFF94 partial atomic charges.

Molecular Interaction Fields (MIFs) produced by the GRID program (grid spacing equal to 0.5 Angstrom) and MxPs fields (MHBPs and MLP) computed using the Sybyl software were imported into the VolSurf program to generate 1D descriptors according to the adequate levels of potential (see Table 6.1).
Statistical analysis

The coefficients of the TLSER model were determined by multivariate regression using the Tsar 3.3 program. PCA and PLS analyses were performed using the Golpe 4.5.12 program. Cross validation was based on a “leave-one-out” deletion pattern (see section 6.5.4).

6.6.3 Results and discussion

Principal Component Analysis (PCA)

Fig. 6.5 shows the results of the principal component analysis (PCA) on the dataset (Table 6.3) described by 88 VolSurf descriptors (Table 6.2). The PCA scores plot is obtained from GRID force field, using four probes (water, DRY, carbonyl and amide), and represents the relative position of the compounds in the two-dimensional space of the principal components (PCs).

The first PC lies along the direction of maximum variance and therefore contains the maximal information (44%), followed by the second PC (15%). Moreover, this plot reveals the presence of four clusters of compounds, namely aliphatic alcohols, barbiturates, phenols and steroids, in addition to heterogeneous drugs. Mannitol (7) and ouabain (86), as significant outliers, were removed from the starting dataset of 86 compounds. These two compounds are very polar and can form many more hydrogen bonds (a maximum of 6 and 12, respectively) than the other chemicals studied. As described in the next sections, hydrogen-bonding capacity is strongly detrimental for skin permeation.

The analysis of a single class of compounds leads to a limited interpretation of the results due to a low molecular diversity in the dataset. While such models have a good statistical fit, their predictivity is poor beyond the dataset from which they were developed. It is therefore necessary to analyze heterogeneous sets of compounds to extract relevant information for predicting skin permeation.
Fig. 6.5. PCA scores plot for 84 compounds reported in Table 6.3. Four clusters of compounds, namely aliphatic alcohols (□), barbiturates (○), phenols (△) and steroids (★) are illustrated in addition to other compounds (●). See text for interpretation.

Theoretical solvatochromic analysis (TLSER)

A set of 63 compounds, measured through human epidermis (see compounds with an asterisk in Table 6.3), was used to establish theoretical solvatochromic models (Eqs 6.15 and 6.16). Seven compounds (48, 50, 53, 54, 66, 70 and 71) were removed from the starting set of 71 chemicals due to badly parameterized fragmental values (f_{ac}). Work is in progress to correct these fragments. Mannitol (7) was also removed as a significant outlier. The calculated TLSER parameters (V_w, Σpi, Σf_{ac}, Σf_{do}, Σf_{ac-ihb} and Σf_{do-ihb}) are given in Table 6.4.

In this dataset no intramolecular hydrogen-bond was detected by the MHBP, except for seven compounds (1, 14, 34, 37, 41, 65, 69). Moreover, as stratum corneum (the outermost and rate-limiting barrier of the skin) contains hydrophilic (keratin-filled cells) and hydrophobic regions (intercellular lipids), the hydrogen-bonding capacity of the solutes should be more important in this skin layer than in non-polar environments. Therefore TLSER models were based on the sum of fragmental values of
hydrogen-bond capacity ($\Sigma f_{ac}, \Sigma f_{do}$) without taking intramolecular hydrogen-bonds into account.

\[
\log K_p = 0.74 \cdot 10^{-2} (\pm 0.33 \cdot 10^{-2}) \cdot V_w + 0.072 (\pm 0.40) \cdot \Sigma \pi
- 0.94 (\pm 0.68) \cdot \Sigma f_{do} - 2.11 (\pm 0.42) \cdot \Sigma f_{ac} - 1.59 (\pm 0.35)
\]

\[n = 63; \quad r^2 = 0.81; \quad q^2 = 0.76; \quad s = 0.52; \quad F = 60 \]

Eq. 6.15

relative contributions: $V_w$ (27%); $\Sigma \pi$ (2%); $\Sigma f_{do}$ (12%); $\Sigma f_{ac}$ (59%)

where $n$ is the number of compounds, $r^2$ is the squared correlation coefficient, $q^2$ is the predictive correlation coefficient, $s$ is the standard deviation, and $F$ is the Fischer's test. Moreover, 95% confidence limits are given in parentheses and the relative contributions of each variable to the model is calculated using Mager's standardization procedure [79].

Removing from Eq. 6.15 the theoretical dipolarity/polarizability parameter ($\Sigma \pi$) which is statistically non-significant leads to Eq. 6.16:

\[
\log K_p = 0.75 \cdot 10^{-2} (\pm 0.33 \cdot 10^{-2}) \cdot V_w - 0.92 (\pm 0.62) \cdot \Sigma f_{do}
- 2.07 (\pm 0.36) \cdot \Sigma f_{ac} - 1.57 (\pm 0.34)
\]

\[n = 63; \quad r^2 = 0.81; \quad q^2 = 0.77; \quad s = 0.51; \quad F = 82 \]

relative contributions: $V_w$ (28%); $\Sigma f_{do}$ (12%); $\Sigma f_{ac}$ (60%)

As described by the above TLSER model, the hydrogen-bonding capacity, and especially the hydrogen-bond acceptor basicity, has a great influence on skin permeation. Although Eq. 6.16 is statistically lower than those obtained by Abraham et al. [33,47] with experimental solvatochromic parameters (see Eqs 3.18 and 3.20 in chapter 3), it underlines the value of theoretical solvatochromic parameters. It should also be said that the datasets analyzed by Abraham et al. were smaller and less diverse. Eq. 6.16 also confirms the relevance of hydrogen-bonding capacity in skin permeation. Finally, it is not surprising that mannitol (7) has an abnormally low permeability coefficient due to its high polarity and the number of external hydrogen bonds it may form (a maximum of 6).
Table 6.4. Calculated solvatochromic parameters for the analyzed compounds.

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<th>N°</th>
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<th>$\Sigma \pi$</th>
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<th>$\Sigma f_{ac}$</th>
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<td>1.23</td>
<td>0.00</td>
<td>0.98</td>
<td>0.00</td>
<td>0.98</td>
</tr>
<tr>
<td>56</td>
<td>210.5</td>
<td>1.23</td>
<td>0.00</td>
<td>0.98</td>
<td>0.00</td>
<td>0.98</td>
</tr>
<tr>
<td>57</td>
<td>125.3</td>
<td>1.23</td>
<td>0.00</td>
<td>0.98</td>
<td>0.00</td>
<td>0.98</td>
</tr>
<tr>
<td>58</td>
<td>166.1</td>
<td>1.63</td>
<td>0.33</td>
<td>1.54</td>
<td>0.33</td>
<td>1.54</td>
</tr>
</tbody>
</table>
3D-QSARs: an initial model based on the GRID field

The GRID force field was chosen to characterize the interaction sites around target molecules using four probes (water, DRY, carbonyl and amide). The calculation of descriptors from the 3D maps so obtained was carried out by the VolSurf procedure. Due to the lack of some well-parameterized fragmental values (in Systahl 1.0) at this time, an analysis based on MxPs fields was not performed with this initial dataset.

The dataset of 84 compounds (see Table 6.3) was used. All data were obtained with human skin either epidermis (70 compounds) or full-thickness of skin (14 compounds). Mannitol (7) and ouabain (86), being significant outliers in previous PCA analyses, were removed from the starting dataset of 86 compounds.

Table 6.5. Statistical parameters of the investigated models.

<table>
<thead>
<tr>
<th>Model</th>
<th>Type of MIFs</th>
<th>n</th>
<th>LVs</th>
<th>r²</th>
<th>q²</th>
<th>SDEP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GRID (water, DRY, carbonyl, amide)</td>
<td>84</td>
<td>3</td>
<td>0.72</td>
<td>0.63</td>
<td>0.71</td>
</tr>
<tr>
<td>2</td>
<td>GRID (water, DRY, carbonyl, amide)</td>
<td>63</td>
<td>2</td>
<td>0.80</td>
<td>0.74</td>
<td>0.57</td>
</tr>
<tr>
<td>3</td>
<td>MLP_{hi}, MLP_{ho}, MHBP_{do} and MHBP_{ac}</td>
<td>63</td>
<td>2</td>
<td>0.84</td>
<td>0.77</td>
<td>0.53</td>
</tr>
<tr>
<td>4</td>
<td>MLP_{hi}, MLP_{ho}, DRY, MHBP_{do} and MHBP_{ac}</td>
<td>63</td>
<td>2</td>
<td>0.84</td>
<td>0.78</td>
<td>0.53</td>
</tr>
</tbody>
</table>
The statistical parameters of the obtained PLS model are summarized in Table 6.5 (Model 1). The good predictive correlation coefficient ($q^2$), obtained from a large and heterogeneous dataset, speaks to the relevance of the model.

The relationship between experimental and predicted skin permeation depicted in Fig. 6.6 shows that a single model can describe the permeation for an heterogeneous dataset. As illustrated, flurbiprofen (79) and ibuprofen (80) have a permeation value much larger than expected, presumably due to a larger random experimental error [67].

![Fig. 6.6. Relationships between experimental and predicted skin permeation using the three-component PLS model from the GRID probes (water, DRY, carbonyl and amide). The dashed line stands for ideal prediction.](image)

The PLS coefficients plot of the model so obtained (Fig. 6.7) shows the contribution of all VolSurf descriptors (Table 6.2). The vertical bars represent the contribution of each single descriptor, with a short bar being an unimportant descriptor and a long bar an important descriptor. The last bar on the right refers to the biological response, here to skin permeation.

From this plot, it can be deduced that volume (V) and surface (S) descriptors are positively correlated with skin permeation. An increase in
molecular and exposed surface is favorable to skin permeation. Rugosity (R) and globularity (G) are also favorable to skin permeation. It means that a spherical molecular shape and a wrinkled surface are better to cross the skin.

The polarity descriptors ($W_n$) are inversely correlated with skin permeability. When the polar regions in a molecule are increased, log $K_p$ values become smaller (they tend towards -5) and percutaneous permeation decreases. The capacity factors ($C W_n$), describing the polar interactions per surface unit, are correlated to the descriptors of polar regions and also decrease skin permeation.

![PLS coefficients (3LVs) plot](image)

Fig. 6.7. PLS coefficients (3LVs) plot for the correlation of VolSurf descriptors obtained from GRID probes (water, DRY, carbonyl and amide) with skin permeation. See text for interpretation and Table 6.2 for the summary of VolSurf descriptors. V S R G refers, in order, to volume (V), surface (S), rugosity (R) and globularity (G).

The spatial distribution of molecular interactions can be characterized by additional descriptors, namely the integy moments. All the integy moments can be visualized in the real 3D molecular space (see
Fig. 6.8). The model underlines that a large integrity moment for polar interactions (IW_n) enhances permeation. Although each of these descriptors are positively correlated with percutaneous penetration, their meaning depends on the energy level considered. The first interaction levels encode dipolarity/polarizability while the last levels encode more specific interactions like hydrogen bonds. As illustrated in Fig. 6.8, interaction regions obtained with the water probe of GRID are concentrated on the -OH and -NO₂ groups of 4-nitrophenol at low energy levels, and on the -OH group only at a higher energy level. Thus, the integrity moment becomes larger when the interaction is more specific and localized.

The carbonyl and amide probe of GRID were used to distinguish specifically the donor and acceptor hydrogen-bond capacity of permeants. The polar descriptors (PW_n), especially those encoding hydrogen bond donor capacity, are inversely correlated with skin permeation. These observations agree with the results of Abraham et al., based on solvatochromic analysis (see chapter 3).

The descriptors of “hydrophobic” regions (D_n) are divided into two groups. The first fifth levels of interaction energy are positively correlated with skin permeation, while the next levels are inversely correlated. As previously described with the water probe, here again specific or non-specific interactions depend on the interaction level. As illustrated in Fig. 6.8, “hydrophobic” regions of 4-nitrophenol are concentrated on the aromatic ring and on the π* system of -NO₂ group at low interaction levels, and on the aromatic stacking specifically at higher interaction levels. Integrity moments are all inversely correlated with skin permeation.

**3D-QSAR: GRID and MxPs fields**

Besides GRID field others additional fields were tested to describe skin permeation. The information coming from the MLP and MHBPs was merged. These two molecular properties are relevant for skin permeation (see chapter 3).
Fig. 6.8. Integy moments (green bars) of 4-nitrophenol calculated at three different energy levels using the water probe of GRID field ($\text{GRID}_{\text{water}}$) and the hydrophobic part of MLP ($\text{MLP}_{\text{ho}}$).
Due to badly parameterized fragmental values (see TLSER analysis), a reduced dataset of 63 compounds (Table 6.3) was investigated. The range of physicochemical properties (see Figs 6.9 and 6.10) was slightly reduced in terms of lipophilicity.

Fig. 6.9. Distribution of molecular weight in the initial and the reduced dataset containing 84 and 63 compounds, respectively.

Fig. 6.10. Distribution of log \( P_{\text{oct}} \) in the initial and the reduced dataset containing 84 and 63 compounds, respectively.
First, a new model based on the GRID field and four probes (water, DRY, carbonyl and amide) was established from the reduced dataset. As summarized in Table 6.5, the statistical parameters of the PLS model (Model 2) are better than those obtained with GRID (Model 1) on a larger dataset. This observation is not surprising due to the smaller and chemically less heterogeneous dataset analyzed.

The relationship between experimental and predicted skin permeation depicted in Fig. 6.11 shows that a simple, single model can describe the permeation of different classes of permeants. The predictivity of this model is however limited to the range of physicochemical parameters studied.

![Fig. 6.11. Relationships between experimental and predicted skin permeation using the two-component PLS model from the GRID probes (water, DRY, carbonyl and amide). The dashed line stands for ideal prediction.](image)

The PLS coefficients plot (Fig. 6.12) shows qualitatively the same information that the previous model based on GRID (Fig. 6.7). Small discrepancies arise from the descriptors of “hydrophobic” regions (Dₙ) which are here all positively correlated with skin permeation.
Fig. 6.12. PLS coefficients (2LVs) plot for the correlation of VolSurf descriptors obtained from GRID probes (water, DRY, carbonyl and amide) with skin permeation. See text for interpretation and Table 6.2 for the summary of VolSurf descriptors. V S R G refers, in order, to volume (V), surface (S), rugosity (R) and globularity (G).

Then, two additional fields (MLP and MHBPs) were merged to establish a new model based on the reduced dataset (63 compounds).

As summarized in Table 6.5, the statistical parameters of the PLS model (Model 3) are slightly better than those obtained with the previous model (Model 2).

The relationship between experimental and predicted skin permeation depicted in Fig. 6.13 shows that a simple model can describe the permeation of different classes of permeants. As indicated, salicylic acid (69) behaves as an outlier due to a permeability value much larger than expected. The influence of conformational effects on skin permeation was tested. Salicylic acid was more deviant using a conformation without intramolecular hydrogen bond, suggesting that another phenomenon than conformation, e.g. its keratolytic property [80], may account for its higher permeation value.
The PLS coefficients plot (Fig. 6.14) shows that volume (V) and surface descriptors (S) are inversely correlated with skin permeation. An increase in molecular and exposed surface is unfavourable to skin permeation. These observations do not agree with the model based on GRID field. As described in chapter 3, the solute's volume may contribute both positively and negatively to skin permeation, depending on the unbalance between the opposite influence of molecular size on lipophilicity and diffusion. Rugosity (R) and globularity (G) are two descriptors with a low statistical weight, as illustrated by very short bars in Fig. 6.14.

Here, all polarity descriptors (Hi, IHi, CHi, PHi and DHi) described by the hydrophilic part of MLP are inversely correlated with skin permeation while the hydrophobic descriptors (Ho, IHo, CHo, PHo and DHo) are positively correlated.

Some descriptors of hydrogen-bonding interactions (Dh, CDh, Ah and CAh) are inversely correlated with skin permeation. While the first energy levels have a statistical significance, most compounds (70-80%) have an interaction energy value equal to zero in the last energy levels so
that their statistical weight is non-significant, as illustrated by short bars in Fig. 6.14. Work is in progress to re-parameterize these energy levels.

![Fig. 6.14. PLS coefficients (2LVs) plot for the correlation of VolSurf descriptors obtained from MxPs fields (MLP_{hi}, MLP_{ho}, MHBP_{do} and MHBP_{ac}) with skin permeation. See text for interpretation and Table 6.2 for the summary of VolSurf descriptors. V S R G refers, in order, to volume (V), surface (S), rugosity (R) and globularity (G).](image)

The comparison with GRID field model (Fig. 6.12) highlights a large discrepancy between the integy moments descriptors. Here the model underlines that a large integy moment for polar interactions (IH_{in}) is detrimental for skin permeation, while a large integy moment for hydrophobic interactions (IH_{on}) enhances permeation. To shed light on the nature of these differences, polar and hydrophobic interactions were analyzed separately.

As illustrated in Figs. 6.15 and 6.16, the information encoded by the water probe of GRID (GRID_{water}) and the hydrophilic (polar) part of the MLP (MLP_{hi}) is not equivalent. Although the calculations based on 4-nitrophenol (Fig. 6.15) and hexyl nicotinate (Fig. 6.16) show that the
localization of interactions are energy level-dependent, GRID\textsubscript{water} encodes mainly polar interactions in terms of hydrogen bonding interactions, while MLP\textsubscript{hi} encodes polarity in terms of $\pi^*$ interactions and essentially hydrogen bond acceptor capacity [23]. The lack of any information on the donor capacity in MLP\textsubscript{hi} arises from the fragmental system based on partitioning in octanol.

Since the parametrization of molecular interaction fields is not identical, the balance between functional groups may change between GRID\textsubscript{water} and MLP\textsubscript{hi}, leading to large discrepancies in integy moments. Thus the size and the direction of their contributions in PLS analysis can also change.

The interactions encoded by the DRY probe of GRID (GRID\textsubscript{DRY}) are also different from those described by the hydrophobic part of MLP (MLP\textsubscript{ho}). While GRID\textsubscript{DRY} encodes specifically $\pi^*$ interactions, as illustrated in Fig. 6.17 with interaction regions concentrated on the aromatic stacking and on the -NO\textsubscript{2} group of 4-nitrophenol, MLP\textsubscript{ho} is concentrated on the aromatic ring characterizing the hydrophobic part of the molecule. Similar observations come from hexyl nicotinate (Fig. 6.18). While GRID\textsubscript{DRY} interacts with the aliphatic chain of the molecule and more specifically with the nicotinate ring via $\pi^*$ interactions, the MLP\textsubscript{ho} surrounds exclusively the aliphatic moiety.

Although the above observations are based on two telling examples, other compounds of the dataset have highlighted similar discrepancies between the probes.

Based on the above observations, the information coming from GRID\textsubscript{DRY} was merged with MxPs fields (MLP and MHBP) in order to build a solvatochromic-type model (see section 6.2). The statistical parameters of the PLS model, as summarized in Table 6.5 (Model 4), are similar to those obtained with MxPs fields alone (Model 3). The relationship between experimental and predicted skin permeation is illustrated in Fig. 6.19.
Fig. 6.15. Integy moments (green bars) of 4-nitrophenol calculated at three different energy levels using the water probe of GRID field (GRID\textsubscript{water}) and the hydrophilic (polar) part of MLP (MLP\textsubscript{hi}).
Fig. 6.16. Integy moments (green bars) of hexyl nicotinate calculated at three different energy levels using the water probe of GRID field ($\text{GRID}_{\text{water}}$) and the hydrophilic (polar) part of MLP ($\text{MLP}_{\text{hi}}$).
Fig. 6.17. Integy moments (green bars) of 4-nitrophenol calculated at three different energy levels using the DRY probe of GRID field (GRID\textsubscript{DRY}) and the hydrophobic part of MLP (MLP\textsubscript{ho}).
Fig. 6.18. Inertiy moments (green bars) of hexyl nicotinate calculated at three different energy levels using the DRY probe of GRID field (GRID_{DRY}) and the hydrophobic part of MLP (MLP_{ho}).
As clearly depicted by the PLS coefficients plot (Fig. 6.20), all polarity descriptors \((H_i\text{, }IHi\text{, }CH_i\text{, }PH_i\text{ and }DHi)\) obtained from the hydrophilic part of MLP are inversely correlated with skin permeation while the hydrophobic descriptors \((Ho\text{, }IHo\text{, }CHO\text{, }PHo\text{ and }DHo)\) are positively correlated. As for the integy moments, GRID\(_{DRY}\) descriptors \((ID)\) are inversely correlated with skin permeation, while those described by MLP\(_{ho}\) \((IHo)\) are positively correlated. This discrepancy is due to the non-equivalence between the information encoded by GRID\(_{DRY}\) and MLP\(_{ho}\), as illustrated by two telling examples (see Figs 6.17 and 6.18).

The descriptors of hydrogen-bonding interactions \((Dh\text{, }CDh\text{, }Ah\text{ and }CAh)\), corresponding to the first energy levels, are detrimental for skin permeation. As mentioned above, high energy levels were badly parameterized and lead to statistically non-significant descriptors.

The results in Fig. 6.20 mean that polar interactions should be decreased and delocalized to improve skin permeation. Although each of these descriptors have the same orientation, their meaning depends on the energy level considered. The first interaction levels encode
dipolarity/polarizability while the last levels encode more specific interactions like hydrogen bonds (see Figs 6.15 and 6.16). In contrast, localized hydrophobic interactions are favorable to permeation. It is the balance of all descriptors, i.e. of molecular properties, and not a single property, which is responsible for skin permeation. Such an interpretation supports the view of the stratum corneum as a lipophilic barrier [56] and highlights the predominance of a pathway rich in lipids (presumably, intercellular) for skin permeation.

Fig. 6.20. PLS coefficients (2LVs) plot for the correlation of VolSurf descriptors obtained from MxPs fields (MLP$_{hi}$, MLP$_{ho}$, MHBP$_{do}$ and MHBP$_{ac}$) and GRID field (DRY probe) with skin permeation. See text for interpretation and Table 6.2 for the summary of VolSurf descriptors. V S R G refers, in order, to volume (V), surface (S), rugosity (R) and globularity (G).

### 6.6.4 Conclusion

The VolSurf approach is very attractive and useful to correlate 3D molecular structures with pharmacokinetic properties. The one-dimensional descriptors are easy to calculate and independent of the
alignment of the molecules, and they allow new molecular interaction fields or MIFs (e.g., MxPs fields) to be implemented. The choice of the MIFs is very important and is related to the type of activity (e.g., skin permeation) and the molecular specificities of the dataset.

While the PLS models obtained from both MxPs (Model 2) and GRID fields (Model 3) are statistically equivalent, MxPs fields are better in terms of mechanistic interpretation for skin permeation. MxPs fields are thus well suited to establish PLS models able to discriminate good and poor skin permeants and they allow a different approach to pharmacokinetic properties.

This computational method is easy to apply, but the interpretation of the results must be made carefully. Conformational effects may influence the predictivity of a model and should be taken into account. Although various algorithms exist to obtain 3D structures from 2D geometries, they are based on specific rules to derive gas phase conformations which are sometimes irrelevant for the analysis of pharmacokinetic data.

The probes are not equivalent and a detailed knowledge of the information encoded in molecular interaction fields (MIFs) is needed to correctly interpret the models. As exemplified in this chapter, the DRY probe mainly describes a part of hydrophobic interactions, namely \(\pi\)-stacking interactions. This limitation may lead to incorrect interpretation and can be overcome by the simultaneous use of GRID\textsubscript{DRY} and MLP\textsubscript{ho} in 3D-QSAR models.

In addition, it must be underlined that rational drug design from VolSurf models is a very difficult task. Indeed the large number of non-equivalent parameters coming from different MIFs produces very complex predictive models and renders nearly impossible the back-translation of the subtle balance of all contributions into 3D structures.
6.7 References


Part III:

Partitioning of ions: influence on the permeation of a series of sulfonamides into bacteria
7 Sulfonamides and bacteria permeation

7.1 Classification and identification of bacteria

7.1.1 Bacteria: a definition

Bacteria are very small, relatively simple unicellular organisms whose genetic material is not enclosed in a special nuclear membrane. For this reason, bacteria are called “prokaryotes”, from Greek words meaning prenucleus. Bacterial cells generally range from 0.20 to 2.0 µm in diameter and from 2 to 8 µm in length and appear in one of several shapes. Bacillus (rodlike), coccus (spherical or ovoid), and spiral (corkscrew or curved) are the most common shapes, but some bacteria are star-shaped or square. Individual bacteria may form pairs, chains, clusters, or other groupings; such formations are usually characteristic of a particular genus or species [1].

Unlike animal cells, bacterial cells are enclosed in cell walls that are largely composed of a polysaccharide called peptidoglycan. These microorganisms generally reproduce by dividing into two equal daughter cells; this process is called binary fission. For nutrition, most bacteria use organic chemicals, which in nature can be derived from either dead or living organisms. Some bacteria can manufacture their own food by photosynthesis, and some can derive nutrition from inorganic substances. Many bacteria can move by using propelling appendages called flagella [2].

7.1.2 Taxonomy

The task of diagnostic microbiology involves detecting and identifying etiologic agents of disease when they are recovered from clinical specimens or from environmental sources. The science of taxonomy includes identification, classification, and naming of these microorganisms. Bacterial identification and all typing schemes, whether they are based on
biochemical, serologic, or other factors, are actually taxonomic classifications at or below the species level. Moreover, reproducible systems for identifying microorganisms are critical, so that a particular strain will be identified correctly and consistently, regardless of the source or the laboratory.

The classification aspects of taxonomy serve to place microorganisms into orderly arrangements of groups so that a new isolate can more easily be characterized by comparison with known organisms. The choice of criteria for assignment into groups is somewhat arbitrary, although there is agreement that the best classifications are those that reflect phylogenic relationships [3]. This arbitrariness is reflected in the genetic definition of a species as strains of bacteria that exhibit 70% DNA relatedness with 5% or less divergence within related sequences [4].

As for the nomenclature, the choice of a microbial name is largely arbitrary, but it must comply with a set of rules contained in the International Code of Nomenclature of Bacteria [5]. Scientific names can, among other things, describe the organism, honor a researcher, or identify the habitat of the species. Thus, the genus of the bacterium *Escherichia coli* is named for a scientist, Theodor Eschrich, whereas its species name, *coli*, reminds us that *E. coli* live in the colon, or large intestine.

From the clinical standpoint, identification uses the attributes developed for a classification scheme to characterize and identify individual bacterial species or strains, to distinguish from all others the organisms being sought, to verify the authenticity or special properties of a microbial clone, or to recognize the etiologic agent of a disease [6]. One of the most useful and cost-effective tests in the clinical microbiology laboratory is the Gram stain [6]. First devised by Hans Christian Gram late in the 19th century, it has remained basically the same procedure and serves to divide bacteria into two main groups: Gram-positive organisms, which retain the primary crystal violet dye and appear deep blue or purple, and Gram-negative organisms, which can be decolorized by alcohol, thereby losing the primary stain and subsequently taking up the counterstain safranin and appearing red or pink. The staining spectrum
includes almost all bacteria, many fungi, and some parasites. Thus, it is not the chemical constituents but the physical structure of the wall that confers Gram-positivity. Indeed, Gram-positive bacteria have very thick cell walls consisting of several layers of peptidoglycan, while Gram-negative bacteria contain an outer wall layer made of lipopolysaccharide (LPS) in addition to a thin peptidoglycan layer.

### 7.2 Antibacterial sulfonamides

#### 7.2.1 Historical background

The story of sulfonamides goes back to the early 20th century when workers of I.G. Farbenindustrie found that the introduction of a sulfamyl group imparted fastness to acid wool dyes, thus indicating affinity for protein molecules. The interest in dyes as possible antimicrobials was stimulated by Ehrlich’s studies on the relationship between selective staining by dyes and their antiprotozoal activity, which led to the testing of azo dyes for antibacterial activity. Investigations at the I.G. Farbenindustrie resulted, in 1932, in a German patent covering “prontosil” (Fig. 7.1) and several other azo dyes containing a sulfonamide group. Domagk carried out the testing of these dyes and he quickly observed that “prontosil” protected mice against streptococcal infections and rabbits against staphylococcal infections, though it was without action in vitro on bacteria [7]. In 1933, the first clinical success with “prontosil” in a case of staphylococcal septicemia was reported by Foerster.

These studies aroused worldwide interest and further developments took place at a very fast rate. One of the earliest systematic investigations of sulfonamides was by Trefouel and co-workers working at the Pasteur Institute in Paris (1935). Their observations pointed to the sulfonamide group as the active structural unit and led to the conclusion that metabolic cleavage of the azo linkage in “prontosil” generates para-aminobenzenesulfonamide (sulfanilamide, Fig. 7.1), which may be responsible for the antibacterial activity.
Some of the other developments in the field of sulfonamides that had effects on future progress of chemotherapy and drug research in general may be mentioned. The standardization by Bratton and Marshall [8] of a simple method for the assay of sulfonamides in body fluids and tissues permitted precise determination of the absorption, distribution, and excretion of these drugs, thus providing a rational basis for calculating proper dosage requirements. Wood’s observation of the competitive and reversal action of sulfonamides by para-aminobenzoic acid (PABA) was the first demonstration of metabolite antagonism as a mechanism of drug action [9].

7.2.2 Chemotherapeutic agents

Sulfonamides, employed herein as a generic name for derivates of para-aminobenzenesulfonamide (sulfanilamide), belong to the class of chemotherapeutic agents. The term “chemotherapy” was coined by Ehrlich at the beginning of the 20th century to describe the use of synthetic chemicals to destroy infective agents. In recent years the definition of the term has been broadened to include antibiotics — substances produced by some microorganisms that kill or inhibit the growth of other microorganisms. The term chemotherapy is now also applied to the use of chemicals (either natural or synthetic) inhibiting the growth of malignant or cancerous cells within the body.
7.2.3 Mechanism of action

Sulfonamides are structural analogs and competitive antagonists of para-aminobenzoic acid (PABA), and thus prevent normal bacterial utilization of PABA for the biosynthesis of dihydrofolate (DHF) and thereby of tetrahydrofolate (THF), which is involved in 1-carbon transfer processes [10]. More specifically, sulfonamides are competitive inhibitors of dihydropteroate synthase (DHPS), the bacterial enzyme responsible for the incorporation of PABA into dihydropteroate, the immediate precursor of folic acid [11]. This latter is required for the biosynthesis of DNA and RNA precursors both in bacteria and man (see Fig. 7.2). Sensitive microorganisms are those that must synthesize their own folic acid; bacteria that can utilize preformed folate are not affected. By inhibiting the metabolism of the bacteria, these drugs inhibit their growth but do not kill them, i.e. they are bacteriostatic rather than bactericidal. Bacteriostasis induced by sulfonamides is preceded by a lag phase, while stored PABA and folic acid are consumed. This bacteriostatic action is counteracted competitively by PABA and by the presence of pus and the products of tissue breakdown that contain thymidine and purines, used by the bacteria to bypass the need for folic acid. Sulfonamides do not affect mammalian cells by this mechanism, since these require preformed folic acid and cannot synthesize it. Man is, therefore, comparable to sulfonamide-insensitive bacteria that utilize preformed folate.

The mechanism presented above, however, does not explain all the known facts concerning the action of sulfonamides on bacteria. Brown [12], using cell-free extracts of E. coli, found that sulfonamides can also be used as alternative substrates by the enzyme system to form products that are probably analogs of reduced forms of pteroate.
Fig. 7.2. Folate biosynthetic pathway and mechanism of action of sulfonamides. The different enzymes are hydroxymethyl dihypteridine pyrophosphokinase (HPPK), dihydropteroate synthase (DHPS), dihydrofolate synthase (DHFS) and dihydrofolate reductase (DHFR).
The synthesis of sulfonamide-containing analogs of folate by intact bacteria has also been demonstrated [10]. These analogs could then exert inhibitory effects.

### 7.2.4 Therapeutic use

The sulfonamides were the first effective chemotherapeutic agents to be employed systematically for the prevention and cure of bacterial infections in man. Since sulfanilamide first came into use, many different derivates have appeared on the market, being chemically modified to achieve a better antibacterial activity, a wider spectrum of microorganisms affected, and/or a more prolonged action [7]. Due to their low cost they are still used in many parts of the world. However, resistance to sulfonamides has emerged among many microorganisms, especially streptococci, meningococci and shigella, making them less effective than formerly [13-15]. Their therapeutic importance has also declined somewhat due to the advent first of penicillin (about 1940) and subsequently of other antibiotics.

Sulfonamides are still used to treat some urinary tract infections, leprosy, and in combination with other drugs, diseases such as toxoplasmosis. Moreover, sulfapyridine was found to have beneficial effects on some inflammatory conditions, unrelated to its antibacterial activity [7]. Thus, salicylazosulfapyridine (sulfasalazine) has been used in the treatment of ulcerative colitis. This drug, which is very poorly absorbed from the gastrointestinal tract, is broken down by intestinal bacteria to sulfapyrine and 5-aminosalicylate. The finding that the anticolitic effect of sulfalazine lies in its 5-aminosalicylic (5-ASA; mesalazine) led to the development of new generations of 5-ASA agents [16]. While most sulfonamides are administrated orally, some of them (sulfacetamide, sulfadiazine and sulfadicramide) are intended for topical use.

In an attempt to synergize the action of sulfonamides and to avoid the development of resistance, the most logical approach is to combine them with agents that block the same metabolic pathway as that blocked
by sulfonamides, but at a different step. The introduction in the mid-1970s of the combination of trimethoprim (i.e. an inhibitor of dihydrofolate reductase) and sulfamethoxazole has added a new dimension to treatment with these agents.

Furthermore, careful observation of side-effects in pharmacological and clinical studies of the early sulfonamides revealed new and unanticipated activities; successful exploitation of these leads opened up new areas in chemotherapy such as oral antidiabetics, carbonic anhydrase inhibitors, diuretics and antithyroid agents.

7.2.5 Nomenclature and Classification

The general term “sulfonamides” has been used for derivates of para-aminobenzenesulfonamide (sulfanilamide), whereas specific compounds are described as N1- or N4-substituted sulfanilamides, depending on whether the substitution is on the amido or aromatic amino group, respectively (Fig. 7.3). Most of the sulfonamides used currently are N1-derivates.

Sulfonamides have been classified in different ways depending on structural and/or pharmacokinetic properties. The classification based on rate of absorption and half-life (the time needed for the concentration of the drug in the blood to be reduced to one-half) appears to be clinically relevant. Thus sulfonamides with a half-life of less than 10h are termed short-acting (e.g. sulfamethizole, sulfisoxazole, sulfanilamide), between 10 and 24h are considered to be medium-acting (e.g. sulfamethoxazole, sulfadiazine), and longer than 24h are long-acting (e.g. sulfadimethoxine, sulfadoxine).

Fig. 7.3. General structure of sulfonamides. See text for the various R-substituents.
Based on a structural classification, N1-sulfonamides of therapeutic use can be divided as follows:

- N1-acyl derivates
- N1-heterocyclic derivates containing six-membered rings (e.g. pyridine, pyrimidines, pyridazines and pyrazines)
- N1-heterocyclic derivates containing five-membered rings (e.g. thiazole, oxazole, isoxazole, 1,3,4-thiadiazole and pyrazole)

7.2.6 Structure and biological activity of sulfonamides

**Qualitative structure-activity relationships**

As sulfanilamide (Fig. 7.1) is a rather small molecule and there are not too many variations that can be carried out without changing the basic nucleus, the following generalizations regarding structure-activity relationships can be made [7]:

- The amino and sulfonyl radicals on the benzene ring must be in 1,4 disposition for activity; the amino group must be unsubstituted or have a substituent that is removed *in vivo*.
- Replacement of the benzene ring by other ring systems, or the introduction of additional substituents on it, decreases or abolishes activity.
- Exchange of the -SO$_2$NH$_2$ by -SO$_2$C$_6$H$_4\cdot$p-NH$_2$ retains activity, while exchange by -CONH$_2$, -COC$_6$H$_4\cdot$p-NH$_2$ markedly reduces it.
- N1-Monosubstitution results in more active compounds with greatly modified pharmacokinetic properties. N1-disubstitution in general leads to inactive compounds.
- The N1-substitution leading to a pK$_a$ value close to the value of the physiological pH is favorable to activity.
Quantitative structure-activity relationships

Several investigators paid attention quite early to the amino and sulfonamido groups in sulfonamides and noted a correlation between the bacteriostatic activity and their degree of ionization. Seydel et al. [17] analyzed infrared (IR) spectra of sulfonamides and concluded that the amount of negative charge on the aromatic amino group is important for activity. However, other workers [18] demonstrated that variation in activity within a series of sulfonamides cannot be attributed to a change in base strength, since all the active compounds have a basic dissociation constant (N1) of about 2, which is close to that of PABA. Thus attention has been focussed mainly on the acidic dissociation constant (N4), which varies widely from about 3 to 11 depending on the R-substituent.

In an extensive study of the relationship between the pKₐ of a series of sulfonamides and their \textit{in vitro} antibacterial activity (MIC, see section 7.3.3) against \textit{E. coli}, Bell and Roblin [19] found a parabolic relationship with the maximal activity observed in compounds whose pKₐ approximated the physiological pH. These authors corroborated Woods and Fildes’s hypothesis [9] regarding the structural similarity of a metabolite and its antagonist, and stated that the more negative the SO₂ group of sulfonamides, so as to resemble as closely as possible the para-aminobenzoate ion, the greater their bacteriostatic activity. Furthermore they supposed that increasing the acidity of a compound, due to a highly electron-withdrawing R-ring, decreased the negativity of the SO₂ group, thus reducing the bacteriostatic activity.

Cowles [20] and Brueckner [21], in a study of the effect of pH of the medium on the antibacterial activity of sulfonamides, found that activity increased with increase in pH of the medium only up to the drug being about 50% ionized, then decreased. While Brueckner assumed different intra- and extra-cellular pH values to explain these observations, Cowles stated that the sulfonamides penetrate the bacterial cell in the unionized form, but once inside the cell, the bacteriostatic activity is due to the ionized form which inhibits dihydropteroate synthase (see section 7.2.3).
Hence for maximal activity, the half-dissociated state appeared to present the best compromise between permeation and activity. This provided an alternative explanation for the parabolic relationship observed by Bell and Roblin between pKₐ and MIC.

In subsequent studies on correlation of physicochemical properties with activity, additional parameters were included such as Hammett sigma values [22] and other electronic data for net charge calculated by molecular orbital methods [23,24], spectral characteristics and hydrophobic constant [25-29].

In these studies it was noticed that some of the sulfonamides had lower antibacterial activity than expected, possibly due to their poor permeation. To define the role of permeability in the antibacterial activity of sulfonamides, investigations were carried on cell-free enzyme systems [30,31]. The comparison of cell-free systems with whole cell systems highlighted that the intracellular ionic concentration of sulfonamides, which is limited by the permeation of the unionized form, governs activity. Subsequent work in this field has fully supported the views expressed quite early in the development of sulfonamides on the predominant role of ionization for antibacterial activity.

However, while much is known on the pKₐ, protein affinity [17] and intrinsic activity of sulfonamides, the intramolecular effects of the R-substituent on acidity and lipophilicity are poorly understood and will be investigated in this study.

### 7.3 Materials and methods

#### 7.3.1 Chemical compounds and reagents

Sulfadiazine, sulfameter, sulfamethazine, sulfathiazole, sulfisomidine and sulfisoxazole were purchased from Aldrich (Buchs, Switzerland). Sulfacetamide, sulfadimethoxine and sulfaguanidine were obtained from Fluka (Buchs, Switzerland). Sulfamonomethoxine and sulfamethizole were provided by ICN Biomedicals (Aurora, USA).
Sulfabenzamide, sulfacarbamide, sulfamerazine, sulfamethoxazole, sulfamethoxypyridazine, sulfamoxole, sulfanilamide, sulfapyridine and sulfaphenazole were purchased from Sigma (Buchs, Switzerland). Sulfaclozine was given by Novartis Animal Health (Basel, Switzerland), sulfacytine by Parke-Davis (Ann Arbor, USA), sulfadicramide by Ciba Vision (Hettlingen, Switzerland) and sulfadoxine by Hoffman-La Roche (Basel, Switzerland). Methanol, n-octanol, KCl, KH$_2$PO$_4$, K$_2$HPO$_4$, LiCl, Li$_2$SO$_4$, NaCl, KOH, NaOH and HCl were purchased from Fluka (Buchs, Switzerland). Analytical grade 1,2-dichloroethane (Merck, Darmstadt, Germany) was used without further purification and handled with all necessary precautions [32].

BTPPATPBCl (bis(triphenylphosphoranylidene)ammonium tetrakis(4-chlorophenyl)borate) was prepared by metathesis of potassium tetrakis(4-chlorophenyl)borate (Fluka, Buchs, Switzerland) and of bis(triphenylphosphoranylidene)ammonium chloride (Aldrich, Buchs, Switzerland).

Dehydrated medium for reconstitution with water of Mueller-Hinton and broth was purchased from Difco® (Brunschwig, Basel, Switzerland). Distilled water was used throughout.

7.3.2 Bacterial strain

*Escherichia coli* (ATCC 25922) was chosen as bacterial strain in this study and was obtained from the American Type Culture Collection (ATCC, Manassas, USA) [33]. *E. coli* is the most common bacterium isolated in clinical microbiology laboratories, the most prevalent microorganism in feces, the most common cause of urinary tract infection, and a common cause of both intestinal and extra-intestinal infections [6]. *E. coli* belongs to the Enterobacteriaceae, a family of Gram-negative, rod-shaped bacteria (157 species grouped into 29 genera) which can grow rapidly by respiratory metabolism in the presence of oxygen and by fermentation in its absence. *E. coli* is classified as mesophile with respect to temperature (normal growth between 21 and 37°C), as neutrophile with
respect to pH (ideal growth between pH 5.0 and 9.0), and as moderately barotolerant with respect to pressure (slow growth above 1 atm) [34,35].

7.3.3 Determination of the minimal inhibitory concentration (MIC)

The method used to quantify the antibacterial activity of sulfonamides was to determine the minimal concentration needed to completely inhibit the growth of a given bacterial strain. This is called the minimal inhibitory concentration (MIC) which was determined using a broth macrodilution method, as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) [33].

Preparation of Mueller-Hinton broth

Mueller-Hinton broth (MHB) is a medium for antimicrobial susceptibility testing which is internationally recognized in standard procedures [33]. Moreover, the low levels of para-aminobenzoic acid (PABA) makes this medium suitable for testing sulfonamides. MHB is composed of dehydrated infusion from beef, casein hydrolysate and starch. The two former ingredients provide amino acids and other useful substances for growth, while starch acts as detoxifying agent. MHB was prepared by dissolving 21 g of powder in 1 L of distilled water. The medium was then sterilized by autoclaving at 121°C for 15 minutes. The broth solution thus obtained was clear and yellowish-brown with a pH of 7.3 ± 0.1 at 25°C.

This medium was used to prepare the inoculum (Escherichia coli, ATCC 25922). It was buffered at pH 7.0 by adding phosphate salts (50 mM) to the broth solution, and sterilized by filtration on a Stericup™ filtration system (Bedford, USA).

Preparation of stock solutions of sulfonamides

Stock solutions of sulfonamides (1 mg/mL or 5 mg/mL) were prepared by dissolving the drugs with a minimal amount of NaOH 2.5 M and by
diluting with a buffered solution at pH 7.0 (50 mM phosphate salts). These solutions were then sterilized by filtration using sterile Millipore filters (Millex-GS, syringe driven filter unit, 0.22 µm pore diameter) mounted on sterile syringes (Omnifix, Luer lock, 20 ml from Braun, Melsungen, Germany), and stored at 4°C until needed.

**Preparation of the inoculum**

The density of inoculum in antimicrobial susceptibility assays is critical for the results so that its adjustment has to be carefully achieved [36]. The inoculum was prepared from a stock culture of *Escherichia coli* (ATCC 25922) stored at -80°C in a rich nutritive medium containing 10% glycerol as antifreeze. An aliquot of the stock culture was streaked onto an agar plate supplemented with sheep blood (nonselective medium) and incubated overnight at 35°C in an ambient air incubator. This preliminary stage was performed to check the purity of the stock culture. Then, one or two colonies from overnight growth were suspended in Mueller-Hinton broth and incubated overnight at 35°C. For rapidly growing bacteria like *E. coli*, overnight broth cultures reach approximately 10^9 CFU/mL (CFU or colony forming units).

A portion of the inoculum suspension was diluted 1:1000 in NaCl 0.9% so that an intermediate inoculum of 10^6 CFU/mL was obtained. When 10 µL of this dilution were added to each tube containing 1 mL of diluted antibacterial agent (see below), a final inoculum of 10^4 CFU/mL was obtained.

Two aliquots of the inoculum suspension at 10^4 CFU/mL were diluted 1:10 and 1:100 in NaCl 0.9%, respectively, and 100 µL of each solution were streaked onto an agar plate supplemented with sheep blood to check for purity and inoculum density (see Fig. 7.4).

The adjusted inoculum suspension was used within 15 minutes of preparation, since longer delays may change the inoculum size.
Determination of antibacterial activities

Experiments were performed as follows:

1. A series of twofold dilutions was prepared in sterile tubes from each stock solution and MHB buffered at pH 7.0 (50 mM phosphate salts) to obtain a final volume of 1 mL per tube.

2. Each tube was inoculated with 10 µL of adjusted inoculum (see above) and covered with plastic parafilm®.

3. All tubes were then incubated overnight at 35°C in an ambient air incubator. In addition, two tubes were filled with buffered MHB and one of them was inoculated to serve, respectively, as a positive and as a negative control for bacterial growth.

4. The tubes from overnight growth were inspected visually to determine where bacteria had grown, as indicated by turbidity. The tubes in which the antimicrobial agent was present in concentrations sufficient to inhibit bacterial growth remained clear (see Fig. 7.5). In experimental terms, the MIC is the concentration of antimicrobial agent present in the “last” clear tube and the smaller the concentration, the greater the activity.

5. The pH was verified in each tube after determination of the MIC, using an Orion micro pH-electrode (Hügli-Labortec, Abtwil, Switzerland).

6. Growth, purity and inoculum density were checked.

7.3.4 \( pK_a \) measurements

The ionization constants were determined by potentiometric titrations using the GLpK\(_a\) apparatus (Sirius Analytical Instruments Ltd, Forrest Row, East Sussex, UK) as previously described [37]. The low aqueous solubility of sulfonamides required \( pK_a \) measurements in the presence of methanol as co-solvent. For these compounds, several separate 20-mL semi-aqueous solutions of ca. 1 mM, in 10-50% (w/w) methanol and 0.15 M KCl (to adjust ionic strength), were initially alkanilinized to an appropriately high pH with standardized KOH.
Fig. 7.4. Inoculation with bacteria (*Escherichia coli* ATCC 25922) of an agar plate supplemented with sheep blood. Dilution 1:10 (left plate) and 1:100 (right plate) of an adjusted inoculum ($10^4$ CFU/ml). Each spot represents a colony forming unit (CFU).

Fig. 7.5. Determination of the minimal inhibitory concentration (MIC) of sulphisoxazole against *Escherichia coli* (ATCC 25922) in Mueller-Hinton broth buffered at pH 7.0 (50 mM phosphate salts).
The solutions were then titrated with HCl 0.5 M to low pH (minimum 1.8). The titrations were conducted under an inert gas atmosphere (Ar) at 25.0 ± 0.1°C. The initial estimates of $p_{S}K_a$ values (the apparent ionization constants in the H$_2$O/co-solvent mixture) were obtained from Bjerrum plots. These values were refined by a weighted non-linear least-squares procedure. The refined values were then extrapolated to zero percent of co-solvent by the Yasuda-Shedlovsky procedure [38].

### 7.3.5 Partition coefficients measurements

The partition coefficients in octanol/H$_2$O and DCE/H$_2$O were determined by the pH-metric method with the GLpK$_a$ apparatus. The principle of the pH-metric method for pK$_a$ and log P measurements has been explained in detail elsewhere [37,38]. At least three separate titrations of sulfonamides (ca. 1 mM) were carried out in the pH range 1.8 to 11.0, using various volumes of octanol or DCE (volume ratios of organic solvent/H$_2$O ranging from 0.2 to 1.0). All experiments were performed under Ar at 25.0 ± 0.1°C. The log P values were estimated from difference Bjerrum plots [39] and refined by a non-linear least squares procedure by including previous determined pK$_a$ values as unrefined contributions.

### 7.3.6 Cyclic voltammetry measurements

The experimental set-up used was a home-made four-electrode potentiostat, as described in reference [40], with ohmic drop compensation [41]. The scanning of the applied potential was performed by a waveform generator (VA-scanner E 612, Metrohm, Herisau, Switzerland), coupled to an X-Y recorder (Bausch & Lomb, Rochester, NY, USA). Both the cell and the four-electrode potentiostat were housed in a Faraday cage. All experiments were carried out at room temperature (25°C).

The electrochemical cell used in all experiments was described in Fig. 7.6.
Water and 1,2-dichloroethane were mutually saturated. The drugs were dissolved in the aqueous phase. The pH of the aqueous solution was adjusted to the desired value with H$_2$SO$_4$ or LiOH.

In this work, an increase of the Galvani potential difference between the two phases (noted $\Delta \phi$) renders the aqueous phase more positive than the organic phase. This increase of $\Delta \phi$ creates a flow of negative charges from 1,2-dichloroethane to water which is taken as a positive current.

All half-wave potentials measured (noted $\Delta \phi_{1/2}^w$) were referred to the half-wave potential of the tetramethylammonium cation (TMA). Thus, the standard transfer potential of an ion $X_i$ (noted $\Delta \phi_{1/2}^w$) can be calculated using Eq. 7.1 [42]:

$$\Delta \phi_{1/2}^w - \Delta \phi_{1/2}^o = \Delta \phi_{1/2}^w - \Delta \phi_{1/2}^o$$

Eq. 7.1

Since the value of $\Delta \phi_{1/2}^o$ is known (160 mV on the tetraphenylarsonium tetraphenylborate scale [43]), the standard Gibbs transfer energy of ion $X_i$ ($\Delta G_{tr,i}^{0,w,o}$) and its standard partition coefficient (log $P_i^0$) can be calculated using Eqs 7.2 and 7.3 [44]:

$$\Delta \phi_{1/2}^o = \Delta G_{tr,i}^{0,w,o} / z_i \cdot F$$

Eq. 7.2
\[
\log p_i^0 = - \frac{\Delta G_{i, l}^{0, w \rightarrow o}}{R \cdot T \cdot \ln 10}
\]

Eq. 7.3

7.4 Results and discussion

The experimental physicochemical parameters and the antibacterial activity of the 24 sulfonamides investigated are summarized in Table 7.1. The chemical structures are illustrated in Fig. 7.8.

7.4.1 Viability of the microorganisms

The bacterial growth of *E. coli* (ATCC 25922) in the broth solution tempered at 35°C and buffered at pH 7.0 (50 mM phosphate salts) was checked spectrophotometrically at 620 nm. As illustrated in Fig. 7.7 the absorbance of the solution, corresponding to the bacterial density, increases exponentially to reach a steady-state after 5 hours. The inoculation of an agar plate with the suspension after 14 hours growth indicated that the inoculum reached 10^9 CFU/mL (CFU or colony forming units). These results show that the broth solution is adequate for good and fast bacterial growth.

![Figure 7.7](image_url)  
Fig. 7.7. Bacterial growth of *E.coli* (ATCC 25922) measured at 620 nm in Mueller-Hinton broth buffered at pH 7.0 (50 mM phosphate salts).
N1-acyl derivates

Sulfanilamide (1)

Sulfacetamide (2)

Sulfadicramide (3)

Sulfabenzamide (4)

Sulfacarbamide (5)

Sulfaguanidine (6)

N1-heterocyclic derivates containing five-membered rings

Sulfathiazole (19)

Sulfamethoxazole (20)

Sulfisoxazole (21)

Sulfamoxole (22)

Sulfamethizole (23)

Sulfaphenazole (24)

Fig. 7.8. Chemical structures of the sulfonamides studied.
N<sup>1</sup>-heterocyclic derivates containing six-membered rings

Fig. 7.8. Continued.
Table 7.1. Experimental and computational physicochemical parameters of sulfonamides.

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<td>0.36</td>
<td>-4.65</td>
<td>4.95</td>
<td>3.32</td>
</tr>
<tr>
<td>24</td>
<td>314.40</td>
<td>1.73</td>
<td>5.89</td>
<td>1.34</td>
<td>2.04</td>
<td>-0.02</td>
<td>1.66</td>
<td>-0.32</td>
<td>-2.99</td>
<td>4.65</td>
<td>3.69</td>
</tr>
</tbody>
</table>

a) Ionization constants for the basic (1) and acidic (2) group determined by potentiometry; $n = 5$; SD < 0.1.

b) Logarithm of n-octanol/water and 1,2-dichloroethane/water partition coefficient of the neutral form determined by potentiometry; $n = 3$; SD < 0.04.

c) Calculated by the CLOGP algorithm [45].

d) Logarithm of n-octanol/water partition coefficient of the neutral form back-calculated from MLP [46].

e) $\Delta\log P^N_{oct-dce} = \log P^N_{oct} - \log P^N_{dce}$.

f) Standard partition coefficient of the anionic form measured by cyclic voltammetry; $n = 6$; SD < 0.1.

g) $diff(\log P^{N-A}_{dce}) = \log P^N_{dce} - \log P^{0,A}_{dce}$.

h) MIC is the minimal inhibitory concentration in [M] (see section 7.3.3).
7.4.2 Antibacterial activity of sulfonamides

Due to the small range of activity (two log units) covered by the set of sulfonamides, a qualitative analysis of the results instead of a quantitative multi-linear analysis was performed.

_Effect of pK_a_

The relationship between antibacterial activity and pK_a of the investigated sulfonamides is illustrated in Fig. 7.9. For convenience in plotting the graph, log (1/MIC) is used rather than MIC. These results do not seem to correlate with the relationship previously described by Bell and Roblin [19], despite the experiments being performed with the same bacterial strain (E. coli) and at the same pH (7.0). Bell and Roblin observed a parabolic response between the antibacterial activity and the acid ionization constant with a maximum laying between pK_a 6 and 7.4 (Fig. 7.10).

![Fig. 7.9. Antibacterial activity of the investigated sulfonamides as a function of pK_a.](image)

Fig. 7.9. Antibacterial activity of the investigated sulfonamides as a function of pK_a. (●) N^1-acyl derivates, (○) N^1-heterocyclic derivates containing six-membered rings and (▲) five-membered rings, respectively.
Fig. 7.10. Relationship of *in vitro* antibacterial activity of sulfonamides to $pK_{a2}$. The data are taken from Bell and Roblin [19].

The discrepancy observed between the results may come from the non-equivalence of the biological activity. However, as depicted in Fig. 7.11 the comparison of the antibacterial activities of 9 compounds measured in common highlights the same qualitative trend. The quantitative differences may be due to variation in experimental conditions, such as broth solution or inoculum density.

Fig. 7.11. Comparison of the antibacterial activity measured in this study (●) with those measured by Bell and Roblin (○)[19]. Compound numbering follows that in Table 7.1.
Based on a set of 50 N\textsuperscript{1}-substituted sulfonamides, Bell and Roblin emphasized the value of this relationship for predicting the MIC of new sulfonamides and thus the pK\textsubscript{a2} of most of the active sulfonamides discovered since then falls in this range. In our study, most of the selected compounds have a pK\textsubscript{a2} value corresponding to an optimal activity and are thus clustered in the top of the parabolic curve depicted in Fig. 7.10.

**Effect of lipophilicity**

To inhibit their enzymatic target, namely dihydropteroate synthase which localized inside the bacteria, sulfonamides must cross biological membranes. Some physicochemical parameters have proven particularly useful for predicting drug permeation, but none has attracted as much interest as lipophilicity [47]. The n-octanol/water system has been one of the most suitable models of lipidic biological membranes, because of the analogy of n-octanol with lipids — its long alkyl chain and the polar hydroxy group.

The antibacterial activity of sulfonamides was thus investigated in terms of lipophilicity measured in the n-octanol/water system. As shown in Fig. 7.12A, an optimal range of lipophilicity corresponding to a good antibacterial activity is observed with compounds having a log P\textsubscript{oct} \textsuperscript{N} > 0. Below this log P\textsubscript{oct} \textsuperscript{N} value, the compounds are too polar and their permeation is thus strongly hindered. Although the inhibition of dihydropteroate synthase is due to the anionic form of sulfonamides, the penetration component is the rate-limiting step for bacteriostasis.

Other different solvent systems yielding partitioning information complementary to n-octanol/water data have been suggested for the modeling of various membranes and tissues [48]. Recently, the 1,2-dichloroethane/water system has been used as an inert solvent intended to mimic the highly hydrophobic region of membranes [49]. The relationship between antibacterial activity and lipophilicity expressed by the 1,2-dichloroethane/water partitioning (Fig. 7.12B) shows the same trend as those described with n-octanol/water system. An optimal range of
lipophilicity \( \log P_{\text{dce}}^N > 0 \) is also observed, thus confirming the importance of this parameter in permeation.

Fig. 7.12. Antibacterial activity of the investigated sulfonamides as a function of lipophilicity measured in terms of \( n \)-octanol/water partitioning (A) and 1,2-dichloroethane/water partitioning (B). (●) \( N^1 \)-acyl derivates, (○) \( N^1 \)-heterocyclic derivates containing \( N^1 \)-heterocyclic derivates containing six-membered rings and (▲) five-membered rings, respectively. The dashed line stands for borderline of optimal lipophilicity.
The $\Delta \log P_{\text{oct-dce}}^N$ parameter, which derives from lipophilicity and encodes the hydrogen-bond donor capacity of a solute, has found useful applications in predicting bio-membrane permeation [50,51]. As revealed by Fig. 7.13, this parameter however is not pertinent to predict antibacterial activity. The $\Delta \log P_{\text{oct-dce}}^N$ parameter varies slightly between the different sulfonamides due to the shedding effect of R-substituent on the hydrogen linked to the amido group (Fig. 7.3). The aromatic amino group, being identical in the investigated compounds, does not account for variation. The largest $\Delta \log P_{\text{oct-dce}}^N$ value observed with sulfacarbamide (5) may be due to the accessibility of the hydrogen linked to the amido group and to the presence of an additional H-bond forming substituent.

![Graph](image)

Fig. 7.13. Antibacterial activity of the investigated sulfonamides as a function of lipophilicity measured by the difference between n-octanol/water and 1,2-dichloroethane/water systems. (●) N1-acyl derivates, (○) N1-heterocyclic derivates containing six-membered rings and (▲) five-membered rings, respectively.

As the anionic form of sulfonamides is responsible for bacteriostasis by inhibiting dihydropteroate synthase, the influence of lipophilicity, measured in terms of the standard ionic partition coefficient ($\log P_{\text{dce}}^{0,A}$), was also investigated (Fig. 7.14). While a large variation in anionic lipophilicity data (3 log units) is observed, the $\log P_{\text{dce}}^{0,A}$ parameter is not relevant to predict antibacterial activity. This result supports the view of
the predominant role of the neutral form partitioning in permeation. In fact, the intracellular ionic concentration of sulfonamides, which is limited by the permeation of unionized compounds, governs activity.

Fig. 7.14. Antibacterial activity of the investigated sulfonamides as a function of their standard ionic partition coefficient measured in 1,2-dichloroethane/water. (●) N1-acyl derivates, (○) N1-heterocyclic derivates containing six-membered rings and (▲) five-membered rings, respectively.

7.4.3 Experimental versus theoretical lipophilicity

Among the computational methods to calculate the n-octanol/water partition coefficient of neutral form (log \( P_{\text{oct}}^N \)) [52], two were investigated in terms of predictivity. The first one is the CLOGP algorithm or the Leo and Hansch approach which is based on the additivity of molecular fragments and the use of numerous correction factors [53]. The second method is based on back-calculated lipophilicity from the Molecular Lipophilicity Potential or MLP (see chapter 6) [46].

CLOGP was calculated for each sulfonamide using the Daylight software 4.41 [45]. The correlation between log \( P_{\text{oct}}^N \) and CLOGP (Fig. 7.15) is not as good as those obtained earlier with other homologous series of compounds [54]. These results may be due to badly parameterized fragmental values for this set of compounds or to the limitation of the
method in predicting conformational effects that occur experimentally in solution. Besides these hypotheses, it might also be possible that the partition coefficients of compounds with $\log P_{oct}^N$ values lower than 1.0 were underestimated using the potentiometric method. An overestimated $\log P_{oct}^N$ value with this method seems less probable.

Here, it is interesting to note that whereas the large and increasing number of correction factors continues to improve the precision of CLOGP for calculating partition coefficients, this number of factors makes it difficult to unravel the origin of discrepancies between experimental and calculated partition coefficients [55].

![Diagram](image)

**Fig. 7.15.** Relationship between experimental lipophilicity measured in the $n$-octanol/water system and the calculated CLOGP value.

The correlation between $\log P_{oct}^N$ and the back-calculated lipophilicity from MLP ($\log P_{MLP}$), as depicted in Fig. 7.16, is not good. It seems that the larger the R-substituent in sulfonamide derivatives, the worst the prediction. Moreover, the results indicate that the atomic system used in MLP is inadequate to predict the lipophilicity of sulfonamide derivatives [52].
7.5 Conclusion

Sufonamides have to penetrate inside the bacteria in order to inhibit their enzymatic target and produce their bacteriostatic activity. Lipophilicity plays a critical role in this process, being the rate-limiting step. An optimal lipophilicity of the neutral form ($\log P_{\text{oct}}^N$ and $\log P_{\text{dce}}^N > 1$) is needed so that sulfonamides penetrate the bacteria. Moreover, acidity is another parameter which strongly influences activity. It follows that a weak acidity is favorable for permeation. However, once inside the bacteria sulfonamides act on dihydropteroate synthase as competitive inhibitors of para-aminobenzoate. This effect being specific for the anionic form, a high acidity is hence desirable. As a result of these two opposite conditions, the most active sulfonamides are moderately acidic, with a compromise $pK_a$ around 6.0. As most of the therapeutic sulfonamides used in this study falls in this range, the investigations are thus limited in terms of acidity properties.
7.6 References


Conclusion
8 General conclusion and perspectives

Interest in transdermal drug delivery has increased in recent years owing to its many advantages over other routes of administration. However, during the preclinical development of transdermal devices, it is difficult as well as unethical to test products in humans initially due to the potential toxicity of pharmaceutical agents. Therefore, traditional skin models from animals have been used for \textit{in vitro} and \textit{in vivo} studies. Practically, it would be advantageous to use human skin for permeation studies but, for most investigators, this skin type is not readily available. Also, skin samples are typically obtained from a variety of anatomical sites and under different disease states, which might alter the percutaneous permeability of drugs.

Most transdermal studies are performed using hairless mouse skin. However, other models are also used including rat, guinea pig, rabbit and shed snake skin, artificial composite membranes, and, more recently, living skin equivalents. Although no model has yet been tested that fully mimics the results obtained with human skin, there are many similar features between these models and human skin.

Because the determination of absorption of chemicals into and through the skin involves ethical difficulties with experiments on animals and human skin, the quantification of transdermal penetration raises great interest in pharmaceutical, toxicological and cosmetic studies.

Quantitative structure-permeation relationships (QSPeRs) relate variations in the permeation of a series of compounds with variations in their physicochemical and/or structural properties. QSPeRs provide insights into permeation mechanisms, prediction of the permeation of novel compounds and allow to limit \textit{in vivo} experiments. Certain limitations of these models, however, have been pinpointed in this thesis. First, an inherent problem lies in the fact that their applicability is restricted by the limited range of polarity and size of the permeants on which they are based. In addition, most of the models use large
Compilations of data from various investigators and laboratories employing different experimental protocols. Thus, these models show fair explanation of variance in the lower molecular size and the middle range of lipophilicity, while the divergences for both very hydrophilic and very lipophilic compounds become conspicuous. Finally, rigorous statistics show that some models are based on non-significant descriptors. As such, they do not afford mechanistic insights and may lead to an over-interpretation of the data.

Understanding the physicochemical factors that control passive skin permeation is a topic of current interest. In this respect, when human or animal skin is difficult to obtain, or when a large number of experiments are to be carried out, synthetic membranes have been widely used. Among them, silicone membranes have been particularly relevant to mimic the hydrophobic nature of the stratum corneum, the outermost epidermal layer being considered as the rate-determining step in skin permeation.

In this thesis, the mechanisms of permeation across silicone membranes were characterized by emphasizing the most distinctive structural parameters in a series of permeants. The results show that the permeation of xenobiotics across silicone membranes is controlled primarily by their H-bond donor capacity, in turn strongly influenced by intramolecular interactions. Thus, a single H-bond donor parameter is shown to correlate with silicone membrane permeability for a congenic set of phenols, whereas a lipophilicity term must be added when heterogeneous drugs are included in the correlation. Interestingly, the permeation of the extended set is also well described by lipophilicity in the 1,2-dichloroethane/water system, which encodes a strong contribution from H-bond donor capacity, in contrast to the n-octanol/water system which does not.

While traditional QSPeRs in skin permeation are based on descriptors for hydrophobic, steric and electronic effects, they usually do not take into account the fact that biological and physicochemical properties of a compound are strongly dependent on its tri-dimensional shape. Moreover, 3D-QSPeRs are often applied to more diverse sets of
compounds than traditional approaches, since molecules are described by properties calculated directly from their tri-dimensional structures, instead of experimental determinations. 3D-QSPeR methods define molecular properties in terms of 3D molecular interaction fields (MIFs).

In this context, a new method called VolSurf, which is able to transform the information present in 3D-MIFs into a limited number of quantitative descriptors, was used to correlate 3D molecular structures of a large and heterogeneous set of compounds with skin permeation. Three different MIFs were investigated, namely GRID which is based on the total energy of interaction between a probe and a target molecule, MLP which is a molecular field of lipophilicity, and MHBPs which take into account hydrogen-bonding capacity. The results indicate that while the models obtained from both MxPs (MLP and MHBPs) and GRID fields are statistically equivalent, MxPs fields are better in terms of mechanistic interpretation for skin permeation.

While this computational method is easy to apply, the interpretation of the results must be made carefully. As illustrated in this thesis, a detailed knowledge of the information encoded in MIFs is needed to correctly interpret the models. In addition, it must be underlined that rational drug design from VolSurf models is a very difficult task. Indeed the large number of non-equivalent parameters coming from different MIFs produces very complex predictive models and renders nearly impossible the back-translation of the balance of all contributions into 3D structures.

To reach their site of action, drugs have to cross biological membranes. As exemplified in this study with sulfonamides, these compounds must cross bacterial membranes to inhibit their enzymatic target, namely dihydropteroate synthase which is localized inside the bacteria. Lipophilicity plays a critical role in this process since an optimal lipophilicity of the neutral form is needed so that sulfonamides penetrate the bacteria. Moreover, acidity is another parameter which strongly influences activity since the anionic form of sulfonamides acts on dihydropteroate synthase. As a result, the most active sulfonamides are
moderately acidic, with a compromise pKₐ around 6.0. As most of the therapeutic sulfonamides used in our study falls in this range, the investigations are thus limited in terms of acidity properties.

The ultimate outcome of any model system is obviously its ability to yield observations in agreement with the more complex process it is meant to mimic. This study has shown that silicone membranes may be a useful trend-predictive model for skin permeation. These models, however, do not incorporate all the structural features needed to evaluate interactions occurring during percutaneous transport. Thus, other polymeric membranes, based on co-polymerization of hydrophilic and hydrophobic constituents, are worthy of further investigations.

Today, the use of combinatorial chemistry in hit and lead finding generates huge numbers of compounds. This in turn produces a need for the fast and reliable characterization of physicochemical properties governing a specific way of permeation. The use of computational tool to predict skin permeation such the VolSurf approach may be very attractive, but a careful analysis of conformational effects should be taken into account since it may influence the predictivity of a model.

Finally, to better characterize the antibacterial activity of sulfonamides, investigations based on the separation of permeation and enzymatic inhibition phenomenon should be carried out. Permeation could be quantified by the determination of sulfonamide uptake after incubation with bacteria, while inhibition constants could be obtained from enzymatic kinetics with isolated dihydropteroate synthase. Such tests should be more precise than the determination of the minimal inhibitory concentration (MIC), for which small differences in bacteriostatic activity (factor of 2) are within experimental error.
Summary

In order to exert its pharmacological and therapeutic effects, a drug must reach its target sites in sufficient concentration, and it is well recognized that the biological activity of drugs mainly depends on their interaction with biological membranes. In this context, the aim of this thesis is to highlight the relevant physicochemical properties responsible for drug permeation across both human skin and into bacteria.

The first part of this work is dedicated to a critical review of skin permeation. The predictive capacity of the different in vivo and in vitro models used to estimate skin permeation is discussed and it is shown that while no model has yet been tested that fully mimics the results obtained with human skin, some models may be trend-predictive. Our studies based on permeation across silicone membranes showed that the mechanism was controlled primarily by the H-bond donor capacity of permeants, which is in turn strongly influenced by intramolecular interactions. Other models to predict skin permeation based on quantitative structure-permeation relationships (QSPeRs) in predicting skin permeation were reviewed and analyzed in terms of their statistical reliability. These models showed fair explanation of variance in the lower molecular size and the middle range of lipophilicity, while divergences for both very hydrophilic and very lipophilic compounds are conspicuous.

As the experimental measurement of transdermal drug permeation is fraught with difficulties, including problems to obtain human skin samples, various computational methods for predicting skin permeation have been developed. In this context a new procedure called VolSurf was investigated. Specific considerations on the nature of molecular interaction fields used in this method were discussed.

In the last part, the antibacterial activity of sulfonamides was investigated in terms of their physicochemical properties. The results indicated that both lipophilicity of the neutral form and acidity were relevant for activity.
Résumé

Pour exercer ses effets pharmacologiques et thérapeutiques, un médicament doit atteindre son site d'action en concentrations suffisantes. Ce processus dépend essentiellement des interactions entre le médicament et les membranes biologiques. Ainsi, le but de cette thèse est d'identifier les propriétés physico-chimiques les plus pertinentes pour la perméation médicamenteuse, tant à travers la peau humaine qu’au travers de bactéries.

La première partie de ce travail consiste en une revue critique de la perméation cutanée. Les limites d’application de différents modèles in vivo et in vitro sont abordées en soulignant le fait que bien qu’aucun modèle ne rende entièrement compte de la complexité de la peau humaine, certains d’entre eux sont utiles comme outils prédictifs. Nos études basées sur la perméation au travers de membranes en silicone ont montré que ce mécanisme dépendait avant tout de la capacité d’un soluté à former des liaisons hydrogènes intramoléculaires. Dans le but de prédire la perméation cutanée, plusieurs modèles basés sur des relations entre la structure et le passage membranaire ont été passés en revue et examinés en fonction de leur fiabilité statistique. Bien que ces modèles soient pertinents pour prédire la perméation de composés de faible poids moléculaire et de lipophilie moyenne, ils sont en revanche peu fiables pour prédire le devenir de composés sortant de ces critères.

Pour faire face aux problèmes liés à l’obtention de tissu cutané d’origine humaine, des méthodes de calculs prévisionnels se sont développées parallèlement aux mesures expérimentales. Dans cette étude, l’algorithme VolSurf a été utilisé et des observations importantes sur la nature des champs moléculaires d’interaction ont été relevées.

Finalement, un travail basé sur l’identification au sein d’une classe de sulfamidés de propriétés physico-chimiques jouant un rôle dans leur activité antibactérienne a montré que tant la lipophilie de la forme neutre que l’acidité de ces composés sont des paramètres importants.