BIOPHARMACEUTICAL ASPECTS OF ORAL DRUG DELIVERY

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Biofarmaceutische aspecten van orale toediening van medicijnen

Met een samenvatting in het Nederlands

Proefschrift ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen, ingevolge het besluit van het College voor Promoties in het openbaar te verdedigen op vrijdag 3 september 2004 des ochtends te 10:30 uur

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Geboren op 17 februari 1964, te Ede

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Dit proefschrift werd mogelijk gemaakt met de financiële steun van Organon NV

ISBN 90-393-3756-X

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1 Biopharmaceutical aspects of oral drug delivery in drug research and development.

1.1 Introduction.

From an historical point of view (bio) pharmaceutical sciences have evolved from alchemy to a science driven approach to seek cure and relief for the ill persons (Rang and Dale, 1987; Pratt and Taylor, 1990). In the 17th century, the scientific founder of chemistry Robert Boyle (1692) was satisfied when dealing with therapeutics to describe and recommend mixtures of worms, dung, urine, and the moss from a dead man's skull. Blood letting, vomiting, and purgatives were standard treatments and many patients died due to these methods. As a reaction Hahnemann introduced homeopathy in the early nineteenth century, stating that the efficacy could be enhanced by dilution. The system rapidly drifted into absurdity as dilutions up to 10^{60} were recommended, being equivalent to one molecule in a sphere with the size of the orbit of Neptune. The dogmatic approaches hindered scientific progress and many therapies were introduced and disappeared (Ryan et al., 2000). This lasted till the end of the 18th century when empirism was replaced by a scientific approach, in a slow and gradual process, until the mid 19th century.

In the second half of the 19th century, medical sciences changed dramatically as the industrial revolution brought technology and chemistry. From the 1860's onwards, the relationship between the chemical structure of a drug and its pharmacological action was studied systematically (Ryan et al., 2000).

The Cambridge physiologist John Newport Langley (1852 - 1925) and the Berlin immunologist Paul Ehrlich (1854 - 1910) developed the receptor theory over the period 1870 – 1910 (Maehle et al., 2002). Nowadays, it is generally accepted that most drugs act by binding to specific macromolecules (receptors), either inside or onto the cells surface, to change their biochemical or biophysical activity and thus their cellular function. The currently known therapeutic targets can be divided into several main classes (Fig. 1).

The theory of receptors was improved through the new quantitative approach developed by the Edinburgh pharmacologist Alfred Joseph Clark (1885 - 1941) in the early 1930's (Maehle et al., 2002). Modifications to this receptor occupancy theory -

by Ariens (Utrecht) in 1954 and by Stephenson (Edinburgh) in 1956 - accounted for the intrinisic activity (efficacy) of a drug (its ability to induce an effect after binding).

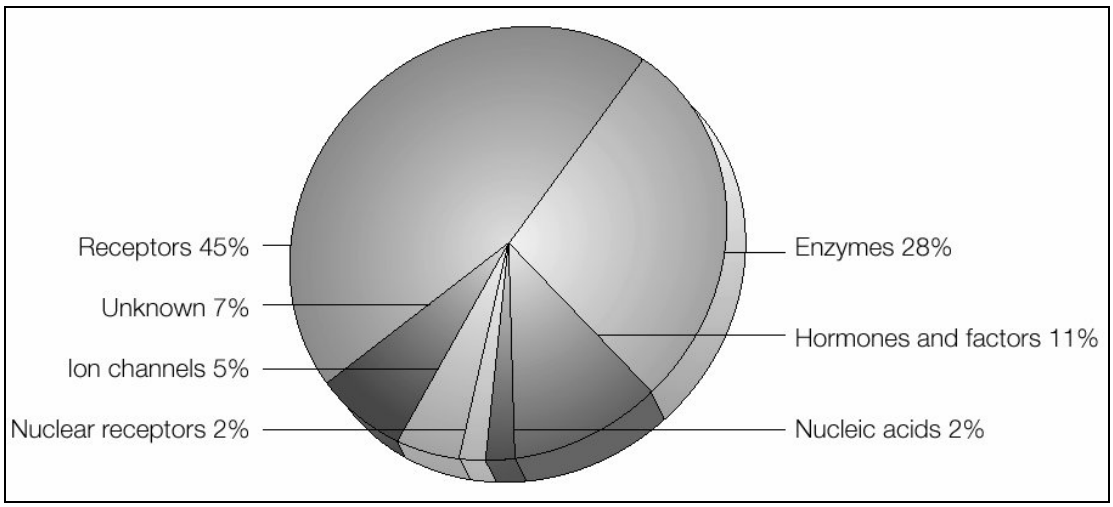


Fig. 1. Therapeutic target classes. All current therapeutic targets can be subdivided into seven main classes, wherein enzymes and receptors represent the largest part. (Bleicher et al., 2003)

In the pharmaceutical industry, new drugs are discovered and developed during a long and costly process. Safety and efficacy need to be evaluated in animal and human studies. However, this is only possible when a drug is absorbed in order to reach the systemic circulation and is transported to the receptors. Hence, the relationship between the physicochemical properties of the drug, dosage form, formulation, and (human) physiology need to be studied in biopharmaceutical studies. The physicochemical properties determine whether a drug can be absorbed into the blood, which transports it to the desired site of action. The dosage form and formulation need to be optimized to facillitate an efficient and reproducible absorption process. Human physiology influences the necessary physicochemical properties, the dosage form, and formulation. The results of the biopharmaceutical studies are used to optimize the pharmacological or therapeutical effectivity of the drugs. To illustrate the position of the biopharmaceutical sciences in the research and development process of new medicines this subject is discussed into more detail.

1.2 Drug Research and Development.

A schematic representation of the research and development process of new drugs is given in Fig. 2. As a first step in the drug research targets need to be identified which are linked to diseases (*Target Discovery*). The total number of targets for drug therapy currently stands at about 500 whereas the search for new targets has merely started. As all targets are encoded by the human genome, the complete sequencing of the human genome has led to the possible identification of approx. 40.000 genes representing 3000 to 10000 new drug targets. As not all targets seem to be suitable for the interaction with small molecular drugs, these numbers may be optimistic (Hopkins and Groom, 2002; Kubinyi, 2003).

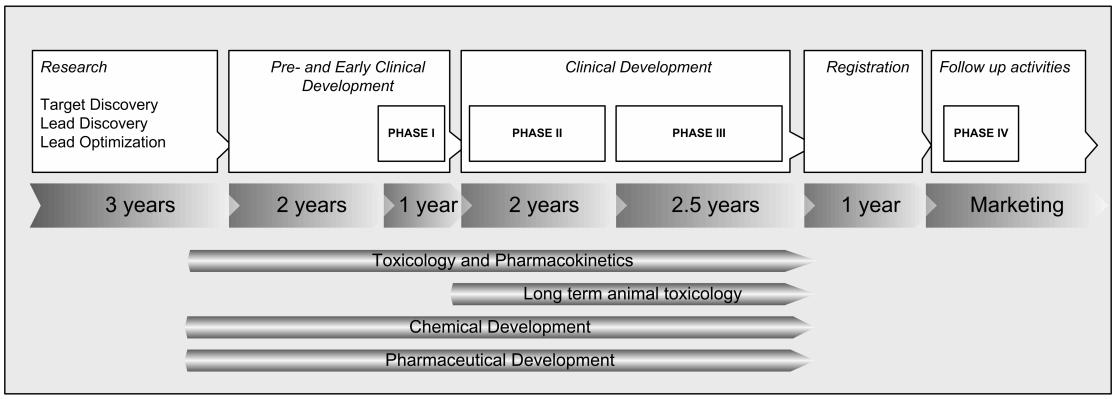


Fig. 2. Schematic representation of the research and development process of new drugs.

Once targets have been identified, assays are developed (*Lead Discovery*) which can be used to screen compound libraries for chemical entities (the so-called 'leads') that have affinitity for the target. Usually the assays detect whether or not a compound exhibits a degree of binding to the target. In this way, hundreds of thousands of compounds can be screened (*High Throughput Screening, HTS*). One of the major pitfalls of the HTS technologies is that the more hydrophobic compounds appear to bind better to targets (the so-called hydrophobic effect) (Tanford, 1973). Hence, leads or drugs discovered in this way tend to be hydrophobic (Lipinski et al., 1997). As a consequence, difficulties arise when the drug needs to be delivered to the systemic circulation. This is usually discovered when the compound is already taken into

development. In the late 1990's, this led to a reaction in the pharmaceutical industry to evaluate the developability of compounds on the basis of biopharmaceutical studies. Here, bioavailability was identified as one of the most important parameters.
The need to evaluate the bioavailability of large numbers of compounds increased the interest to calculate physicochemical parameters (Polar Surface Area, log P, hydrogen donor and acceptor sites etc.) during the 1990's (Lipinski et al., 1997; Palm et al, 1997; Testa et al., 2001). Furthermore, relationships between the physicochemical properties and biopharmaceutical parameters (fraction absorbed, absolute bioavailability etc.) were studied and found (Rodrigues, 2002; van de Waterbeemd et al., 2003). Logically, these approaches cannot be expected to generate predictions that are more accurate than the variability in the biological measurements (van de Waterbeemd et al., 2003). Hence, the accuracy is not extremely high.
The quality of a lead usually requires further improvement (*Lead optimization*). Here the focus is placed on the design and synthesis of compounds with improved properties. Rational decision making is assisted by computer aided drug design (*cheminformatics*) and pharmacology establishes whether the desired improvement is achieved. The effects of drugs are evaluated in in vitro cell systems and animal experiments. Examples of in vitro cell tests are Caco-2 studies (evaluation of the drugs' transport across the gastrointestinal epithelium and P-glycoprotein transport), metabolism experiments with hepatocytes and toxicological screening assays (e.g. cytotoxicity, genotoxicity (vitotox), mutagenicity (Ames II), and nucleo receptor tests) (Toxicology Tutor, 2003). As the number of compounds to be tested is ever increasing the development of computational ADMET (Absorption Distribution Metabolism Excretion Toxicology) methodologies finds a lot of interest in the last couple of years (Bursi, 2003).
Despite the massive increase in research and development investments for the discovery of new drugs, the number of drugs which are approved for the market is decreasing (Fig. 3) (Renfrey and Featherstone, 2002). This is in contrast with the irresistible idea of the late 1990s that once all the disease targets were characterized, drugs for each would eventually follow (Hopkins and Groom, 2002). Paradoxically, the chances for failure have incresased as target validation, i.e. evidence of the

working mechanism of a target, is lagging behind (Ulrich and Friend, 2002; Kubinyi, 2003).

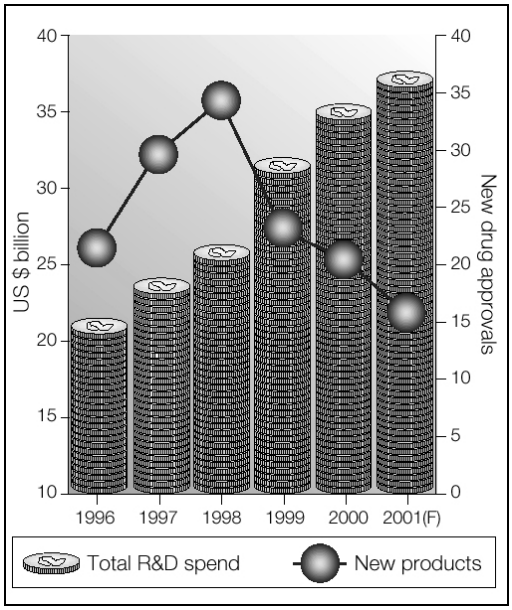


Fig. 3. Research and development spending versus productivity for the top 20 pharmaceutical companies (Renfrey and Featherstone, 2002)

1.2.1 Preclinical and early clinical development.

Once a lead compound enters the preclinical and early clinical development stage, the most important objective of further studies is a full evaluation of its safety. Possible toxicity of the substance is examined extensively, and its pharmacological properties are further investigated. Studies are also performed to examine phamacokinetics, dosage forms, and stability. Animal studies are an important part of this work, to obtain a profile of the drug in vivo and to provide further insight into the therapeutic action (pharmacology), adverse effects on the body (toxicology), and metabolism (pharmacokinetics).

Toxicity of the drug candidate (e.g. mutagenicity, cytotoxicity, QTc prolongation), poor biopharmaceutical properties (inappropriate pharmacokinetics), and lack of efficacy are some of the primary reasons for failure of drugs in development (Fig. 4).

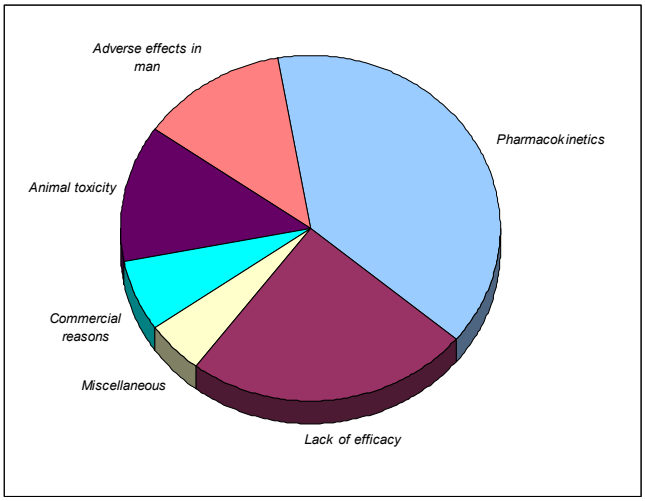


Fig. 4. Reasons of failure for drugs during development (van de Waterbeemd and Gifford, 2003).

Poor biopharmaceutical properties are often attributed to poor chemical stability in the gastrointestinal tract, low solubility in relation to the administered dose, poor permeability (*absorption*), or a poor metabolic and/or elimination profile. With the aid of extensive (bio)pharmaceutical studies part of the encountered problems can be solved (e.g. increased absorption or solubility, application of different routes of administration to minimize metabolism etc.). Bioavailability is an important parameter and safety studies must represent a sufficient systemic exposure in the test models.

Furthermore, it should be ensured that a sufficient oral bioavailability is to be expected in man. Systemic exposure is determined by the absorption potential of the compound and its metabolic stability. However, interspecies differences in metabolic profiles are common (Chiou et al., 1998, 2000; Chiou and Barve, 1998; Chiou and Buehler, 2002), and early knowledge of human metabolic pathways and human specific metabolites of a drug candidate is important.

1.2.2 Clinical development.

Compounds that survive preclinical testing need to be tested in human clinical studies. At first, the safety and pharmacokinetics are evaluated in healthy volunteers (phase I). These studies are then extended to a limited number of patients (phase IIA). Dose

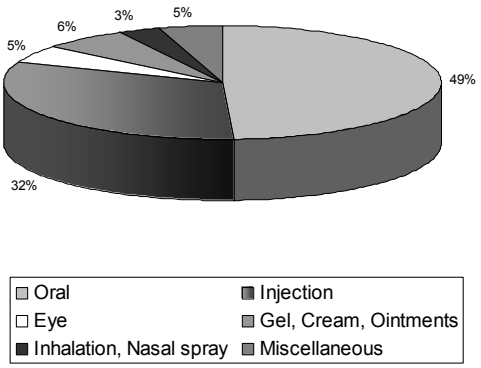
related efficacy and safety are determined in a group of approx. 100 to 300 patients (phase IIB). Finally, the compound's efficacy, safety and tolerance are compared against placebo and/or active compounds in a group of 8000 to 10000 subjects who are representative of the population that will ultimately receive the treatment (phase III). Typically, clinical studies are performed over a time period of 3 to 6 years. Often different pharmaceutical formulations are used in the clinical studies. For example, in phase I studies aqueous solutions / suspensions are used while in phase III studies the final market formulation is administered. Hence, bioequivalence studies are helpful to determine whether the pharmacokinetic properties of both formulations are still the same. Similarly, bioequivalence studies are also required for generic products prior to their introduction on the market.

1.2.3 Registration.

All clinical, chemical, safety, and (bio)pharmaceutical information is compiled and submitted as a dossier for regulatory approval. The information, which fills 400.000 or more pages, is checked by the regulatory agencies to ensure that all experiments have been performed according to the regulations and that the drug meets the claimed and required levels of efficacy, safety, and quality. Each product has to be registered separately and different dosage forms go through the registration process individually. This process takes about one year.

1.2.4 Oral drug delivery.

As biopharmaceutical studies form an integral part of the development process a closer analysis of the issues involved is needed. Drugs may be administered via different routes (oral, transdermal, nasal etc.) in order to reach the systemic circulation. Once the drug arrived in the blood it can be transported to the targets. Irrespective of the route of administration the involved issues are the same: release from the dosage form, absorption of the drug into the blood and finally removal of the drug from the body (metabolism and elimination). Oral administration of drugs is by far the most convenient and widely applied (Fig. 5). The physicochemical and physiological parameters involved and their effect(s) upon oral administration are summarized in Table 1.



Number of products evaluated: 763
Total number of formulations evaluated: 1005

Oral formulations:

Tablets	*332*
Capsules	*85*
Oral solutions	*74*
Sublingual tablets	*2*

Fig. 5. Most popular dosage forms in drug administration.
The evaluation is based on Queensland Hospitals Standard Drug List (SDL) 2000 Version 19 (1 August 2003). For many products more than one dosage form was available.

Table 1. Physicochemical and physiological parameters involved in oral drug delivery.

Physicochemical parameter	***Effect***
Solubility	Dissolution
pKa	Solubility, Dissolution, Absorption
Molecular size	Absorption
Hydrophobicity	Solubility, Dissolution, Absorption
Hydrogen bonding capacity	Solubility, Dissolution, Absorption
Particle size, Crystal form	Solubility, Dissolution
Complexation	Solubility
Chemical stability	
Physiological parameter	***Effect***
Permeability of the gastrointestinal membrane	Absorption
Surface area at the administration site (differences in surface area of stomach, duodenum, small and large intestine)	Absorption
Mucus and unstirred water layer (UWL): present at the gastrointestinal surface.	Absorption
Water fluxes. Water is (re)absorbed in the colon.	Solubility
Transporters: efflux (P-glycoprotein) as well as influx transporters. Regional variations in expression in the gastrointestinal tract.	Absorption, Excretion
Metabolism: Regional variations in expression along the gastrointestinal tract.	Exposure, Half life
Food may influence metabolism and transporters . Food components may influence transport and metabolism (*Rodrigues, 2003; Sandt, 2001*).	Solubility, Absorption, Exposure, Half life
First Pass Effect (FPE): metabolism during absorption in the gastrointestinal tract and wall and first passage of the liver.	Exposure
Enterohepatic circulation: after absorption the drug is excreted by the liver via bile into the gallbladder and then again excreted into the duodenum.	Exposure
Intestinal secretions: *bile* (solubilizing effect; food stimulates excretion of bile).	Solubility
Pancreas (secretion of *enzymes*: e.g. lipases, proteases, amylases).	Stability
Transit time (stomach 60 - 90 min, duodenum 30 - 40 min, jejunum 1.5 - 2 hours, ileum 5 - 7 hours colon 35 - 36 hours). Small particles leave stomach much faster than large particles. Presence of food is of influence on the transit time, fat reduces gastric emptying.	Absorption
Gastrointestinal pH (pH in stomach: 1 - 3 and in intestine: 5 - 7.5 depending on the fasted and fed state).	Solubility
Composition of food (solubilization of drugs by fat and bile and reduction of gastrointestinal motility).	Solubility, Transit time

1.3 Aim of thesis.

In the last 10 years, biopharmaceutical science has given a large contribution to the research and development of new medicines. Relationships between the physicochemical properties, the formulation, and the in vivo behavior (pharmacokinetics / pharmacodynamics) are studied, and the results have been used to optimize the pharmacologic or therapeutic efficacy of drugs. The aim of the present thesis is to give a contribution to some relevant biopharmaceutical issues in the field of oral drug delivery.

In the first part of the thesis (chapters 2 through 4), the physicochemical properties required for delivery of the drug to the systemic circulation are studied. First the influence of the route of administration on the physicochemical properties of drugs is determined (chapter 2), followed by a more detailed analysis of the properties needed for an effective absorption of steroids (chapter 3). Steroids were chosen as a group of model drugs as current research is mainly focussed on the pharmacological effects and pharmacokinetics. These studies were extended by determining the gastrointestinal absorption and P-glycoprotein (Pgp) efflux transport of heterocyclic drugs (chapter 4). In this study, an evaluation is also made of the predictive value of in vitro screening tests for P-glycoprotein transport by comparing Pgp transport to in vivo data.

Upon oral administration, an alternative route for absorption is the lymphatic uptake. The major advantage is found in the fact that first pass metabolism is circumvented. Additionally, it offers a possibility to deliver very hydrophobic drugs to the systemic circulation. Currently it is unknown if the potential of lymphatic drug delivery can be predicted a priori for new drugs. In Chapter 5 it is investigated if this is possible on the basis of the physicochemical properties of a drug, binding affinity to the components present in the lymphatic fluids or with the in vitro Caco-2 cell system. In the second study involving lymphatic drug delivery, the contribution of lymphatic absorption of a very hydrophobic drug to the systemic exposure of testosterone was determined (chapter 6).

Finally, a scientific approach is developed to evaluate the possibility for waiving bioequivalence studies on the basis of standard human pharmacokinetic data (chapter 7). With this work a contribution is given to the recent call for (new scientific)

approaches for waiving bioequivalence studies in order to reduce the number of (clinical) studies.

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2 Physicochemical properties required for an optimal systemic availability of drugs in relation to the route of administration.

Abstract

The physicochemical properties of drugs determine whether a drug can reach the systemic circulation. For the oral route of administration the 'ideal' set of physicochemical properties is well established. However, by comparison the other routes have received much less attention. For this reason it was evaluated how the necessary physicochemical properties are influenced by the route of administration (oral, buccal/sublingual, transdermal, nasal and vaginal).

For all studied routes of administration (oral, transdermal, buccal/sublingual, nasal and vaginal) the barriers to the systemic circulation are formed by multiple layered epithelia, with the exception of the gastrointestinal epithelium which consists of a single layer of enterocytes. The different epithelia are made up of a wide variety of cell types contributing to the specialized functions of these tissues. Irrespective of the route of administration the majority of drugs are absorbed either transcellularly or paracellularly (intercellularly).

The calculated physicochemical properties of transdermally, buccally/sublingually, nasally and vaginally delivered drugs showed that they are all within the generally accepted range required for a good oral absorption. For the solubility it was found that the available volume to dissolve the drug is very important. The available 'physiological' volume is much smaller for the non-oral routes. As a result the solubility is the most critical parameter for the transdermal, buccal/sublingual, nasal and vaginal drug delivery.

It is concluded that the required properties to achieve permeation through epithelia are independent on the route of administration. If a non-oral route of administration is chosen the solubility becomes a very critical parameter as less volume is available to dissolve the drug. Hence, a change in route of administration is not suitable to increase exposure in case of hydrophobic drugs.

2.1. Introduction.

The physicochemical properties of drugs are important variables which determine whether the systemic circulation can be reached. One of the tasks of the medicinal chemist is to vary the physicochemical characteristics of new drugs in order to optimize absorption, pharmacological activity, metabolism and to minimize possible toxic effects.

In the 1990s considerable progress was achieved concerning the required physicochemical properties needed for oral absorption. Analyzing the key properties of compounds in the World Drug Index (WDI) the now well-established 'rule of five' was derived (Lipinski et al., 1997). Similar analyses led to refinement of the criteria for the physicochemical properties of CNS (Central Nervous System) drugs (Kelder et al., 1999) and also 'drug likeness' of NCEs is currently being assessed (Ajay et al., 1998; Wagener and van Geerenstein, 2000).

In the last 5 years attention has shifted towards the prediction of ADME (Absorption, Distribution, Metabolism, Elimination) and toxicological properties (van de Waterbeemd and Gifford, 2003; Bursi, 2003). This was based on two investigations which indicated that poor ADME properties and lack of efficacy are the main reasons for failure in clinical development (Prentis et al., 1988; Kennedy, 1997). Recently doubts have arisen about these conclusions as within the group of 'failed' drugs a large fraction of poorly bioavailable anti-infectives was present (Kubinyi, 2003). Also the in silico prediction of the required structural elements for P-glycoprotein transport and CYP3A4 metabolism of drugs received a lot of attention as both parameters were identified to be limiting to systemic exposure (Testa et al., 2001; Seelig, 1998; van de Waterbeemd and Gifford, 2003; van de Waterbeemd et al., 2001, 2003).

Besides oral administration the transdermal, buccal/sublingual, vaginal and nasal route of administration are popular and frequently applied. To reach the systemic circulation, absorption via these routes will take place through physiologically different epithelia. In comparison to oral administration these alternative routes offer the advantage of circumventing first pass metabolism or other possible problems associated with passage through the gastrointestinal tract (e.g. microbial and/or chemical degradation, variable physiological conditions, food effect etc.) (Rowland and Tozer, 1994).

This chapter evaluates the required physicochemical properties for each route of administration. The oral route of administration is included as a reference point and compared with transdermal, buccal/sublingual, vaginal and nasal drug administration. Invasive routes of administration like injections are excluded from the present evaluation, as formally no 'absorption' barrier is present.
At first instance an inventory is made of the physiological barriers. Subsequently, the physicochemical properties of marketed and investigational drugs are evaluated. From both approaches the required physicochemical properties are derived for each route of administration. Finally, the results are evaluated in relation to the consequences for pharmaceutical development.

2.2 Experimental.

To calculate the physicochemical properties of drugs for transdermal, buccal/ sublingual, nasal and vaginal drug delivery, lists were compiled on the basis of literature and internet surveys (see for example: transdermal (Yamashita and Hashida, 2003; Panchagnula, 1997), buccal / sublingual (Zhang et al., 2002; Motwani and Liptworth, 1991), nasal (Behl et al, 1998; Dahlin, 2000; Dale et al., 2002), vaginal (Gursoy et al., 1989; Gursoy and Bayhan, 1992)). Marketed as well as investigational drugs were included. The chemical structures of drugs were retrieved from several in-house drug databases at Organon. The ClogP, Molecular Weight (MW), number of rotatable bonds (RB), number of hydrogen bond donor (HD) and acceptor sites (HA) and the static Polar Surface Area (PSA, $Å^2$) were calculated using the methods described in Kelder et al. (1999). The static PSA does not take into account the different conformations of the chemical structures, but gives essentially the same results as the dynamic PSA. It has the advantage of a much shorter time of calculation (Kelder et al., 1999). Averages of the calculated parameters were calculated as well as the 10 to 90 percentile ranges, i.e. the range that contains 80% of the drugs evaluated.

2.3 Results.

2.3.1 Physiological / Anatomical overview.

2.3.1.1 Absorption barriers.

Epithelial tissues, differing in cellular composition and functionality, form the barrier for drugs to the systemic circulation. Table 1 summarizes the relevant epithelial tissues for the different routes of administration. A more detailed analysis of the physiological barriers is made down below.

Table 1. Epithelial tissues forming the barriers to the systemic circulation for different routes of administration (Williams, 1995).

Route	***Epithelial characteristics***	
Oral	Single layered epithelium of enterocytes (columnar epithelium) covered with mucus.	
Transdermal	Multilayered epithelium with sebaceous and sweat glands and hair follicles. The top layer consists of cornified cells.	
Buccal / Sublingual	Multilayered epithelium covered with mucus. Depending on the position in the mouth the epithelium is keratinized or non-keratinized.	
Vaginal	Multilayered epithelium covered with mucus. The epithelium is non-keratinized.	
Nasal	*Vestibular region*	Pseudostratified multilayered epithelium containing hairs.
	Respiratory region	Pseudostratified multilayered epithelium with many ciliated columnar cells. Covered with a thick layer of mucus.
	Olfactory region	Pseudostratified columnar cells, sensory receptors, olfactory nerves and subepithelial olfactory glands. Covered with a thick layer of mucus.

Oral. When a drug is administered orally it passes the mouth, esophagus, stomach, duodenum, jejunum (small intestine), colon (large intestine) and finally leaves the body if not absorbed. The first requirement for absorption is that the drug dissolves (*dissolution*). I.e. only drug that is dissolved has the ability to permeate the gastrointestinal membrane. As a result the solubility of the drug is an important parameter. In the duodenum bile is secreted by the bile bladder, which may enhance the in vivo solubility to a large extent (Wiedmann and Kamel, 2002; Bakatselou et al., 1991; Horter and Dressman, 1997). Once dissolved the drug is available in the intestine to be absorbed.

To facilitate efficient absorption of nutrients the surface area of the intestine is very large (small intestine ± 462 m^2, large intestine ± 1 m^2). The small intestine is folded and contains *villi*, and additional surface area is available through the *microvilli* present on the apical side of the *enterocytes*. The enterocytes are covered by mucus and an unstirred water layer.

The biological membrane of the enterocytes, which consists of a bilayer of phospholipids, forms the main barrier for absorption. In the membrane also other structures and molecules are present such as proteins (e.g. transporters), cholesterol etc. (Alberts et al., 1989; Lee, 2003; McConnell and Radhakrishnan, 2003). As phospholipids are most abundantly present and have specific physicochemical properties, they will be of influence on the required characteristics of drugs in order to pass through the biological membrane.

A drug may pass the layer of enterocytes in different ways of which *passive diffusion* is the most important. Besides passive diffusion, facilitated diffusion, paracellular and active transport, endocytosis are possible mechanisms to reach the systemic circulation (Duizer, 1999).

Before reaching the systemic circulation a drug may be metabolized during absorption by enzymes present in the enterocytes. Furthermore, when the drug has entered the portal vein it is directly transported to the *liver* where also metabolism may take place. After passage through the liver the drug reaches the *systemic circulation* and is transported by the blood towards the targets.

Transdermal. The skin is, with a surface of about 1.8 m^2, the largest organ of the human body by mass (Williams, 1995). It has metabolic, immunologic and sensory capabilities. The primary purpose of the skin is the protection against environmental influences and xenobiotics. Furthermore, it plays an important role in maintaining the body's water balance. The skin is composed of the epidermis, dermis and the underlying subdermal tissue (hypodermis).

The dermis and the epidermis are separated by a basement membrane, whereas the dermis remains continuous with the subcutaneous and adipose tissues. The epidermis consists of five layers of cells types, beginning from the outside of the skin: the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum germinativum (basale) (Williams, 1995). Within the dermis varying numbers of hair follicles, sebaceous and sweat glands are present, all supported by a rich vascular network.

The cornified layer of the stratum corneum provides the rate limiting step for drug penetration. The stratum corneum consists of 10 to 15 layers of flattened cornified cells and a lipid rich intercellular matrix. The matrix contains a complex mixture of lipids, ceramides, sterols, sterol esters and free fatty acids, which are organized in multilaminated sheets. The bilayers in the intercellular spaces in the stratum corneum consist of straight, closely packed hydrocarbon chains. The hydrocarbon chains alternate with 'aqueous channels' which comprise the ‘polar’ route through the intercellular space.

With respect to absorption a distinction is made between the transappendageal and the transepidermal route. In the transappendageal route the drug is absorbed via the eccrine (sweat) glands or via the pilosebaceous unit (the hair follicles and the sebaceous glands). The transappendageal route is considered to be of minor importance because of the relatively small surface area (less than 0.1% of the total surface area of the skin).

Transepidermal transport of polar and non-polar drugs occurs via transcellular and intercellular routes. The lipid matrix (keratin - phospholipid complex) of the stratum corneum plays a significant role in determining the permeability of drugs across the skin. The polar molecules mainly diffuse through the polar pathway consisting of the aqueous 'channels' within the hydrated stratum corneum. The non-polar molecules are

transported either transcellularly or through the non-aqueous lipoidal intercellular matrix.

Buccal / Sublingual. The barrier for drugs in the oral cavity is formed by the mucosa, which is covered by mucus. Mucus serves as lubricant and as a mechanical barrier. It's main component is mucin, a large glycoprotein, giving the mucus the viscoelastic and adhesive properties. The thickness of the mucus layer varies from 5 to 200 μm, with an average value of approximately 80 μm.
The upper layer of the mucosa is formed by multiple layers of squamous (i.e. scaly) cells which from the epithelium (Williams, 1995). At the proximal side the epithelium is bound to the connective tissue by the basal lamina, which is a protein-like fibrous matrix with a thickness of approx. 1 μm. In the connective tissue (lamina propria and submucosa) blood and lymph vessels are present. As the primary function of the oral mucosa is protection rather than absorption, different functions emerge in the organization of the mucosa.
Oral mucosa can be distinguished into five major regions: (1) the floor of the mouth (sublingual), (2) the buccal mucosa (cheeks), (3) the gum (gingiva), (4) the palatal mucosa and (5) the inner side of the lips. The total surface area of the mucosal membrane is approx. 200 cm^2, and shows differences in structure, thickness, permeability and blood flow.
Oral mucosa contains several lipids, called keratines, which have an approx. molecular weight of 40 - 70 kDa. Depending on the localization in the oral cavity, the top layer of oral mucosa can be keratinized (comparable to the stratum corneum of the skin). Non-keratinized epithelium contains low molecular weight (MW) lipids in contrast to the keratinized epithelium (high MW lipids). Furthermore, variations in thickness and bloodflow are present. The characteristics are summarized in Table 2.
As mentioned oral mucosa is covered with mucus, which is continuously provided with fresh serous and mucous saliva. The rate of secretion is approx. 0.2 - 0.4 mL/min at rest upto 2 mL/min at maximum secretion. The volume of saliva after swallowing is approx. 0.9 mL. The pH of saliva varies from 5.8 to 7.1 (mean 6.8). Besides water and mucin saliva contains inorganic salts (sodium, chloride, calcium, phosphate and bicarbonate) and enzymes like α-amylase etc. At higher flow rates of saliva the pH

Table 2. Oral epithelium characteristics. (de Vries, 1991)

Tissue	***Structure***	***Thickness (µm)***	***Bloodflow (mL/min/cm^2)***
Buccal	Non keratinized	500 - 600	2.4
Sublingual	Non keratinized	100 - 200	0.9
Gingival	Keratinized	200	1.5
Palatal	Keratinized	250	0.9

increases due to the lower resorption of sodium, chloride and bicarbonate.
In the absorption process of drugs the mucus layer may act rate limiting in the diffusion of lipophilic drugs. Furthermore, due to negatively charged mucin, mucus is able to bind positively or repulse negatively charged drugs. In the keratinized epithelia the upper layer seems to be the major barrier to absorption. This in contrast to the non-keratinized epithelia where the entire epithelium functions as a barrier. Principally, drugs can be absorbed transcellularly and paracellularly. The resistance to transcellular transport is determined by the biological membrane of the cells.
Less lipophilic drugs or hydrophilic drugs can permeate paracellularly. However, lipophilic components can be present in the intercellular fluid (Squier, 1973). The amount of lipophilic components is higher in the upper layers of the epithelium as compared to the tissue near the basal lamina. While tight junctions are present throughout oral mucosa, they are rarely seen in the buccal mucosae (de Vries et al., 1991) which might be beneficial for paracellular transport. Active transport and pinocytosis have not been reported for oral drug absorption and almost all low molecular weight, lipophilic drugs penetrate the epithelium by passive diffusion.

Vaginal. The vagina is a tubular canal with a length of approximately 10 to 15 cm, extending from the vestibule to the uterus. The vaginal wall consists of a non keratinized stratified squamous epithelium and a muscular layer. The membrane is folded into numerous microridges of which the morphology changes throughout the reproductive cycle. Porous structures appear within the microridges during midcycle (Ludwig and Metzger, 1976; Waltz et al., 1978).

The thickness of the epithelium varies during the menstrual cycle. A few days before ovulation the epithelium is the thickest (± 250 μm) and becomes thinner (± 150μm) during the secretory phase of the menstrual cycle. Upon natural aging the thickness of the vaginal wall decreases resulting in an increased permeability of the epithelium after menopause.
Although no glands are present in the epithelium, it is covered with a layer of fluid (approximately 100 - 200 μm). Vaginal fluid is composed of cervical secretions, uterine, follicular, and peritoneal fluid as well as exfoliated epithelial cells, bacteria and bacterial products. The pH of vaginal fluid varies from 4 to 5 (Brannon-Peppas, 1993; Schmidt and Beller, 1978). The pH is maintained by the action of bacteria. The cells in the vaginal epithelium produce large amounts of glycogen, which is stored in the cytoplasm. Glycogen from exfoliated cells is converted into lactic acid by bacteria. The relatively low pH is of influence on the microflora.
Absorption of drugs through the vaginal epithelium is by the transcellular or the paracellular pathway (Sayani and Chien, 1996). The main advantage of the vaginal route of administration is avoidance of the gastrointestinal first pass metabolism. Although a first pass effect (FPE) has been reported in literature, this should not be confused with the gastrointestinal first pass effect (Cicinelli et al., 2000). I.e. the uterine FPE is probably a local effect.

Nasal. When a drug enters the nasal cavity it will arrive in one of the three functionally distinct regions: the vestibular, respiratory or olfactory region (Williams, 1995). The vestibular area, usually containing long hairs filtering airborne particles, is located at the openings of the nasal passages. The respiratory area is the largest of the three regions and its ciliary epithelium serves as a removal system for deposited particles on the mucous layer. The olfactory region is located at the very top of the nasal cavity and is free of airflow and is responsible for the sensory function of smell (Williams, 1995). Direct entry of drugs into the brain is possible through the olfactory region (Dahlin, 2000). In adult humans the nasal cavity volume is 15 - 20 mL, the surface area 150 - 180 cm^2 and is covered by a 2 to 4 mm thick mucosa. Approx. 5 - 10 cm^2 is olfactory and the remaining 145 - 170 cm^2 respiratory. The main advantage of nasal drug delivery is found in the circumvention of the First Pass Effect (FPE).

The vestibular region, covered by pseudostratified epithelium showing resemblance to skin, is the least important to absorption. The respiratory epithelium is the most important for systemic drug absorption due to the high vascularisation (Behl et al., 1998; Dale et al., 2002, Dahlin, 2000). It consists of columnar, goblet and basal cells. Ciliated columnar cells are most predominantly present. The goblet cells secrete mucus, are columnar in shape and have microvilli on their surface. The basal cells are present on the basal membrane consisting of a layer collagen fibrils along with the columnar and goblet cells. Near the basal membrane the junctions between the cells are not tight. Consequently the cells tend to be surrounded by regions of intercellular fluid. A subepithelial connective tissue layer, composed of branched fibrils surrounded by a highly viscous substance, is present under the porous basal membrane.

Human olfactory epithelium is similar in organization and morphology to that of most vertebrate species (Dahlin, 2000). The epithelium is pseudo stratified columnar and consists of receptor (also called olfactory or primary olfactory neuron or cranial nerve), supporting and basal cells. The presence of the receptor cells differentiates olfactory epithelium from the other epithelia. Large interspecies differences are present in the surface area of the olfactory region (e.g. humans: ± 10 cm^2, dog: 150 - 200 cm^2) and the number of receptor cells. In the nasal cavities of mice and rats respectively 47% and 50% of the total nasal epithelium consists of olfactory epithelium, in comparison to 3% in humans.

The mucus layer of the respiratory epithelium (thickness 20 - 30 μm) is removed and replaced every 10 to 25 minutes and the pH ranges from 5.5 to 6.5. In the olfactory epithelium mucus (thickness 60 to 70 μm) is not removed by the movement of cilia, but by overproduction by the mucus glands. Few solute particles enter the olfactory region due to the lack of airflow. Once a drug contacts the mucous layer it will not be removed quickly due to the low mucus flow and it may be able to contact the olfactory epithelium.

Transport across mucosa is generally thought to occur either transcellularly or paracellularly. The transcellular pathway is often referred to as the lipoidal and the paracellular pathway as the aqueous pathway. I.e. hydrophilic permeants do not partition well into the cell membrane and are therefore transported via the intercellular

space (paracellular transport). Lipophilic permeants are transported transcellular. The size of the pores between cells for paracellular transport in mucosa are larger as compared to the gastrointestinal tract, although exact measurements could not be found in literature (Chien, 1991).

Drugs sprayed onto the olfactory mucosa can be absorbed by three routes: (1) the olfactory neurons, (2) the supportive cells and the surround capillary bed and (3) the cerebrospinal fluid (CSF) (Dahlin, 2000). Several xenobiotics, including viruses, metals and amino acids, have been reported to be transferred from the nasal mucosa into the olfactory bulb via the olfactory pathways (Dahlin, 2000). The potential delivery of drugs to the CNS via the olfactory pathways to the brain has received an increasing interest (Dahlin, 2000).

2.3.1.2 Metabolism for the different routes of administration.

Many drugs are metabolized in the liver and sometimes also at extrahepatic sites before final excretion by renal, biliary or other routes. Depending on the products formed upon metabolism, metabolic reactions can be classified as functionalisation (phase I) or conjugation (phase II) reactions (la Du et al., 1971; Benford et al., 1987). In phase I metabolism the cytochrome P450 monooxygenases are the most widely studied. However, other enzymes are involved as well (dehydrogenases, oxidases, esterases, reductases). The most important phase II enzymes are the UDP (uridine 5'-diphosphate) glucuronosyltransferases (UGT) but other enzymes are involved as well (sulfotransferases, glutathione S-transferases etc.). Upon glucuronidation the aqueous solubility of the substrate is increased and renal and biliary excretion is facilitated. Depending on the route of administration drugs may undergo first pass metabolism during absorption depending on the expression level in the involved tissues. The involvement of cytochrome P450 upon oral administration is well documented and also involvement of UGT metabolism has been postulated (Tukey and Strassburg, 2000). Table 3 shows the distribution of the P450 and UGT enzymes in the tissues that are relevant for oral, buccal/sublingual, transdermal, vaginal and nasal drug administration.

Table 3. Presence of P450 enzymes and UGTs in human tissues relevant for absorption of drugs after oral, buccal/sublingual, transdermal, vaginal and nasal drug administration.

	Tissue	***Types expressed***
P450	Liver [1]	3A4, 3A5, 2C8, 2C9, 2C18, 2C19 [2]
		1A2, 2E1, 2A6, 2D6, 2B6 [3]
		2B1, 2B2, 4A11 [4]
	Buccal / Sublingual [5]	1A1, 1A2, 2C, 2E1, 3A4, 3A7, 3A5, 2D6
	Esophagus [6]	2A6, 2A13, 2B6, 2S1
	Intestine [6]	1A1, 1B1, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5
		2J2, 4B, 2S1
	Colon [6]	1A1, 1A2, 1B1, 2J2, 3A4, 3A5
	Skin [7]	1B1, 2B6, 2D6, 3A4 [2]
		2A6, 2C18, 2C19, 3A5 [3]
	Endometrium [8]	2C, 2E1, 3A4, YP3A5, 4B1, 1B1, 11A, 3A7
	Nasal mucosa	2A6, 2A13, 2B6, 2C, 2J2, 3A
UGT [9]	Liver	1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, 2B10,
		2B11, 2B15
	Esophagus	1A7, 1A8, 1A10, 2B7, 2B10, 2B15
	Intestine	1A8, 1A10, 2B7
	Colon	1A1, 1A3, 1A4, 1A6, 1A8, 1A9, 1A10, 2B7
	Skin	2B11, 2B17
	Olfactory epithelium	2A11
	Ovary	1A6

[1] (Lin and Lu, 2001), [2] High expression, [3] Low expression, [4] (Brandon et al., 2003), [5] (Vondracek et al., 2001), [6] (Ding and Kaminsky, 2003), [7] (Yengi et al., 2002), [8] (Hukkanen et al, 1998), [9] (Tukey and Strassburg, 2000).

Oral administration. Enzymes with metabolic activity, P450 as well as UGTs, are primarily localized in epithelial cells. The activity varies with the position of the cells in the villi. Metabolic activity is generally higher in the duodenum and jejunum than in the ileum and colon, i.e. metabolism decreases when the drug passes through the

gastrointestinal tract (Paine et al., 1997; de Wazier et al., 1990; Thummel et al., 1997). A wide range of metabolic enzymes is present in the gastrointestinal tract (Krishna and Klotz, 1994). Intestinal microorganisms also metabolize drugs, but this topic is beyond the scope of this chapter. Recent studies suggested that the contribution of intestinal metabolism to the overall first pass metabolism of drugs is quantitatively more important than that of hepatic metabolism, although this is being debated in literature (Lin and Lu, 2001). Nonetheless, it is thought that glucuronidation most likely represents a metabolic barrier function of the gastrointestinal mucosa (Tukey and Strassburg, 2000). Large inter-individual variations in response to cytochrome P450 inhibition have been observed in vivo (Lin and Lu, 2001).

Transdermal administration. In the human skin many metabolizing enzymes have been found to be expressed. P450 and UGTs have been reported to be present as well as other enzyme systems. Although many drugs are reported to undergo metabolism in the skin (e.g. estradiol, testosterone or vidarabine), it is questionable whether this 'presystemic' metabolism plays an important role as the amount of enzymes present is much less as the amounts expressed in liver and intestine (Krishna and Klotz, 1994; Gonzalez et al., 2001; Janmohamed et al., 2001). However, local tissue specific metabolism may be responsible for specific cytotoxic effects.

Buccal/Sublingual administration. Several P450 enzymes have been reported to be present in the oral mucosa. However, the expression levels remain unclear in comparison to hepatic or intestinal levels. The presence of UGTs in oral mucosa has not been reported up to now. The limited data available seem to suggest that the expressed P450 and UGT enzymes do no give a substantial contribution to so-called first pass metabolism but is limited to local metabolism.

Vaginal administration. Metabolic enzymes expression was found to be variable during the menstrual cycle. E.g. expression of CYP3A7 in endometrium is significantly greater in the proliferative phase compared with the secretory phase (Sarkar et al., 2003). CYP3A4 expression was comparable between the two phases. Expression of both CYP3A7 and CYP3A4 is minimal in the cervix. An indication was found for localized expression of CYP3A enzymes in the glandular epithelium as well as the stroma.

Nasal administration. In many mammalian species cytochrome P450 expression is highest in nasal mucosa of all extrahepatic tissues, although apparently not in humans (Ding and Kaminsky, 2003). UGT conjugation enzymes are also present but the contribution to metabolism is probably low (Hamilton et al., 2002). Only a few studies on human nasal drug metabolism are available. It is not known whether these enzymes are inducible in nasal mucosa, although they are inducible in other tissues. The extent and clinical significance of nasal metabolism is unknown, and an actual human nasal 'first pass' drug metabolism has not been investigated (Dale et al., 2002).

In summary, in many, if not all, tissues which form the barrier for drugs administered via the oral, transdermal, buccal/sublingual, vaginal or nasal route cytochrome P450 and UGT enzymes are present. The P450 metabolic enzymes are relevant to first pass metabolism in the gastrointestinal tract, which is well documented in literature. For the oral route of administration the UGT metabolic enzymes are postulated to be relevant for first pass metabolism as they are expressed in the small intestine and colon and they are also involved in the enterohepatic circulation (Tukey and Strassburg, 2000). Based on the available literature it seems that in the other tissues the metabolic enzymes are only relevant for local cytotoxic effects, due to the relatively low expression levels. This observation would be consistent with the view that the gastrointestinal first pass effect can be circumvented by the use of alternative routes of administration. In literature one case of a substantial first pass effect upon transdermal administration was reported. In vivo data suggest a first pass effect for nitroglycerin of 10 to 20% after transdermal administration (Guy et al., 1987).

2.3.1.3 (Active) Transport for the different routes of administration.

Throughout the human body many different type of transporters are present (Sun et al., 2003; Rodrigues, 2002). At present the P-glycoprotein (Pgp) transporter is the most widely studied one (Rodrigues, 2002). Pgp transports a wide variety of chemically unrelated endogenous and exogenous compounds out of cell membranes. In normal tissues, Pgp is found on the apical surface of several epithelial cell types and high expression levels are found in cells of protective tissues such as the blood-brain barrier, the placental barrier, the blood-testis barrier, the adrenal gland, placenta,

and kidney. Lower expression levels are found in the liver, lung, skeletal muscle and heart. Extremely high expression levels are found in many cancer cells and this is thought to be the reason why cytostatics do not enter tumors.
With respect to oral administration Pgp is the most frequently cited transporter that is thought to be limiting to the bioavailability. Pgp mRNA levels increase longitudinally along the intestine, with the lowest levels in the stomach and highest in the colon, an observation that may have implications for controlled release technology (Lin, 2003; Fojo et al., 1987; Brady et al., 2002; Makhey et al., 1998). On the basis of the overlap between both the substrates and inhibitors of CYP3A4 and Pgp (Wacher et al., 1995; Kim et al., 1999), tissue localization, and coinducibility it has been hypothesized that these two proteins/systems work together to protect the body from absorption of harmful xenobiotics including drugs (Wacher et al., 1998). This was recently shown for verapamil where the residence time in the intestinal membrane was increased, resulting in a significantly greater intestinal metabolism (Johnson et al., 2001).
In the human skin the expression of Pgp is only reported to be expressed in the case of malignant melanomas. Therefore it is unlikely that Pgp plays a role under normal conditions in transdermal drug delivery. For the buccal / sublingual, nasal and vaginal route of administration no information is available whether Pgp is present in the mucosal tissues. Hence, it is unknown if Pgp could be limiting to drugs for reaching the systemic circulation.

2.3.2 Physicochemical properties.

The physicochemical properties of drugs delivered by the transdermal, buccal / sublingual, nasal and vaginal route of administration are shown in Table 4. In addition the criteria which have been derived for oral absorption are included as well (Lipinski et al., 1997; Palm et al., 1997).

2.4 Discussion.

For the different routes of administration the different epithelia were shown to have a large degree of similarity. Generally the barrier to reach the systemic circulation is multilayered with the exception of the intestinal membrane. In all cases drugs are absorbed by the same mechanisms: transcellular diffusion or paracellular transport. In

Table 4. Criteria for the physicochemical properties of orally administered drugs and the properties of drugs administered by the transdermal, buccal / sublingual, nasal and vaginal route of administration.

Route	***MW***	***HA***	***HD***	***PSA***	***RB***	***ClogP***
Oral [1]	0<X<500	0<X<11	0<x<6	0<X<110	0<X<13	-2<X<5
Transdermal (n = 42)	295.9 [2] (188.0 – 411.7) [3]	3.36 (1 - 6)	1.24 (0 – 2.9)	45.1 (8.9 – 75.7)	3.86 (0 - 8)	2.25 (-0.16 – 4.70)
Buccal/Sublingual (n = 46)	275.1 (163.3 – 342.3)	2.93 (1 - 5)	1.26 (0 – 2.5)	38.1 (13.0 – 66.0)	3.30 (0.5 - 6)	2.39 (0.58 – 4.16)
Nasal (n = 53)	391.6 (227.5 – 575.7)	4.77 (2 – 8.8)	2.83 (0 - 4)	73.6 (23.4 – 119.5)	6.11 (1 – 12.4)	2.05 (-0.61 – 4.14)
Vaginal (n = 27)	321.3 (171.8 – 416.1)	2.93 (1.6 - 5)	1.33 (0 - 3)	43.2 (18.6 – 69.3)	3.70 (0 – 7.4)	3.04 (0.13 – 5.81)

MW = molecular weight, HA = number of hydrogen bond acceptor sites, HD = number of hydrogen bond donor sites, PSA = Polar Surface Area (Å^2), RB = number of rotatable bonds, ClogP = calculated octanol/water partition coefficient. [1] Criteria for oral absorption. The criteria are based on (Lipinski et al., 1997; Palm et al., 1997). [2] Average value. [3] The 10 to 90 percentile range.

case of transcellular diffusion the biological membrane determines whether a drug is absorbed or not. If the barrier is formed by multiple layered epithelia (transdermal, buccal/sublingual, vaginal or nasal administration) also the thickness is of influence. For paracellular transport of a drug over an epithelial barrier the composition of the intercellular matrix is an important parameter. In the gastrointestinal tract the cells are closely connected by the tight junctions which limits drug transport. On the other hand, in the epithelia of the non-oral routes hydrophobic as well as hydrophilic domains are present. E.g. in the skin the intercellular matrix contains very hydrophobic ceramides resulting in a very effective barrier for hydrophilic molecules. Nevertheless, it has been shown possible to deliver hydrophilic drugs to the systemic circulation via mucosal transport (buccal/sublingual, nasal and vaginal), however, absorption enhancers are often needed (Bernkop-Schnurch and Clausen, 2002; Thanou et al., 2001; Bauerova et al., 2001; Heineman et al., 2001; Senel and Hincal, 2001; Sayani and Chien, 1996).

The physicochemical properties of drugs required to reach the systemic circulation show also a large degree of similarity for the evaluated routes of administration. In all cases the physicochemical properties comply with the criteria for good oral absorption (Lipinski et al., 1997; Palm et al., 1997). In other words, irrespective of the route of administration (oral, transdermal, buccal/sublingual, vaginal or nasal) the set of physicochemical properties needed for a drug to reach the systemic circulation is the same.

Analysis of the chemical composition of the cellular membrane reveals that there is a large degree of similarity between the different cell types as well. The biological membrane consists of proteins, cholesterol, carbohydrates and phospholipids, the latter being most abundantly present (Alberts et al., 1989). Phospholipids are amphiphilic in nature and consist of two hydrophobic fatty acid hydrocarbon chains (ClogP approximately 6 to 8.5) and a hydrophilic group. The physicochemical properties are ideal to form the bilayers of the cellular membrane (Tanford, 1973). If phospholipids were much more hydrophilic the membrane would be instable and simply dissolve in water. On the other hand, if the phospholipids were too hydrophobic, not enough material would be present in the aqueous phase to form membranes. Hence, with respect to the composition of biological membranes the

physicochemical properties can simply not vary to a large extent. As phospholipids have specific physicochemical properties, they will dictate the required characteristics of drugs in order to permeate through the biological membrane. As a result the physicochemical properties of drugs are independent of the route of administration. The lipid composition of intestinal, buccal, dermal and vaginal epithelia indicates as well that this is indeed the case (Lampe et al., 1983; Ganem- Quintanar et al., 1997; Thompson et al., 2001; Dudeja et al., 1989).

Up to now one of the most important parameters has not been discussed: the solubility of drugs in relation to the amount of solvent available. Before a drug is able to pass through a physiological barrier it must be in a dissolved state. Several simple models are available which include the solubility of the drug in the evaluation of absorption: the Dose (D_o) and Dissolution number (D_n) (Capsugel, 1997), Maximum Absorbable Dose (MAD) (Johnson and Swindell, 1996) and the Absorption Potential (AP) (Dressman et al., 1985). The equations to calculate the parameters in these models are given in Table 5.

The dose number evaluates whether the Anticipated Human Dose (AHD) of the drug can be dissolved in the available volume. The (physiological) volumes to dissolve the drugs are shown in Table 6 for the different routes of administration. Clearly the volume of fluid available for dissolving the drug is the most critical. For the oral route of administration a large volume is available, especially considering the fact that in vivo solubilization by bile can be very large (Bakatselou et al., 1991; Horter and Dressman, 1997; Wiedmann and Kamel, 2002). If a non-oral route is chosen it is immediately clear that the solubility is a very restrictive parameter. Values of the Dose number below 0.1 indicate that solubility is not a problem, while values about 10 show that the solubility is troublesome.

The Maximum Absorbable Dose (MAD) represents the amount of drug that can permeate across a barrier. Again the volume available to dissolve the drug is a critical parameter for the non-oral routes of administration. However, if the absorption rate constant is sufficiently high and a longer residence time can be achieved a considerable amount of drug can still be absorbed. The absorption rate constant can be calculated from the permeability of the epithelium involved or estimated by the ratio

of C_{max} and AUC. If the MAD is higher than the AHD no problems are to be expected with respect to absorption.

Table 5. Evaluation of solubility. Equations to calculate the Dose and Dissolution number, Maximum Absorbable Dose and Absorption Potential.

Model	***Application***	***Formula***
Dose number (D_o)	Oral, Transdermal, Buccal / Sublingual, Nasal, Vaginal	$Do = \frac{D}{V \cdot C_s}$
Maximum Absorbable Dose (MAD)	Oral, Transdermal, Buccal / Sublingual, Nasal, Vaginal	$MAD = C_s \cdot V \cdot K_a \cdot t_{res}$ $K_a = \frac{C_{\max}}{AUC}$
Dissolution number (D_n)	Oral (Buccal/Sublingual)	$Dn = t_{res} \cdot \frac{6 \cdot Diff \cdot C_s}{\rho \cdot d^2} = \frac{t_{res}}{t_{dis}}$
Absorption Potential (AP)	Oral	$AP = \log\left(\frac{m_{ow} \cdot C_s \cdot V \cdot f_u}{D}\right)$ *Acid:* $pH = pKa + \log\left(\frac{f_i}{f_u}\right)$ *Base:* $pH = pKa + \log\left(\frac{f_u}{f_i}\right)$

D_o = Dissolution number, D = Dose, V = Volume available to dissolve the drug (see Table 6), C_s = solubility, MAD = Maximum Absorbable Dose, K_a = absorption rate constant = C_{max}/AUC), C_{max} = maximum concentration in the time versus concentration profile, AUC = Area Under the Curve, D_n = Dissolution number, t_{res} = residence time, Diff = Diffusion coefficient, ρ= density, d = particle size diameter, t_{dis} = time needed for dissolution, AP = Absorption Potential, m_{ow} = partition coefficient, f_u = fraction unionized, f_i = fraction ionized.

Table 6. Physiological (aqueous) volumes available for dissolving drugs for the different routes of administration.

Route	***Fluid***	***Volume (mL)***
Oral	Small intestinal water volume or glass of water	250
Transdermal	Material of the patch containing the drug	± 0.2 [1]
Buccal / Sublingual	Saliva	± 0.9
Vaginal	Mucus covering the wall	± 1.2 [2]
Nasal	Liquid sprayed into the nasal cavity	± 0.2

[1] *Volume of patch = surface area (10 cm^2) x thickness (0.02 cm).*

[2] *Volume of vaginal fluid = surface area vaginal wall (78.5 cm^2) x thickness fluid layer (0.015 cm) = 1.2 mL. It is assumed that the total surface area contributes to the volume of fluid available for dissolving the drug.*

For oral or buccal / sublingual drug administration the Dissolution number can be used to evaluate whether the drug can dissolve within the available time frame (= residence time). The unknown parameter to estimate the Dissolution number is the diffusion coefficient of the drug which can be calculated from permeability data which are readily available in literature (e.g. Sayani and Chien, 1996). High values of the Dissolution number (i.e. larger than 10) show that the residence time is long enough for drugs to dissolve and the dissolution rate is not a critical parameter. Finally, for oral administration the absorption potential was introduced (Dressman et al., 1985). If the calculated absorption potential is larger than unity no problems are expected for oral administration. The absorption potential is also suitable for acids and bases as it takes the degree of ionization of the drug into account.
On the basis of the presented results a set of parameters can be derived for the oral, transdermal, buccal/sublingual, vaginal and nasal route of administration. The criteria presented in Table 7 address the solubility as well as the permeability of drugs, which are both crucial to obtain a sufficient systemic exposure.

Table 7. Criteria for drugs to reach the systemic circulation.

Physicochemical criteria for oral, transdermal, buccal/sublingual, nasal and vaginal drug delivery.	
Molecular Weight (MW)	$0 < MW < 500$
Number of hydrogen bond donor sites (HA)	$0 < HA < 11$
Number of hydrogen bond acceptor sites (HD)	$0 < HD < 6$
Polar Surface Area (PSA)	$0 < PSA < 110$
Number of rotatable bonds (RB)	$0 < RB < 13$
ClogP	$-2 < ClogP < 5$

Good absorption:	all properties within range
Moderate absorption:	1 or 2 calculated properties outside indicated range
Poor absorption:	3 or more properties outside indicated range

Model	***Route***	***Absorption criteria***	
Dose number [1] (D_o)	Oral, Transdermal, Buccal / Sublingual, Nasal, Vaginal	D_o <0.1 $0.1 < D_o < 10$ $D_o > 10$	Good Moderate Poor
Maximum Absorbable Dose (MAD) [1]	Oral, Transdermal, Buccal / Sublingual, Nasal, Vaginal	MAD < 0.1*AHD [2] 0.1*AHD < MAD < AHD MAD > AHD	Poor Moderate Good
Dissolution number [1] (D_n)	Oral (Buccal/Sublingual)	$D_n < 0.1$ $0.1 < D_n < 10$ $D_n > 10$	Poor Moderate Good
Absorption potential [1] (AP)	Oral	AP > 1	Good

[1] See Table 5 for equations, [2] AHD = Anticipated Human Dose

As the properties of drugs are similar for all routes of administration one could question what the real advantage is of the evaluated alternatives compared to oral drug delivery. In drug discovery it is often thought that a different route of administration is useful to overcome non-ideal properties of the new drugs. However, the problem in drug discovery is that the new compounds tend to be more and more hydrophobic by nature, as the current High Throughput Screening (HTS) technologies are based on binding assays to determine whether a drug binds to a target (Lahana, 1999). I.e. one of the major pitfalls is that the more hydrophobic compounds appear to bind better to the targets (the so-called hydrophobic effect) (Tanford, 1973). The current analysis emphasizes that the solubility is one of the key parameters. It becomes immediately clear that in case of hydrophobic drugs a different route of administration cannot be used. As a matter of fact, the criteria for the solubility are even more restrictive for the non-oral routes of administration.

The main advantage of the non-oral routes of administration is found in the circumvention of the First Pass Effect (FPE) in comparison to the oral route. Although metabolic enzymes are present in almost all epithelia, first pass metabolism is clearly dependent on the expression levels in the involved tissues. Only a limited number of reports are available in literature where a 'First Pass Effect' is reported for the non-oral routes of administration (e.g. the FPE of nitroglycerin when administered transdermally (Guy et al., 1987)). Nonetheless, in cases where the administered dose is low, e.g. in the µg range, presystemic metabolism might be considerable even when the expression levels of the metabolic enzymes are low.

Finally, the current analysis underlines the renewed interest in the biopharmaceutical sciences. In the pharmaceutical industry the new trend is to integrate pharmaceutics in drug discovery as early as the lead optimization phase. Screening assays are frequently applied to determine the solubility and to study the possibilities for drug solubilisation. Prodrug approaches are employed to increase the aqueous solubility or to target the lymphatics (Charman and Stella, 1992). Also attempts are made to influence the permeability using absorption enhancers. Although frequently applied in transdermal drug delivery, absorption enhancers seem to be nearly of academic interest (e.g. Sayani and Chien, 1996), as the number of non-transdermal market products containing absorption enhancers is very small.

2.5 Conclusions.

For all studied routes of drug administration (oral, buccal / sublingual, transdermal, nasal and vaginal) the barriers to the systemic circulation are formed by multiply layered epithelia, with the exception of the gastrointestinal epithelium which consists of a single layer of enterocytes. The different epithelia are made up of a wide variety of cell types contributing to the specialized functions of these tissues. Irrespective of the route of administration the majority of drugs are absorbed either transcellularly of paracellularly (intercellularly).

For the transdermal, buccal/sublingual, nasal and vaginal route of administration the physicochemical properties of drugs were calculated. Strikingly, the ranges of the molecular weight, number of hydrogen bond donor and acceptor sites, polar surface are, number of rotatable bonds and ClogP are all within the generally accepted ranges for passive transcellular transport after oral administration.

Evaluating the solubility of drugs in terms of the 'physiological' volume available for dissolution showed that this is a very critical parameter. The volume available to dissolve the drug decreases from oral to buccal/sublingual, vaginal, transdermal and nasal drug delivery. This clearly shows that the solubility is more critical for the non-oral routes of administration.

It is concluded that, for permeation across epithelia, the required properties are independent on the route of administration. If a non oral route of administration is chosen the solubility becomes a very critical parameter as less volume is available to dissolve the drug. Hence, for the medical chemist a change in route of administration is not suitable to increase exposure in case of hydrophobic drugs. The main advantage of non oral routes of administration is found in circumventing the first pass effect.

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3 Physicochemical properties and transport of steroids across Caco-2 cells.

Abstract

In steroid research, the focuss has been placed on pharmacology and pharmacokinetics. As a result, the biopharmaceutical properties received relatively little attention. This study evaluates the physicochemical properties of steroids in relation to gastrointestinal absorption.

Thousands of chemical structures were retrieved from the Organon compound database and physicochemical properties calculated (molecular weight, ClogP (octanol/water partition coefficient), static Polar Surface Area (PSA) etc.). Within this series of steroids different pharmacological groups were defined and ClogP was the most discriminative descriptor. Based on the outcome of this survey steroids were selected for the Caco-2 permeability study.

The steroids were well transported over the cell monolayers and the P_{app} was independent of the concentration and the transport direction. No relationship was found with the PSA, however, the small differences in the P_{app} values showed a weak inverse correlation with ClogP. The hydrophilic steroids (ClogP approximately 0 - 2) tend to diffuse faster over the cell monolayers compared to the more hydrophobic steroids (ClogP approximately 5). The relationship with ClogP suggests that partitioning of steroids between the biological membrane and the surrounding aqueous phase is one of the main mechanisms for absorption.

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3.1 Introduction.

In the development of New Chemical Entities (NCEs), the optimization of the biological activity plays an important role. In the first instance this is accomplished by chemical modifications of the lead compound(s) (Lead Optimization). When an NCE is taken into development, the formulation of the product is developed and optimized. The hydrophobic properties (e.g. octanol/water partition coefficients (logP) up to 5) and low aqueous solubility of steroids have led to the general perception, and maybe prejudices, that steroids are 'problem' drugs. Although the amount of data available in literature is limited, steroids may be considered as a class with a good gastrointestinal absorption representing a good passive transcellular permeability (Hardman and Limbird, 1996). To estimate gastrointestinal absorption Caco-2 cell monolayers are frequently used in the pharmaceutical industry. Recently, multiple publications showed that the Caco-2 permeability coefficient was sigmoidally related to the oral absorption in humans (Norinder et al., 1997; Gres et al., 1998; Yazdanian et al., 1998; Yee, 1997). Since it is assumed that steroids are readily absorbed, this should result in high permeability coefficients, which is indeed the case for testosterone, progesterone, corticosterone, estradiol and dexamethasone (Norinder et al., 1997; Gres et al., 1998; Yazdanian et al., 1998).

With respect to the absorption of steroids, P-glycoprotein (Pgp) and P450/17βHSD (17β-hydroxysteroid dehydrogenase) metabolizing enzymes are of importance. P-glycoprotein is expressed in Caco-2 cells depending on the stage of differentiation of the cells (Duizer, 1999). It is not known whether the amount of Pgp present in Caco-2 cells is representative for the in vivo situation. Metabolism, in general, is one of the main variables in the pharmacological action of all steroids. In a Caco-2 monolayer experiment the medium used at the beginning of confluence is of influence on the final metabolic capability of the cells (Schmiedlin-Ren et al., 1997). Depending on the cell-line used, differences might occur in P450 metabolism and Pgp activity (Raeissi et al., 1999). However, normally Caco-2 cells have a low CYP3A4 expression (Gres et al., 1998). The group of 17βHSD enzymes are essential for both the synthesis and the metabolism/inactivation of C_{19} and C_{18} steroid hormones (androgen and estrogens) (Labrie et al., 1997; Andersson et al., 1997). They play a key role in the development, growth and function of all reproductive tissues in both

males and females. In the literature no information is available whether these enzymes are present in Caco-2 cells.

The objective of the current study is to make an inventory of the physicochemical properties (solubility, logP etc.) of steroids and to evaluate which properties could be relevant for absorption. In the first instance the molecular descriptors (ClogP, molecular weight (MW), Polar Surface Area (PSA) etc.) of the steroids in Organon's structure database were calculated. In the different (defined) pharmacological groups of steroids, the discriminating descriptors were determined and on the basis of these results a set of steroids was chosen for measuring the Caco-2 apparent permeability coefficients. Using the measured Caco-2 permeability coefficients the relevant properties for absorption were analyzed.

3.2 Methods.

3.2.1 Calculation of the molecular descriptors of steroids.

The chemical structures of all steroids held in the Organon compound database were retrieved. Testosterone esters were excluded since they represent a special class of compounds with respect to their chemical structure and physicochemical properties. The ClogP, MW, charge, number of rotatable bonds, number of H-bond donors and acceptors and the static Polar Surface Area (PSA, $Å^2$) were calculated for the retrieved steroids using the methods described in Kelder et al. (1999). The static PSA does not take into account the different conformations of the chemical structures, but gives essentially the same results as the dynamic PSA. It has the advantage of a much shorter time of calculation (Kelder et al., 1999).

Based on general structural features of androgens, antiprogestagens, glucocorticoids, mineralocorticoids, estrogens and progestagens, the set of retrieved steroids was refined (Zeelen, 1990). The structural elements used are shown in Fig. 1. On the basis of the results of the theoretical calculations a selection of steroids was made, to be tested in the Caco-2 system.

3.2.2 Compounds tested in the Caco-2 cell system.

The molecular structures of the steroids and reference compounds to be tested are shown in Fig. 2. The steroidal structures represent the defined pharmacological

groups (Fig. 1). Since the majority of the steroids often possess mixed pharmacological properties, it is difficult to choose compounds with a single biological effect. However, the chemical elements shown in Fig. 1 for the different groups of steroids do in general hold and were therefore applied (Zeelen, 1990). All steroids and mannitol were supplied by NV Organon (Oss, The Netherlands). Polyethylene glycol 4000 (PEG4000), cephalexin, antipyrine, verapamil and D-glucose were supplied by Fluka and Sigma-Aldrich. The purity of all the used chemicals was higher than 98%. For the measurement of glucose, mannitol and PEG4000 radiolabeled compounds were used: ^{14}C-glucose (ICN), ^{14}C-PEG4000 (Amersham PB) and ^{3}H-mannitol (Amersham PB).

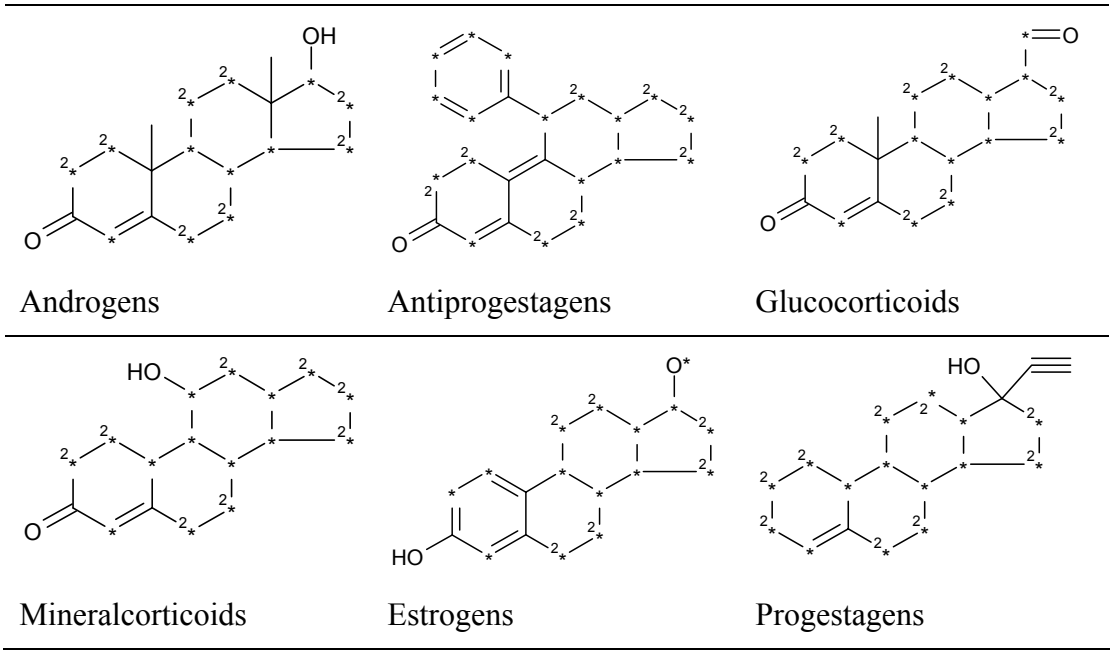


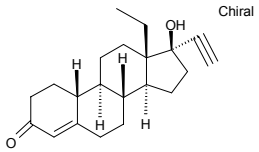
Fig. 1. Structural features of steroids belonging to different pharmacological subclasses.

** is a free site and the number indicates the number of free sites.*

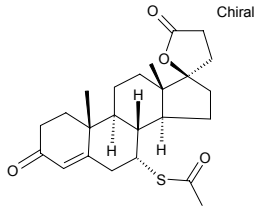
3.2.3 Caco-2 cell system.

Cell culture. The Caco-2 cells (American Type Culture Collection, code HTB 37, human colon adenocarcinoma, passage number 33 - 40) were grown in culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM), supplemented

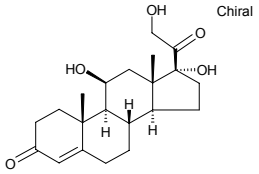
with heat-inactivated foetal calf serum (10 % v/v), non-essential amino acids (1% v/v), L-glutamine (2 mM) and gentamicin (50 µg/mL). The Caco-2 cells were cultured by seeding about 2,000,000 cells in 75 cm^2 tissue culture flasks containing culture medium. Near confluent Caco-2 cell cultures were harvested by trypsinisation and resuspended in culture medium. The cells were routinely cultured in a humidified incubator at 37 °C in air containing 5% CO_2.



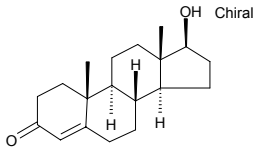
1. norgestrel



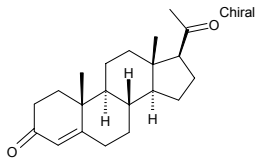
2. spironolactone



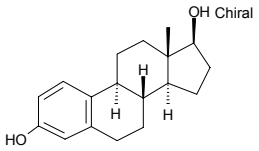
3. hydrocortisone



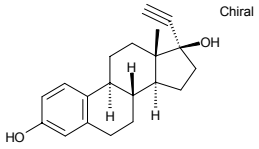
4. testosterone



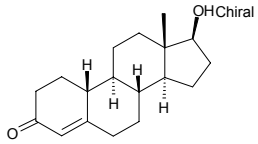
5. progesterone



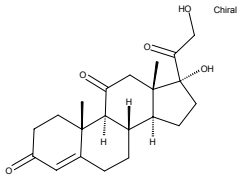
6. estradiol



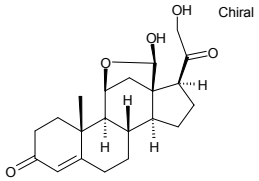
7. ethinylestradiol



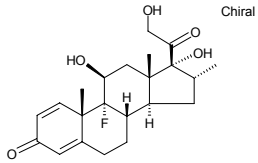
8. nandrolone



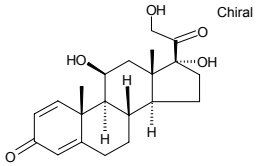
9. cortisone



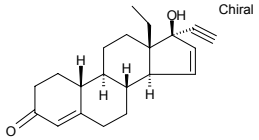
10. aldosterone



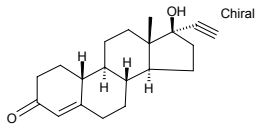
11. dexamethasone



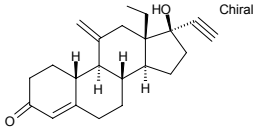
12. prednisolone



13. gestodene

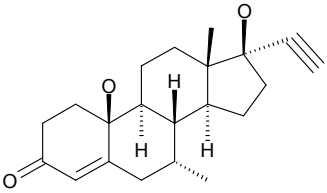


14. norethisterone

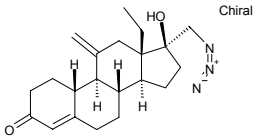


15. etonogestrel

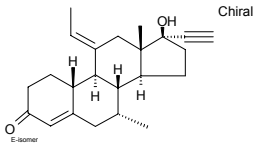
Fig. 2. Molecular structures of the steroids and reference compounds tested in the Caco-2 study.



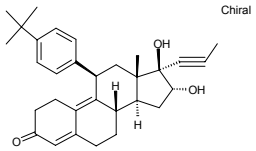
16. Org OM 08



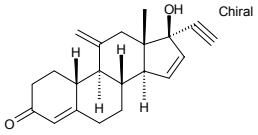
17. Org 32540



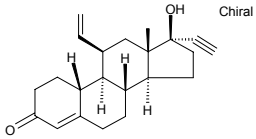
18. Org 34694



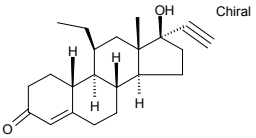
19. Org 36410



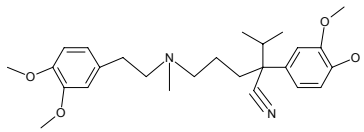
20. Org 30659



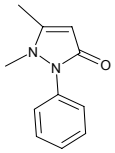
21. Org 4325



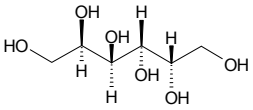
22. Org 4060



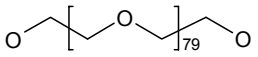
23. Verapamil



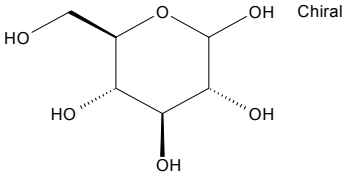
24. antipyrine



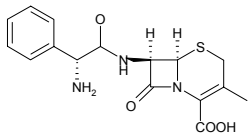
25. mannitol



26. PEG4000



27. D-glucose



28. cephalexin

Fig. 2. Cont'd.

Caco-2 cells were seeded on semi-permeable filter inserts (12 wells Transwell plates, Costar) at approximately 100,000 cells per filter (growth area 1.1 cm^2 containing 2.5 mL culture medium). The cells on the insert were cultured for 17 to 24 days at 37 °C in a humidified incubator containing 5% CO_2 in air. To check the differentiation status of the formed monolayer the transepithelial electrical resistance (TEER) was measured (Millicell-ERS epithelial voltohmmeter, Millipore Co., Bedford, USA). The TEER of the cell monolayers was calculated according to the following equation: TEER = ($R_{monolayer}$ - $R_{empty\ filter}$) x A ($\Omega.cm^2$), $R_{monolayer}$ is the resistance measured, $R_{empty\ filter}$ is the resistance of control filters without cells (approximately 140 $\Omega.cm^2$)

and A is the surface area of the filter insert (1.1 cm^2). After two to three weeks in cell culture, the monolayers developed a TEER of approximately 500 $\Omega.cm^2$.

Transport study. All test substances were tested at a high and low concentration. For the high concentration the maximum aqueous solubility was chosen and the low concentration was set at one tenth of the aqueous solubility. Antipyrine and PEG4000 were tested at 10 and 100 µM, mannitol and D-glucose at a concentration of 1 and 10 mM. Three filter inserts were used per concentration. Transport of the test substances was assessed after apical and basolateral exposure.
It was decided not to use BSA in the transport medium of the Caco-2 cells, since most steroids do not bind equally to proteins (e.g SHBG, HSA, BSA) (Kragh-Hansen, 1981; Watanabe and Sato, 1996; Baker, 1998). Furthermore, the Caco-2 permeability might be influenced by the presence of BSA in the receptor compartment as is the case for midazolam and dexamethasone (Fischer et al., 1999; Yamashita et al., 2000).
For apical exposure, culture medium was removed from the filter insert prior to moving them to a new 12 wells plate containing 1.8 mL fresh transport medium (Hanks Balanced Salt Solution, pH = 7.4, 25 mM D-Glucose, 50 mM HEPES (N-(2-hydroxyethyl) piperazine-N'-2-ethanesolphonacid), 1.25 mM $CaCl_2$, 0.5 mM $MgCl_2$). The transport study started by filling the apical chambers with 500 µL of the test solution (dissolved test substance in Hanks Balanced Salt Solution, pH = 7.4, 25 mM D-Glucose, 50 mM HEPES, 1.25 mM $CaCl_2$, 0.5 mM $MgCl_2$). In the case of D-glucose transport medium without D-glucose was prepared.
For basolateral exposure, culture medium at the apical side was replaced by 500 µL fresh transport medium (pH = 7.4) and the transport study started by transferring the filter inserts to new 12 wells plates containing 1.8 mL test solution (pH = 7.4). In the case of D-glucose transport medium without D-glucose was prepared.
All cultures were incubated on a rotating platform (approx. 30 rpm) in a humidified incubator containing 5% CO_2 in air at 37 °C. 400 µL samples were collected from the receptor compartment at 1, 2 and 4 hours after application of the test substances. Directly after each sampling the original volume was restored by adding 400 µL fresh transport medium.

3.2.4 Calculations.

The Apparent Permeability Coefficient (P_{app}, cm/s) was calculated using the following equation: $P_{app} = (dQ/dt)/(1000*A*C_0)$, where dQ/dt = initial permeability rate (mol/s), A = surface area filter insert (1.1 cm^2), and C_0 = initial concentration (mol/L).

3.2.5 Solubility.

The aqueous solubility of the steroids was measured by adding approximately 5 mg of steroid to 2 mL of demineralized water followed by agitation on a roller bench overnight. The solutions were filtrated to remove the undissolved compound. The concentration was assessed by means of HPLC as described later (see next section).

3.2.6 Concentration measurements.

All concentration measurements were determined by HPLC (HP1100 with DAD detection and temperature controlled column compartment). The following columns were used: Luna C8 (Phenomenex), Luna C18 (Phenomenex), Phenyl-Hexyl (Phenomenex) and Supelcosil LC-NH2 (Supelco). The temperature of the column was held at 30 °C. Detection was performed at 210, 250, 280, 300, and 310 nm. The injection volume was 5 – 40 µL depending on the peak area of the 0.1*Cs (= 10% of the saturation concentration Cs) peak area. The runtimes were typically between 5 and 6 min.

The concentrations of [^{14}C]-glucose, [^{14}C]-PEG4000 and [^{3}H]-mannitol were measured by using a LKB/Wallac S1409 scintillation Counter and Packard Ultima Gold scintillation liquid.

3.3 Results and discussion.

All steroids which could not be classified uniquely within the defined pharmacological subclasses (Fig. 1) were removed from the orginal database. Table 1 shows the number of steroids found in each subclass together with the number of duplicates of the other pharmacological subclasses. E.g. in the subclass of glucocorticoids, 45 androgens were present.

Analyses of the molecular descriptors of the pharmacological groups showed that there are only minor differences in the Polar Surface Areas and the steroids in general

fulfill the criterion for oral absorption: MW < 500, ClogP < 5, PSA < 110 $Å^2$ (Kelder et al., 1999; Lipinski et al., 1997). ClogP seems to be more discriminating between the defined groups of steroids, although the differences are small. Within the defined pharmacological subclasses, the androgens and mineralocorticoids tend to be more hydrophilic compared to the estrogens and glucocorticoids. Based on these results it was decided to include ClogP in the decision criteria for the selection of the steroids to be tested in the Caco-2 cell monolayer experiments.

Table 2 shows the calculated molecular descriptors of the compounds tested in the Caco-2 cell system. As expected from the theoretical evaluation of the structure database, only 1 of the steroids (Org 36410) is predicted of having a moderate oral bioavailability (i.e. the ClogP is outside the range for good oral absorption in Table 2). Fig. 3 shows that the experimental water solubility of steroids is inversely related to ClogP.

Fig. 4 shows the measured Caco-2 apparent permeability coefficients of the tested compounds for the saturated solution in the donor compartment. The permeability data from this study are comparable to Caco-2 data reported in literature (Norinder et al., 1997; Gres et al., 1998; Yazdanian et al., 1998).

Table 1. Number of steroids found in each pharmacological subclass together with the number of duplicates of the other pharmacological subclasses.

	androgens	***antiproges-tagens***	***gluco-corticoids***	***mineralo-corticoids***	***estrogens***	***progestagens***
androgens (131) *						
antiprogestagens (444) *	0					
glucocorticoids (265) *	45	0				
mineralocorticoids (100) *	21	0	52			
estrogens (364) *	0	0	0	0		
progestagens (463) *	3	0	0	0	3	

* *Total number of compounds in the indicated group.*

Table 2. Solubility and molecular descriptors of the tested compounds in the Caco-2 system.

	S	***ClogP (-2<X<5)*** [1]	***MW (X<500)*** [1]	***PSA (X<110)*** [1]	***HD (X<6)*** [1]	***HA (X<11)*** [1]
1	5.4	3.3	312.45	33.6	1	2
2	78.5	2.3	416.57	56.8	0	4
3	896.6	1.7	362.47	78.3	3	5
4	160.5	3.2	288.43	38.2	1	2
5	24.8	3.8	314.47	31.4	0	2
6	11.0	3.8	272.39	43.8	2	2
7	34.4	3.9	296.41	42.7	2	2
8	1002.9	2.7	274.4	38.1	1	2
9	776.8	1.3	360.45	77.1	2	5
10	1776.7	-0.1	360.45	70.1	2	5
11	254.8	1.8	392.47	73.8	3	5
12	481.1	1.4	360.45	77.7	3	5
13	24.2	3.0	310.44	35.3	1	2
14	17.8	2.8	298.42	37.1	1	2
15	13.3	3.3	324.46	34.0	1	2
16	674.7	1.7	328.45	50.6	2	3
17	22.5	4.5	355.48	70.5	1	4
18	2.1	3.7	338.49	37.0	1	2
19	6.5	5.9	458.64	55.2	2	3
20	162.1	2.5	308.42	38.2	1	2
21	33.9	3.3	324.46	37.2	1	2
22	2.8	3.8	326.48	37.4	1	2
23		4.5	454.61	61.9	0	6
24		0.8	188.23	21.6	0	3
25		-2.1	182.17	107.9	6	6
26		0.1	1061.26	181.5		
27		-2.2	180.16	105.6	5	6
28		-1.6	347.39	93.0	4	7

*S = Solubility (mol/L, * 10^6), ClogP = calculated log P (octanol / water), MW = Molecular Weight, PSA = Polar Surface Area ($Å^2$), HD = number of hydrogen bond donor sites, HA = number of hydrogen bond acceptor sites,* [1] *= criteria for good oral absorption. The numbers in the first column correspond to the compounds in Fig. 2.*

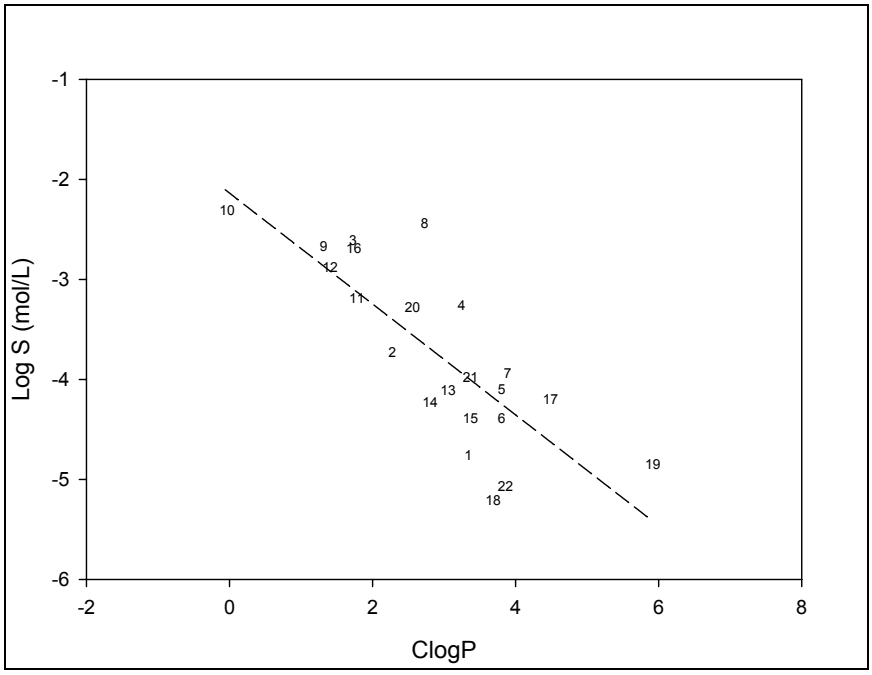


Fig. 3. Aqueous solubility of steroids as function of ClogP.

Regression line: Y = -0.55X –2.14 (r^2 = 0.64). The numbers correspond to the compounds of Fig. 2.

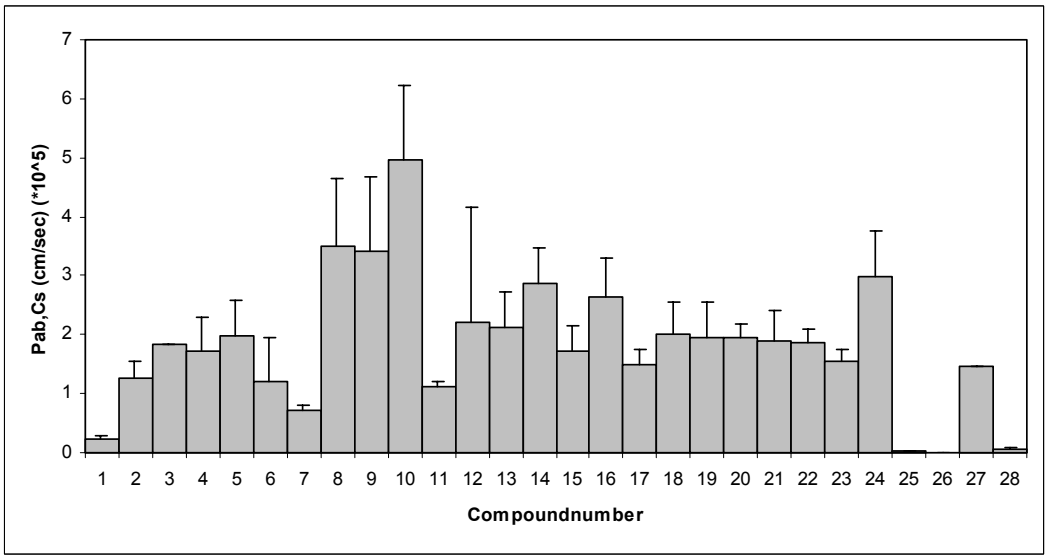


Fig. 4. Caco-2 permeability coefficients of the compounds tested.

$P_{ab,Cs}$ = apparent permeability coefficient from the apical to the basolateral side, C_s = at saturation concentration. The numbers correspond to the compounds of Fig. 2. The error bars indicate the standard deviation (n=3).

Comparing the permeability coefficients of the tested steroids with the reference compounds having a low permeability coefficient (mannitol and PEG4000: $P_{app} < 1*10^{-6}$ cm/s) shows that the steroids are all well transported (i.e. $P_{app,steroid} > \pm 1*10^{-5}$ cm/s). However, based on the permeability coefficient three groups of steroids can be distinguished. First, the group of steroids with the highest permeability coefficient, comparable to antipyrine ($P_{app,steroid} > 2*10^{-5}$ cm/s) (nandrolone, cortisone, aldosterone, prednisolone, gestodene, norethisterone, Org OM08 and Org 34694). Secondly, the group of steroids with an average permeability coefficient, comparable to verapamil ($1*10^{-5} < P_{app,steroid} < 2*10^{-5}$ cm/s) (spironolactone, hydrocortisone, testosterone, progesterone, estradiol, dexamethasone, etonogestrel, Org 32540, Org 36410, Org 30659, Org 4325 and Org 4060). And finally, the group of steroids with the lowest permeability coefficient ($P_{app,steroid} < 10^{-5}$ cm/s) (norgestrel and ethinylestradiol). The permeability coefficient for this last group is still much greater than for compounds such as mannitol or PEG4000, known for their low permeability. The high permeability coefficients of the diverse group of steroids tested are consistent with the general opinion that they are well absorbed. This is confirmed by the observation that the direction of transport (Fig. 5) and the concentration of steroid in the donor compartment (C_s or $0.1*C_s$; results not shown) have no influence on the measured permeability coefficients.

Although several reports are available in which the metabolic capability of Caco-2 is described, no metabolism was detected in this study (Schmiedlin-Ren et al., 1997; Raeissi et al., 1999; Lampen et al., 1998; Prueksaritanont et al., 1996). Metabolite formation would lead to differences in the steroid retention time in the HPLC chromatograms due to changes in the chemical structure or the appearance of secondary peaks. No differences in retention time or secondary peaks were detected, hence, the high permeability of steroids cannot be attributed to metabolism.

The results in Fig. 5 clearly show that the ratio of the apical to basolateral (ab) and basolateral to apical (ba) transport rates are all well within the 0.5 (influx) to 2 (efflux) bandwidth, which is generally considered as the margin for active transport. The Caco-2 cells used in this study have a high expression of Pgp (Versantvoort et al., 2002). Active transport is only present for glucose, which has a transporter in the intestinal membrane (Duizer, 1999), resulting in a high permeability coefficient. The

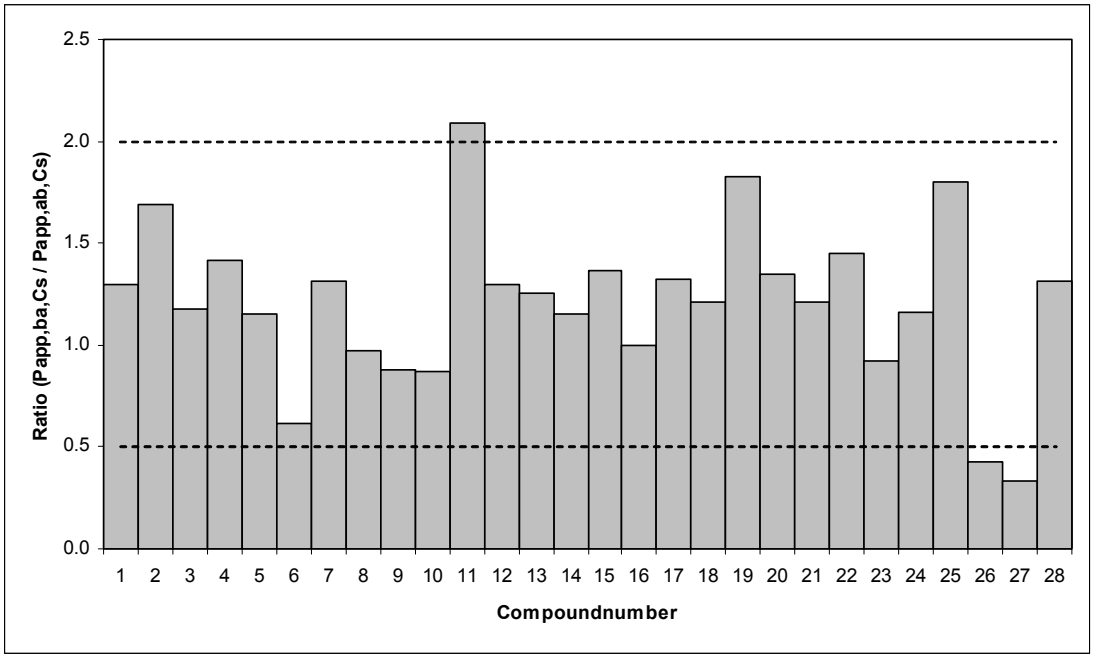


Fig. 5. Ratio of the basolateral to apical ($P_{ba,Cs}$) and apical to basolateral ($P_{ab,Cs}$) Caco-2 permeability coefficients.

C_s = at saturation concentration. The dashed lines indicate the transitions towards influx (0.5) or efflux transport (2.0). The numbers correspond to the compounds of Fig. 2.

ratio of approx. 0.5 for PEG4000 is probably caused by the extremely low transport, with the low concentrations measured during the permeability experiment affecting the accuracy. From the present study it cannot unambiguously be deduced whether steroids are actively transported, since the reference drug verapamil was tested at concentrations which were too high (15 and 150 mM), resulting in saturation of the Pgp transporter (Sandstrom et al., 1998). However, the results are consistent with the view that steroid absorption occurs by passive diffusion. In several literature surveys no indication was found for the presence of (active) influx transporters for steroids in the intestinal membrane.

However, there are many indications of interactions between steroids and P-glycoprotein (Pgp) (Ecker et al., 1999; Ernest and Bello-Reuss, 1998; Barnes et al., 1996). Steroids are known for their potency of antagonistic action on Pgp (Barnes et al., 1996; Deliconstantinos and Fotiou, 1986). Estradiol, corticosterone, aldosterone,

cortisol and dexamethasone are (potential) substrates for P-glycoprotein (Barnes et al., 1996). In this particular study human colon carcinoma cells which had been treated to obtain an unusually high Pgp expression (SW620 Ad300 cells) were used. No literature data are available on the amount of Pgp present in the intestine / colon in comparison with the cell lines used in in vitro experiments. Studies using rat intestine or pig kidney showed the Pgp transport of methylprednisolone, aldosterone, hydrocortisone, estriol and dexamethasone (Saitoh et al., 1998). In the present study there is no indication of steroid transport by P-glycoprotein, although it is known that Pgp is abundantly expressed in the used Caco-2 cells (Versantvoort et al., 2002). Due to the high permeability coefficients of the steroids it may be possible that Pgp efflux transport is not observed (Lentz et al., 2000). In Fig. 6 the Caco-2 permeability is plotted as function of the Polar Surface Area. All the steroids are located in the high permeability part of Fig. 6, corresponding to a high absorption (Norinder et al., 1997; Gres et al., 1998; Yazdanian et al., 1998). No sigmoidal relation is found between the apparent permeability coefficient and the Polar Surface Area, since all the steroids are located in the high permeability part of Fig. 6. The absence of this relationship is in line with several reports (Winiwarter et al., 1998; Oprea and Gottfries, 1999) which questioned the suggested presence of such a relationship (Norinder et al., 1997; Gres et al., 1998; Yazdanian et al., 1998).

Fig. 7 shows the apparent permeability coefficient as function of the calculated ClogP. As shown, the small differences in permeability observed within this series of steroids have a weak inverse correlation with ClogP: the hydrophilic steroids (ClogP approx. 0 – 2) tend to diffuse faster over the cell monolayers compared to the more hydrophobic steroids (ClogP approx. 5). In vivo the slower transport of the more hydrophobic steroids may be more prominent since the gastrointestinal membrane is covered with a mucous layer. The found weak correlation with ClogP is consistent with the absence of systematic variations in molecular size (MW) and hydrogen bonding capacity (number of hydrogen bond donor and acceptor sites) in this set of steroids (Table 2). I.e. since MW, hydrogen bonding capacity and lipophilicity (ClogP) are often intercorrelated an apparent linear relationship is found between permeability and one of these three descriptors (van de Waterbeemd et al., 1996).

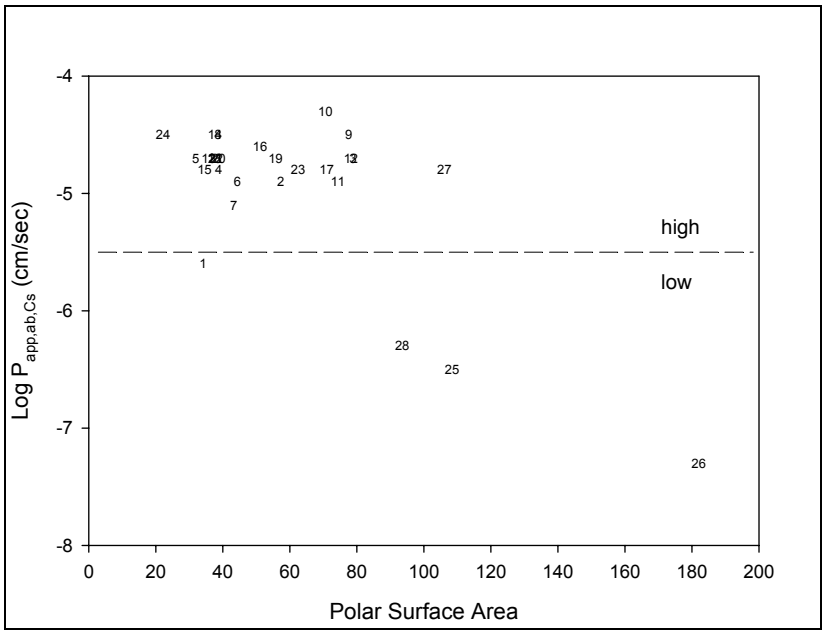


Fig. 6. Caco-2 permeability coefficient as function of the Polar Surface Area.
$P_{ab,Cs}$ = apparent permeability coefficient apical to basolateral transport at saturation concentration. The dashed line indicates the transition of a high to a low permeability. The numbers correspond to the compounds of Fig. 2.

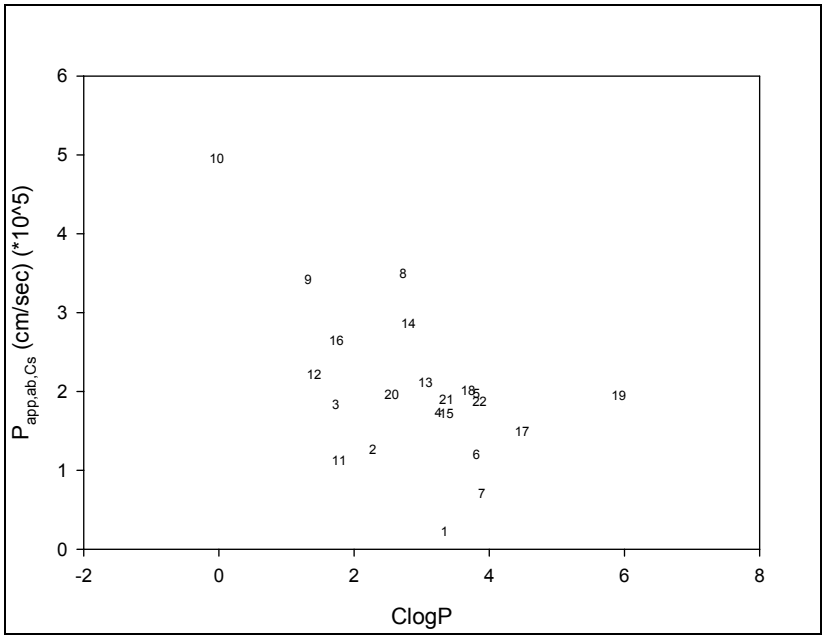


Fig. 7. Caco-2 permeability coefficient of steroids as function of the calculated ClogP.
$P_{ab,Cs}$ = apparent permeability coefficient apical to basolateral transport, C_s = at saturation concentration. The numbers correspond to the compounds of Fig. 2.

The presented results clearly show that despite the hydrophobic character and low water solubility, steroids are well transported over the Caco-2 cell monolayer.
The results of the Caco-2 permeability study are consistent with the analyses of the steroid database. The Polar Surface Area is more or less the same for all the steroids tested and is (probably) only indicative for absorption (all steroids fall within the same range of the Caco-2 permeability). With ClogP a trend can be seen that the more hydrophilic steroids are better absorbed than the more hydrophobic ones. However, compared to the compounds with a low apparent permeability coefficient (mannitol, PEG4000 and L-dopa) steroids are still transported well.

In the present study it is clearly shown that steroids are readily absorbed. But what is the underlying mechanism? It is well known that steroids are able to influence the fluidity of biological membranes, as does cholesterol (Stryer, 1995; Lamche et al., 1990; Mahesh et al., 1996). Since cholesterol contains a steroid skeleton and is a 'major' constituent of biological membranes, the preference of steroids for biological membranes might be explained. Therefore, one could argue that absorption might be facillitated due to an increased membrane fluidity. In the literature no data are available concerning the absorption of steroids and membrane fluidity. However, pharmacological effects of steroids are reported to be related to a change in membrane fluidity (Mahesh et al., 1996; Golden et al., 1999; Godstein, 1984). The so-called steroid anesthetics (very hydrophilic steroids ClogP << 0) are a special case where the pharmacological action is linked to changes in membrane fluidity (Goldstein, 1984). Even though membrane fluidity is frequently linked to steroid action no literature is available on absorption and membrane fluidity.
Furthermore, there were no indications concerning active transport (influx as well as efflux). Therefore, the only way to explain the good absorption of steroids, in a mechanistic way, is their 'natural' preference for the biological membrane, i.e. the steroids partition between the membrane and the surrounding aqueous phase. This is substantiated by the relationship found between the apparent permeability coefficient and the ClogP. Hence, transport takes place by means of the concentration difference over the membrane (= passive diffusion).

3.4 Conclusions.

Analyses of the molecular descriptors of the steroids in Organon's database showed that the compounds generally reflect the properties needed for a good oral absorption. Minor variations were found for the Polar Surface Area, molecular weight, number of Hydrogen bond donor and acceptor sites and the number of rotatable bonds. The calculated logP was more discriminative.

The Caco-2 cell monolayer system gave permeability data which are comparable to other literature sources, indicating that the system worked well. Generally, steroids are all well transported, although differences in the permeability coefficients were present. In the present study, no indications were found for active transport (influx as well as efflux) or metabolism.

The high apparent permeability coefficient of most steroids is in agreement with the expected behavior for compounds with small Polar Surface Areas (below 110 $Å^2$). The small differences in permeability observed within this series of steroids have a weak inverse correlation with ClogP: the hydrophilic steroids tend to diffuse faster over the cell monolayers in comparison to the hydrophobic steroids, which diffuse more slowly. The relationship with ClogP suggests that partitioning of the steroids between the biological membrane and the surrounding aqueous phase is one of the main mechanisms for absorption, indicating passive diffusion. This is supported by the observation that the apparent permeability coefficient was not influenced by the concentration. Since steroids with a lower ClogP tend to a higher permeability, it might be of interest for the synthesis and selection of NCEs to choose the compound with the lower ClogP.

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4 Caco-2 permeability, P-glycoprotein transport ratios and brain penetration of heterocyclic drugs.

Abstract

In this study the gastrointestinal absorption and P-glycoprotein (Pgp) efflux transport of heterocyclic drugs was investigated with the Caco-2 cell model. As the possibility for Pgp transport is routinely tested in High Throughput Screenings (HTS) assays, the Caco-2 data were compared to the results of in vivo tests. I.e. since Pgp is expressed at the Blood Brain Barrier as well, it can be expected that CNS penetration will be impaired if a drug is a Pgp substrate.

Based on the calculation of the physicochemical properties a good oral absorption was predicted for all the drugs tested in this study which corresponded well with the measured Caco-2 permeabilities (P_{app}). Generally a high permeability was measured, being in agreement with earlier published human in vivo absorption data.

Based on the transport data of domperidone and verapamil it was found that the Pgp efflux transporter was expressed in the Caco-2 cells. Many of the drugs tested were indicated to be potential Pgp efflux substrates. However, no correlation could be found between brain penetration in rats and the Pgp efflux ratio as measured with the Caco-2 cells.

From the data it is concluded that Pgp efflux ratio's as determined in in vitro High Throughput Screening tests, where the transport conditions are fixed (pH gradient, concentration etc.), cannot routinely be used to predict a possible affected brain penetration.

Int. J. Pharm. 263 (2003) 113-122.

4.1 Introduction.

In the discovery and development of New Chemical Entities (NCE's) the absorption of the drug and possible interactions with transporter systems are important variables (Testa et al., 2001). Transporters are of special interest since they can be of influence on the absorption and distribution of drugs. Drugs intended to treat depression and psychotic disorders need to cross firstly the enterocytes in the gastrointestinal (gi) tract and secondly the Blood Brain Barrier (BBB) to enter the Central Nervous System (CNS) (Sandt, 2001; Pratt and Taylor, 1990, Williams, 1995).
In the gi-tract and in the BBB transporter systems are present, from which the P-glycoprotein (Pgp) transporter is the most frequently studied (Cordon-Cardo et al., 1989; Thiebaut et al., 1987; Rodrigues, 2002). Pgp serves as an ATP dependent efflux pump that exports a large number of structurally unrelated substrates out of the cell. In general it is thought that Pgp limits intestinal absorption as well as penetration of the CNS, since this transporter is widely expressed in the gi-tract and the BBB (Rodrigues, 2002).
Since in Caco-2 cells the Pgp transporter is strongly expressed, due to their tumorous nature (Romsicki and Sharom, 1999; Rodrigues, 2002), this cell system is widely used in in vitro screening technologies to predict gastrointestinal absorption and possible Pgp transport of NCE's (Stoner et al., 2000; Sandt, 2001; Testa et al., 2001). However, little information is available which links Pgp transport to possible in vivo effects (Trouman and Thakker, 2001; Chiou et al., 2001; Ahmed et al., 2000).
The objective of this study is to evaluate the Caco-2 permeability and possible (Pgp) efflux transport of a series of heterocyclic drugs. Furthermore, using the (Pgp) efflux transport data of the Caco-2 experiments it is examined whether (Pgp) efflux influences brain penetration in rats in vivo.

4.2 Materials and Methods.

4.2.1 Compounds tested.

The chemicals were supplied by the following manufacturers. Sigma / Aldrich: salicylic acid, acetylsalicylic acid, carbamazepine, imipramine hydrochloride, caffeine, verapamil, amitriptyline hydrochloride, ranitidine hydrochloride, clonidine hydrochloride, desipramine hydrochloride, PEG4000, pyrilamine maleate, antipyrine,

haloperidol, acetaminophen and indomethacin. Janssen Research Foundation: risperidone and domperidone. NV Organon: quinidine, ibuprofen, morphine, Org 25907 (3-OH-4,4-dimethyl-1-[4-[4-(2-pyrimidinyl)-1-piperazinyl]butyl]-2,6-piperidinedionemonohydrochloride), Org 9935 (4,5-Dihydro-6-(5,6-dimethoxybenzo[*b*]thien-2-yl)-5-methyl-3(2H)-pyridazinone), Org 12962 (1-[6-Chloro-5-(trifluoromethyl)-2-pyridinyl]piperazine hydrochloride), Org 23430 (4-[(4-Fluorophenyl)-4-chlorothienyl-2-methylene]-methylpiperidinebutane-1,4-dioate), Org 5222 (asenapine) (trans-DL-5-Chloro-2,3,3*a*,12*b*-tetrahydro-2-methyl-1H-dibenz[2,3:6,7]oxepino[4,5-c]pyrrole (Z)-2-butenedioate), Org 13011 (1-[4-[4-[4-(Trifluoromethyl)-2-pyridinyl]-1-piperazinyl]butyl]-2-pyrrolidinone(E)-2-butenedioate), Org 33062 (4,4-Dimethyl-1-[4-[4-(2-pyrimidinyl)-1-piperazinyl]butyl]-2,6-piperidinedionemonohydrochloride), Org 23366 (1-[4-[4-[bis(4-fluorophenyl)methylene]-1-piperidinyl]-1-oxobutyl]pyrrolidine methanesulfonate), Org 34037 (R(-)-6-(4-Chlorophenyl)-2,3,5,6-tetrahydroimidazo[2,1-a]isoquinoline(E)-2-butenenedioate), Org 32782 (Methyl 2,6-di-deoxy-2,6-di-amino-alpha-D-mannopyranosyl-(1-2)-O-alpha-D-mannopyranoside diacetate), Org 34167 ((-)-2-(1,2-Benzisoxazol-3-yl)-alpha-(2-propenyl)benzenemethanamine hydrochloride) and mannitol. The purity of all the used compounds was higher than 98%.

The radio labeled compounds were supplied by the following manufacturers. ICN: [^{3}H]-mannitol. Amersham: [^{14}C]-PEG 4000. Perkin Elmer Life Science: [^{3}H]-verapamil and [^{14}C]-caffeine. Janssen Research Foundation: [^{3}H]-domperidone and [^{3}H]-risperidone. NV Organon: [^{3}H]-Org 12962, [^{14}C]-Org 23430, [^{3}H]-Org 5222, [^{3}H]-Org 13011, [^{14}C]-Org 23366, [^{3}H]-Org 34037 and [^{3}H]-Org 32782.

4.2.2 Calculation of the physico chemical properties.

The chemical structures of the drugs were retrieved from the Organon compound database. The ClogP, MW and the static Polar Surface Area (PSA, Å^{2}) were calculated using the methods described in (Kelder et al., 1999). The static PSA does not take into account the different conformations of the chemical structures, but gives

essentially the same results as the dynamic PSA. It has the advantage of a much shorter time of calculation (Kelder et al., 1999).

4.2.3 Cell culture.

The Caco-2 cells (ATCC, code HTB 37, human colon adenocarcinoma, passage number 29-33) were grown in culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM), supplemented with heat-inactivated foetal calf serum (10 % v/v), non-essential amino acids (1% v/v), L-glutamine (2 mM) and Penicilline/Streptomycine (100 IU µg/mL and 0.1 mg/mL respectively). The Caco-2 cells were cultured by seeding about 2,000,000 cells in 80 cm^2 tissue culture flasks containing culture medium. Near confluent Caco-2 cell cultures were harvested by trypsinisation and resuspended in culture medium. The cells were routinely cultured in a humidified incubator at 37 °C in air containing 5% CO_2.

Caco-2 cells were seeded on semi-permeable filter inserts (Costar 24-well Transwell plates) at ca. 21,000 cells per filter growth area 0.33cm^2 (containing 0.1 mL culture medium). The cells on the insert are cultured for 22 to 24 days at 37 °C in a humidified incubator containing 5% CO_2 in air.

To check the differentiation status of the formed monolayer the transepithelial electrical resistance (TEER) was measured (Millicell-ERS epithelial voltohmmeter, Millipore Co., Bedford, USA). The TEER of the cell monolayers was calculated according to the following equation:

TEER = ($R_{monolayer}$ - $R_{empty\ filter}$) x A ($\Omega.cm^2$), where $R_{monolayer}$ is the resistance measured, $R_{empty\ filter}$ is the resistance of control filters without cells (approximately 140 $\Omega.cm^2$) and A is the surface area of the filter insert (0.33 cm^2). After two to three weeks in cell culture, the monolayers developed a TEER of approximately 600 $\Omega.cm^2$.

4.2.4 Drug transport experiments.

All test substances were tested at a high (1 mM) and low (0.1 mM) concentration. Antipyrine and PEG4000 were tested at 100 and 10 µM, whereas mannitol, verapamil, caffeine, Org 12962, Org 5222 and Org 13011 at 10 and 1 mM respectively. Three filter inserts were used per concentration. Transport of the drugs

was assessed after apical exposure and after basolateral exposure. Per drug tested all Caco-2 tests were performed with cells of the same passage number.

It was decided not to use BSA in the transport medium of the Caco-2 cells, since most drugs do not bind equally to proteins (e.g SHBG, HSA, BSA) (Kragh-Hansen, 1981; Wanatebe and Sato, 1996; Baker, 1998). In our study protein binding of the drugs varied from 0 - 99% (Hardman and Limbird, 1996). Furthermore, the Caco-2 permeability might be influenced by the presence of BSA in the receptor compartment as is the case for midazolam and dexamethasone (Fischer et al., 1999; Yamashita et al., 2000), i.e. in a diffusion experiment the permeability is mainly determined by the free fraction.

In the current study a physiologically relevant pH gradient (pH = 6.5 / pH = 7.4) was applied since the apparent permeability values in the apical to basolateral direction are reportedly more predictive of human in testinal absorption than using pH 7.4 on both sides (Boisset et al., 2000; Yamashita et al., 2000).

For apical exposure, culture medium was removed from the filter insert prior to moving them to a new 24 wells plate containing 0.6 mL fresh transport medium (Hanks Balanced Salt Solution, pH = 7.4, 25 mM D-Glucose, 50 mM HEPES, 1.25 mM $CaCl_2$, 0.5 mM $MgCl_2$). The transport study starts by filling the apical chambers with 100 µL of the test solution (in Hanks Balanced Salt Solution, pH = 6.5, 25 mM D-Glucose, 50 mM HEPES, 1.25 mM $CaCl_2$, 0.5 mM $MgCl_2$). After 1, 2 and 4 hours the inserts were transferred to new 24 wells plates containing fresh transport medium. Samples were withdrawn of the receptor compartments. The applied pH gradient was present during the complete course of the experiment.

For basolateral exposure, culture medium at the apical side was replaced by 100 µL fresh transport medium (pH = 6.5) and the transport study started by transferring the filter inserts to new 24 wells plates containing 0.6 mL test solution (pH = 7.4). All cultures were incubated on a rotating platform in a humidified incubator containing 5% CO_2 in air at 37 °C. Samples of the receptor compartments were collected at 1, 2 and 4 hours after application of the test substances and directly after sampling the original volume was restored by adding fresh transport medium. The applied pH gradient was present during the complete course of the experiment.

4.2.5 Calculations.

The Apparent Permeability Coefficient (P_{app}, cm/s) was calculated using the following equation: $P_{app} = (dQ/dt)/(1000*A*C_0)$, where dQ/dt = initial permeability rate (mol/s), A = surface area filter insert (0.33 cm^2), C_0 = initial concentration (mol/L).
The permeability ratio was calculated according to: $P_{ratio} = P_{app,ba} / P_{app,ab}$
where $P_{app,ba}$ = permeability from the basolateral to the apical side (blood to intestine) (cm/s) and $P_{app,ab}$ = permeability from the apical to the basolateral side (intestine to blood) (cm/s).

4.2.6 Concentration measurements.

All the concentrations of the non-radiolabeled compounds were determined by HPLC (HP1100 with DAD detection and temperature controlled column compartment). The following columns were used: Luna C8 (Phenomenex), Luna C18 (Phenomenex), Phenyl-Hexyl (Phenomenex) and Supelcosil LC-NH2 (Supelco). The temperature of the column was held at 30 °C. Detection was performed at 210, 250, 280, 300 and 310 nm. The injection volume was 5 – 40 µL depending on the peak area of the lowest concentration used. The runtimes were typically between 5 and 6 min.
The concentrations of the ^{14}C- and ^{3}H-labeled compounds were measured by using a LKB/Wallac S1409 scintillation Counter and Packard Ultima Gold scintillation liquid.

4.3 Results and discussion.

4.3.1 Caco-2 permeability data.

Calculations of the physicochemical properties show that most compounds used in this study are predicted to have a good oral absorption, except for mannitol and PEG4000 (Table 1). Both compounds are highly polar and are known for their bad oral absorption. Similar results are obtained for the prediction of drugs into the CNS. The wide majority of the tested drugs is well transported over the Caco-2 cell monolayers which is consistent with the Polar Surface Area data (Tables 1 and 2) (Kelder et al., 1999; Palm et al., 1997). I.e. the permeability is approximately 10^{-5} cm/s or higher, corresponding to a high absorption (antipyrine (97%), caffeine (100%), desipramine (95-100%), imipramine (100%), ranitidine (50%), clonidine (100%), acetominophen (60-70%), acetyl salicylic acid (68-100%), quinidine (100%),

indomethacin (100%), salicylic acid (100%), ibuprofen (100%)) (Yazdanian et al., 1998; Norinder et al., 1997; Gres et al., 1998). Even the low permeability compounds (morphine (bioavailability 80% (Yee, 1997)) and Org 9935) are transported at a much higher rate than the reference compounds with a known low permeability (mannitol and PEG4000 having a bioavailability of 13% and 1% respectively) (Gres et al., 1998). The Caco-2 permeability data from this study are comparable to data reported in literature indicating that the cell system used in this study worked adequate (Yee, 1997; Yazdanian et al., 1998; Camenish et al., 1998). For the drugs which did not show polarized transport (see section 3.2) the apical to basolateral permeability was not influenced by the concentration in the donor compartment (log $P_{ab,low}$ = 0.97 x log $P_{ab,high}$; r^2 = 0.82).

Although several reports are available in which the metabolic capability of Caco-2 cells is described, no metabolism was detected in this study (Schmiedlin-Ren et al., 1997; Raeissi et al., 1999; Lampen et al., 1998; Prueksaritanont et al., 1996). I.e. metabolite formation would lead to differences in the drug retention time and extra peaks in the HPLC chromatograms, due to changes in the chemical structure. Since the retention time did not change, during transport over the cells, it is concluded that metabolism was absent.

4.3.2 P-glycoprotein transport.

To investigate possible efflux transport of the CNS drugs by the P-glycoprotein (Pgp) transporter the permeability ratio was calculated. The calculation of the permeability ratio is explained in detail in paragraph 4.2.5. In general it is assumed that a permeability ratio of 2 and higher is indicative for Pgp transport, corresponding to a net efflux transport of the drug (Karlsson et al., 1993). I.e. drugs are preferentially transported towards the gastrointestinal (apical) side of the Caco-2 cells. The Caco-2 cells used in this study have a high expression of Pgp (Versantvoort et al., 2002). The calculated permeability ratios are given in Table 2.

Table 1. Calculated physico chemical parameters of the drugs tested.

	Compound	***Clog P***	***PSA***	***MW***	***Prediction of absorption***		***pK_a***
		(0 < X < 6) [a] ***(-2< X <5)*** [b]	***(0 < X < 70)*** [a] ***(0 < X <110)*** [b]	***(0 < X < 450)*** [a] ***(0 < X < 500)*** [b]	***Oral***	***CNS***	
1	Mannitol	-2.1	97.9	182.2	Moderate	Bad	
2	PEG 4000	0.1	181.5	1061.3	Bad	Bad	
3	Verapamil	4.5	54.5	454.6	Good	Bad	8.8 [e]
4	Antipyrine	0.2	20.2	188.2	Good	Good	1.4 [c]
5	Caffeine	-0.04	45.8	194.2	Good	Moderate	0.2 [c]
6	Desipramine HCl [f]	4.5	14.0	266.4	Good	Good	10.1 [c]
7	Imipramine HCl [f]	5.0	8.0	280.4	Good	Good	9.5 [c]
8	Amitriptyline HCl [f]	4.9	4.0	277.4	Good	Good	9.4 [c]
9	Carbamazepine [f]	2.0	34.9	236.3	Good	Good	3
10	Ranitidine HCl	0.6	70.8	314.4	Good	Moderate	8.2
11	Domperidone [f]	4.3	56.5	425.9	Good	Good	7.6 / 11.1 / 11.8 [d]
12	Clonidine HCl	1.4	29.8	230.1	Good	Good	8.3 [c]
13	Pyrilamine Maleate	3.2	26.8	285.4	Good	Good	4.0 / 8.9 [c]
14	Haloperidol [f]	3.9	32.6	375.9	Good	Good	8.3 [c]
15	Acetaminophen	0.5	38.6	151.2	Good	Good	9.5 [c]
16	Acetylsalicylic acid	1.0	49.6	180.2	Good	Good	3.5 [d]
17	Quinidine HCl	2.8	39.1	324.4	Good	Good	4.2 / 8.3 [c]
18	Morphine	0.6	45.4	285.3	Good	Good	9.9 [c]

Table 1. Cont'd.

	Compound	***Clog P*** ***(0 < X < 6)*** [a] ***(-2< X < 5)*** [b]	***PSA*** ***(0 < X < 70)*** [a] ***(0 < X <110)*** [b]	***MW*** ***(0 < X < 450)*** [a] ***(0 < X < 500)*** [b]	***Prediction of absorption***		***pK_a***
					Oral	***CNS***	
19	Indomethacin	4.2	53.0	357.8	Good	Good	4.5 [c]
20	Salicylic acid	2.2	44.9	138.1	Good	Good	3.0 [c]
21	Ibuprofen	3.7	28.6	206.3	Good	Good	5.2 [c]
22	Risperidone [f]	2.6	49.8	410.5	Good	Good	3.1 / 8.2 [d]
23	Org 12962 [f]	1.7	24.0	265.7	Good	Good	8.4 [d]
24	Org 23430 [f]	5.0	4.0	321.8	Good	Good	9 [d]
25	Org 5222 [f]	4.6	12.8	285.8	Good	Good	8.6 [d]
26	Org 33062 [f]	0.8	56.5	359.5	Good	Good	7.3 [d]
27	Org 25907 [f]	0.4	72.8	375.5	Good	Moderate	
28	Org 13011 [f]	1.4	34.2	370.4	Good	Good	7.3 [d]
29	Org 34037 [f]	4.6	13.8	282.8	Good	Good	9.8 [d]
30	Org 23366 [f]	4.7	20.2	424.5	Good	Good	8.6 [d]
31	Org 9935	3.2	49.7	304.4	Good	Good	
32	Org 32782	4.5	27.4	268.1	Good	Good	9 [d]
33	Org 34167 [f]	3.1	38.5	264.3	Good	Good	8.4 [d]

ClogP = calculated log P, PSA = Polar Surface Area, MW = Molecular weight.

[a] Criteria for CNS penetration. [b] Criteria for oral absorption. The criteria for oral and CNS penetration are based on (Lipinski et al., 1997) and (Kelder et al., 1999). [c] (Newton and Kluza, 1978) [d] own measurement [e] (Hasegawa et al., 1984) [f] CNS drug

Table 2. Caco-2 permeability and transport ratio of the drugs tested

No.	Drug	High concentration			Low concentration		
		$P_{app, ab}$	(Sdev [a])	P_{ratio}	$P_{app,ab}$	(Sdev [a])	P_{ratio}
1	Mannitol	6.30E-07	(0.51)	0.7	7.06E-07	(0.99)	1.4
2	PEG 4000	3.22E-07	(0.39)	1.0	2.70E-07	(0.29)	1.2
3	Verapamil	6.24E-05	(0.45)	0.6	9.17E-06	(15.4)	6.5
4	Antipyrine	5.56E-05	(0.45)	1.6	9.68E-05	(0.60)	1.0
5	Caffeine	6.05E-05	(0.47)	1.2	4.23E-05	(0.42)	1.6
6	Desipramine HCl	2.12E-05	(0.62)	4.0	-	(-)	-
7	Imipramine HCl	1.89E-05	(0.22)	4.8	1.37E-05	(0.48)	6.0
8	Amitriptyline HCl	2.10E-05	(0.04)	3.9	1.73E-05	(0.43)	4.5
9	Carbamazepine	2.70E-05	(0.27)	1.3	5.01E-05	(0.00)	1.3
10	Ranitidine HCl	4.59E-06	(0.47)	0.7	2.43E-05	(0.00)	0.5
11	Domperidone	3.18E-06	(1.17)	15.1	1.48E-06	(0.63)	36.3
12	Clonidine HCl	2.18E-05	(0.18)	3.0	3.40E-05	(0.00)	3.0
13	Pyrilamine Maleate	1.87E-05	(0.13)	0.3	2.51E-05	(0.00)	-
14	Haloperidol	3.93E-06	(0.49)	-	1.68E-05	(0.00)	-
15	Acetaminophen	3.16E-05	(0.05)	1.4	6.16E-05	(0.46)	1.8
16	Acetylsalicylic acid	6.67E-05	(0.39)	0.4	3.35E-05	(0.00)	0.5
17	Quinidine HCl	1.28E-05	(0.13)	3.2	4.17E-06	(-)	9.9
18	Morphine	2.54E-06	(0.00)	3.5	-	(-)	-
19	Indomethacin	6.17E-05	(0.85)	0.6	3.93E-05	(0.49)	0.9
20	Salicylic acid	4.35E-05	(0.46)	0.5	5.80E-05	(1.00)	0.5
21	Ibuprofen	5.64E-05	(0.32)	0.4	5.83E-05	(0.00)	0.5
22	Risperidone	1.42E-05	(0.11)	5.0	1.12E-05	(0.08)	5.7
23	Org 12962	2.62E-05	(0.08)	1.3	2.40E-05	(0.22)	3.2
24	Org 23430	6.10E-06	(-)	6.6	6.10E-06	(1.00)	6.4
25	Org 5222	2.85E-05	(0.20)	1.0	9.11E-06	(0.44)	5.9
26	Org 33062	1.49E-05	(0.27)	4.3	2.53E-05	(0.00)	4.0
27	Org 25907	1.74E-05	(0.30)	3.3	8.42E-06	(0.00)	12.3
28	Org 13011	1.39E-05	(0.15)	1.1	2.22E-05	(0.10)	2.3

Table 2. Cont'd.

No. Drug	***High concentration***			***Low concentration***		
	$P_{app, ab}$	***(Sdev [a])***	P_{ratio}	$P_{app,ab}$	***(Sdev [a])***	P_{ratio}
29 Org 34037	2.12E-05	(0.03)	3.8	1.66E-05	(0.20)	5.6
30 Org 23366	1.38E-05	(0.17)	3.5	3.54E-06	(0.76)	13.7
31 Org 9935	1.96E-06	(1.28)	0.5	8.42E-06	(0.00)	0.7
32 Org 32782	2.58E-05	(0.17)	3.0	1.25E-05	(0.07)	5.7
33 Org 34167	1.42E-05	(-)	1.0	1.25E-05	(-)	0.9

P_{app} = apparent Caco-2 permeability (cm/s), ab = apical to basolateral transport, ba = basolateral to apical transport, High conc. = high concentration in the donor compartment, Low conc. = low concentration in the donor compartment, P_{ratio} = permeability ratio = ($P_{app,ba}$ / $P_{app,ab}$), - = no data available.

[a] *Standard deviation must be multiplied by the exponent of the P_{app} value.*

For domperidone, a well known Pgp transported dopamine antagonist (Schinkel, 1999), permeability ratios are found of 15.1 and 36.3. This indicates that Pgp was expressed in the Caco-2 cells. Also the verapamil data show that Pgp was expressed (Hendrikse, 1999; Sandstrom et al., 1998). At a low concentration in the donor compartment a ratio of approx. 6 is found. At higher concentrations, however, the transporter becomes saturated resulting in a permeability ratio of approx. 0.6 (Sandstrom et al., 1998).

Since Pgp is widely expressed in the BBB one would expect a low brain / CNS penetration when a high permeability ratio is measured. Therefore it is interesting to compare the permeability ratios from this study with brain penetration data in rats (Table 3) (Kelder et al., 1999). Brain penetration is expressed as the ratio between the maximum concentration in the brain and the maximum concentration in the blood (= C_{brain} / C_{blood}). The following criteria are generally accepted for CNS penetration and Pgp transport:

- if the C_{brain}/C_{blood} ratio is higher than 1 CNS penetration is considered to be good,
- if the permeability ratio is higher than 2 a drug is considered to be transported by Pgp.

When the brain penetration (C_{brain} / C_{blood}) is plotted as function of the permeability ratio it can be seen if CNS penetration is in agreement with the supposed Pgp transport of the drug (this is indicated with the shaded areas in Figure 1).

Table 3. Brain penetration data in rats (Kelder et al., 1999).

Compound	***C_{brain}/C_{blood}*** [a]	***Compound***	***C_{brain}/C_{blood}***
3 Verapamil	10.0	12 Clonidine HCl	1.3
6 Desipramine HCl	10.0	22 Risperidone	1.0
7 Imipramine HCl	11.2	23 Org 12962	43.7
8 Amitriptyline HCl	9.5	25 Org 5222	10.7
9 Carbamazepine	1.0	27 Org 25907	1.8
10 Ranitidine HCl	0.06	28 Org 13011	1.4
11 Domperidone	0.2	33 Org 34167	1.0

[a] *Radiolabeled drugs were orally administered to male Wistar rats. Blood and brain samples were taken at fixed time points. The brains were perfused via the aorta with saline until free of blood (within 1 minute). Parent compound peak values were used to calculate the C_{brain} / C_{blood} ratio.*

From the presented data it becomes clear that brain penetration data are not 'always' consistant with the measured efflux ratios (Yamazaki et al., 2001; Adachi et al., 2001; Fricker et al., 1996). Several drugs are supposed to be transported by Pgp (permeability ratio higher than 2) and still show a good brain penetration (C_{brain}/C_{blood} > 1). In a HTS experiment a drug with an efflux ratio higher than 2 would be typically

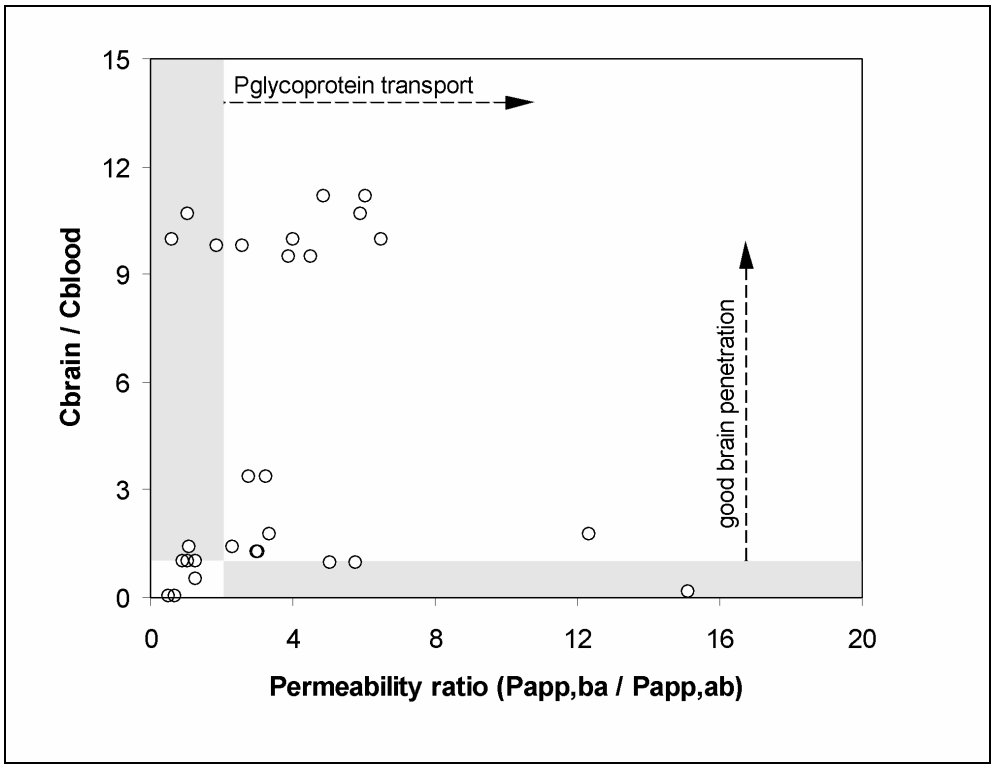

Fig. 1 Brain penetration plotted as function of the permeability ratio.
The shaded areas indicate where Pgp and brain penetration data are consistent with each other. At a low Pgp efflux ratio a high brain penetration and at a high Pgp efflux ratio a low brain penetration is expected.

considered to be a potential Pgp substrate. Recently it has been reported that multiple efflux systems of the ABC superfamily are expressed in differentiated Caco-2 cell monolayers (Taipalensuu et al., 2001), i.e. other transporters than Pgp may contribute to the measured efflux ratio. Also a possible species difference in the Pgp substrate susceptibility might be present, as was shown by Yamazaki et al. (2001) in Pgp-mediated transport studies using Pgp of mouse and human origin. In the current study Pgp of rat (brain penetration data) and human (Caco-2 data) origin are compared. Also experimental variables may contribute to possible variations in the measured (Pgp) efflux ratio. Recently it has been reported that the basolateral to apical permeability of alkaline drugs may be influenced by the pH gradient over the Caco-2 cells (Mizuuchi et al., 2000; Baker et al., 2002), i.e. the permeability of the basolateral to apical transport is overestimated for drugs with high-pK_a values (pK_a > 8-9). Since many of the drugs in Table 3 have an alkaline nature (Table 1), this may have been a

factor of influence. However, the data of verapamil and domperidone, both being bases, are in line with earlier published results (Sandstrom et al., 1998; Hendrikse, 1999; Schinkel, 1999). Also the applied concentration in the donor compartment could be of influence on the measured permeability ratio, since the Pgp transporter can become saturated (Sandstrom et al., 1998). Furthermore, if the P_{app} is high, as reported in this study for most of the tested drugs (Table 2), the influence of Pgp may become less pronounced (Lentz et al., 2000).

Comparing the data of this study with Mahar Doan et al. (2002) and Baker et al. (2002) shows that antipyrine, carbamazepine, domperidone, indomethacin, risperidone and verapamil are correctly predicted. The CNS drugs desipramine, imipramine, amitriptyline and clonidine give opposite results, whereas the imipramine results possibly can be explained by the presence of the pH gradient over the Caco-2 cell monolayer (Baker et al., 2002).

All the above discussed variables may contribute to false negative and false positive predictions when standard transport conditions are used in the Caco-2 transport experiments. Hence, the Pgp efflux ratio's derived from High Throughput Screening (HTS) experiments, where the transport conditions are fixed (pH gradient, concentration etc.), cannot be routinely used to predict a possible limited brain penetration. Positive identification of Pgp efflux transport clearly needs to be established by performing a transport experiment in the presence of a known Pgp inhibitor.

Besides Pgp efflux of CNS drugs also the physicochemical properties are of influence on brain penetration. Comparing the molecular descriptors to differentiate between CNS and non-CNS drugs (Table 1) shows that the CNS group had fewer hydrogen bond donor sites (data not shown), greater lipophilicity and a lower polar surface area. This corresponds well with the results of Mahar Doan et al. (2002). However, a relationship between molecular weight and possible Pgp transport (Mahar Doan et al., 2002) could not be established as the large majority of drugs in this study had molecular weight lower than 400.

In the evaluation of the significance of Pgp efflux data from in vitro tests, it is interesting to review several recent reports on the clinical relevance of Pgp on the oral absorption in the gastrointestinal tract (Trouman and Thakker, 2001; Sakaeda et al.,

2001; Chiou et al., 2001). Drugs which are known Pgp substrates show an average bioavailability of 47%, suggesting that being a substrate for Pgp does not always result in poor bioavailability (Sakaeda et al., 2001). On average the studied Pgp transported drugs had the same pharmacokinetic parameters as other drugs (fraction absorbed, bound fraction, urinary excretion, total, renal and hepatic clearance) (Sakaeda et al., 2001). In a recent review it was shown that marketed drugs, which are known Pgp substrates, all had linear pharmacokinetics in humans (C_{max} and AUC linearly related to dose), indicating that the absorption was not impaired by Pgp (Chiou et al., 2001). In the light of these results it is clear that the role of Pgp in gastrointestinal absorption might be overestimated and the results of the current study indicate that the same may apply to CNS penetration.

4.4 Conclusions.

Based on the calculation of the physico chemical properties a good oral absorption was predicted for all the heterocyclic drugs tested in this study. This corresponds well with the measured Caco-2 permeabilities. For almost all the drugs tested a high permeability was measured. Even the low permeability drugs (morphine and Org 9935) were transported at a much higher rate as the reference compounds with a known low permeability (mannitol and PEG4000). The high Caco-2 permeability was in agreement with earlier published human in vivo absorption data. Comparison of the Caco-2 permeability with data reported in literature showed that the Caco-2 cell system worked well. For the drugs which did not show polarized transport the apical to basolateral permeability was not influenced by the concentration in the donor compartment.

Based on the transport data of domperidone and verapamil it was found that the P-glycoprotein (Pgp) efflux transporter was expressed in the Caco-2 cells used. Approximately 50% of the drugs tested were indicated to be potential Pgp substrates (desipramine, amitriptyline, clonidine, risperidone, Org 23430, Org 33062, Org 34037, verapamil, imipramine, cimetidine, domperidone, quinidine, Org 12962, Org 5222, Org 25907, Org 23366 and Org 32782). Since Pgp is expressed at the Blood Brain Barrier as well, CNS penetration might be impaired if a drug is a Pgp substrate.

However, no correlation could be found between brain penetration and the efflux ratio.

This may be caused by physiological (species difference, presence of multiple efflux systems) and experimental (e.g. overestimation of the P_{app} in the presence of a pH gradient, concentration in the donor compartment etc.) variables contributing to possible variations in the measured Pgp efflux ratio.

From the data it is concluded that Pgp efflux ratios as determined in in vitro HTS screening tests, where the transport conditions are fixed (pH gradient, concentration etc.), cannot be routinely used to predict a possible limited brain penetration.

Acknowledgements.

The authors would like to thank Dr. Willem Meuldermans and Dr. Pieter Annaert from the Janssen Research Foundation for the donation of the [^{3}H] labeled risperidone and domperidone and the discussions. Furthermore Dr. Jan Kelder of the Molecular Design and Informatics Department of NV Organon is thanked for his contribution to the selection of the set of drugs to be tested.

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5 A feasibility study into the prediction of lymphatic drug delivery.

Abstract

In this study it was investigated whether it is feasible to predict the potential of drugs for lymphatic absorption. At present the octanol / water partition coefficient is the only parameter available for this purpose, however, the amount of data is limited and alternatives are not available. An extensive dataset, which received hardly any attention in literature, was used to study whether relationships exist between molecular descriptors and the extent of lymphatic drug transport. In addition, partitioning to the lipid fraction of ex vivo dog lymph and Intralipid as well as the suitability of the Caco-2 cell system were explored.

Using the lymphatic drug transport data of a chemically diverse set of compounds, it was shown that the (octanol/water) partition coefficient is a suitable parameter for assessing the potential for lymphatic transport of drugs. Partitioning of drugs towards the lipid fraction of ex vivo dog lymph or Intralipid offers an useful alternative methodology. The prediction becomes more accurate when dealing with drugs within a chemically similar series.

The Caco-2 transport data showed that the formation and secretion of chylomicrons (CM) is the limiting factor for this test system. Unless the problem of CM formation and secretion can be solved, Caco-2 cells are not suitable for assessing the potential of drugs for lymphatic absorption. This may be related to the malignant origin of the Caco-2 cells.

5.1 Introduction.

The oral route of administration is considered as the most convenient one and is therefore most frequently applied. After oral administration a drug is absorbed by the enterocytes and reaches the systemic circulation via the portal vein. Inside the enterocytes (very) lipophilic food components (e.g. vitamins and lipids), xenobiotics (e.g. DDT) and drugs (e.g. testosterone esters and halofantrine) can be incorporated into lipoproteins (chylomicrons (CM) and Very Low Density Lipoproteins (VLDL)) (Charman and Stella, 1992). The incorporation of the drug into CM is dependent on the physicochemical properties of the particular drug and the composition of the dosage form. These lipoproteins are then excreted by a process of exocytosis and enter the mesenteric lymphatics through the lacteals which extend into the villi. Lipoproteins cannot enter the blood capillaries due to their size. The lymph fluid, containing the lipoproteins, is collected into larger vessels and drains via the thoracic lymph duct into the systemic circulation at the junction with the left internal jugular vein. Following the lymphatic route drugs circumvent first pass metabolism. Lipoproteins are formed of fatty acids (FA) and monoglycerides (MG) after absorption into the enterocytes (Porter and Charman, 1997). FA and MG originate from digested triglycerides (TG) present in food or the administered vehicle (Charman et al., 1997). The short chain FA enter the systemic circulation directly. The long chain FA are re-esterified via the monoacyl glycerol or phosphatidic pathway and are 'built' into CM and VLDL, with CM being the major transport lipoprotein.

Although the exact mechanism by which lipophilic drugs enter the lymphatics is not fully known, most drugs are associated with the CM. Based on the relative ratio of the blood and lymph flow (approximately 500) and a lipid content of approx. 1-2% it can be calculated that a drug needs to have a log P (octanol / water partitioning coefficient) higher than 5 to assure a considerable lymphatic transport (Charman and Stella, 1992). Furthermore, the solubility in the lipid core of the lipoproteins needs to be sufficient to promote the partitioning process. As a result it is expected that log P plays an important role with respect to lymphatic drug delivery. This was shown for a homologous series of four testosterone esters where lymphatic transport was linearly related to log P (Noguchi et al., 1985).

Up to now most studies involving lymphatic drug transport have been conducted with animal models; rats and dogs being the most popular species. The flow rates on the basis of body weight are remarkably similar in rat, dog and human (Landis, 1962). To study intestinal drug absorption a wide variety of in vitro systems are available for which the Caco-2 cell system is the most commonly used model. Recently, Caco-2 cells have also been used to study intestinal lipid metabolism.

The ability of Caco-2 cells to synthesize TG rich lipoproteins followed by polarized secretion is well established (van Greevenbroek et al., 1996, 1998, 2000; Levy et al., 1995, 1999; Luchoomun and Hussain, 1999; Spalinger et al., 1998a, 1998b; Field et al., 1988; Dulfer et al., 1996; Trotter and Storch, 1991; Mehran et al., 1994, 1997; Traber et al 1987). In Caco-2 cells the formation and secretion of chylomicrons and VLDL are stimulated optimally by the addition of long chain unsaturated fatty acids, such as oleic acid, whereas saturated fatty acids (e.g. palmitic or stearic acids) result predominantly in secretion of low-density lipoproteins (LDL) and intermediate density lipoproteins (IDL) (van Greevenbroek et al., 2000). This is in line with in vitro experiments which showed that triglycerides containing oleic acid are the most powerful stimulators of CM production (Caliph et al., 2000; Field et al 1988). However, important differences between the lipid metabolism in the human intestine and Caco-2 cells are present. The monoacyl glycerol pathway is nearly inactive in Caco-2 cells and Caco-2 cells produce apoB-100 instead of the apoB-48 in the human intestine (Levy et al., 1995; Luchoomun and Hussain, 1999). ApoB-48 is present on the surface of lipoproteins and plays a role in the assembly and secretion process (Phillips et al., 1997). Furthermore, the synthesized lipoproteins are secreted to a much lesser extent by the Caco-2 cells as do the enterocytes in the human intestine (Levy et al., 1995).

The aim of the current study was to determine whether it is feasible to predict lymphatic drug delivery. At present the octanol / water partition coefficient is the only parameter available for this purpose, however, the amount of data is limited and alternatives are not available. The first approach was to study whether relationships exist between molecular descriptors and the lymphatic transport of a chemically diverse group of xenobiotics. Secondly, surrogate log P's were measured to determine the partitioning of testosterone esters to the lipid fraction of Intralipid and ex vivo dog

lymph. Thirdly, Caco-2 cells were used to study the formation and excretion of lipoproteins in relation to the lymphatic transport of testosterone esters.

5.2 Materials and Methods.

5.2.1 Calculation of molecular descriptors.

The ClogP, MW and the static Polar Surface Area (PSA, $Å^2$) were calculated using the methods described in (Kelder et al., 1999). The static PSA does not take into account the different conformations of the chemical structures, but gives essentially the same results as the dynamic PSA. It has the advantage of a much shorter time of calculation (Kelder et al., 1999).

5.2.2 Materials.

Testosterone (T), testosterone heptanoate (TH), testosterone undecanoate (TU), testosterone palmitate (TP), testosterone oleate (TO), testosterone propionate (TProp) and methylnortestosterone bucyclate (MB) were supplied by NV Organon, Oss, The Netherlands. Oleic acid (OA) was purchased from Sigma-Aldrich (St Louis, MO). All chemicals were of analytical grade.

Intralipid 10% (purified soy bean oil, purified egg phospholipids, glycerol, sodiumchloride and water for injection) was purchased from Pharmacia & Upjohn (Woerden, The Netherlands). These chemicals were of reagent grade.

The following radiolabeled compounds were used in the studies: [^{3}H]-testosterone (>89% radiochemical purity, 23 Ci/mmol), [^{3}H]-testosterone undecanoate (>93% radiochemical purity, 24 Ci/mmol) and [^{3}H]-methylnortestosteron bucyclate (>95% radiochemical purity, 5 Ci/mmol) were obtained from NV Organon. [^{14}C]-oleic acid (>98% radiochemical purity, 50 mCi/mmol) was purchased from Sigma-Aldrich (St Louis, MO).

5.2.3 Ex vivo lymph from beagle dogs.

Lymph was collected from the ductus thoracicus during autopsy on 18 month old male beagle dogs who were used as placebo control group in toxicological studies. At least 1 hour prior to autopsy each dog was fed a standard meal of 250 grams of pelleted dog food (Witham, Essex, UK). The total amount of lymph collected was

approximately 15 mL. After collection of the samples the collected lymph was pooled. Immediately after collection 5% EDTA was added to the sample tube as anticoagulant and protease inhibitor. The lymph samples were stored at 4 °C for a maximum of seven days prior to the experiment and analysis. All animal experiments were conducted in accordance to protocols approved by the Animal Ethics Committee.

5.2.4 Partitioning of drugs with Intralipid and lymph.

10% Intralipid was diluted with demineralised water to 2% (v/v). A 2% Intralipid emulsion was chosen as the lipid content of lymph is approximately 2% (Charman and Stella, 1992). A solution of 1.25 mg/mL of the testosterone ester was prepared in ethanol and 100 µL of this solution was added to 500 µL of 2% Intralipid. The incubation was carried out at 37°C for 16 hr under continuous shaking (Kilian, 1973). After incubation the sample was subjected to sequential density gradient ultracentrifugation as described below. The two fractions with the density of chylomicrons were further analysed for the concentration of the testosterone esters. The concentration measurement is described below.

To measure the partitioning of testosterone esters with ex vivo dog lymph the same protocol as for the 2% Intralipid solution was followed.

5.2.5 Density gradient ultracentrifugation.

Density gradient ultracentrifugation was used to separate the oily phase and lipoprotein fractions in the Intralipid / lymph partitioning and Caco-2 experiments. To this extend lipoproteins were separated into different density classes by sequential ultracentrifugation on a KBr density gradient (Rickwood, 1978; van Greevenbroek et al., 1995).

In the partitioning experiments with Intralipid and ex vivo dog lymph the volume of the samples was, prior to ultracentrifugation, adjusted to 2.5 mL with a KBr-salt solution of 1.250 g/mL, in open top polyallomer tubes (Beckman, California). The medium was then overlaid with 3 mL each of a KBr-salt solution with a density of 1.063, 1.019 and 1.006 g/mL. This was done by placing a 5 mL syringe, without the plunger and with a curved hose attached to the needle, just above the surface of the

solution. The solution with the lower density was pipetted into the syringe and allowed to flow by gravity into the centrifuge tube. Distinct layers could be observed. To obtain the large CM fraction, samples were ultracentrifuged for 33 min, 40.000 rpm, 15 °C in a Sorvall (Connecticut, USA) TH 641 rotor. The top 1 mL was collected and replenished with 1 mL of d = 1.006 g/mL solution. Small CM were obtained from the top after a second ultracentrifugation (3 h and 28 min, 40.000 rpm). The separations were carried out at 15 °C.

In the Caco-2 experiments the total amount of lipoproteins present in the basolateral compartment was isolated by the density gradient ultracentrifugation using conditions described previously (Luchoomen and Hussain, 1999; Seaballuck et al., 2003).

5.2.6 Concentration measurement of testosterone esters with HPLC.

For the measurement of the partitioning of testosterone esters the following analytical method was used (Kilian, 1975). 100 µL of an internal standard solution was added to the sample and the testosterone ester and internal standard were extracted with 5 mL ether. The log P of the testosterone esters and the internal standard were in the same order of magnitude. After freezing the aqueous layer in dry-ice-acetone, the ether layer was decanted into a centrifuge tube and the ether evaporated under a gentle stream of nitrogen gas. The residue was resuspended in 1 mL of 5% (w/v) sodium chloride solution and the testosterone ester was extracted with 0.5 mL of cyclopentanone. An aliquot of the cyclopentanone solution was subjected to HPLC analysis under the following conditions: C18 column 150 x 3.9 mm, 5 µm particles (Waters, Ireland), flow 1 mL/min, injection volume 10 µL, column temperature 40 °C, UV detection at 240 nm. The mobile phases, gradients and internal standards that were used are given in Table 1.

Table 1. Mobile phases, gradients and internal standards for the chromatographic analysis of the testosterone esters.

Compound	***Mobile phase***	***Internal standard***
Testosterone	Acetonitrile / water gradient [2]	Testosterone propionate
Testosterone heptanoate	Acetonitrile / water gradient [1]	Testosterone undecanoate
Testosterone undecanoate	Acetonitrile / water gradient [1]	Testosterone heptanoate
Testosterone palmitate	Methanol	Testosterone undecanoate
Testosterone oleate	Methanol	Testosterone undecanoate

Gradients: [1] 0-5 min water (W) / acetonitrile (A) = 60/40 (% v/v), 5-20 min W/A = 10/90, 20-25 min (W/A) = 60/40. [2] 0-10 min W/A = 60/40, 10-15 min W/A = 10/90, 20-25 min W/A = 60/40. All gradients were linear.

5.2.7 Concentration measurement of [^{3}H] and [^{14}C] labeled drugs.

Samples of 50 µL were mixed with 3 mL of Ultima Gold (Packard, Meriden, CT) and counted in a Packard 1900 CA Tri Carb liquid scintillation counter.

5.2.8 Caco-2 experiments.

5.2.8.1 Cell culture.

In this study the Biocoat Intestinal Epithelial Differentiation Enviroment (BIEDE) HTS (High Throughput Screening) Caco-2 system (Beckton Dickinson, Cat. no. 354802) was used. The Caco-2 cells (ATCC, code HTB 37, human colon adenocarcinoma, passage number 30 - 34) were grown in culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM), containing glucose supplemented with heat-inactivated foetal calf serum (20 % v/v), non-essential amino acids (1% v/v), L-glutamine (2 mM) and Penicilline/Streptomycine (100 IU µg/mL and 0.1 mg/mL respectively). The Caco-2 cells were cultured by seeding about 2,000,000 cells in 80 cm^2 tissue culture flasks containing culture medium. Near confluent Caco-2 cell cultures were harvested by trypsinisation and resuspended in culture medium (serum free DMEM with MITO+™ serum extender) to a concentration of 400,000 cells/mL. MITO+™ serum extender consists of EGF, transferrin, insulin, ECGS, triiodothyronine, hydrocortisone, progesterone, testosterone, estradiol-17β, selenium and o-phopsphorylethanolamine (Chong et al., 1997). Caco-2 cells were seeded by

adding 0.5 mL of the cell suspension to the semi-permeable filter inserts (HTS fibrillar collagen multiwell insert system; 1 μm pore size; 24 wells; filter area 0.33 cm^2). The culture medium was replaced after 24 hours with 0.5 mL Entero-STIM™ (butyric acid containing serum free DMEM). Butyric acid reduces cell proliferation and induces cell differentiation. At day 4 the medium is replaced with 0.5 mL fresh medium. The transport study is conducted at day 5. The cells are cultured at 37 °C in a humidified incubator containing 5% CO_2 in air.

To check the differentiation status of the formed monolayer the transepithelial electrical resistance (TEER) was measured (Millicell-ERS epithelial voltohmmeter, Millipore Co., Bedford, USA). The TEER of the cell monolayers was calculated according to the following equation: TEER = ($R_{monolayer}$ - $R_{empty\ filter}$) x A ($\Omega.cm^2$), where $R_{monolayer}$ is the resistance measured, $R_{empty\ filter}$ is the resistance of control filters without cells (approximately 140 $\Omega.cm^2$) and A is the surface area of the filter insert (0.33 cm^2).

5.2.8.2 Induction of CM secretion in Caco-2 cells.

The production of CM was measured after incubation for 20 hr with the transport medium (pH = 7.4) containing 0.5 mM oleic acid bound to 0.125 mM Bovine Serum Albumin (BSA). This vehicle was prepared as previously described (van Greevenbroek et al., 1996; Field et al., 1988; Seeballuck et al., 2003). Briefly, the medium was prepared from 50 mM oleic acid stock solution in ethanol and the required amounts of oleic acid stock solution were dried under nitrogen and labeled with trace [^{14}C]-oleic acid (50 mCi/mmol). The sodium salt was prepared by neutralization with sodium hydroxide (100 mM) in ethanol solution. After drying, the fatty acid salt was reconstituted in hot water (> 80 °C). BSA in serum-free culture medium was added to 50% of the final volume. After 2 h at 37 °C the resulting clear solution was diluted to full volume with serum-free culture medium to final working concentration. A 'fasted' state vehicle was prepared containing 0.125 mM BSA labeled with trace [^{14}C]-OA, as outlined above. The concentration of [^{14}C]-OA was 0.01 mM in the 'fed' and 'fasted' medium.

5.2.8.3 Transport study of T, TU and MB.

The transport medium containing OA (0.5 mM) and BSA (0.125 mM) was prepared as described in the previous section. T, TU and MB, and the corresponding radiolabels, were added at the final dilution step. The final concentration of T, TU and MB was 20 μM for all drugs. The radiolabeled drugs were present at a concentration of 0.15 μM ([^{3}H]-T), 0.14 μM ([^{3}H]-TU) and 0.67 μM ([^{3}H]-MB).
The apical compartment received 300 μL of the OA-BSA-drug solution (pH = 7.4). The basolateral compartment received 1 mL of serum-free culture medium (pH = 7.4). After 20 hours incubation apical and basolateral media were collected. Basolateral medium was subjected to density gradient ultracentrifugation. The cell monolayer was rinsed three times and cut out of the filterholder.

5.3 Results and Discussion.

5.3.1 Prediction of lymphatic drug delivery with molecular descriptors.

Table 2 shows the calculated physicochemical properties of a series of drugs for which lymphatic drug transport has been studied (Kilian, 1975). For all drugs the Polar Surface Area (PSA) and Molecular Weight (MW) comply with the criteria for a good oral absorption (Lipinski et al., 1997; Kelder et al., 1999; Palm et al., 1997). However, many drugs have a very high ClogP. Figure 1 shows that the amount of drug which is transported through the lymphatics increases with ClogP. A similar relationship between log P and lymphatic transport has been reported for testosterone esters (Noguchi et al., 1985). In this case a much better correlation was found, probably because the drugs were chemically more similar.
A drug may be considered well transported through the lymphatics if more than 10% of the total dose is transported in the lymph fluid. The results in Figure 1 show that this is the case when the ClogP is larger than approx. 6. This is in line with the derived criterion for the log P on the basis of the blood and lymph flow ratio (approx. 500) and the lipid content of the lymph fluid (approx. 1 - 2%) (Charman and Stella, 1992). The relative large variation in lymphatic transport may partly be caused by the cannulation of the thoracic lymph duct in rats, which also contains lymphatic fluid from other tissues (Charman and Stella, 1992). As not all compounds distribute in the same way over the different density fractions of lipoproteins and fatty tissues in the

body this may contribute to the variation (Kilian, 1975). Finally, as the extent of lymphatic transport is also dependent on the used vehicle this might be a contributing factor as well (Kilian, 1975; Noguchi et al., 1985).

Table 2. Calculated physicochemical properties of the Kilian dataset for lymphatic transported compounds (Kilian, 1975).

Compound		***Clog P***	***PSA (Å^2)***	***MW***
1	DDT	6.8	1.4	354.5
2	DDD	6.1	1.4	320.0
3	DDE	6.7	1.4	318.0
4	Aldrin	5.4	1.4	364.9
5	Dieldrin	3.7	12.6	380.9
6	Lindane	3.8	1.4	290.8
7	Hexachlorbiphenyl	7.6	1.4	360.9
8	Hexadecane	9.2	1.4	226.4
9	3-Methylcholantrene	6.6	1.4	268.4
10	Δ1-Tetrahydrocannabinol	7.2	24.3	314.5
11	Vitamin D3	9.5	22.7	384.6
12	Quinestrol	7.0	23.5	378.6
13	Progesterone	3.9	31.2	314.5
14	Nandrolone	2.7	38.1	274.4
15	Lynestrenol	5.1	19.2	284.4
16	Diethylstilbestrol	5.0	49.7	268.4
17	Cyclandelate	4.6	30.9	276.4
18	Dioctylphthalate	8.7	26.7	390.6
19	p-Dimethylaminoazobenzene	4.5	16.7	225.3
Average		6.0	16.1	298.8
Min		2.7	1.4	226.4
Max		9.5	49.7	390.6

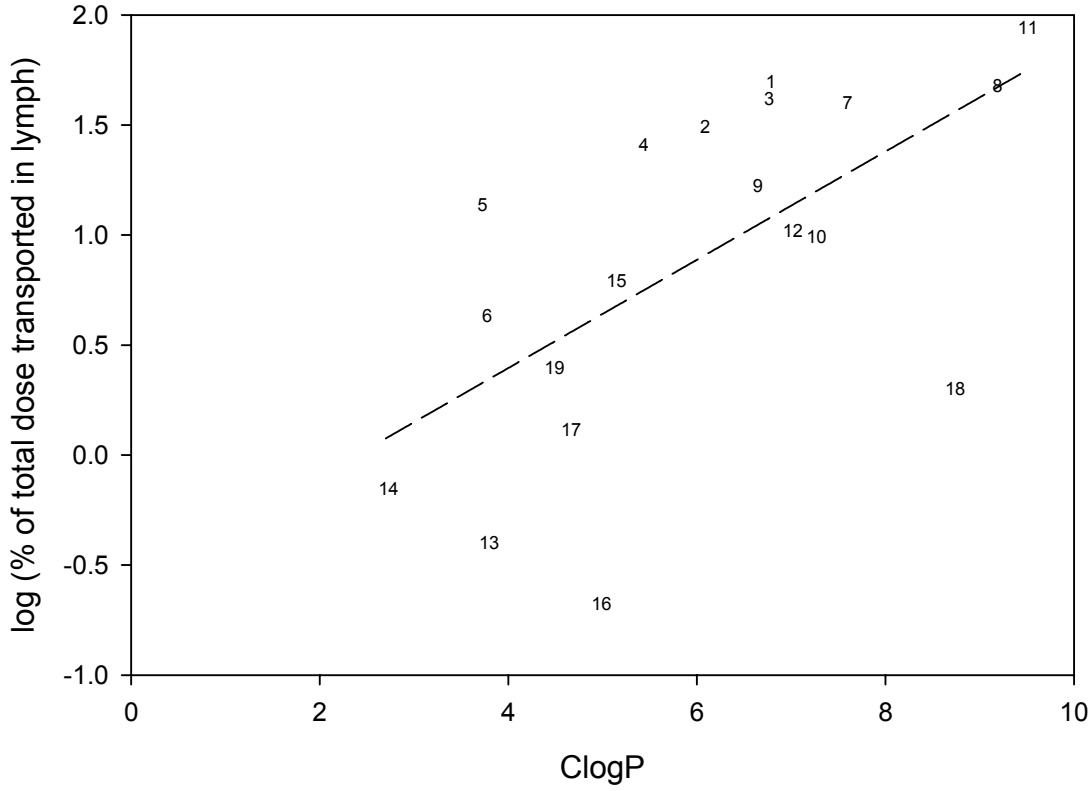

Fig. 1. Lymphatic transport as function of the ClogP after cannulation of the thoracic lymph duct in rats using a 0.3 mL triolein vehicle in the fasted state. (Kilian, 1975). *1: DDT, 2: DDD, 3: DDE, 4: Aldrin, 5: Dieldrin, 6: Lindane, 7: Hexachlorbiphenyl, 8: Hexadecane, 9: 3-Methylcholantrene, 10: Δ1-Tetrahydrocannabinol, 11: Vitamin D3, 12: Quinestrol, 13: Progesterone, 14: Nandrolone, 15: Lynestrenol, 16: Diethylstilbestrol, 17: Cyclandelate, 18: Dioctylphthalate, 19: p-Dimethylamino-azobenzene. The dotted line is the linear fit.*

5.3.2 Partitioning of testosterone esters to the lipid fraction of Intralipid and ex vivo dog lymph.

As it is evident that the partition coefficient plays an important role, it was determined to what extent testosterone esters partition to the lipid fraction of Intralipid and ex vivo dog lymph. Furthermore, the relationship between partitioning and reported

lymphatic transport was determined (Noguchi et al., 1985). The results are shown in Fig. 2.

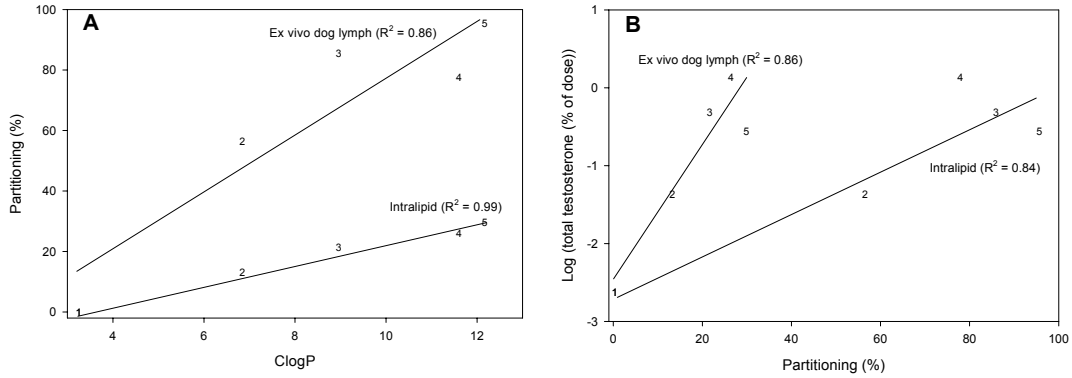

Fig. 2. Partitioning and lymphatic transport of testosterone esters.
Panel A: Partitioning of testosterone esters to the lipid phase of Intralipid and ex vivo dog lymph as function of ClogP. Partitioning is expressed as the percentage of the testosterone ester present in the lipid fraction. Panel B: Lymphatic transport of testosterone esters (Noguchi et al., 1985) (unanesthetized, mesenteric lymph duct cannulated rat model) as function of the partitioning to the lipid fraction of Intralipid and ex vivo dog lymph. Lymphatic transport is expressed as percentage total testosterone of the administered dose (ester + free testosterone). The drawn lines represent the linear fit. 1 = testosterone (ClogP = 3.22), 2 = testosterone heptanoate (6.81), 3 = testosterone undecanoate (8.93), 4 = testosterone palmitate (11.57), 5 = testosterone oleate (12.14).

Linear relationships are found between the percentage of drug present in the lipid phase with ClogP for Intralipid as well as ex vivo dog lymph. Clearly more drug partitions towards the lipoproteins present in the lymph samples than to the soybean oil droplets of the Intralipid emulsion. This reflects a better solubility of the testosterone esters in the cores of the lipoproteins (e.g. the triglycerides in the core of CM). Whether the partitioning of drugs to the chylomicrons represents the in vivo situation remains to be established, as the distribution of drug over the various lymph fractions (CM, VLDL, LDL, HDL and proteins) depends on the way in which the

experiment has been performed. E.g. the distribution of vitamin D3, dieldrin, quinestrol and mestranol over the lymph fractions is different when measured after oral administration or in in vitro partitioning experiments (species: rat) (Kilian, 1975). It has been suggested that the interaction between the drug and the proteins present in the shell of lipoproteins may be different in vivo and in vitro, reflecting possible differences in protein binding (Kilian, 1975).

5.3.3 Caco-2 experiments.

To test whether Caco-2 cells are suitable for assessing lymphatic drug transport, experiments were performed to study the formation of CM and the transport of drugs. First the transport of oleic acid (OA) was studied and a mass balance was made. I.e. the amount of drug was determined in respectively the apical compartment, the monolayer, and the basolateral compartment. In the latter a separation was made between the aqueous fraction (W_f) and the secreted phospholipids (CM) by the Caco-2 cells.

As model drugs testosterone (T), testosterone undecanate (TU) and methyl nortestosterone bucyclate (MB) were used. T was chosen as reference drug as it is known that this compound is not transported through the lymphatics (Sieber, 1971). Furthermore, T is a highly permeable drug in Caco-2 monolayers in the absence of OA (Faassen et al., 2003). From TU and MB it is known that they are transported through the lymphatics (Noguchi et al., 1985). The physicochemical properties of OA and the drugs are summarized in Table 3.

The properties comply with the required properties for good oral absorption (Lipinski et al., 1997; Palm et al., 1997) except for the ClogP, which is very high for OA, TU and MB. Although the ClogP of TU, MB and OA is high, the aqueous solubility in the same order of magnitude as is common for steroids (in the µg/mL range) (Faassen et al., 2003).

To identify a possible 'lymphatic' transport of drugs, oleic acid (OA) is needed to induce CM formation. Two different types of OA vehicles were used, namely a 'Fed' state vehicle (0.5 mM OA / 0.013 mM [^{14}C]-OA / 0.125 mM BSA) to induce the formation of CM, and a 'Fasted' state vehicle (0.013 mM [^{14}C]-OA / 0.125 mM BSA)

to study OA absorption by Caco-2 cells as such. The Caco-2 transport data of OA are shown in Table 4.

Table 3. Physicochemical properties of the drugs used in the Caco-2 experiments.

Drug	***C_s***	***MW***	***Clog P***	***PSA***
T	46.3	288.4	3.2	28.6
TU	3.0	456.7	8.9	33.3
MB	15.0	454.7	8.1	33.3
OA	< 100	282.5	7.8	28.6

T = testosterone, TU = testosterone undecanoate, MB = methylnortestosterone bucyclate, OA = oleic acid, Cs = aqueous solubility (µg/mL), MW = molecular weight, ClogP = calculated octanol/water partitioning coefficient, PSA = Polar Surface Area ($Å^2$).

Table 4. Caco-2 transport data of oleic acid.

	Apical	***Cell***	***W_f***	***CM***	***Recovery***
Apical to basolateral transport					
OA Fed (n=3)	66.5 [1] *(1.4)* [2] *(65.4 - 68.1)* [3]	28.6 *(4.0)* *(25.4 - 33.0)*	0.27 *(0.21)* *(0.1 - 0.5)*	0.009 *(0.004)* *(0.006 - 0.011)*	95.4 *(3.5)* *(91.6 - 98.6)*
OA Fasted (n=3)	75.4 *(10.7)* *(63.4 - 84.1)*	25.2 *(1.9)* *(23.1 - 26.4)*	0.83 *(0.47)* *(0.3 - 1.2)*	0.029 *(0.036)* *(0.004 - 0.011)*	101.5 *(12.2)* *(87.6 - 110.6)*
Basolateral to apical transport					
OA Fed (n=2)	0.1 *(0.0)* *(0.1)*	4.0 *(0.3)* *(3.8 - 4.2)*	102.8 *(1.5)* *(101.7 - 103.8)*	-	106.9 *(1.8)* *(105.6 - 108.1)*
OA Fasted (n=2)	0.5 *(0.14)* *(0.4 - 0.6)*	3.7 *(0.6)* *(3.3 - 4.1)*	105.7 *(2.3)* *(104.1 - 107.3)*	-	109.9 *(1.8)* *(108.6 - 111.2)*
Cell viability data					
Mannitol	94.9 *(13.5)* *(84.5 - 110.1)*	-	5.2 *(2.1)* *(2.8 - 6.9)*	-	100.1 *(13.7)* *(91.4 - 115.9)*
TEER values:	Prior to transport experiment:		1505 ± 247		
	After transport experiment:		1315 ± 94		

The amounts of oleic acid present in the apical compartment (Apical), Caco-2 cells (Cell), aqueous fraction basolateral compartment (Wf) and chylomicron fraction basolateral compartment (CM) are expressed as percentage of the total amount of radiolabeled oleic acid added at the start of the test. Recovery = Apical + Cell + W_f + CM. TEER in $\Omega.cm^2$.
[1] amount of oleic acid in %, [2] SD (%), [3] minimum and maximum values.

The mannitol data show that during the 20 hours of the experiment only a small amount of mannitol permeates through the cell layers, indicating that the Caco-2 cell layers remained intact and viable during the experiment. This was also confirmed by comparing the TEER values of control filters, i.e. the TEER value did not change during the course of the experiment. The recovery of the labeled oleic acid varied between approximately 90 and 100% indicating good mass balances.

Only a small fraction of the administered oleic acid is present in the CM that are secreted into the basolateral compartment. The low amount of CM formed is a well described problem in the use of Caco-2 cells (Mehran et al, 1997; Levy et al., 1995) but the consequences are not discussed in literature. For the secretion of CM's from enterocytes the apoB48 cofactor is needed and in Caco-2 cells apoB100 is present instead of apoB48 (Levy et al., 1999). It has been reported that upto 5% of the total amount of labeled oleic acid can be secreted into the basolateral compartment (Seaballuck et al., 2003). In those studies a 21 day culture period was maintained which may result in Caco-2 cells which may have different properties with respect to lipid processing capabilities. In the current study the BIEDE system was used in which a 5 day differentiation period is applied, and fully differentiated and mature Caco-2 cells are obtained as judged on microscopical morphological (apical microvillular surface, cell/cell junctional complexes and cellular interdigitation) and biochemical (alkaline phosphatase, aminopeptidase N and dipeptidyl peptidase IV activity, P-glycoprotein function) characteristics (Woods and Asa, 1997; Asa and LaRocca, 1997). Differences in the ability of Caco-2 cells to form CM have only been described *during* the differentiation process (Mehran et al., 1997). Therefore it is not to be expected that the 5 and 21 day culture periods will be different.

A considerable amount of oleic acid accumulates in the Caco-2 cells suggesting that CM are formed. It is remarkable that this occurs to the same extent in the 'fasted' as well as in the 'fed' state conditions. I.e. the amount of oleic acid present in the 'fasted' state vehicle is much less compared to the 'fed' state vehicle and this should imply that there is a minimal formation of CM's. It is possible that in the 'fasted' state condition OA is present in an inactive protein bound form inside the cells.

The transport data from the *basolateral to the apical* compartment show that oleic acid is only transported in minimal amounts. Also no accumulation takes place inside the
Caco-2 cells. This applies to the 'fasted' as well as the 'fed' state. This suggests that for the absorption of oleic acid into the cells a transporter is involved. The presence of fatty acid transporters in the apical membrane of enterocytes has been described in literature (Schaffer and Lodish, 1994; Abumrad et al., 1998, 1999; Stahl et al., 1999; Chen et al., 2001; Martin et al., 2000; Hirsch et al., 1998; Hermann et al., 2001), although there is still controversy with respect to the functionality in the fatty acid (FA) absorption process (Chen et al., 2001; Hamilton and Kamp, 1999).

The Caco-2 transport data of T, TU and MB in the presence of OA are shown in Table 5. The mannitol data and TEER values indicate that the Caco-2 cell layers remained intact and viable.
Also in this experiment the amount of drug associated with the CM fraction in the basolateral compartment after ultracentrifugation is very small. The data indicate that either a very small amount of CM has been formed and secreted or that the affinity of the drugs for the CM is very low. The latter is less likely as Fig. 2 shows that a considerable amount of drug partitions to the lipoproteins present in lymph fluid. The CM data are consistent with the OA transport data (Table 4) and it is clear that the amount of CM formed and secreted by the Caco-2 cells is very small.
The accumulation of drugs inside the Caco-2 cells is limited, which is in contrast with the substantial accumulation of OA (Table 4). This is also an indication that the CM formation inside the cells was small. Hence, if many CM were present inside the cells it is expected that a considerable amount of the hydrophobic drugs should have been present in the core of lipoproteins and consequently been accumulated. Finally, the more hydrophobic drugs (T and MB) accumulated to a larger extent in the Caco-2 cells as the more hydrophilic drug (T), suggesting that at least some chylomicrons were present inside the cells.
Surprisingly the aqueous fraction of the basolateral compartment contains approx. 50% of the drug initially present at the apical side. As CM formation and secretion was limited, this means that the drugs were transported over the cell layers by means

of passive transcellular diffusion. Clearly, the long duration of the experiment, (20 hours), needed to allow CM to be formed and secreted (e.g. Dulfer et al., 1996; van Greevenbroek et al., 1996; Seeballuck et al., 2003), is beneficial for passive diffusion of the hydrophobic drugs. In the end an equilibrium is reached between the apical and basolateral compartment. Strikingly, in vivo it takes only approximately 15 min to form, secrete and transport lipoproteins to the intestinal lymph (Tso et al., 1985). The large difference in time between CM secretion in vivo and in Caco-2 cells shows that the latter is not representative from a physiological point of view.

Table 5. Caco-2 transport data of T, TU, MB and Mannitol in the presence of oleic acid.

	Apical	***Cell***	***Wf***	***CM***	***Recovery***
Apical to basolateral transport					
T	34.2 [1]	0.275	51.4	0.0275	85.9
(n=4)	*(0.5)* [2]	*(0.15)*	*(2.6)*	*(0.005)*	*(2.8)*
	(33.5-34.5) [3]	*(0.1 - 0.4)*	*(48.0 - 53.8)*	*(0.02 - 0.03)*	*(81.9 - 88.5)*
TU	45.1	0.65	55.3	0.05	101.0
(n=4)	*(1.3)*	*(0.17)*	*(6.6)*	*(0.05)*	*(7.8)*
	(44.1 - 47.0)	*(0.5 - 0.8)*	*(50.2 - 64.9)*	*(0.0 - 0.12)*	*(95.1 - 112.5)*
MB	45.8	1.8	41.5	0.044	89.2
(n=4)	*(5.1)*	*(0.48)*	*(3.6)*	*(0.033)*	*(8.5)*
	(39.0 - 50.1)	*(1.1 - 2.2)*	*(37.4 - 45.0)*	*(0.0 - 0.08)*	*(78.2 - 96.3)*
Cell viability data					
Mannitol	97.2	-	3.85	-	101.0
(n=2)	*(0.5)*		*(0.07)*		*(0.6)*
	(96.8 - 97.5)		*(3.8 - 3.9)*		*(100.4 - 101.4)*
TEER values:	Prior to transport experiment:		1469 ± 91		
	After transport experiment:		1560 ± 366		

The amounts of T, TU and MB present in the apical compartment (Apical), Caco-2 cells (Cell), aqueous fraction basolateral compartment (Wf) and chylomicron fraction basolateral compartment (CM) are expressed as percentage of the total amount of radiolabeled drug added at the start of the test. Recovery = Apical + Cell + W_f + CM. T, TU and MB were tested at a concentration of 20 µM. TEER in $\Omega.cm^2$.[1] = amount of drug in %, [2] = SD (%), [3] = minimum and maximum values.

5.4 Conclusions.

The results of the analysis of the molecular descriptors show that the (octanol/water) partition coefficient is a suitable parameter for assessing the potential for lymphatic

transport of drugs. For this purpose calculated as well as measured log P values can be used and it is clear that the prediction becomes more accurate when dealing with drugs within a chemically similar series (Noguchi et al., 1985).

For the same purpose partitioning experiments to ex vivo lymph as well as Intralipid can be used. However, in this study it was found that the relationship between lymphatic drug transport and partitioning varied strongly between the Intralipid and ex vivo lymph system (Fig. 2B), which is probably caused by a different solubility of the drugs in the oily phase of lymph and Intralipid. To assess the solubility component of the incorporation of drugs into lipoproteins a triglyceride rich oil could be used such as olive or soybean oil (Sober, 1970).

The Caco-2 transport data show that the formation and secretion of CM is the limiting factor in the suitability of this system for studying the potential for lymphatic drug delivery. Surprisingly, although known this major limitation has hardly been discussed in literature (Levy et al., 1995; Field et al., 1988). Unless the problem of CM formation and secretion can be solved, Caco-2 cells are not suitable for assessing the potential of drugs for lymphatic absorption. In this respect it is necessary to take into account the malignant origin of Caco-2 cells. Short as well as long chain fatty acids are thought to be modulating factors in the possible protection to and initiation of tumor formation as well as its progress (Busstra et al., 2003; Augenlicht et al., 2002). Hence, the malignant nature of Caco-2 cells may make this cell system unsuitable for assessing lymphatic drug delivery as lipoprotein formation and secretion are compromised (Levy et al., 1995; Luchoomun and Hussain, 1999; Field et al., 1988).

Finally, the current study shows that when Caco-2 cells are used, mass balances need to be made in order to understand what happened to the drug. From a physiological point of view isolated rat intestine segments (non malignant material) might be a better model to perform experiments as described in this report.

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6 Contribution of lymphatically transported testosterone undecanoate to the systemic exposure of testosterone after oral administration of two andriol formulations in conscious lymph duct-cannulated dogs.

Abstract

Orally administered testosterone (T) is ineffective in the treatment of male androgen deficiency syndromes due to extensive presystemic first-pass metabolism. In contrast, the lipophilic long-chain ester testosterone undecanoate (TU) exhibits androgenic activity that has been attributed to formation of T via systemic hydrolysis of lymphatically transported TU. However, there are no definitive data regarding the oral bioavailability of TU or the extent to which lymphatically transported TU contributes to the systemic availability of T after oral TU administration.

This report describes the application of stable isotope methodology in a thoracic lymph duct-cannulated dog model to study the oral bioavailability and lymphatic transport of TU after postprandial administration. When administered as either Andriol or Andriol Testocaps, the mean (±S.E., n = 4) absolute bioavailability of TU was 3.25 ± 0.48 and 2.88 ± 0.88%, respectively, and lymphatically transported TU accounted for between 91.5 and 99.7% of the systemically available ester. Model-independent pharmacokinetic analysis indicated that 83.6 ± 1.6 and 84.1 ± 8.2% of the systemically available T, resulting from Andriol or Andriol Testocaps, respectively, was due to systemic hydrolysis of lymphatically transported TU. These data demonstrate that intestinal lymphatic transport of TU produces increased systemic exposure of T by avoiding the extensive first-pass effect responsible for the inactivation of T after oral administration.

J. Pharmacol. Exp. Ther. 306 (2003) 925-933.

6.1 Introduction.

The oral administration of testosterone (T) is ineffective in the treatment of male androgen deficiency syndromes because T is subject to almost quantitative presystemic first-pass metabolism mediated by the gut wall and liver (Daggett et al., 1978). Conversely, the lipophilic ester prodrug testosterone undecanoate (TU) demonstrates androgenic activity after oral administration to rats (Coert et al., 1975) and humans (Hirschhauser et al., 1975; Maisey et al., 1981; Skakkebaek et al., 1981). Because oral administration of TU results in the appearance of TU (and the metabolite 5α-dihydrotestosterone undecanoate; DHTU) in lymph of thoracic duct-cannulated rats (Coert et al., 1975; Noguchi et al., 1985) and humans (for whom a thoracic duct cannula was inserted after neck dissection surgery; Horst et al., 1976), the androgenic activity of orally administered TU is generally attributed to T (and 5α-dihydrotestosterone; DHT) formed during the systemic metabolic elimination of TU, which escaped the presystemic first pass effect due to intestinal lymphatic absorption and transport (Coert et al., 1975; Horst et al., 1976).

Although it is accepted that lymphatic absorption of TU likely contributes to systemic T exposure after oral TU administration, the extent of that contribution has not been quantitatively determined. Indirect evidence of lymph transport of TU in humans was reported where the systemic exposure of T increased after oral TU administration in the fed state, compared with administration in the fasted state (Frey et al., 1979; Bagchus et al., 2003). However, human studies cannot determine the extent of lymphatic transport of TU, or the extent to which lymphatically transported TU leads to an increase in systemic T exposure, because it is not practical or possible to cannulate the thoracic lymph duct.

Therefore, to characterize the absorption and bioavailability of orally administered TU, we have used a new lymph duct-cannulated dog model (Khoo et al., 2001) (a more representative model of the human than alternative animal models such as the rat; Edwards et al., 2001) to conclusively determine whether the intestinal lymphatics are responsible for the quantitative delivery of TU into the systemic circulation and to test the hypothesis that lymphatically transported TU is responsible for the majority of systemic T exposure after postprandial oral administration of TU. The current study used stable isotope methodology (to minimize intraindividual differences in drug

distribution and elimination and provide the necessary data in a minimal number of dogs; Heck et al., 1979) to compare the relative performance of two commercially available TU formulations (Andriol and Andriol Testocaps) with respect to the amount of TU absorbed via the intestinal lymphatics and the resulting systemic exposure of T after oral administration of the TU formulations.

6.2 Materials and methods.

6.2.1 Materials.

Deuterium-labeled TU ([^{2}H]-TU, batch DOFO0100A, labeled exclusively at C_{16} and C_{17}) was supplied by Organon NV (Oss, The Netherlands) as a mixture of [^{2}H]$_3$-TU (79%), [^{2}H]$_2$–TU (20%), and [^{2}H]$_1$-TU (1%). Andriol (batch AN39477001), Andriol Testocaps (batch A39337001/387205), testosterone decanoate (TD), and methyl nortestosterone (MENT) were also supplied by Organon NV. Intralipid (10%) was purchased from Baxter Healthcare (Sydney, Australia) and Tri-Sil reagent was purchased from Pierce Chemical (Rockford, IL). All other chemicals were of analytical reagent grade and solvents were of HPLC grade. Water was obtained either from a Nanopure (Barnstead, Dubuque, IA) or Milli-Q (Millipore Corporation, Bedford, MA) water purification system.

6.2.2 Extemporaneous preparation of an Intralipid formulation of [^{2}H]-TU.

[^{2}H]-TU for intravenous administration was incorporated into the Intralipid lipid emulsion by adaptation of a previous method (Humberstone et al., 1996). Briefly, 12 mg of [^{2}H]-TU (equivalent to 9.6 mg of [^{2}H]$_3$-TU) was dissolved in an N,N-dimethylacetamide/triacetin mixture (3:5 (v/v), 320 µL), and the resultant solution was added dropwise into 15 mL of Intralipid. Incorporation of [^{2}H]-TU into the emulsion droplets was achieved by 3x1-min periods of sonication with a probe sonicator (XL-Series operated at a power setting of 6; Misonix, Inc., Farmingdale, NY). During sonication, the temperature of the emulsion was kept below 10°C by cooling in a bucket of ice water. After the complete addition of [^{2}H]-TU, the concentration of [^{2}H]-TU in the emulsion was determined before and after centrifugation to determine the extent of incorporation of [^{2}H]-TU in the lipid droplets. The resulting emulsion was sterilized before administration by filtration

through a sterile 0.22-μm filter. Immediately after administration in the dog study, a 50-μL aliquot of the filtered emulsion was collected and stored frozen at -80°C for determination of the exact [^{2}H]-TU concentration.

6.2.3 Oral formulations of TU.

The two TU formulations assessed in this study were Andriol and Andriol Testocaps. Andriol was formulated as soft gelatin capsules containing a 18.2% (w/w) solution of TU in oleic acid with the individual capsule composition being 40 mg of TU, oleic acid, gelatin, glycerol, Karion 83, sodium ethyl hydroxybenzoate, sodium propyl hydroxybenzoate, titanium dioxide (E171), and iron oxide red (E172). Andriol Testocaps soft gelatin capsules contained a 12.0% (w/w) solution of TU in lauroglycol FCC/castor oil (40:60% (w/w)) with the individual capsule composition being 40 mg of TU, lauroglycol FCC, castor oil, gelatin, glycerol, and sunset yellow (E110).

6.2.4 Surgical procedures.

All surgical and experimental procedures were approved by the local Institutional Animal Experimentation Ethics Committee. After induction of surgical anesthesia, the thoracic duct and portal vein of healthy adult female greyhound dogs (27–30 kg) was cannulated as described previously (Khoo et al., 2001). After surgery, the dogs were allowed to recover unrestrained for a period of 14 to 16 h, and during this time, they returned to normal ambulatory movement. An intravenous catheter was inserted into the cephalic vein before drug administration to enable serial sampling of peripheral blood during the study period. No attempt was made to control dogs with regard to their position relative to oestrus.

6.2.5 Experimental procedures.

Each dog was fed a standard can of commercial dog food (680 g) containing 5% crude fat (maximum) approximately 30 to 45 min before the intravenous administration of the [^{2}H]-TU/Intralipid emulsion, which was administered at a constant rate infusion (5.0 mL over 10 min) via the cephalic vein cannula. At the end of the infusion period, heparinized saline (2 mL) was rapidly flushed through the

cannula to ensure complete delivery of the i.v. dose, and each dog was immediately administered an 80 mg oral dose of TU as either two Andriol capsules *(n* = 4 dogs) or two Andriol Testocaps capsules *(n* = 4 dogs). In addition to the dogs having access to drinking water ad libitum, 25 mL of normal saline was administered via the portal vein cannula at hourly intervals to limit any possible dehydration due to continuous collection of thoracic lymph.

Samples (5 mL) of systemic and portal blood were obtained simultaneously via the cephalic vein and portal vein cannulae, respectively, at the following times: -10 (i.e., predose), -5 (i.e., mid-infusion), 0 (i.e., end of infusion), 10, 20, 30, 40, 50, 60, 75, 90, 105, and 120 min, and every 30 min thereafter until 12-h postdose. Blood samples were collected into individual glass tubes containing potassium fluoride (50 μL of 10% (w/v) solution) as an inhibitor of nonspecific esterases. Once the blood had clotted, samples were centrifuged and the resultant serum stored at -80°C before analysis.

Lymph was collected continuously into 50-mL polypropylene tubes containing 75 mg of disodium EDTA (anticoagulant) for the 12-h postdosing period. All lymph samples collected per hour were pooled, and the total volume of lymph that had been collected was determined gravimetrically (assuming a specific gravity of 1 g/mL). Aliquots (50 μL) of the pooled lymph samples from each hourly collection period were dispensed into Eppendorf tubes and stored at -80°C before analysis.

6.2.6 Analytical procedures.

The techniques used to determine the concentration of labeled and unlabeled TU, T, DHT, and DHTU in serum and lymph, the concentration of TU in the intravenous formulation, and the concentration of triglyceride in lymph are described below.

6.2.7 Serum concentrations of TU/DHTU.

TU, [^{2}H]-TU, DHTU, [^{2}H]-DHTU, and the structurally related internal standard TD were isolated from serum by solid-phase extraction before analyte quantification by liquid chromatography coupled to mass spectrometry (LC-MS/MS) using electrospray ionization. Serum samples (200 μL) were mixed with internal standard solution (100 ng/mL in acetonitrile) and acetonitrile (70 μL) in glass tubes and transferred into SPE

tubes (Bond Elut C_{18} ; Varian Medical Systems, Palo Alto, CA), which had been preconditioned with methanol (3 mL) and water (3 mL) on a vacuum manifold. Samples were introduced dropwise onto the extraction column using minimal vacuum, and the column was then washed once with 3 mL of water. Analytes were slowly eluted from the column into a glass autosampler vial with acetonitrile/tetrahydrofuran (75:25 (v/v), 1.0 mL), and the eluate was then reduced to dryness within the autosampler vial using a vacuum centrifuge. The residue was reconstituted in acetonitrile before LC-MS/MS analysis.

The LC system was a Hewlett Packard Series 1100 equipped with an LC analytical column (Supelcosil LC-8-DB, 50 mm x 4.6 mm, 5-µm particle size) running at a column temperature of 40°C. The flow was 0.5 mL/min and an 8-min gradient was used in which the mobile phase composition varied (linearly) from 10% water (0.2% (v/v) acetic acid) in methanol to 100% methanol over 6.9 min. The mass spectrometer was an API 3000 (Applied Biosystems, Foster City, CA) operated at a vaporizer temperature of 200°C and an ionization spray voltage of 5,500 V. In the MS/MS mode the protonated $[M + H]^+$ molecules of TU, $[^2H]_3$ -TU, DHTU, $[^2H]_3$ -DHTU, and TD were used as the precursor ion and measured as product ions at *m/z* ratios of 270.75, 274.09, 254.91, 258.15, and 270.86, respectively. The limits of quantitation (LOQ) for TU and DHTU (and the corresponding related labeled analytes) in serum were 0.5 and 1 ng/mL, respectively.

6.2.8 Lymph concentrations of TU/DHTU.

TU, $[^2H]$-TU, DHTU, $[^2H]$-DHTU, and the structurally related internal standard TD were isolated from lymph by liquid-liquid extraction before quantification by LC-MS/MS using electrospray ionization. Lymph samples (20 µL) were thoroughly mixed with internal standard solution (100 ng/mL in acetonitrile) and 0.1 M HCl solution (20 µL) in glass tubes. A 300-µL aliquot of 2-butanol was added with thorough mixing, after which the samples were centrifuged and the 2-butanol transferred to glass autosampler vials. The eluate was evaporated to dryness using a vacuum centrifuge, and the residue was reconstituted in acetonitrile (25 µL) before analysis. The LC analytical column (Chromolith SpeedROD RP18e, 50 mm x 4.6 mm; Merck, Darmstadt, Germany) was maintained at a column temperature of 40°C,

and separations conducted at 1.00 mL/min where an 8-min gradient program was used in which the mobile phase composition remained constant (7% water, 0.2% (v/v) acetic acid in methanol) for 5 min, after which it changed to 100% methanol over 0.1 min where it remained for a further 0.9 min before returning to the starting conditions over a further 0.1 min. The mass spectrometer was an API 3000 (Applied Biosystems), and the operating conditions were identical to those for determination of TU in serum, except that the vaporizer temperature was set at 250°C. In the MS/MS mode, the protonated $[M + H]^+$ molecules of TU, $[^2H]_3$-TU, DHTU, $[^2H]_3$-DHTU, and TD were used as precursor ion and measured as product ions at *m/z* 271.10, 274.30, 255.20, 258.10, and 271.20, respectively. The LOQ for TU and DHTU (and the corresponding related labeled analytes) in lymph was 2 and 5 ng/mL, respectively.

6.2.9 Lymph and serum concentrations of T/DHT.

T, $[^2H]$-T, DHT, $[^2H]$-DHT, and the structurally related internal standard MENT were isolated from dog serum and lymph samples by solid phase extraction. The isolated compounds were converted to their trimethylsilyl (TMS) derivatives, extracted with n-hexane, and quantified using capillary gas chromatography (GC) coupled with mass spectrometry detection in the positive ion chemical ionization mode. Serum (200 µL) and lymph (25 µL) samples were mixed with internal standard solution (50 ng/mL in acetonitrile) and 10% acetonitrile in 0.08 M HCl solution (200 and 450 µL for serum and lymph, respectively) in glass tubes. After a 30-min equilibration period, the solutions were transferred into SPE tubes (Isolute C_{18} extraction column (Separtis, Grenzach-Wyhlen, Germany), preconditioned with methanol (1 mL) and water (1 mL)) on a vacuum manifold. Samples were introduced onto the extraction columns and each column was washed twice with 1 mL of water. Extraction columns were then placed in centrifuge tubes and centrifuged (4 min at 3500 rpm) before eluting each column with 1 mL of methanol into a 4-mL glass tube. The eluate was evaporated to dryness using a vacuum centrifuge, 25 µL of Tri-Sil derivatizing reagent was added, and the tubes were then mixed and allowed to stand for 15 min at 60°C. Water (0.5 mL) and n-hexane (1.2 mL) were then added, and after thorough vortex mixing and centrifugation, the n-hexane layer was transferred into a glass vial before evaporation to dryness using a vacuum centrifuge. The residue was

reconstituted in acetonitrile and an aliquot injected onto the GC. The GC system was a HP 5890 Series II gas chromatograph equipped with a fused-silica capillary column (DB-17, 20 m x 0.18 mm, 0.30-µm film thickness; Agilent Technologies, Palo Alto, CA). Helium was the carrier gas at a column head pressure of 200 kPa, and the injector and transfer line temperatures were set at 260 and 300°C, respectively. A 7-min program was used where the oven temperature remained constant for 1 min before increasing linearly (25.0°C/min) to 300°C over the subsequent 6-min period. The mass spectrometer was a MAT SSQ-7000 (Thermo Finnigan, San Jose, CA) operated with an electron energy of -70 V, an emission current of 200 µA, and an ion source temperature of 200°C. The TMS derivatives of T, DHT, $[^2H]_3$ -T, and $[^2H]_3$ -DHT were measured at *m/z* 361.0, 363.0, 364.0, and 366.0, respectively, whereas the TMS derivative of MENT was measured at *m/z* 361.0. The LOQ of T and DHT in serum (and the corresponding related labeled analytes) were 0.2 and 0.4 ng/mL, respectively, whereas the corresponding values in lymph were 2 and 4 ng/mL.

6.2.10 $[^2H]$-TU in Intralipid.

A 50-µL aliquot of the Intralipid emulsion *(n* = 5) was diluted in propan-2-ol (950 µL), and the total TU concentration of the resulting solution was determined using an HPLC system consisting of a Spectra System P4000 pump, Spectra System AS3000 autosampler, and Spectra System UV2000 UV detector equipped with an LC analytical column (Kromasil C_{18} , 100 mm x 4.6 mm, 5-µm particle size; Varian Medical Systems) operated at a column temperature of 30°C. The mobile phase was 100% methanol flowing at 1.0 mL/min, the injection volume was 10 µL, and the detection wavelength was 254 nm. The concentration of $[^2H]_3$-TU in the Intralipid emulsion was equivalent to 79% of the total TU concentration.

6.2.11 Acceptance criteria for HPLC and GC assays.

Each analytical run included blank serum or lymph samples, duplicate calibration samples, authentic study samples, and triplicate quality control samples at three different concentrations spanning the calibration range. The results of each analytical run were accepted when the correlation coefficient of the calibration curve exceeded 0.99, when at least two-thirds of the calibration standards were accepted and the

accuracy of all accepted calibration points was between 80 and 120%, and when the accuracy of at least two-thirds of the QC samples at each concentration was between 80 and 120%.

6.2.12 Lymph concentrations of triglyceride.

After a 1 in 10 dilution of lymph samples in Milli-Q water, triglyceride (TG) concentrations were determined using a standardized clinical chemistry analyzer (Roche Cobas Mira, Basel, Switzerland) and a commercial enzyme-based colorimetric assay kit (Roche Diagnostics, Mannheim, Germany).

6.2.13 Data analysis.

The mass of analyte transported into thoracic lymph during each sampling period was calculated as the product of the analyte concentration and total volume of lymph collected during the sampling period. The fraction of the dose transported (F_{lymph}) was calculated as the ratio of cumulative mass of analyte transported and oral dose.

6.2.14 Analysis of serum pharmacokinetic profiles.

The maximum serum concentration (C_{max}), and the time to reach the maximum (t_{max}) were noted directly from the individual profiles. The area under the serum concentration-time profiles from time 0 up to the last measured concentration ($AUC^{0\text{-}tlast}$) was calculated by WinNonLin version 4 (Pharsight, Apex, NC) using the linear trapezoidal method. Where the apparent terminal phase of the serum concentration-time profiles was evident (i.e., for [^{2}H]-TU and [^{2}H]-T), the area obtained by extrapolation to infinite time was added to $AUC^{0\text{-}tlast}$ to obtain the area from time 0 to infinity $AUC^{0\text{-}\infty}$. Serum clearance of [^{2}H]-TU ($CL_{[2H]\text{-}TU}$) was calculated as the ratio of intravenous dose and $AUC^{0\text{-}\infty}$, i.e.,

$$CL_{[2H]\text{-}TU} = D_{[2H]\text{-}TU}/AUC^{0\text{-}\infty}{}_{[2H]\text{-}TU} \quad (1)$$

The proportion of the dose absorbed into the systemic circulation via the portal route ($F_{portal\ blood}$) was calculated from the ratio of the dose-normalized AUC values for TU and [^{2}H]-TU, i.e.,

$$F_{portal\ blood} = (AUC_{TU}^{0\text{-}tlast} / D_{TU}) \times (D_{[2H]\text{-}TU} / AUC_{[2H]\text{-}TU}^{0\text{-}\infty}) \times 100\% \quad (2)$$

The proportion of the dose absorbed into the systemic circulation via the thoracic lymph (F_{lymph}) was calculated from the ratio of the cumulative mass of TU transported in lymph and the dose of orally administered TU, i.e.,

$$F_{lymph} = \text{Cumulative mass of TU transported in lymph}/D_{TU} \quad (3)$$

The predicted systemic serum AUC for TU due to lymphatically transported TU ($AUC_{TU}^{lymph\ derived}$) was calculated as follows:

$$AUC_{TU}^{lymph\ derived} = \text{cumulative mass of TU transported in lymph}/CL_{[2H]\text{-}TU} \quad (4)$$

where it was assumed that 1) there was no isotope effect, i.e., TU behaves in an identical manner to [^{2}H]-TU in vivo; 2) TU entering the systemic circulation via the lymph is subject to the same dispositional and elimination events as [^{2}H]-TU administered i.v. in Intralipid; and 3) that the pharmacokinetics of TU and related compounds were linear.
The predicted systemic exposure to T, due to lymphatically transported TU ($AUC_T^{lymph\ derived}$), was calculated as follows:

$$AUC_T^{lymph\ derived} = AUC_{TU}^{lymph\ derived} * (AUC_{[2H]\text{-}T}^{0\text{-}\infty} / AUC_{[2H]\text{-}TU}^{0\text{-}\infty}) \quad (5)$$

where it was assumed that the factors determining the systemic exposure to T due to lymphatically transported TU are identical to those determining the systemic exposure to [^{2}H]-T after intravenous administration of [^{2}H]-TU.

6.2.15 Statistical analysis.

Statistically significant differences in pharmacokinetic parameters and lymph transport arising from administration of the Andriol or Andriol Testocaps formulations were analyzed by Student's *t* test at a significance level of $\alpha = 0.05$.

6.3 Results.

6.3.1 Systemic serum concentrations after i.v. administration of [^{2}H]-TU.

Fig. 1 presents the mean systemic serum concentration versus time profiles for [^{2}H]-TU, [^{2}H]-DHTU, [^{2}H]-T, and [^{2}H]-DHT after i.v. administration of [^{2}H]-TU to thoracic lymph duct-cannulated dogs, and the calculated pharmacokinetic parameters are given in Table 1. Pharmacokinetic parameters were not calculated for [^{2}H]-DHTU because there were no measurable serum concentrations in five of the eight dogs. After i.v. administration of the same [^{2}H]-TU formulation to each dog, there was no statistically significant difference *(*$p > 0.05$) in any calculated i.v. pharmacokinetic parameter between the groups of dogs that simultaneously received oral administration of Andriol or Andriol Testocaps (Table 1). Consequently, the mean value for each parameter determined after i.v. administration was calculated using data from all eight dogs.

Maximum serum concentrations of [^{2}H]-TU were observed at the end of the infusion period (i.e., $t_{max} = 0$) with maximum concentrations of [^{2}H]-T and [^{2}H]-DHT being observed at later times with the mean t_{max} values of 26 ± 3 and 49 ± 6 min for [^{2}H]-T and [^{2}H]-DHT, respectively. The mean C_{max} value for [^{2}H]-TU was 1,234 ± 65 nM, with lower values observed for [^{2}H]-T (77.7 ± 4.1 nM) and [^{2}H]-DHT (7.8 ± 1.1 nM). In the apparent terminal phase, serum concentrations of [^{2}H]-TU declined with a $t_{1/2}$ approximately 2-fold shorter than that of [^{2}H]-T and [^{2}H]-DHT, the mean $t_{1/2}$ values being 15.8 ± 0.5, 32.1 ± 2.7, and 37.5 ± 5.8 min for [^{2}H]-TU, [^{2}H]-T, and [^{2}H]-DHT, respectively. The calculated $AUC^{0-\infty}$ values were 36,429 ± 2,024, 5,466 ± 264, and 589 ± 99 nM•min for [^{2}H]-TU, [^{2}H]-T, and [^{2}H]-DHT, respectively. The mean clearance (CL) and V_D values of [^{2}H]-TU were 0.222 ± 0.012 L/min and 5.08 ± 0.31 liters, respectively.

6.3.2 *Lymphatic transport and portal concentrations after oral administration of TU.*

The mean rate of lymphatic transport of TG, TU, and DHTU into thoracic lymph after postprandial oral administration of 80 mg of TU as Andriol or Andriol Testocaps is presented in Fig. 2, A to C, together with the mean cumulative recovery of each compound in thoracic lymph (Fig. 2, D-F). Lymph concentrations of T and DHT were

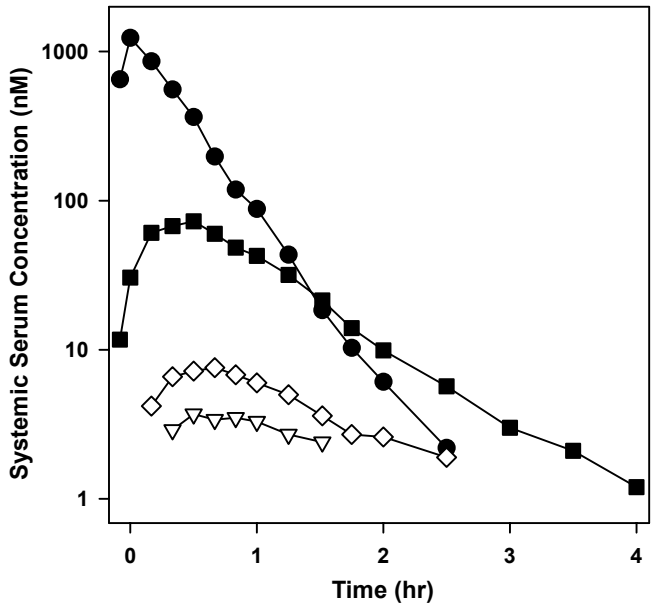

Fig. 1. Mean (n = 8) systemic serum concentrations (nanomolar) of [^{2}H]-TU (●), [^{2}H]-DHTU (▽), [^{2}H]-T (■), and [^{2}H]-DHT (◇) after i.v. administration of [^{2}H]-TU to postprandial lymph duct-cannulated dogs as a 10-min infusion in Intralipid.
Data are presented as the mean of all study dogs as there was no statistically significant difference in the i.v. pharmacokinetic parameters for groups of dogs receiving either oral Andriol or Andriol Testocaps. S.E. error bars are within the size of the individual symbols.

below the LOQ (or where measurable, so low as to be considered negligible) for the entire sampling period. The maximum rate of lymphatic TU transport was observed during the 1- to 2-h postdosing period, whereas the corresponding maxima for TG and DHTU transport occurred during the 2- to 3-h postdosing period. Recovery of TG in thoracic lymph corresponded to >80% of the administered mass of lipid confirming the integrity of each cannulated dog.

In terms of drug transport, the cumulative recoveries of TU and DHTU in thoracic lymph accounted for 3 and 0.12% of orally administered TU, respectively. The mean portal serum concentration versus time profiles of TU, T, and DHT are presented in Fig. 3. Portal serum concentrations for DHTU are not available because the concentrations were below the LOQ (i.e., <1 ng/ml) for the entire sampling period in five of the eight dogs.

Table 1. Mean (± S.E.) systemic serum pharmacokinetic parameters for [^{2}H]-TU, [^{2}H]-T, and [^{2}H]-DHT determined after a 10 min i.v. infusion (3.91 mg) of [^{2}H]-TU to dogs that simultaneously received an oral dose of 80 mg of TU administered as either Andriol (n = 4) or Andriol Testocaps (n = 4).

Compound	***i.v. Parameter***	***Oral Andriol®*** ***Group (n=4)***	***Oral Andriol® Testocaps™*** ***Group (n=4)***	***p value*** [a]	***Combined i.v. Data*** [b] ***(n=8)***
[^{2}H]-TU	C_{max} (nM)	1,250 ± 116	1,217 ± 77	0.81	1,234 ± 65
	$AUC^{0-\infty}$ (nM.min)	36,137 ± 3,835	36,720 ± 2,089	0.90	36,429 ± 2,024
	$t_{1/2}$ (min)	15.2 ± 0.7	16.4 ± 0.7	0.25	15.8 ± 0.5
	CL (L/min)	0.228 ± 0.022	0.218 ± 0.012	0.70	0.222 ± 0.012
	V_D (L)	4.98 ± 0.50	5.17 ± 0.44	0.78	5.08 ± 0.31
[^{2}H]-T	C_{max} (nM)	74.3 ± 6.5	81.1 ± 5.3	0.44	77.7 ± 4.1
	t_{max} (min)	25 ± 5	26 ± 4	0.90	26 ± 3
	$AUC^{0-\infty}$ (nM.min)	5,187 ± 452	5,746 ± 261	0.33	5,466 ± 264
	$t_{1/2}$ (min)	32.3 ± 4.5	31.8 ± 3.5	0.93	32.1 ± 2.7
^{2}H]-DHT	C_{max} (nM)	6.3 ± 1.3	9.3 ± 1.4	0.16	7.8 ± 1.1
	t_{max} (min)	42 ± 11	37 ± 6	0.68	49 ± 6
	$AUC^{0-\infty}$ (nM.min)	418 ± 73	760 ± 145	0.08	589 ± 99
	$t_{1/2}$ (min)	38.7 ± 11.7	36.3 ± 4.7	0.86	37.5 ± 5.8

[a] *p values are the results of the two-tailed Student's t test of the mean parameter estimates between the two formulations.*

[b] *The mean ± S.E. values for all study dogs (n = 8) was calculated because there was no statistically significant difference (p > 0.05) between Andriol and Andriol Testocaps for any of the measured parameters.*

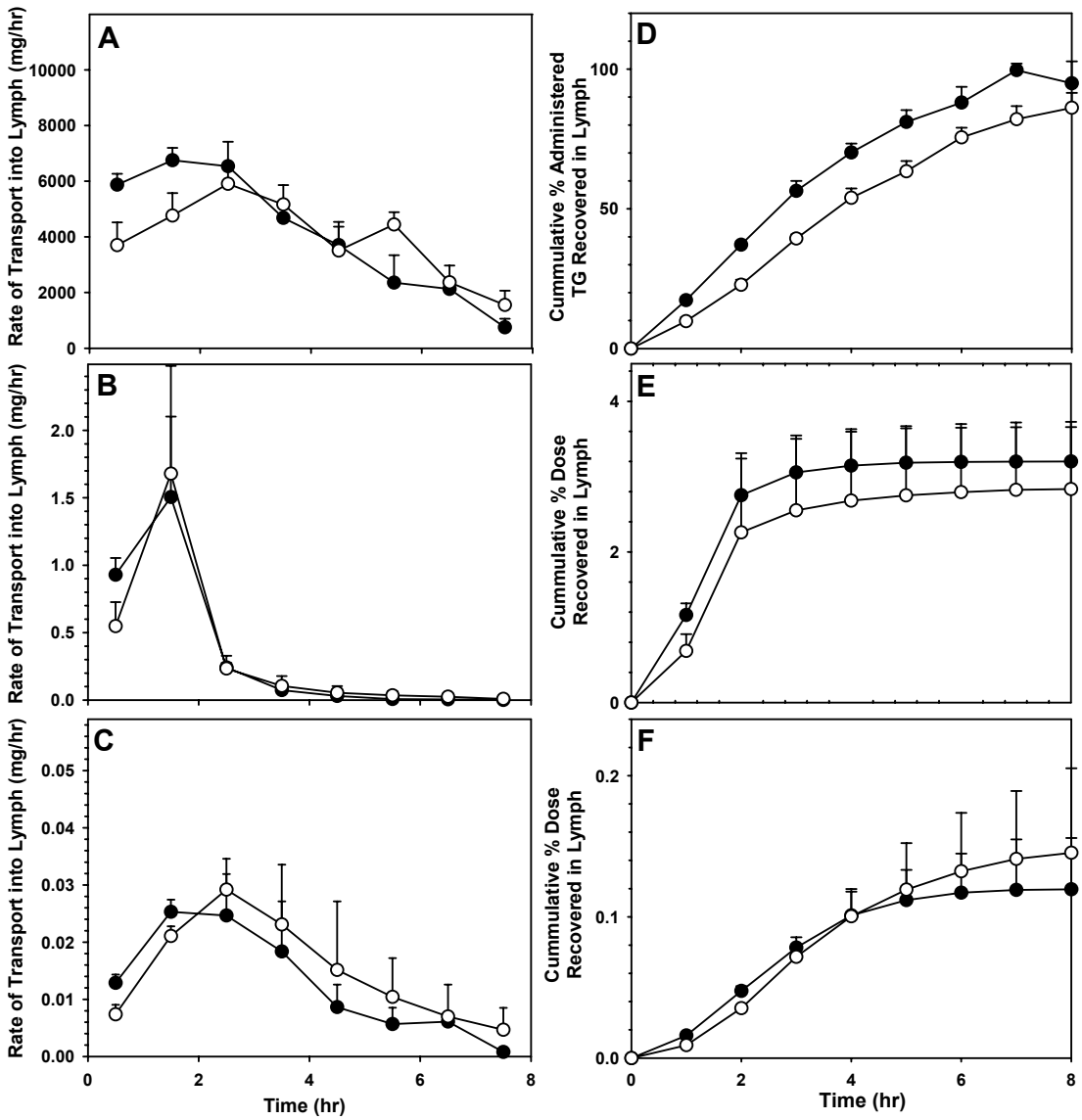

Fig. 2. Mean (± S.E.) rate (milligrams per hour) and cumulative (percentage of dose) thoracic lymphatic transport of triglycerides (TG) (A and D), TU (B and E), and DHTU (C and F) after oral administration of 80 mg of TU to postprandial lymph duct-cannulated dogs as either Andriol (closed symbols, n = 4) or Andriol Testocaps (open symbols, n = 4).

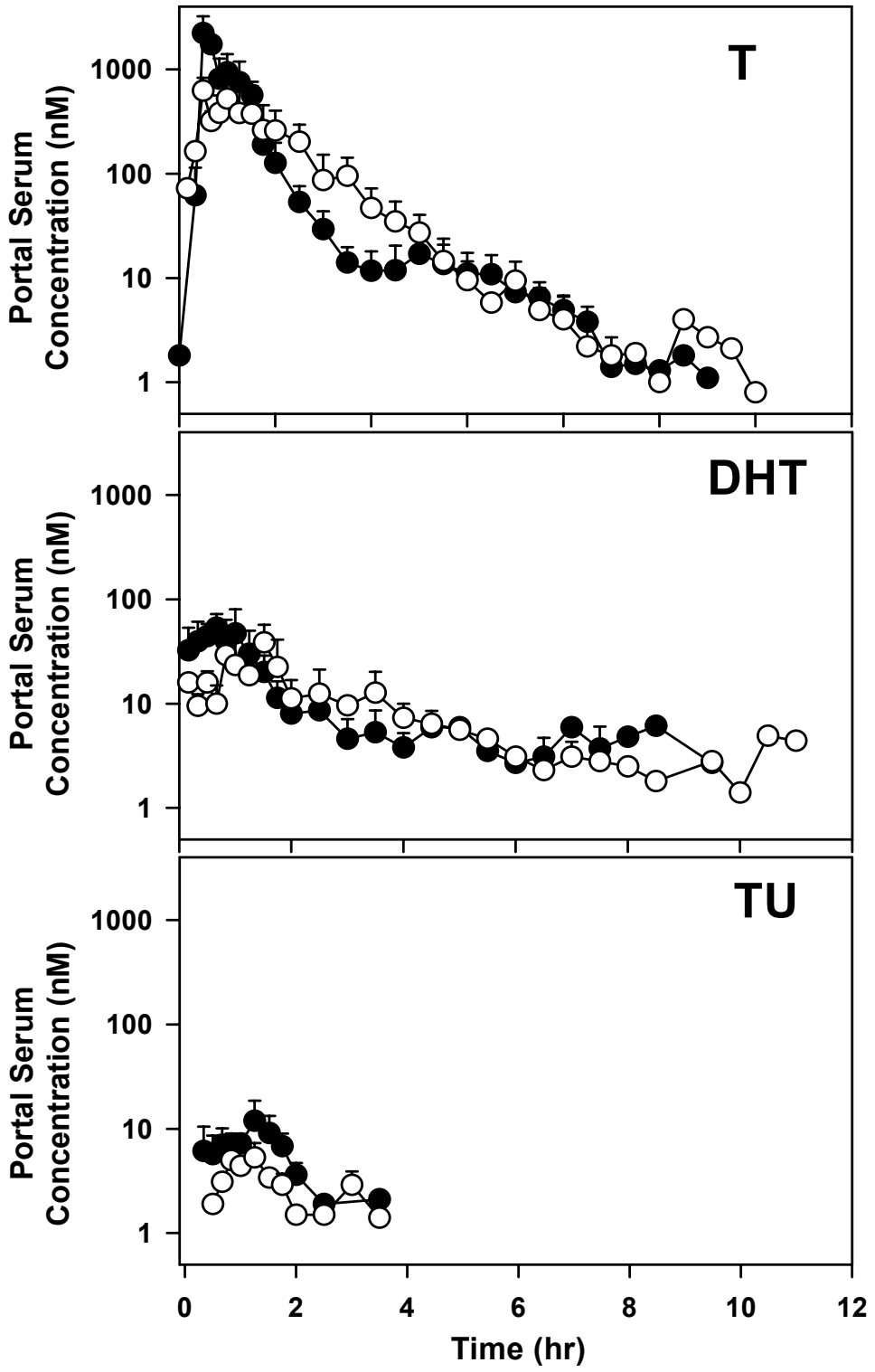

Fig. 3. Mean (± S.E.) portal serum concentrations (nanomolar) of T, DHT, and TU after oral administration of 80 mg of TU to postprandial lymph duct cannulated dogs as either Andriol (closed symbols) or Andriol Testocaps (open symbols).

6.3.3 Systemic serum concentrations of TU, DHTU, T, and DHT.

The mean systemic serum concentration versus time profiles of TU, T, and DHT after postprandial oral administration of 80 mg of TU as Andriol or Andriol Testocaps to thoracic lymph duct-cannulated dogs are presented in Fig. 4. Profiles for DHTU are not presented as systemic serum DHTU concentrations were below the LOQ for the entire sampling period in six of the eight dogs. Pharmacokinetic parameters determined for TU and T in each dog are presented in Table 2, together with the mean values for all dogs receiving the same oral formulation. There was considerable intersubject variability in the systemic C_{max} , t_{max} , and AUC values for TU and T, which gained access to the systemic circulation via the portal blood (i.e., in the lymph cannulated dogs). There was no statistically significant difference ($p > 0.05$) in any pharmacokinetic parameter in the Andriol or Andriol Testocaps groups.

6.3.4 Systemic exposure of TU and T.

The mean contribution of intestinal lymphatic transport and portal blood absorption to the systemic availability (expressed as a percentage of the oral dose) of TU administered as Andriol or Andriol Testocaps is presented in Table 3. Irrespective of the administered formulation, the lymphatic route was responsible for the majority of systemic TU exposure (>95%), even though the total mass of TU reaching the systemic circulation represented approximately 3% of the orally administered TU. Andriol and Andriol Testocaps were indistinguishable in terms of either the total oral TU availability or the fractional contributions of the two absorption routes to oral availability. The contribution of lymphatically derived T (i.e., T resulting from lymphatically absorbed TU) and portally absorbed T to systemic T exposure is presented for each dog in Table 4 as the lymph derived and measured systemic AUC values of T, respectively (i.e., $AUC^{lymph\ derived}$ and $AUC^{measured}$, respectively). For all dogs, lymph-derived T constituted the major proportion of systemically available T, and there was no statistically significant difference ($p > 0.05$) in the total systemic exposure to T (where exposure is evaluated on the basis of AUC^{total}) between Andriol and Andriol Testocaps (Table 4). Although the mean $AUC^{measured}$ of T after administration of Andriol Testocaps was approximately 30% lower compared with Andriol (i.e., T arising from portal blood absorption), the difference was not

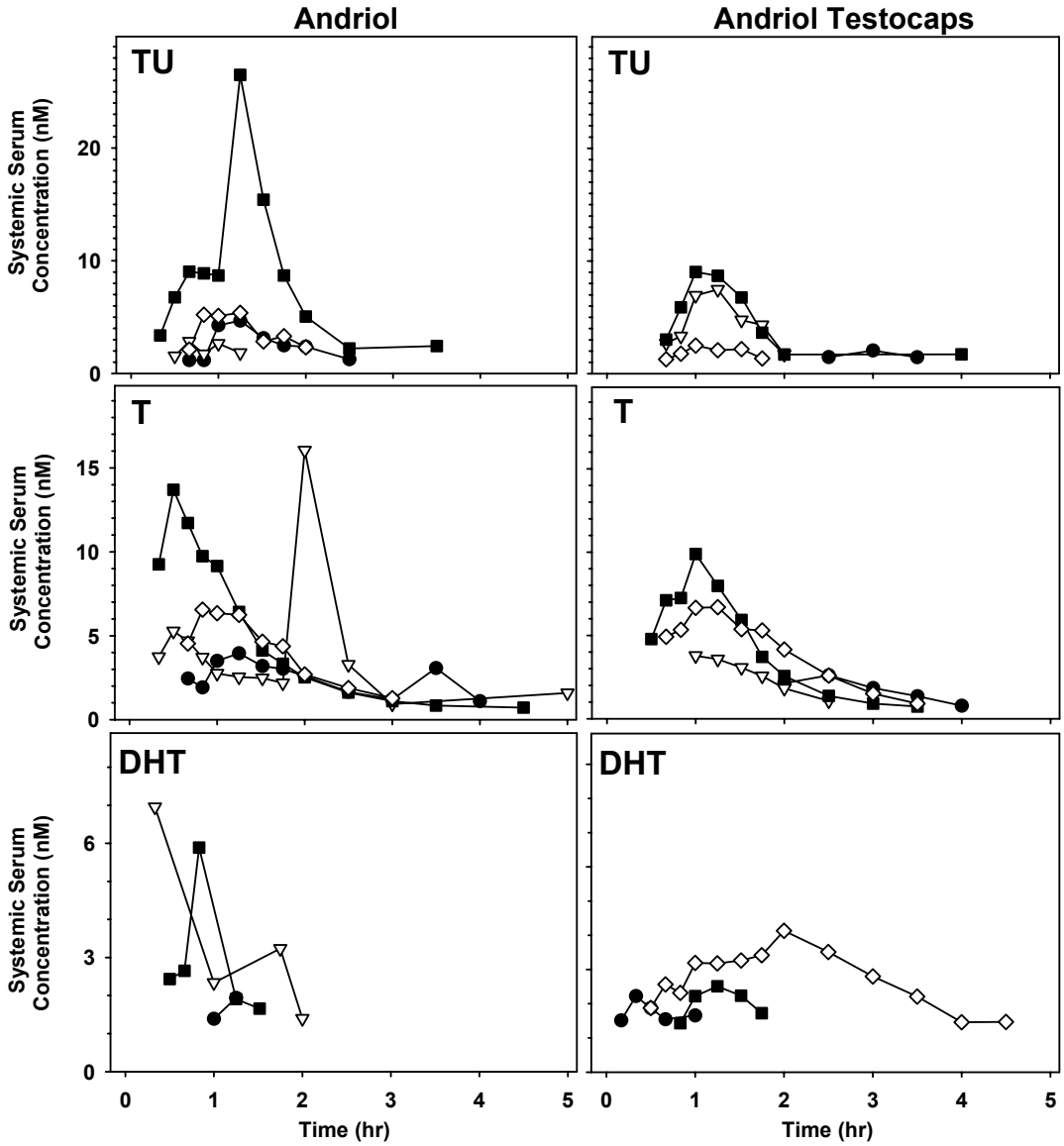

Fig. 4. Systemic serum concentration (nanomolar) versus time profiles of TU, T, and DHT in individual lymph duct cannulated dogs after post-prandial oral administration of 80 mg of TU as either Andriol (dog 2, ●; dog 4, ▽; dog 5, ■; and dog 7, ◇) or Andriol Testocaps (dog 1, ●; dog 3, ▽; dog 6, ■; and dog 8, ◇). Data have been omitted where analyte concentrations were below the LOQ.

Table 2. Systemic serum pharmacokinetic parameters for testosterone undecanoate and testosterone determined in lymph duct cannulated dogs following oral postprandial administration of 80 mg TU as either Andriol® or Andriol® Testocaps™.

Formulation	***Dog Number***	***Testosterone Undecanoate*** [a]			***Testosterone*** [a]		
		C_{max} (nM)	***t_{max} (min)***	***$AUC^{0\text{-}tlast}$ (nM.min)***	***C_{max} (nM)***	***t_{max} (min)***	***$AUC^{0\text{-}tlast}$ (nM.min)***
Andriol®	Dog 2	4.69	74	305	3.95	84	493
	Dog 4	2.85	40	108	16.05	130	792
	Dog 5	26.49	75.25	1434	13.69	40.5	968
	Dog 7	5.39	77.5	326	6.55	60.75	550
	Mean ± SE	9.86 ± 5.57	67 ± 9	543 ± 301	10.06 ± 2.86	69 ± 19	701 ± 110
Andriol® Testocaps™	Dog 1	2.05	183	127	2.6	162	239
	Dog 3	7.47	76	412	3.78	70	246
	Dog 6	9.02	60	715	9.88	70	723
	Dog 8	2.5	61.25	134	6.69	85	677
	Mean ± SE	5.26 ± 1.75	95 ± 30	347 ± 139	5.74 ± 1.63	87 ± 22	471 ± 132

[a] *no significant difference ($p>0.05$) in any parameter between Andriol® and Andriol® Testocaps™*

Table 3. The systemic availability of TU expressed as a percentage of the administered dose (mean ± SE, n=4) arising from lymphatic transport (F_{lymph}) and portal blood transport ($F_{portal\ blood}$), and as the total transport (F_{total}) in thoracic duct cannulated dogs after postprandial administration of 80 mg TU as either Andriol® (n=4) or Andriol® Testocaps™ (n=4).

Availability of TU [a]	***Andriol®***	***Andriol® Testocaps™***
F_{lymph} (% dose)	3.20 ± 0.46	2.85 ± 0.89
$F_{portal\ blood}$ (% dose)	0.054 ± 0.029	0.036 ± 0.015
F_{total} (% dose)	3.25 ± 0.48	2.88 ± 0.88

[a] *No significant difference (p > 0.05) between any parameter.*

statistically significant (p > 0.05) and the absolute values are of minor relevance because the predicted systemic T exposure arising from lymphatically derived TU (which accounts for approximately 85% of total systemic T exposure) was similar for the two formulations.

6.4 Discussion.

Testosterone undecanoate exhibits androgenic activity after oral administration in 1975 (Coert et al., 1975; Hirschhauser et al., 1975). Oral administration of TU results in the lymphatic appearance of TU and DHTU in rat (Coert et al., 1975; Noguchi et al., 1985) and humans (Horst et al., 1976), and it is generally accepted that the androgenic activity originates from T and DHT formed after systemic hydrolysis. Both TU and DHTU avoid the hepatic first-pass effect by absorption via the lymphatics (Coert et al., 1975; Horst et al., 1976). Over the past 25 years, there have been numerous reports of the efficacy and safety of orally administered TU in T replacement therapy (Luisi and Franchi, 1980; O'Carroll et al., 1985; Carani et al., 1990; Mårin et al., 1992; Gooren, 1994; Morales et al., 1997; Geurts and Coelingh Bennink, 2000); however, few studies have attempted to characterize efficacy in terms of T bioavailability due to TU administration. Although some studies have attempted such an assessment, they have not provided direct evidence of the

Table 4. The contribution of lymphatically transported Testosterone Undecanoate (TU) to the total systemic exposure of Testosterone (T) following postprandial oral administration of 80 mg TU as either Andriol® or Andriol® Testocaps™.

Formulation	***Dog Number***	***Testosterone Undecanoate***	***Testosterone***			***% contribution of lymphatically transported TU to the AUC^{total} for T***
		$AUC^{lymph\ derived}$ (nM.min) [a]	***$AUC^{lymph\ derived}$ (nM.min)***	***$AUC^{measured}$ (nM.min)***	***AUC^{total} (nM.min)***	
Andriol®	Dog 2	22,217	2,016	493	2,509	80.3
	Dog 4	22,067	4,035	792	4,827	83.6
	Dog 5	36,330	4,516	968	5,474	82.4
	Dog 7	19,338	4,054	550	4,604	88.1
	Mean ± SE	24,988 ± 3,838	3,655 ± 558	701 ± 110	4,354 ± 642	83.6 ± 1.6 [b]
Andriol® Testocaps™	Dog 1	14,650	2,544	239	2,787	91.4
	Dog 3	26,025	4,652	246	4,898	95.0
	Dog 6	7,696	1,073	723	1,796	59.7
	Dog 8	46,321	6,453	677	7,130	90.5
	Mean ± SE	23,673 ± 8,442	3,681 ± 1,180	471 ± 132	4,153 ± 1,185	84.1 ± 8.2 [b]

[a] *The $AUC^{lymph\ derived}$ value for TU was used to determine the $AUC^{lymph\ derived}$ value for T according to Equation (4).*

[b] *No significant difference ($p>0.05$) in any parameter between Andriol® and Andriol® Testocaps™*

contribution of lymphatic TU to increased systemic T exposure. For example, Frey et al. (1979) compared the relative bioavailabilities of orally administered T (in the form micronized T, crystalline TU, and TU in arachis oil) and reported an increase in systemic T exposure when TU was administered in conjunction with a high-fat meal. Although this result is consistent with enhanced lymphatic drug transport due to the simultaneous absorption of dietary lipids (Geurts and Coelingh Bennink, 2000; Khoo et al., 2001, 2002; Bagchus et al., 2003), the data are not conclusive nor quantitatively insightful. Similarly, Tauber et al. (1986) reported that the mean absolute bioavailability of T after oral administration of TU to women was 6.83 ± 3.32%, whereas the mean absolute bioavailability of orally administered T after oral T administration was 3.64 ± 2.45%. Again, these results suggest that TU leads to increased systemic T exposure (relative to oral T administration); however, they do not conclusively demonstrate the role of lymphatic TU absorption because they do not determine the relative contributions of the lymphatic and portal absorption pathways. To determine the contribution of lymphatic and portal blood absorption to the systemic bioavailability of highly lipophilic drugs, our laboratory developed and validated a thoracic lymph duct-cannulated dog model (Khoo et al., 2001). The current study was undertaken to determine the contribution of intestinal lymphatic transport of TU to systemic availability after postprandial administration of two TU formulations (Andriol or Andriol Testocaps) and to examine the hypothesis that lymphatic TU transport provides an advantage in oral T therapy because it provides for the majority of systemic T exposure after postprandial administration.

6.4.1 Systemic serum concentrations after i.v. administration of [^{2}H]-TU.

After the i.v. infusion, serum concentrations of [^{2}H]-TU declined rapidly in a monoexponential manner with a mean half-life of 15.8 ± 0.5 min with the concomitant formation of [^{2}H]-DHTU, [^{2}H]-T, and [^{2}H]-DHT arising from the respective hydrolysis and/or reduction of [^{2}H]-TU due to systemic esterase and 5α-reductase activity. Because the serum clearance of the relevant TU metabolites was not individually determined in this study, it was not possible to determine the fractional conversion of [^{2}H]-TU to [^{2}H]-DHTU, [^{2}H]-T, and [^{2}H]-DHT. However,

the data qualitatively confirm that the therapeutic benefit derived from TU is likely mediated by T and DHT.

The low variability in the serum concentration versus time profiles of [^{2}H]-TU, [^{2}H]-T, and [^{2}H]-DHT after i.v. administration of [^{2}H]-TU indicated excellent intersubject consistency in the systemic conversion of [^{2}H]-TU to [^{2}H]-T and [^{2}H]-DHT (Fig. 1). Furthermore, there was no significant difference in the i.v. profiles for the two groups that received the different oral formulations, indicating that compositional differences in the oral TU formulations did not affect the systemic pharmacokinetics of TU (Table 1).

6.4.2 Absorption into lymph after oral TU administration.

TU and DHTU were observed in thoracic lymph after oral administration of TU to postprandial lymph duct-cannulated dogs, consistent with previous reports in rats (Coert et al., 1975; Noguchi et al., 1985) and humans (Horst et al., 1976). The profile of TU transport in lymph was essentially the same after oral administration of either the Andriol or Andriol Testocaps formulations (Fig. 2, B and E). Although of minor qualitative importance in terms of the mass transported, the profile of lymphatic TU transport was different compared with that observed for TG and DHTU transport. For example, although the maximum rate of lymphatic TU transport occurred 1 to 2 h postdosing, the maximal rate of transport of TG and DHTU occurred 2 to 3 h postdosing (Fig. 2, A and C). Furthermore, the rate of lymphatic TU transport declined rapidly after reaching its maximal transport rate, whereas the rate of TG and DHTU transport declined more gradually (Fig. 2, A–C). These profiles provide possible insight into the disposition of TU (and DHTU) within the gastrointestinal tract and enterocyte before absorption into lymph. For example, because TU is metabolized within the intestinal lumen and gut wall (Coert et al., 1975; Horst et al., 1976), which is the basis for the high serum concentrations of T and DHT observed in portal blood in the present study (Fig. 3), the change in the relative rates of TG and TU transport after the 1- to 2-h postdosing period likely reflects the reduction in the available mass of TU within the enterocyte for incorporation into chylomicrons. If a limiting factor in the kinetics of TU lymph transport was rapid hydrolysis within the intestinal lumen, then the observation of the prolonged transport of DHTU in concert

with TG transport suggests that DHTU (formed by 5α-reductase metabolism of TU) may have a longer residence time within the enterocyte lipid-processing microdomains which could arise from either differences in the metabolic stability or enterocyte-based processing mechanisms for DHTU and TU.

6.4.3 Contribution of lymphatic and blood absorption to systemic availability of TU.
In the intact animal, thoracic duct lymph empties directly into the systemic circulation at the junction of the internal jugular and brachiocephalic vein. Therefore, the cumulative mass of drug recovered in thoracic lymph during the experimental period is equivalent to the mass of drug otherwise systemically available after absorption via the lymphatic route. In the lymph duct cannulated dog, the apparent bioavailability calculated from the ratio of the dose normalized systemic serum AUC values after oral and i.v. administration (determined using a stable TU isotope, which is not expected to exhibit an in vivo isotope effect; Baba et al., 1979, 1980; Shinohara et al., 1980, 1988; Fujioka et al., 1986, 1989; Shinohara and Baba, 1990) is equivalent to the fraction of the dose systemically available after absorption via the portal vein. Based on the ratio of the dose-normalized serum AUC values for TU and $[^2H]_3$-TU, the mean fraction of the TU dose reaching the systemic circulation via the portal vein was similar and extremely low at 0.054 ± 0.029 and 0.036 ± 0.015% from Andriol and Andriol Testocaps, respectively (Table 3). In terms of the lymphatic transport, the mean fraction of the orally administered dose of TU recovered in thoracic lymph was 3.20 ± 0.46 and 2.85 ± 089% from Andriol and Andriol Testocaps, respectively (Table 3). There was no statistical difference ($p > 0.05$) in the extent of portal blood or lymphatic transport of TU after administration of either Andriol or Andriol Testocaps. When the total systemic TU availability is calculated as the sum of the availabilities due to portal and lymphatic TU absorption (i.e., $F_{portal\ blood} + F_{lymph}$), it is obvious that lymphatic absorption was responsible for 93.0 to 99.8% of the TU that ultimately reaches the systemic circulation (Table 3). Because the total systemic availability of TU after postprandial administration of Andriol (3.25 ± 0.48% of the dose) was not significantly different ($p > 0.05$) compared with Andriol Testocaps (2.88 ± 0.88% of the dose), the two formulations are indistinguishable in terms of their systemic TU exposure.

6.4.4 Factors contributing to systemic T exposure.

It is evident from Fig. 1 that [^{2}H]-TU delivered into the systemic circulation is rapidly converted to [^{2}H]-T. However, it was not possible to estimate the fractional conversion of [^{2}H]-TU to [^{2}H]-T without an estimate of the serum clearance of [^{2}H]-T and this precluded calculation of the mass of T reaching the systemic circulation as a consequence of lymphatic transport of TU. This was addressed by using eqs. 4 and 5 to calculate a value for the term $AUC_T^{\text{lymph derived}}$ to estimate the systemic exposure of T arising from lymphatic transport of TU (i.e., the systemic exposure of T arising from the contents of the thoracic duct emptying into the systemic circulation).
After postprandial administration of 80 mg of TU as Andriol, the mean value of $AUC_T^{\text{lymph derived}}$ was 4,354 ± 642 nM•min and the corresponding value for Andriol Testocaps was 4,153 ± 1,185 nM•min (Table 4). Because the mean systemic T serum AUC values (AUC^{measured}) after administration of Andriol and Andriol Testocaps were 701 ± 110 and 471 ± 132 nM•min, respectively (Table 4), it is clear that T derived from systemic hydrolysis of lymphatically absorbed TU was the major contributor to the total systemic T exposure (i.e., $AUC_T^{\text{lymph derived}} + AUC^{\text{measured}}$). In addition, there was no statistically significant difference ($p > 0.05$) in AUC^{total} between dogs administered Andriol or Andriol Testocaps (Table 4). Consequently, the two formulations were indistinguishable in terms of the systemic exposure to T.

6.5 Conclusion.

Numerous strategies (typically involving administration of 17-α-alkylated testosterone prodrugs via the oral and injected routes) have previously been applied to circumvent the fact that direct oral T administration is ineffective in androgen replacement therapy. However, orally administered testosterone undecanoate is both safe and efficacious upon oral drug administration (Luisi and Franchi, 1980; O'Carroll et al., 1985; Carani et al., 1990; Mårin et al., 1992; Gooren, 1994; Morales et al., 1997; Geurts and Coelingh Bennink, 2000). The results from the current study demonstrate conclusively that the therapeutic advantage associated with oral TU administration is a consequence of lymphatic transport of TU to the systemic circulation. Furthermore, the study demonstrated conclusively that there is no difference in the systemic

exposure of T, or TU, resulting from postprandial oral administration of Andriol or Andriol Testocaps.

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7 Biowaivers for oral immediate release products: Implications of linear pharmacokinetics.

Abstract

Bioequivalence of drug formulations plays an important role in drug development. Recently the Biopharmaceutical Classification System (BCS) has been implemented for waiving bioequivalence studies on the basis of the solubility and gastrointestinal permeability of drug substance. This study aims to give a contribution to a recent call for new approaches for waiving bioequivalence studies.

Using the rationale of the BCS it can be argued that biowaivers can also be granted on the basis of standard pharmacokinetic data. If a drug exhibits dose linear pharmacokinetics and the dosage form shows a sufficiently fast dissolution profile, it can be concluded that this drug appears to pose no problem with respect to absorption. It should be noted that a change of an immediate release tablet formulation can only lead to a deviating rate and/or extent of absorption when the release of the active from the formulation is altered. Logically, the dissolution profiles of the different formulations should be equal to guarantee bioequivalency. Thus, both BCS and the alternative linear pharmacokinetics approach require an evaluation of dissolution profiles. The justification of BCS is found in the permeability classification of the compound, that of the linear pharmacokinetics lies in the apparent lack of a permeability problem. In this context e.g. P-glycoprotein (Pgp) transported drugs form a interesting class of compounds, which may be treated likewise when complying to the aforementioned requirements. Furthermore, poorly soluble compounds may be less troublesome than expected. It is shown that linear kinetics can be explained by the solubilizing activity of e.g. bile salts. Here linear pharmacokinetics shows that elevated doses appear not to exhibit a limitating role on the dissolution. Hence, a change in formulation without any effect on the dissolution profile is not expected to cause a change in availability. It is clear that the formulations to be compared should not contain excipients that display an effect on (presystemic) drug metabolism.

Accepted Clin. Pharmacokinet.

7.1 Introduction.

In the 1960's several case histories started to attract the attention of the scientific community on bioequivalence of medicinal products. It became evident that differences in rate and extent of absorption could result in either undermedication or intoxication (Wijnand, 1994). Well known are the prednisone, phenytoin and digoxin cases (Wijnand, 1994). For prednisone dramatic differences were found in the dissolution profiles, while for phenytoin a change in the excipients was the cause for bioinequivalence. In 1967 the FDA defined criteria for possible 'problem' drugs, recognizing that bioequivalence is an important issue in drug development (Wijnand, 1994). Bioequivalence is even more important in the case of Narrow Theurapeutic Index (NTI) drugs. Examples of drugs with a NTI and reported bioequivalence problems are phenytoin, digoxin and warfarin (Wijnand, 1994; Rowland and Tozer, 1994; Schultz, 1995; PMSB/ELD Notifications, 2000).

A generally accepted practical definition of bioavailability is understood to be the extent and the rate at which a drug is delivered from a pharmaceutical form and becomes available in the general circulation (CPMP, 2001; Guidance for Industry, 2000a). Two oral dosage forms are considered to be bioequivalent if both rate and extent of absorption are the same. In clinical development of New Chemical Entities (NCE), bioequivalence studies need to be performed when the formulation of the pharmaceutical dosage form has been changed. Typical examples are changes in the composition, production parameters or process technology. However, for NCEs with a high solubility (i.e. the administered dose is soluble in the gastrointestinal fluids) and a high permeability of the gastrointestinal membrane (e.g. measured in Caco-2 permeability studies) it is fair to expect that (small) formulation changes, which hardly affect the dissolution behavior of tablets or capsules, do not have a significant influence on the in vivo absorption behaviour. Hence, drugs with these properties are expected to be bioequivalent. These arguments formed the scientific basis for the Biopharmaceutical Classification System (BCS) which is extensively described in literature (Amidon et al., 1995). Recently a guidance on obtaining biowaivers on the basis of solubility and permeability data has been published by the FDA (Guidance for Industry, 2000b). Also the European regulatory agencies are working with these

concepts, but extra requirements were defined (e.g. the bioavailability should be higher than 90% and the absence of metabolism) (CPMP, 2001).

In the BCS, drugs are classified on the basis of the solubility and gastrointestinal permeability. Four classes of compounds can be distinguished: I (high solubility, high permeability), II (low solubility, high permeability), III (high solubility, low permeability) and IV (low solubility, low permeability). Class I compounds are typical examples for waiving bioequivalence studies. In the selection process of NCE's, compounds with a low aqueous solubility and low permeability are preferably filtered out since they might pose problems during pharmaceutical development. In the BCS the emphasis is placed on what happens with the formulation in the gastro intestinal tract. The so called dose, dissolution, and absorption number are estimated on the basis of Fick's first law to evaluate respectively the solubility, dissolution, and absorption of the drug substance. Basically, the BCS thus predicts a theoretically expected outcome.

The main assumption in the BCS is that, if the fraction of the dose absorbed is the same, the human body should always do the same with the absorbed compound (i.e. distribution and elimination will not be changed). Even in a disease state this argument is still a valid argument.

Using the scientific rationale, developed to set up the Biopharmaceutical Classification System, it can be argued that biowaivers may also be granted on the basis of standard pharmacokinetic data as obtained within drug development, e.g. single dose and single rising dose pharmacokinetic data as obtained in clinical phase 1 studies. When a drug exhibits dose linearity (a linear relation between AUC and/or C_{max} with the dose) and a reasonably fast dissolution profile, it can be argued that these compounds do not pose problems with respect to absorption. The approach is based on actual measurements of the amount of drug which has reached the systemic circulation. Linearity in fact reflects that changes in neither solubility nor permeability are critical in the dose range tested. It should be noted that the standard pharmacokinetic data used for this approach represent the performance of one particular type of formulation. Alternative formulations can only be biopharmaceutically similar when the rate and extent of release of the drug substance from this formulation is equal. Comparison of two different formulations should be

done on the basis of dissolution testing, which is basically the same as for the BCS approach. Hence, in vivo pharmacokinetic data can be used as surrogate parameters for in vivo solubility and permeability data.

This paper aims to substantiate a claim for obtaining biowaivers on the basis of standard human pharmacokinetic data. With this, we would like to give a contribution for new approaches for waiving bioequivalence studies, as called for recently (Chen et al., 2001; Yu et al., 2002).

As will be shown down below, both the BCS and the dose linear pharmacokinetic approach are complementary to each other, and can be used vice versa to support the case for obtaining biowaivers**.**

7.2 Experimental.

7.2.1 Materials.

Ketoprofen and naproxen were purchased form Sigma Chemical Co. (St.Louis, MO). HPLC grade acetonitrile and methanol were obtained from Fischer Scientific (Fair Lawn, NJ). All other chemicals were of analytical reagent (AR) grade. The steroids (11beta,17alpha)-11-ethyl-17-hydroxy-19-norpregn-4-en-20-yn-3-one (Org 4060) and (6beta,11beta,17beta)-11-[4-(dimethylamino)phenyl]-4',5'-dihydro-6-methylspiro[estra-4,9-diene-17,2'(3'H)-furan]-3-one (Org 31710) were supplied by NV Organon.

7.2.2 Bile fluids beagle dogs.

Ex vivo bile fluids from beagle dogs were obtained during section experiments in toxicological experiments. After retrieving the bile fluids the samples were immediately stored at -70 °C. All animal experiments were conducted in accordance to protocols approved by the local Animal Ethics Committee.

7.2.3 Solubility determination in bile fluids.

Prior to the determination of the solubility the bile fluids were thawed at room temperature. Aliquots (2-3 mL) of 5 dogs were pooled and diluted with phosphate buffer 1:1 (v/v) (pH=6.5). Small alliquots (3 mL) were placed in 10 mL glass test tubes and an excess of drug (150 mg) was then added to either the phosphate buffer or

bile solution and shaken (250 rpm) for 6 hours at 37 °C in a warm air tank. Subsequently, the solutions were filtered (0.45 µm GHP Acrodisc filter), diluted (if necessary) to suitable concentrations and analyzed by HPLC. One of the duplicate samples was filtered for a second time to determine adsorption of drug substance to the filter. All samples were assayed by HPLC for the drug content. The content of the second sample was used to determine the filter recovery.

7.2.4 High performance liquid chromatography.

All concentration were determined by HPLC (HP1100 with DAD detection and temperature controlled column compartment). A Symmetry C18 column (length: 250 mm, internal diameter: 4.6 mm, 5 µm particle size) was used for the separation and the temperature was held at 40 °C. A MilliQ-water (pH=3.2):acetonitrile:methanol (40:17:13 v/v) buffer and methanol were used as mobile phase, which was run in a gradient:

1. 0 - 10 min : isocratic buffer (buffer:methanol = 100:0)
2. 10-20 min methanol gradient (buffer:methanol= 100:0 → 25:75)
3. 20-25 min isocratic buffer (buffer:methanol = 25:75)

The flow rate was 1 mL/min and detection was performed at 244, 254 and 300 nm (bandwidth 8 nm). The injection volume was 10 µL. The retention times were typically between 5 and 6 min.

The detection limits of the used analytical method are: 0.0000079 (ketoprofen), 0.010 (naproxen), 0.0000083 (Org 4060) and 0.0027 (Org 31710) mg/mL.

7.2.5 Single dose pharmacokinetic study with Org 4060.

The study was performed at ASTER, Paris, France, and was conducted in compliance with the Declaration of Helsinki (18th World Medical Assembly 1964, amended in Tokyo 1975, Venice 1983, and Hong Kong 1989) and with Good Clinical Practice guidelines. The study protocol was approved by the local ethics committee, and all thirty healthy postmenopausal volunteers (age: 49 - 61 years; body weight: 50 - 78 kg) signed informed consent before participating in the study.

The study was performed double blind and was placebo controlled. Per dose group, five subjects received Org 4060 (0.25, 0.5, 1.0, 2.0 or 10.0 mg) and one subject

received a placebo tablet. The tablets contained 0, 0.25, or 1.0 mg Org 4060 and corn starch, hydroxypropylcellulose, lactose and magnesium stearate as excipients. The study was conducted in the fasted state. No serious adverse events were reported during the trial period.

Venous blood samples were collected before and 0.25, 0.5, 075, 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 20, 24, 48 and 72 hours following oral intake of Org 4060. After collection, blood samples were processed to serum and stored in separate tubes at -20 °C until analysis.

Org 4060 plasma concentrations were measured by use of a validated gas chromatographic assay with mass spectrophotometric detection. Based on plasma Org 4060 concentrations the pharmacokinetic data were calculated.

7.3 Results.

7.3.1 Reflections on solubility: low solubility drugs.

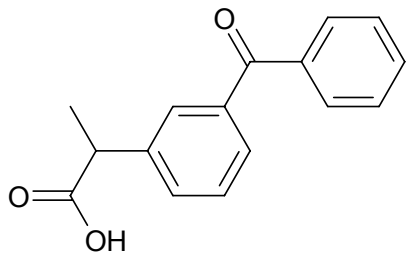
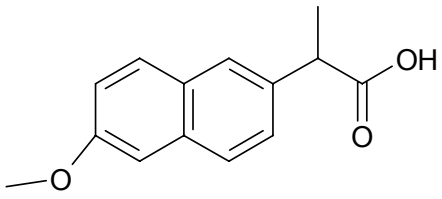
7.3.1.1 Ketoprofen and naproxen (reference drugs from the Biopharmaceutical Classification System).

Ketoprofen and naproxen are both reference Class II drugs in the BCS. They are both non-steroidal anti-inflammatory drugs (NSAID) of the 2-arylpropionic acid class and have been used clinically for decades in the treatment of rheumatoid disorders (arthritis and osteoarthritis), dysmenorrhea and pain (Hardman and Limbird, 1996). The chemical structures, dose regimens and a summary of the pharmacokinetic parameters are shown in Table 1.

The absorption of ketoprofen and naproxen is rapid and almost complete when given orally (Hardman and Limbird, 1996; Davies and Anderson, 1997; Jamali and Brocks, 1990). Absorption of these drugs is essentially a passive process primarly determined by the physicochemical properties of the drug. The time to peak maximum in the plasmaconcentration versus time curve varies from 1 tot 4 hours and the volume of distribution is the same for both compounds.

Table 1. Chemical structures, dose regimens and pharmacokinetic parameteres of ketoprofen and naproxen.

Chemical structures and solubilities.

	
ketoprofen	*naproxen*

pK_a and solubility in aqueous solutions and bile (mg/mL)

pK_a	4.8 [1]	pK_a	4.2 [1]
Water pH = 1	0.012	Water pH = 1	0.030 [1]
Phosphate buffer pH = 6.5	3.31	Phosphate buffer pH = 6.5	1.40
Bile pH = 6.5	8.36	Bile pH = 6.5	3.40

Dose regimens. [2]

	ketoprofen	*naproxen*
Rheumatoid disorders	50 - 75 mg	250- 500 mg
Dysmenorrhea	25 - 50 mg	250- 500 mg
Pain	20 - 50 mg	250- 500 mg

Pharmacokinetic parameters. [2]

	ketoprofen	*naproxen*
t_{max} (h)	1.2 – 2	2 - 4
C_{max} (mg/L)	3.9 and 2.0 [3]	37 - 79 [4]
$F_{absorbed}$ (%)	90	100
V_d (L/kg)	0.1	0.16
$t_{1/2}$ (h)	2 – 4	12 – 15

[1] (McFarland et al., 2000) [2] (Hardman and Limbird, 1996; Davies and Anderson, 1997; Jamali and Brocks, 1990) [3] After a 50 mg dose four times daily in the fasted respectively fed state.[4] After a 250 and 500 mg single dose.

Ketoprofen shows dose linearity for the R and S enantiomers over the complete therapeutic dose range (50 to 200 mg) after administration of racemic ketoprofen

(Jamali and Brocks, 1990; Geisslinger et al., 1995; Rudy et al., 1998). The AUC of naproxen is dose linear for doses up to 500 mg (Davies and Anderson, 1997; Runkel et al., 1974). Multiple dose administration yields absorption characteristics similar to those seen after single doses. Doses of naproxen higher than 500 mg show that the AUC increases non linearly (Runkel et al., 1974; Runkel et al., 1976). This is caused by saturation of the plasma protein binding of naproxen, which causes the fraction of unbound naproxen to increase. Hence, the clearance of naproxen is also increased with the dose.

The solubility experiments (Table 1) show that both ketoprofen and naproxen are practically insoluble in the aqueous media with a low pH (approx. 1). However, when the pH is raised to 6.5, a pH value representative for the gastrointestinal tract, the solubility increases dramatically for both NSAID's (Dressman, 1998). This is caused by the pH value of the buffers in relation to the pKa values (Table 1). When bile is present the solubility is increased with an additional factor of 2 to 3. In vivo this will result in a complete dissolution of the compounds which makes them readily available for absorption. This is consistent with the observation that both ketoprofen and naproxen are rapidly and completely absorbed within the dose linear range (Table 1).

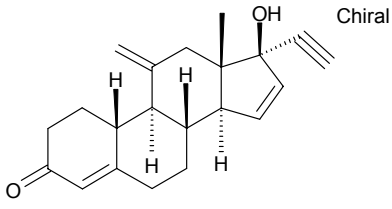
7.3.1.2 Steroids.

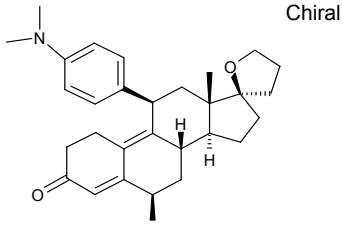
The hydrophobic properties and low aqueous solubility of steroids have led to the general perception that steroids are 'problem' drugs. Although the amount of data in literature is limited, steroids may be considered as a class with a good gastrointestinal absorption representing a good passive transcellular permeability (Hardman and Limbird, 1996; Faassen et al., 2003). Since steroids are generally well absorbed, they would typically be classified as Class I or II drugs according to the BCS, depending on the dose. Table 2 shows the pharmacokinetic data of Org 4060. The chemical structures, solubility in bile fluid and the dose-AUC relationship of Org 4060 and Org 31710 are given in Figure 1.

Table 2. Pharmacokinetic parameters of Org 4060 (mean ± SD).

Dose (mg)	***t_{max} (h)***	***C_{max} (ng/mL)***	***$t_{1/2}$ (h)***	***$AUC_{0\rightarrow\infty}$ (ng.h/mL)***	***nC_{max} (ng/mL/mg)***	***$nAUC_{0\rightarrow\infty}$ (ng.h/mL/mg)***
0.25	0.65 ± 0.22	1.19 ± 0.81	35.3 ± 15.6	14.0 ± 7.6	4.77 ± 3.24	56.0 ± 30.4
0.50	0.65 ± 0.22	2.27 ± 1.25	37.1 ± 14.9	25.6 ± 9.1	4.55 ± 2.50	51.2 ± 18.1
1.00	0.70 ± 0.27	4.06 ± 1.69	38.9 ± 17.2	49.5 ± 16.2	4.06 ± 1.69	50.0 ± 16.2
2.00	0.70 ± 0.45	5.15 ± 1.73	29.7 ± 6.7	84.6 ± 57.0	2.57 ± 0.87	42.3 ± 28.5
10.00	0.65 ± 0.22	9.31 ± 4.12	65.2 ± 1.32	481.9 ± 178.7	0.93 ± 0.41	48.2 ± 17.9

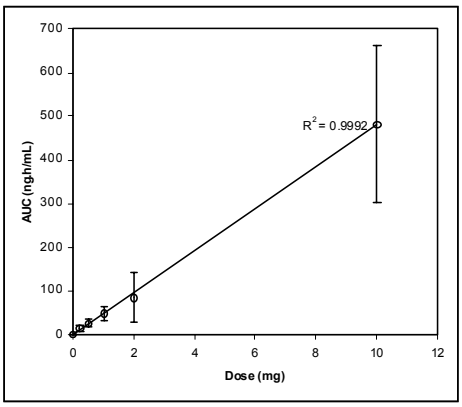
Chemical structures and solubility (mg/mL) in phosphate buffer and bile fluid.

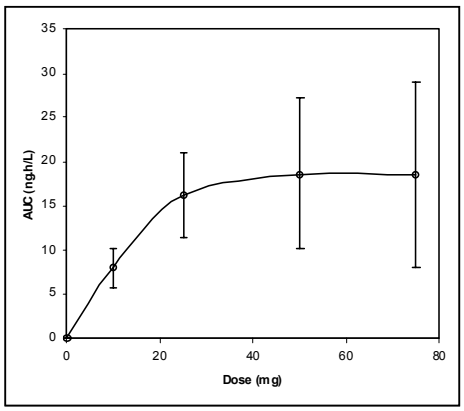




Phosphate buffer pH = 6.5 Bile pH = 6.5	0.00001 0.069	Phosphate buffer pH = 6.5 Bile pH = 6.5	n/d 1.74

AUC versus dose plots.





Org 4060	*Org 31710* [1]

Figure 1. Chemical structures, solubility in bile fluid and the AUC versus Dose relationship for Org 4060 and Org 31710. Error bars indicate standard deviation. n/d = not detectable.

[1] *(Mulder et al., 1998)*

A dose-linear relationship was found for the AUC of Org 4060 in the dose range studied (0 to 10 mg). However, at doses higher than 1 mg, C_{max} becomes non-linear which is most probably due to a somewhat slower dissolution rate. Although the mean estimated $t_{1/2}$ for the highest dose group (10 mg) was considered to be long (65.2 ± 1.3 h), the ANOVA on the log-transformed $t_{1/2}$ values showed no statistically significant dose effect. For Org 31710 a statistically significant deviation from linearity was observed in the pharmacokinetic parameters after administration of single oral doses of 10 to 75 mg (Mulder et al., 1998). The nC_{max} and nAUC values were lower in the higher dose groups, whereas there is a tendency for $t_{1/2}$ to increase and for t_{max} to decrease with increasing dose.

The pharmacokinetic data show that for Org 4060, in spite of the low aqueous solubility, (Figure 1) absorption is not impaired. I.e. the dose linear response shows that the in vivo solubility has not been limited and was followed by a good absorption. These findings are further supported by the in vivo solublization by bile salts (Figure 1) and the generally accepted idea that steroids are well absorbed (Faassen et al., 2003; Bakatselou et al., 1991; Horter and Dressman, 1997; Wiedmann and Kamel, 2002). However, the dose non-linearity of Org 31710 indicates that at (extremely) high doses absorption will become dissolution limited.

7.3.2 Reflections on permeability: P-glycoprotein (Pgp) transported drugs.

P-glycoprotein, an intestinal transmembrane efflux transporter, is extensively described as a possible cause for reducing gastrointestinal absorption (Schinkel, 1999; Chiou et al., 2001). Because of saturation phenomena, it is often thought that Pgp transported drugs will not have dose linear pharmacokinetics. However, in a recent publication the contrary was shown to be true and Table 3 shows 14 commercially available Pgp transported drugs which all have linear pharmacokinetics (Chiou et al., 2001; Heykants et al., 1981; Huang et al., 1986).

Since dose linear pharmacokinetics are indicative for a non-problematic in vivo dissolution and absorption of drugs, the clinical significance of P-glycoprotein on the gastro intestinal absorption seems to be limited (Chiou et al., 2001). This is inherently caused by the design of clinical studies during development. In case that no exposure is found the dose will be increased in discrete intervals resulting in saturation of the

Pgp transporter (Sandstrom et al., 1998) with the matching dose linear range. Secondly, when the permeability of a drug is high, Pgp efflux may become less important due to the long gastrointestinal transit time (Lentz et al., 2000). I.e. absorption will take place in due time, irrespective whether the Pgp transporter is present. Finally, on average the pharmacokinetic parameters (fraction absorbed, bound fraction, urinary excretion, total, renal and hepatic clearance) of Pgp transported drugs do not differ significantly from non Pgp transported drugs (Sakaeda et al., 2001).

Table 3. Reported in vitro polarized transport properties (Caco-2) and oral doses used in humans of 14 P-glycoprotein or other efflux transporter substrates that indicate dose-independent pharmacokinetics (Chiou et al., 2001; Heykants et al., 1981; Huang et al., 1986).

Drug	***Efflux ratio***	***Dose employed in oral studies (mg)***
Digoxin	26.4	0.25, 0.5, 1.0
Etoposide	4.9	25, 100, 200
Felodipine [1]	1.7	5, 15, 40
Fexofenadine [1]	3.5	20, 60, 120
Furosemide	9.7	40, 80
Indinavir	13	400, 800
Losartan	4	10, 25, 50, 100, 200, 300
Nadolol	2	60, 120
Propranolol [1]	1.5	40, 80, 160
Ritonavir	15	200, 400, 600,
Saquinavir	25	75, 200, 600, 1200, 1800
Tacrolimus	4.8	1, 3, 5, 7, 10
Verapamil [1]	1.4	40, 80, 120
Domperidone	15 - 36 [2]	10, 60

The efflux ratio is defined as the ratio of the permeability from the basolateral to apical compartment and the permeability from the apical to basolateral compartment.
[1] Also marketed as an extended release formulation (Micromedex, 2003).
[2] Unpublished data on file.

7.4 Discussion.

In order for a drug to become systemically available after oral administration it needs to dissolve in the gastrointestinal tract, followed by absorption. If two formulations are claimed to be bioequivalent it needs to be ensured that both rate and extent of absorption are the same. In a typical dose linearity study the same formulation will be used for the complete dose range. However, it is not to be expected that a change in formulation will influence dose linearity as long as the dissolution profiles are similar. I.e. the same amount of drug will dissolve in the gastrointestinal tract followed by absorption. It should be noted that the same arguments hold for changes in the dose within the dose linear range. In the CPMP guideline on bioequivalence, these dose changes are used to waive BE studies for the intermediate strengths in the dose linear range(CPMP, 2001). The advantage of using pharmacokinetic data is that the waiver for bioequivalence studies is based on in vivo human data instead of in vitro data. Ketoprofen and naproxen, both Class II and reference compounds in the BCS, have dose linear pharmacokinetics. In the BCS no biowaiver will be granted for both drugs. However, under intestinal in vivo conditions the solubility is so high that the complete dose can readily be dissolved (Table 1). As indicated by the dose linearity, the absorption of ketoprofen and naproxen is an efficient process in the clinically relevant dose range. This is confirmed by earlier published human jejunal permeability data for ketoprofen and naproxen (Lennernas et al., 1995). As a result, linear pharmacokinetics provides justification for a waiver for both ketoprofen and naproxen in the dose linear range. By analogy with the BCS, pharmacokinetic data are used as surrogate parameters for in vivo solubility and permeability. When linear pharmacokinetics is understood by the solubility enhancement of intestinal fluid, there is obviously a justification to apply these principles to Class ll drugs (low solubility, high permeability).

In the concept of dose linearity no biowaiver can be granted for naproxen when the applied dose is higher than 500 mg. Since non linearity is caused by changes in clearance (Davies and Anderson, 1997; Runkel et al., 1974; Runkel et al., 1976) and not by changes in dissolution or absorption the prediction of the dose linearity concept fails under these particular conditions.

The pharmacokinetic data and ex vivo bile fluid solubility data of steroids and P-glycoprotein transported drugs show that also for these drugs biowaivers can be granted on the same arguments. The steroid Org 4060 displays linearity over the complete dose range tested
(0 - 10 mg) and a biowaiver is possible provided that the dissolution profiles are equal. Dose linearity of Pgp transported drugs seems to indicate that the clinical significance of Pgp on the gastro intestinal absorption may be limited (Chiou et al., 2001).
On the other hand Org 31710 displays non-linear pharmacokinetics at the high doses tested (upto 75 mg, Figure 1). The observed dose non linearity is caused by an incomplete dissolution due to the high doses tested. As a result no biowaiver can be granted for Org 31710.
As already indicated the dissolution profiles of the different formulations should be equal to guarantee bioequivalence. For ketoprofen and naproxen the solubility data (Table 1) indicate that the pH of the dissolution medium is the most important parameter. As a result dissolution experiments for the comparison of the reference and test product could be conducted in an aqueous medium with a pH of 6.8, mimicing the intestinal pH, to test the similarity of the dissolution profiles. It is clear that the applied dissolution method must be adjusted to the physicochemical properties of the drug and that the discriminating power of the method should be high enough to detect products with poor in vivo performance (Guidance for Industry, 1997). In the current guidelines this is handled by defining a criterion for rapid dissolution (85% of the drug dissolves in 15 to 30 min (CPMP, 2001; Guidance for Industry, 2000b)), assuming that in this particular case dissolution is a non critical parameter and that the formulation behaves like a solution (Guidance for Industry, 1997). In the preface to USP 24 it is stated that 'There is no known medically significant bioinequivalence problem with articles where 75% of an article is dissolved in water or acid at 37 °C in 45 minutes in the official basket or paddle apparatus operated at the usual speed, that is, USP First Case. It obviates wastefull biostudies' (USP 24). At present the development of in vivo relevant dissolution media for discriminating dissolution methods is still being discussed in the scientific literature (Yu et al., 2002; Dressman, 1998; Dressman and Reppas, 2000; Kostewicz et al., 2002).

One clear assumption in the dose linear approach is that the formulation should not affect the absorption process itself. When an excipient would change for example intestinal metabolism, the extent of absorption could be altered. In this case a drug like Saquinavir, which has dose linear pharmacokinetics in the fasted state and is presystemically metabolised by CYP3A4, would not be bioequivalent (Chiou et al., 2001; Muirhead et al., 1992; Shaw et al., 1992). However, excipients which interfere with presystemic metabolism are not known and, in general, excipients should not exhibit a pharmacological effect.

For granting biowaivers in general we propose the following strategy. In the early phase of development of NCE's the dose linearity of single rising dose studies can be used for biowaivers in combination with supporting permeability (Caco-2) and solubility (aqueous and bile solutions) data. When clinical development proceeds (e.g. multiple dose, food interaction and ADME studies) the dose linearity principle can be further substantiated. In the end a scientific case can be presented for obtaining biowaivers on the basis of human clinical data and supporting in vitro permeability and solubility results.

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Summary.

Most drugs display their therapeutic activity on specific places in the human body, the so-called receptors. Drugs administered via any route (oral, transdermal, nasal etc.) should reach the systemic circulation in order to be transported towards the receptors. Irrespective of the route of administration the same sequence of steps are of relevance for the exposure to a drug: release from the dosage form (*dissolution*), absorption into the blood (*permeation through a biological membrane*) and finally removal from the body (*metabolism and elimination*).

From the preceeding it is clear that a drug is only active when it reaches the central circulation. Biopharmaceutics is a field of scientific interest, which studies the relationship between the physicochemical properties of the drug, the characteristics of the dosage form and the (human) physiology (chapter 1). The physicochemical properties determine whether a drug dissolves and can permeate through a biological barrier. The dosage form and formulation enable an efficient absorption process. Finally, human physiology dictates the necessary physicochemical properties, the dosage form and formulation in relation to the route of administration.

The first part of this thesis (chapters 2 through 4) focusses on the physicochemical properties of drugs. Chapter 2 evaluates the physicochemical properties of drugs required to achieve appropriate absorption upon several routes of administration. For the oral route of administration the 'ideal' set of physicochemical properties is well established, however, the other routes have received much less attention. For all studied drug delivery routes (oral, transdermal, buccal/sublingual, nasal and vaginal) the barriers to the systemic circulation are formed by multiple layered epithelia, with the exception of the gastrointestinal epithelium which consist of a single layer of enterocytes. The different epithelia are made up of a wide variety of cell types contributing to the specialized functions of these tissues. Irrespective of the route of administration the majority of drugs are absorbed either transcellularly or paracellularly (intercellularly).

Despite of the physiological differences in the barriers, it appears that the physicochemical properties required for acceptable permeability through these tissues are all within the generally accepted range needed for good oral absorption. Permeation is however only possible when the compound has been dissolved. For

this, the solubility must be evaluated with regard to the 'physiological' volume available for dissolution. In general, this volume is much smaller for the non-oral routes. Consequently, solubility is one of the most critical parameters for transdermal, buccal/sublingual, vaginal and nasal drug delivery. This is why the formulation becomes of dominant importance. Eventually, a change in route of administration is not suitable to increase the bioavailability/exposure in case of drugs with less optimal physicochemical properties.

In steroid research the focuss has been placed on pharmacology and pharmacokinetics. As a result the biopharmaceutical properties received relatively little attention. In the second study the physicochemical properties of steroids were evaluated in relation to gastrointestinal absorption (chapter 3). The chemical structures of all steroids in the Organon compound database were retrieved and the physicochemical properties calculated (molecular weight, number of hydrogen bond donor and acceptor sites, number of rotatable bonds, Polar Surface Area and ClogP). Based on general structural features of androgens, antiprogestagens, glucocorticoids, mineralocorticoids, estrogens and progestagens, the set of steroids was further refined. Between the defined groups of steroids ClogP was the most discriminative descriptor and this parameter was used to select a set of steroids to be tested in a Caco-2 permeability study. The measured apparent permeability coefficients (P_{app}) were related to the calculated and measured physicochemical properties. All steroids were well transported over the cell monolayers and the P_{app} was independent of the concentration and the transport direction. No relationship was found with the PSA. The small differences in the P_{app} values showed a weak inverse correlation with ClogP: the hydrophilic steroids (ClogP approx. 0 - 2) tend to diffuse faster over the cell monolayers compared to the more hydrophobic steroids (ClogP approx. 5). The relationship with ClogP suggests that partitioning of steroids between the biological membrane and the surrounding aqueous phase is one of the main mechanisms for absorption.

In the next study the gastrointestinal absorption and P-glycoprotein (Pgp) efflux transport of heterocyclic drugs was investigated with the Caco-2 cell model (chapter 4). As the possibility for Pgp transport is routinely tested in High Throughput Screenings (HTS) assays, the Caco-2 data were compared to the results of in vivo

tests. Since Pgp is expressed at the Blood Brain Barrier as well, it can be expected that CNS penetration will be impaired if a drug is a Pgp substrate.

Based on the calculation of the physicochemical properties a good oral absorption was predicted for all the drugs tested, which corresponded well with the measured Caco-2 permeabilities (P_{app}). The transport data of domperidone and verapamil showed that the Pgp efflux transporter was expressed in the Caco-2 cells. Many of the drugs tested were indicated to be potential Pgp efflux substrates. Since Pgp is expressed at the Blood Brain Barrier as well, it was expected that CNS penetration will be impaired if a drug is a Pgp substrate. However, no correlation could be found between brain penetration in rats and the Pgp efflux ratio as measured with the Caco-2 cells. In this respect species differences as well as experimental conditions (pH, concentration etc.) could be of influence as well. The data showed that Pgp efflux ratio's as determined in in vitro High Throughput Screening tests, where the transport conditions are fixed (pH gradient, concentration etc.), cannot routinely be used to predict a possible affected brain penetration.

An alternative route for intestinal absorption is lymphatic uptake. The major advantages are found in the fact that first pass metabolism is circumvented and the possibility to deliver very hydrophobic drugs to the systemic circulation. It was investigated whether it is feasible to predict the potential of drugs for lymphatic absorption (chapter 5). At present the octanol / water partition coefficient is the only parameter available for this purpose, however, the amount of data is limited and alternatives are not available. An extensive dataset, which received hardly any attention in literature, was used to study whether relationships exist between molecular descriptors and the extent of lymphatic drug transport. In addition, partitioning to the lipid fraction of ex vivo dog lymph and Intralipid as well as the suitability of the Caco-2 cell system were explored.

Using the lymphatic drug transport data of a chemically diverse set of compounds it was shown that the (octanol/water) partition coefficient is a suitable parameter for assessing the potential for lymphatic transport of drugs. Partitioning of drugs towards the lipid fraction of ex vivo dog lymph or Intralipid offer an useful alternative methodology. The prediction becomes more accurate when dealing with drugs within a chemically similar series. The Caco-2 transport data showed that the formation and

secretion of chylomicrons (CM) is the limiting factor for this test system. Unless the problem of CM formation and secretion can be solved, Caco-2 cells are not suitable for assessing the potential of drugs for lymphatic absorption. This may be related to the malignant origin of the Caco-2 cells.

In the second study involving lymphatic drug delivery, the contribution of lymphatic absorption of testosterone undecanoate, a very hydrophobic drug, to the systemic exposure of testosterone was determined (chapter 6). Orally administered testosterone (T) is ineffective in the treatment of male androgen deficiency syndromes due to extensive presystemic first-pass metabolism. In contrast, the lipophilic long-chain ester testosterone undecanoate (TU) exhibits androgenic activity that has been attributed to formation of T via systemic hydrolysis of lymphatically transported TU. However, there are no definitive data regarding the oral bioavailability of TU or the extent to which lymphatically transported TU contributes to the systemic availability of T after oral TU administration.

In a thoracic lymph duct-cannulated dog model, the oral bioavailability and lymphatic transport of TU after postprandial administration was determined. When administered as either Andriol or Andriol Testocaps, two available commercial formulations, the mean absolute bioavailability of TU was 3.25% and 2.88%, respectively, and lymphatically transported TU accounted for between 91.5 and 99.7% of the systemically available ester. Model-independent pharmacokinetic analysis indicated that 83.6 and 84.1% of the systemically available T, resulting from Andriol or Andriol Testocaps, respectively, was due to systemic hydrolysis of lymphatically transported TU. These data demonstrate that intestinal lymphatic transport of TU produces increased systemic exposure of T by avoiding the extensive first-pass effect responsible for the inactivation of T after oral administration.

Finally, a novel methodology was proposed to evaluate the possibility for waiving bioequivalence studies on the basis of standard human pharmacokinetic data (chapter 7). With this work a contribution is given to the recent call for new scientific approaches for waiving bioequivalence studies in order to reduce the number of (clinical) studies.

Recently the Biopharmaceutical Classification System (BCS) has been implemented for waiving bioequivalence studies on the basis of the solubility and gastrointestinal

permeability of drug substance. Using the rationale of the BCS it can be argued that biowaivers can also be granted on the basis of standard pharmacokinetic data. If a drug exhibits dose linear pharmacokinetics and a sufficiently fast dissolution profile, it can be concluded that this drug appears to pose no problem with respect to absorption. It should be noted that a change of an immediate release tablet formulation can only lead to a deviating rate and/or extent of absorption when the release of the active from the formulation is altered. Logically, the dissolution profiles of the different formulations should be equal to guarantee bioequivalency. Thus, both the BCS and the alternative linear pharmacokinetics approach require an evaluation of dissolution profiles. The justification of BCS is found in the permeability classification of the compound, those of the linear pharmacokinetics lies in the apparent lack of a permeability problem. In this context e.g. Pgp transported drugs form an interesting class of compounds, which may be treated likewise when complying to the aforementioned requirements. Furthermore, poorly soluble compounds may be less troublesome than expected. I.e. in vivo the bile salts solubilize low solubility drugs which results in dose linear pharmacokinetics. Here linear pharmacokinetics shows that elevated doses appear not to exhibit a limiting role on the dissolution. Hence, a change in formulation without any effect on the dissolution profile is not expected to cause a change in availability.

Samenvatting.

De meeste medicijnen werken op receptoren die zich op specieke plaatsen in het menselijk lichaam bevinden. Voor elke toedieningsroute (oraal, transdermaal, nasaal etc.) moet de actieve stof de centrale bloedcirculatie bereiken om naar de receptoren getransporteerd te worden. Onafhankelijk van de toedieningsroute zijn de processen die zich afspelen bij het toedienen van medicijnen hetzelfde: afgifte door de doseringsvorm (*dissolutie, oplossen*), absorptie van de actieve stof in het bloed (*permeatie door een biologisch membraan*) en uiteindelijk verwijdering uit het lichaam (*metabolisme en eliminatie*).

Uit het voorgaande wordt duidelijk dat een medicijn alleen werkzaam kan zijn wanneer de centrale circulatie bereikt wordt. In de biofarmaceutische wetenschappen worden de relaties bestudeerd tussen de fysisch chemische eigenschappen van de actieve stof, de eigenschappen van de doseringsvorm en de (humane) fysiologie. De fysisch chemische eigenschappen bepalen of een medicijn kan oplossen en een biologische barrière kan passeren. De doseringsvorm en de samenstelling hebben invloed op het absorptieproces. Afhankelijk van de toedieningsroute van het medicijn stelt de humane fysiologie specifieke eisen aan de benodigde fysisch chemische eigenschappen, doseringsvorm en de samenstelling van het preparaat.

Het eerste deel van dit proefschrift (hoofdstukken 2 tot en met 4) gaat in op de fysisch chemische eigenschappen van actieve stoffen. In hoofdstuk 2 wordt geëvalueerd welke eigenschappen nodig zijn om een goede absorptie te bewerkstelligen via verschillende toedieningsroutes. Voor de orale toediening zijn de ‘ideale’ fysisch chemische eigenschappen welbekend. Aan alternatieve toedieningsroutes is echter in vergelijking veel minder aandacht besteed. Bij de bestudeerde routes (transdermaal, buccaal / sublinguaal, vaginaal en nasaal) wordt de fysiologische barrière gevormd door meerlagige epithelia. Dit in tegenstelling tot het maagdarmkanaal waar een enkelvoudige laag van enterocyten de barrière vormt. De epithelia bevatten een grote verscheidenheid aan verschillende soorten cellen die bijdragen aan de specifieke functionaliteit van de weefsels. Onafhankelijk van de toedieningsroute wordt het merendeel van de medicijnen ofwel transcellulair of paracellulair (tussen de cellen door) geabsorbeerd. Ondanks de verschillen in de fysiologische barrières zijn de

benodigde fysisch chemische eigenschappen van medicijnen hetzelfde als voor orale toediening.

Absorptie is echter alleen mogelijk wanneer de stof in opgeloste vorm 'aangeboden' wordt aan het epitheel. Daarom moet voor de evaluatie van de oplosbaarheid ook het 'fysiologisch' beschikbare volume voor het oplossen van de actieve stof meegenomen worden. In het algemeen geldt dat dit volume veel kleiner is in geval van de niet orale toedieningsroutes. Dit heeft tot gevolg dat de oplosbaarheid een van de meest kritische parameters is voor transdermale, buccale / sublinguale, vaginale en nasale toediening van medicijnen. Om deze reden is voor deze toedieningsroutes de formulering van groot belang. Bovendien is een verandering van de toedieningsroute niet geschikt om de biobeschikbaarheid te verhogen wanneer een medicijn niet de optimale fysisch chemische eigenschappen bezit.

In het huidige onderzoek naar steroïden is de aandacht voornamelijk gericht op de farmacologische effecten en de farmacokinetische eigenschappen. Als gevolg hiervan is relatief weinig aandacht besteed aan de biofarmaceutische eigenschappen. In de tweede studie zijn de fysisch chemische eigenschappen van steroïden geëvalueerd met betrekking tot de absorptie in het maagdarmkanaal (hoofdstuk 3). Alle chemische structuren van steroïden werden opgezocht in de Organon database en de fysische chemische eigenschappen berekend (molecuulgewicht, aantal waterstof brug donor en acceptor plaatsen, aantal roteerbare bindingen, polair oppervlak (PSA) en de octanol/water partitiecoëfficiënt (ClogP)). Aan de hand van algemene structuurelementen van androgenen, antiprogestagenen, glucocorticoïden, mineraalcorticoïden, estrogenen en progestagenen, werd de verzameling steroïden verder verfijnd. De partitiecoëfficiënt bleek de meest discriminerende parameter te zijn en op basis hiervan werd een set steroïden geselecteerd die werd onderzocht in een Caco-2 permeabiliteitsstudie. De gemeten permeabiliteitscoëfficiënten (P_{app}) werden gerelateerd aan de berekende en gemeten fysisch chemische eigenschappen. Alle steroïden werden goed getransporteerd door de cellen en de P_{app} was onafhankelijk van de concentratie en de transportrichting. Er was geen relatie met het polaire oppervlak. Een zwakke omgekeerd evenredige relatie was aanwezig tussen de Papp waarden en ClogP: de hydrofiele steroïden (ClogP tussen 0 en 2) hebben de neiging om sneller over de cellagen te diffunderen dan de hydrofobere steroïden

(ClogP ongeveer 5). De relatie met ClogP geeft aan dat partitie van steroïden met het biologische membraan een rol speelt in het mechanisme van de absorptie.
Het onderzoek werd vervolgd met een studie naar de absorptie en het P-glycoproteïne (Pgp) efflux transport van heterocyclische medicijnen met het Caco-2 model (hoofdstuk 4). Omdat de mogelijkheid voor Pgp transport routinematig getest wordt met dit model in zogenaamde 'High Throughpout Screening' experimenten, werden de gegevens vergeleken met de resultaten van in vivo testen in ratten. Op basis van de berekende fysisch chemische eigenschappen werd een goede absorptie voorspeld, wat in overeenstemming was met de gemeten permeabiliteit in Caco-2 cellen. In het algemeen was de permeabiliteit van de getestte stoffen hoog, wat in overeenstemming is met in de literatuur gerapporteerde absorptie gegevens. Aan de hand van de transport data van domperidon en verapamil kon aangetoond worden dat de Pgp efflux transporter aanwezig was in de Caco-2 cellen. De transport gegevens gaven aan dat een groot aantal van de geteste stoffen potentiële Pgp substraten zijn. Omdat Pgp ook in hoge mate in de bloed hersen barrière voorkomt werd verwacht dat voor Pgp getransporteerde medicijnen de absorptie in de hersenen nadelig beïnvloed kan worden. Opmerkelijk genoeg kon er geen relatie aangetoond worden tussen breinpenetratie in ratten en de Pgp efflux ratio's gemeten in Caco-2 cellen. Mogelijkerwijs spelen hierbij zowel species verschillen (Pgp in ratten wordt hier vergeleken met het humane Pgp transport eiwit in Caco-2 cellen) alsmede de experimentele condities (pH, concentratie etc.) een beïnvloedende rol. De gegevens lieten zien dat de Pgp efflux data, zoals bepaald in 'High Throughput Screening' testen (gefixeerde transportcondities zoals pH en concentratie etc.), niet routinematig toegepast kunnen worden om een mogelijk effect op de breinpenetratie te voorspellen.
Een alternatieve absorptieroute bij orale toediening is het zogenaamde lymfatische transport. De belangrijkste voordelen van lymfatische toediening van medicijnen worden gevonden in het vermijden van het zogenaamde 'First Pass Effect' (FPE) en de mogelijkheid om zeer hydrofobe stoffen in de systemische circulatie te krijgen. Onderzocht is of lymfatisch transport van medicijnen voorspeld kan worden (hoofdstuk 5). Op dit moment is de octanol / water partitiecoëfficiënt de enige parameter die hiervoor beschikbaar is. Echter, de hoeveelheid gegevens is gelimiteerd en alternatieven zijn niet voorhanden. Een uitgebreide dataset, waaraan in de

literatuur weinig aandacht besteed is, werd gebruikt om na te gaan of er relaties zijn tussen het lymfatisch transport en de berekende fysisch chemische eigenschappen. Bovendien werd de toepasbaarheid bepaald van experimenteel bepaalde partitiecoëfficiënten met Intralipid en ex vivo lymfvloeistof van honden. Tot slot is onderzocht of het Caco-2 model toegepast kan worden om lymfatisch drug transport te evalueren. Voor een heterogene set van modelverbindingen kon aangetoond worden dat er een relatie bestaat tussen de berekende octanol / water partitiecoëfficiënt en de mate waarin stoffen via de lymfe getransporteerd worden. De voorspellingen worden beter als de stoffen tot dezelfde chemische reeks behoren. In de plaats van de berekende partitiecoëfficiënten kunnen ook de partitiecoëfficiënten gebruikt worden die gemeten zijn met Intralipid (olie in water emulsie) of de lipoproteïne fractie van ex vivo lymfvloeistof. De Caco-2 transport data lieten zien dat de vorming en uitscheiding van chylomicronen (CM) de limiterende factoren zijn voor dit testsysteem. Caco-2 cellen zijn niet geschikt om mogelijk lymfatisch transport van medicijnen te voorspellen, tenzij het probleem van de vorming en uitscheiding van chylomicronen opgelost kan worden. Mogelijk wordt dit veroorzaakt door de tumorachtige oorsprong van de Caco-2 cellen.

In de tweede studie is onderzocht wat de bijdrage van lymfatisch transport is aan de systemische beschikbaarheid van testosteron (T) na orale toediening van het zeer hydrofobe testosteron undecanoaat (TU) (hoofdstuk 6). Oraal toegediend T is niet effectief gebleken bij de behandeling van androgeen deficiënte mannen. Dit wordt veroorzaakt door het grote ‘First Pass Effect’. TU wordt lymfatisch getransporteerd na orale inname en hydrolyse tot T vindt plaats in de bloedbaan. Tot op heden was de orale biobeschikbaarheid van TU niet bekend evenals de bijdrage van het lymfatische transport aan de systemische beschikbaarheid van T.

In een gecanuleerd hondenmodel (borstbuis = ductus thoracicus) werd de orale biobeschikbaarheid en het lymfatisch transport van TU bepaald na inname van een maaltijd. Twee verschillende, commercieel verkrijgbare formuleringen werden getest (Andriol en Andriol Testocaps). De gemiddelde absolute biobeschikbaarheid van Andriol en Andriol Testocaps was respectievelijk 3.25% en 2.88%. Van deze hoeveelheid werd respectievelijk 91.5% en 99.7% lymfatisch getransporteerd. Model onafhankelijk pharmacokinetische berekeningen lieten zien dat 83.6% (Andriol) en

84.1% (Andriol Testocaps) van het systemisch beschikbare T gevormd werd door de hydrolyse van TU. Deze resulaten toonden aan dat de systemische blootstelling aan T toeneemt door intestinaal lymfatisch transport van TU. Het vermijden van het First Pass Effect was hiervan de oorzaak.

Tot slot wordt in dit proefschrift een nieuwe werkwijze gepresenteerd om, op basis van standaard humane farmacokinetische gegevens, bioequivalentie studies in de farmaceutische ontwikkeling overbodig te maken (hoofdstuk 7). Met dit werk is een bijdrage geleverd aan een recente oproep voor nieuwe wetenschappelijke benaderingen om het aantal bioequivalentiestudies te reduceren.

Bioequivalentiestudies spelen een belangrijke rol in de farmaceutische ontwikkeling om aan te tonen dat, wanneer de samenstelling van een medicijn gewijzigd wordt, de werking niet nadelig beïnvloed wordt. Recentelijk is hiervoor het Biofarmaceutisch Classificerings Systeem (BCS) geïmplementeerd. Hierdoor is het mogelijk om, op basis van oplosbaarheid en intestinale permeabiliteitsgegevens van de actieve stof, toestemming te verkrijgen van de autoriteiten om bioequivalentiestudies niet uit te hoeven voeren. Gebruik makende van de rationale van het BCS kan beargumenteerd worden dat dit ook mogelijk is op basis van humane farmacokinetische gegevens. Wanneer de farmacokinetische respons van een medicijn dosis lineair en de dissolutie onveranderd is, kan geconcludeerd worden dat er geen problemen te verwachten zijn met betrekking tot de absorptie. Hierbij moet opgemerkt worden dat een wijziging in de samenstelling van een orale toedieningsvorm (tablet of capsule) alleen tot afwijkingen van de absorptie(snelheid) kan leiden wanneer de afgifte (*dissolutie*) uit de formulering veranderd wordt. Logischerwijs moeten de dissolutieprofielen van de verschillende formuleringen gelijk zijn om bioequivalentie te garanderen. Zowel het BCS als de lineair farmacokinetische benadering behoeven de evaluatie van het dissolutieprofiel. De rechtvaardiging van het BCS wordt gevonden in een beoordeling van de permeabiliteit (*absorptie*) en die van de farmacokinetische benadering in de klaarblijkelijke afwezigheid van een permeabiliteitsprobleem. In deze context vormen Pgp getransporteerde medicijnen een interessante klasse van verbindingen, die op dezelfde manier behandeld kunnen worden. Hierbij gelden uiteraard de vooraf genoemde criteria. Verder zijn hydrofobe, slecht wateroplosbare, verbindingen minder moeilijk dan vaak wordt gedacht. In vivo zorgen de aanwezige galzouten

ervoor dat de oplosbaarheid van slecht oplosbare verbindingen verhoogd wordt waardoor hogere doseringen nog steeds kunnen oplossen. In dit geval toont de lineaire farmacokinetiek aan dat de in vivo oplosbaarheid niet limiterend is geweest voor de dissolutie / oplosbaarheid van hoge doseringen. Hierom is het niet te verwachten dat een verandering in de formulering, die het dissolutieprofiel niet beinvloedt, invloed heeft op de biobeschikbaarheid. Evenwel is het duidelijk dat de te vergelijken formuleringen geen hulpstoffen mogen bevatten die het (presystemisch) metabolisme van het medicijn kunnen beïnvloeden.

Dankwoord.

Dit proefschrift zou niet mogelijk zijn geweest zonder de bijdrage en steun van vele collega's. Het door hen uitgedragen enthousiasme en de gestelde uitdagingen vormen de basis van dit proefschrift.
In de eerste plaats wil ik een speciaal dankwoord richten tot Herman Vromans, mijn promotor en bovenal collega. Zonder jouw uitdagende karakter en kritische houding zou dit proefschrift niet geworden zijn wat het nu is. Met name jouw bijdrage aan de 'oplossing' van het 'Michigan incident' wordt ten zeerste gewaardeerd. Dit heeft uiteindelijk geresulteerd in de door ons fel begeerde publicatie in Clinical Pharmacokinetics. Ik wil niet onvermeld laten dat ik jouw oprechte belangstelling voor mij als privépersoon erg op prijs stel.
Bij de diverse onderzoeken hebben vele collega's van Organon's research afdelingen een prominente rol gespeeld. Zonder iemand tekort te willen doen wil ik hier vermelden dat ik goede herinneringen bewaar ik aan de samenwerking met Gerard Vogel, Henry Spanings, Jan Kelder, Martin Smit en Martijn Rooseboom. Hopelijk gaat dit nog vele jaren door inclusief het uitwisselen van de 'roddels'. Gerard en Martijn moeten soms haast wel gek geworden zijn als ik weer eens kwam 'buurten', maar ik kan met gepaste trots vermelden dat ik nooit het kantoor uitgegooid ben. Ook de medeauteurs van de diverse publicaties, Natalie Houwing, Holger Lass, Rob Onderwater en Johan Lenders, mogen niet onvermeld blijven.
Ook de rol van de directe collega's binnen de 'Farmacie' speelden een belangrijke rol. In het bijzonder wil ik Kees van der Voort Maarschalk en Sytske Moolenaar noemen die in elk stadium van de totstandkoming van dit proefschrift zeer enthousiast waren. Het is fijn om dit te mogen ervaren en de oprechtheid van de belangstelling en interesse is zeer stimulerend. Naar aanleiding van jullie 'proeflezing' is het waarschijnlijk toch aan te raden om naar een cursus Engels te gaan.
Ook de collega's binnen de sectie (Joop Zwinkels, Joost Verheezen, Onno de Vegt, Edwin Burger, Theo Bouwman, Anton Hooymans, Annieke Groen, Monique van Veldhoven en Mari Janssen) speelden op de achtergrond een ondersteunende rol. Hoewel niet altijd direct betrokken ervaar ik de getoonde belangstelling en nieuwsgierigheid naar de resultaten van het onderzoek als stimulerend.
Eveneens hebben diverse studenten van de Universiteit van Utrecht hun bijdrage geleverd aan de gepresenteerde onderzoeken. Met name de bijdragen van Jesse Swen (hoofdstuk 5) en Naomi Tielen (hoofdstuk 2) mogen niet onvermeld blijven. Helaas heb ik jullie niet kunnen verleiden om ook te promoveren maar ik ben ervan overtuigd dat jullie ervaringen nog eens van pas zullen komen. Hoewel het onderzoek van Kaspar van den Dries als student niet in dit proefschrift is terecht gekomen is hij wel een bijzondere deelgenoot in de lotgevallen van een 'promovendus' geweest.
Hoewel niet altijd even duidelijk hebben ook Victor Nickolson en Andreas Lohmann op de achtergrond hun bijgedrage geleverd. Al gedurende vele jaren is het mogelijk

om het zogenaamde ‘Biopharmaceutics’ project binnen Organon uit te voeren. Hoewel het promotieonderzoek niet de doelstelling was heeft het algemene onderzoek, ten behoeve van de research en development afdelingen van Organon, een belangrijke rol gespeeld bij de totstandkoming van de publicaties.
Ook de samenwerking met de collega’s van de diverse universiteiten heb ik als stimulerend en uitdagend ervaren. Een speciaal woord van dank dient dan ook uit te gaan naar Bill Charman (Melbourne, Australie), Patrick Augustijns (Leuven, Belgie), Per Artursson (Uppsala, Zweden), en Erik Frijlink (Groningen). Met name het zogenaamde ‘Van der Valk’ overleg met Erik was naar mijn mening een bijzondere gebeurtenis.
Tot slot wil ik Mayra, mijn ouders, Ko, en Thijs bedanken voor hun support op de achtergrond. Uiteindelijk doe je het toch voor ‘thuis’.

Normaal gesproken worden in een dankwoord alleen personen genoemd. Echter, ik zou graag de aandacht willen vestigen op de rol die de werkomgeving speelt. De spirit, het enthousiasme, de uitdagingen en het plezier ten aanzien van werk en leven bepalen of je je lekker voelt en of het uiteindelijk leuk is om naar het ‘werk’ te gaan. De sfeer wordt gemaakt door de collega’s en ik realiseer mij dat ik enorm veel geluk heb dat ik omringd ben door een fantastisch stel collega’s. Mijn werkomgeving ervaar ik als zeer stimulerend en uitdagend en ik geniet er elke dag weer van. Hoewel het niet altijd ‘koek en ei’ is, is het van het grootste belang om te proberen deze ‘spirit’ te behouden. Anders gaat er een hoop lol in het dagelijkse werk verloren. Ik ga altijd met enorm veel plezier naar de teamvergaderingen van Male Contraceptie, Org 33628 en Org 39970 en ervaar ik het plezier wat wij samen hebben als een enorme stimulans.

Curriculum vitae.

Fried Faassen was born in Ede on February 17th 1964. After finishing secondary school in 1981 (Marnix College, Ede), he studied Analytical Chemistry at the HLO 'STOVA' in Wageningen. In 1986 he started to study Molecular Sciences at the Agricultural University in Wageningen. A specialization was taken in Physical and Colloid Chemistry and in 1990 the Master Degree was obtained. In 1990 he joined NV Organon to work on the physical characterization of polymers (Technology Department). After two years a change was made towards pharmaceutical unit operations (high shear and fluid bed granulation, roller compaction, development and scaling up of new production processes, purchasing production equipment for pharmaceutical manufacturing etc.). In 1996 he accepted a position within the Department of Pharmaceutics where he worked on formulatory issues in pharmaceutical development. In 1998 he started the so-called 'Biopharmaceutics' project within Organon and became specialized in the Biopharmaceutical Sciences. In 2000 he was appointed as Director in the Department of Pharmaceutics for 'Oral dosage forms'. Currently he acts as intermediary between the research scientists from Oss and Newhouse and the pharmaceutical development departments, to optimize the developability of new drugs. This Ph.D. thesis was conducted as an extension of the biopharmaceutical research within the Department of Pharmaceutics.

Fried Faassen werd geboren in Ede op 17 februari 1964. Na het afsluiten van de middelbare school in 1981 (Marnix College te Ede) studeerde hij analytische chemie op het HLO ('STOVA') te Wageningen. In 1986 werd de studie Moleculaire Wetenschappen aangevangen op de Landbouwuniversiteit te Wageningen. Het doctoraalexamen werd in 1990 afgesloten met als hoofdrichting fysische- en kolloidchemie. Sinds 1990 is hij werkzaam bij NV Organon. In de beginjaren binnen het Technology Department werkte hij aan de fysische karakterisering van polymeren en later aan farmaceutische unit operations (high shear en fluid bed granulatie, walsen, ontwikkelen en opschalen van nieuwe productieprocessen en het inkopen van procestechnologische apparatuur). In 1996 werd een positie aangenomen binnen het Department of Pharmaceutics waar hij werkte aan formuleringsvraagstukken in de farmaceutische ontwikkeling. In 1998 werd het zogenaamde biofarmacie project opgezet binnen Organon waar hij zichzelf specialiseerde in de biofarmaceutische wetenschappen. In 2000 werd hij Director van de sectie 'Orale toedieningsvormen'. Momenteel functioneert hij als intermediair tussen de wetenschappers uit Oss en Newhouse en de farmaceutische ontwikkelingsafdelingen om de ontwikkelbaarheid van nieuwe medicijnen te optimaliseren. Dit proefschrift werd uitgevoerd in het verlengde van het biofarmaceutische onderzoek binnen het Department of Pharmaceutics.