Physicochemical Properties and Transport of Steroids across Caco-2 Cells

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Purpose. The purpose of this work was to study the relevant physicochemical properties for the absorption of steroids.
Methods. Various physicochemical properties of steroids were calculated (molecular weight, ClogP, static polar surface area [PSA], etc.). Within this series of steroids, different pharmacological groups were defined. Based on the outcome of this survey, steroids were selected for the Caco-2 permeability study. The apparent permeability coefficients (P_{app}) were related to the calculated and measured physicochemical properties.
Results. Between the defined groups of steroids, ClogP was the most discriminative descriptor. 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 P_{app} showed a weak inverse correlation with ClogP.
Conclusions. The molecular descriptors and P_{app} values showed that all steroids are well transported. 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 with the more hydrophobic steroids (ClogP approximately 5). The relationship with ClogP suggests that partitioning of steroids between the biologic membrane and the surrounding aqueous phase is one of the main mechanisms for absorption.

KEY WORDS: steroids; physicochemical properties; absorption; Caco-2 permeability.

INTRODUCTION

In the development of new chemical entities (NCEs), the optimization of the biologic activity plays an important role. In the first instance, this is accomplished by chemical modifications of the lead compound(s) (in the synthesis and testing phase). When an NCE is taken into development, the formulation of the product is developed and optimized.

The hydrophobic properties (e.g., ClogP up to 5) and low aqueous solubility of steroids have led to the general perception, and maybe prejudices, that steroids are so-called "problem drugs." Although the amount of data available in literature is limited, steroids may be considered as a class with good gastrointestinal absorption, representing a good passive transcellular permeability (1). 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 (2–5). Because 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 (2–4).

With respect to the absorption of steroids, P-glycoprotein (P-gp) and P450/17βHSD (17β-hydroxysteroid dehydrogenase) metabolizing enzymes are of importance. P-gp is expressed in Caco-2 cells depending on the stage of differentiation of the cells (6). It is not known whether the amount of P-gp 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 (7). Depending on the cell line used, differences might occur in P450 metabolism and P-gp activity (8). However, normally Caco-2 cells have a low CYP3A4 expression (3). The group of 17βHSD enzymes is essential for both the synthesis and the metabolism/inactivation of C_{19} and C_{18} steroid hormones (androgen and estrogens; 9,10). 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 as to whether these enzymes are present in Caco-2 colon cells.

The objective of the current study was to make an inventory of the physicochemical properties (e.g., 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]) 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.

METHODS

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 because 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 PSA ($Å^2$) were calculated for the retrieved steroids using the methods described previously (11). 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 (11).

Based on general structural features of androgens, antiprogestagens, glucocorticoids, mineralocorticoids, estrogens, and progestogens, the set of retrieved steroids was refined (12). The structural elements used are shown in Fig. 1. On the

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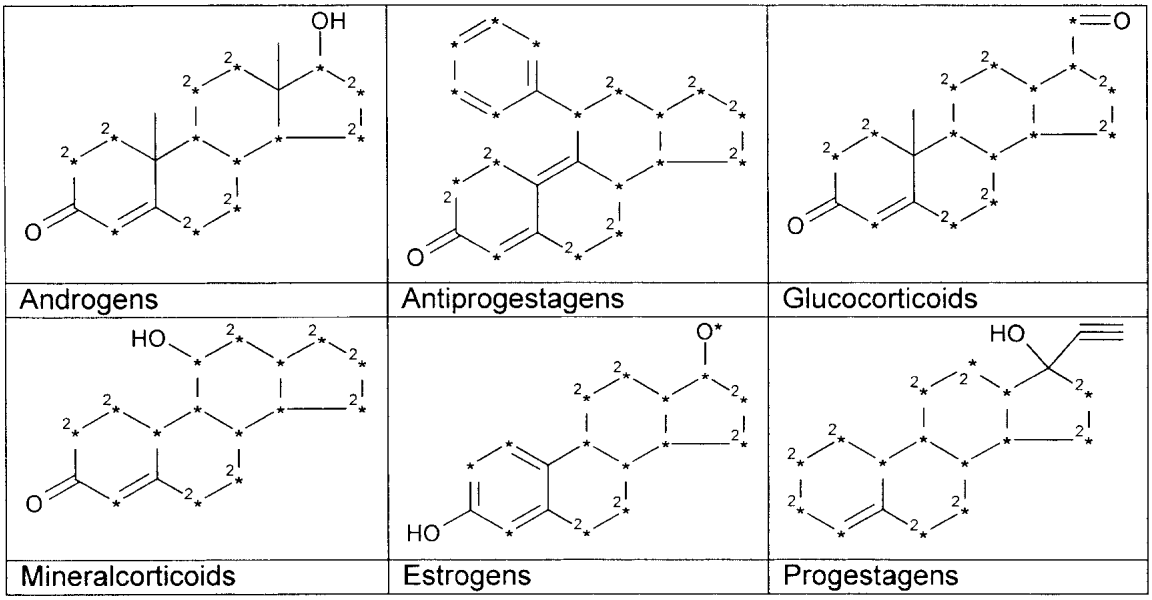


Fig. 1. Structural features of steroids belonging to different pharmacological subclasses. *Free site. The number indicates the number of free sites.

basis of the results of the theoretical calculations, a selection of steroids was made to be tested in the Caco-2 system.

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). Because the majority of the steroids often possess mixed pharmacological properties, it is difficult to choose compounds with a single biologic effect. However, the chemical elements shown in Fig. 1 for the different groups of steroids do in general hold and were therefore applied (12). 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).

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 supplemented with heat-inactivated fetal calf serum (10% v/v), nonessential 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 trypsinization 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 (12-well Transwell plates, Costar) at approximately 100,000 cells per filter (growth area 1.1 cm^2 containing 2.5 mL of 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, MA, USA). The TEER of the cell monolayers was calculated according to the following equation:

$$\text{TEER} = (R_{\text{monolayer}} - R_{\text{empty filter}}) \times A \ (\Omega.\text{cm}^2)$$

where $R_{\text{monolayer}}$ is the resistance measured, $R_{\text{empty filter}}$ is the resistance of control filters without cells (approximately 140 Ω.cm^2), and A is the surface area of the filter insert (1.1 cm^2). After 2 to 3 weeks in cell culture, the monolayers developed a TEER of approximately 500 Ω.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 and 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 bovine serum albumin (BSA) in the transport medium of the Caco-2 cells because most steroids do not bind equally to proteins (e.g., hormone binding globuline (HBG), human serum albumine (HSA), BSA; 13–15). 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 (16,17).

For apical exposure, culture medium was removed from the filter insert before moving them to a new 12-well 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 of fresh transport medium (pH 7.4) and the transport study started by transferring the filter inserts to new 12-well plates containing 1.8 mL of 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. Samples (400 μL) were collected from the receptor compartment at 1, 2, and 4 h after application of the test substances. Directly after each sampling the original volume was restored by adding 400 μL of fresh transport medium.

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).

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 high-performance liquid chromatography (HPLC) as described later (see next section).

Concentration Measurements

All concentration measurements were determined by HPLC (HP11000 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.

RESULTS AND DISCUSSION

All steroids that could not be classified uniquely within the defined pharmacological subclasses (Fig. 1) were removed from the original database. Table I 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 PSA and that the steroids in general fulfill the criterion for oral absorption: MW < 500, Clogp < 5, PSA < 110 $Å^2$ (11,18). 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 with 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 II 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 one of the steroids (Org 36410) was predicted of having a moderate oral bioavailability (i.e., the ClogP is outside the range for good oral absorption in Table II). Figure 3 shows that the experimental water solubility of steroids is inversely related to ClogP.

Figure 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 (2–4).

Comparing the permeability coefficients of the tested steroids with the reference compounds having a low P_{app} (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 P_{app}, three groups of steroids can be distinguished: first, the group of steroids with the highest P_{app}, comparable to antipyrine ($P_{app,steroid} > 2*10^{-5}$ cm/s; e.g., nandrolone, cortisone, aldosterone, prednisolone, gestodene, norethisterone, Org OM08, and Org 34694); second, the group of steroids with an average P_{app}, comparable with verapamil ($1*10^{-5} < P_{app,steroid} < 2*10^{-5}$ cm/s; e.g., spironolactone, hydrocortisone, testosterone, progesterone, estradiol, dexamethasone, etonogestrel, Org 32540, Org 36410, Org 30659, Org 4325, and Org 4060); and third finally the group of steroids with the lowest P_{app} ($P_{app,steroid} < 10^{-5}$ cm/s; e.g., norgestrel and ethinylestradiol). The P_{app} value for this last group is still much greater than for compounds, such as mannitol or PEG4000, which are known for their low permeability. The high P_{app} of the diverse group of steroids tested is 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 (Cs or 0.1*Cs; 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 (7,8,19,20). Metabolite formation would lead to differences in the steroid retention time in the HPLC chromatograms because of 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.

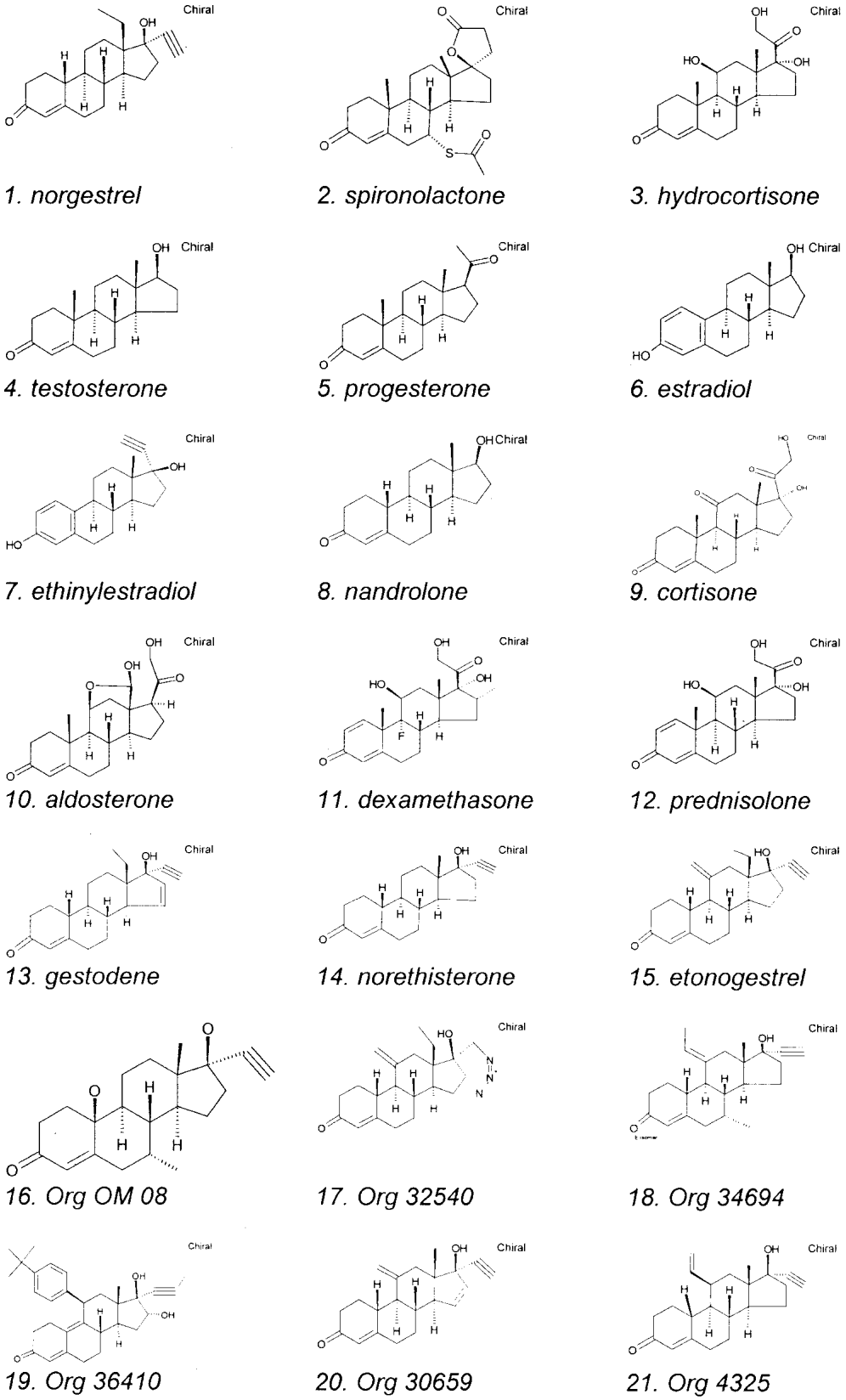
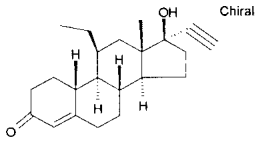
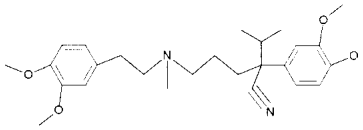


Fig. 2. Molecular structures of the steroids and reference compounds tested in the Caco-2 study.

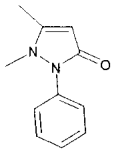
The results in Fig. 5 clearly show that the ratio of the apical to basolateral (A-to-B) and basolateral to apical (B-to-A) transport rates are all well within the 0.5 (influx) to 2 (efflux) bandwidth, which is generally considered as the margin for active transport. The Caco-2 cells used in this study have a high expression of P-gp (21). Active transport is only present for glucose, which has a transporter in the intestinal membrane (6), resulting in a high $P_{app,ab}$. The ratio of approx.



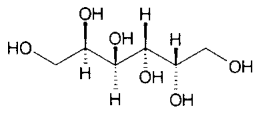
22. Org 4060



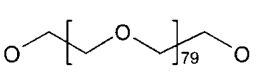
23. Verapamil



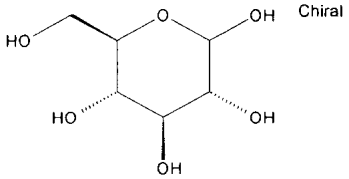
24. antipyrine



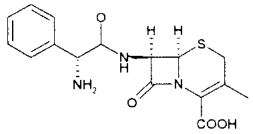
25. mannitol



26. PEG4000



27. D-glucose



28. cephalexin

Fig. 2. Continued.

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, because the reference drug verapamil was tested at concentrations that were too high (15 and 150 mM), resulting in saturation of the P-gp transporter (22). 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-gp (23–25). Steroids are known for their potency of antagonistic action on P-gp (25,26). Estradiol, corticosterone, aldosterone, cortisol, and dexamethasone are (potential) substrates for P-gp (25). In this particular study, human colon carcinoma cells that had been treated to obtain an unusually high P-gp expression (SW620 Ad300 cells) were used. No literature data are available on the amount of P-gp 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 P-gp transport of methylprednisolone, aldosterone, hydrocortisone, estriol, and dexamethasone (27). In the present study, there is no indication of steroid transport by P-gp, although it is known that P-gp is abundantly expressed in the used Caco-2 cells (21). Because of the high permeability coefficients of the steroids it may be possible that P-gp efflux transport is not observed (28).

In Fig. 6, the Caco-2 permeability is plotted as function of the PSA. All the steroids are located in the high permeability part of Fig. 6, corresponding to a high absorption (2–4). No sigmoidal relation is found between the apparent permeability coefficient and the PSA because all the steroids are located in the high permeability part of Fig. 6. The absence of this relationship is comparable with several reports (29,30), which questioned the suggested presence of such a relationship (2–4).

Figure 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 approximately 5). *In vivo*, the slower transport of the more hydrophobic steroids may be more prominent because the gastrointestinal membrane is covered with a mucous layer. The found weak correlation with ClogP is consistent

Table I. Number of Steroids Found in Each Pharmacological Subclass together with the Number of Duplicates of the Other Pharmacological Subclasses

	Androgens	Antiprogestagens	Glucocorticoids	Mineralocorticoids	Estrogens	Progestagens
Androgens (131)[a]						
Antiprogestagens (444)[a]	0					
Glucocorticoids (265)[a]	45	0				
Mineralocorticoids (100)[a]	21	0	52			
Estrogens (364)[a]	0	0	0	0		
Progestagens (463)[a]	3	0	0	0	3	

[a] Total number of compounds in the indicated group in parentheses.

Table II. Solubility and Molecular Descriptors of the Tested Compounds in the Caco-2 System

	S	ClogP (−2 < X < 5)[a]	MW (X <500)[a]	PSA (X <110)[a]	H-DON (X <6)[a]	H-ACCEPT (X <11)[a]
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

Note: S = Solubility (mol/L, * 10^6), ClogP = calculated log P (octanol/water), MW = molecular weight, PSA = polar surface area (Å^2), H-DON = number of hydrogen bond donor sites, H-ACCEPT = number of hydrogen bond acceptor sites.

[a] Criteria for good oral absorption. The numbers in the first column correspond to the compounds in Fig. 2.

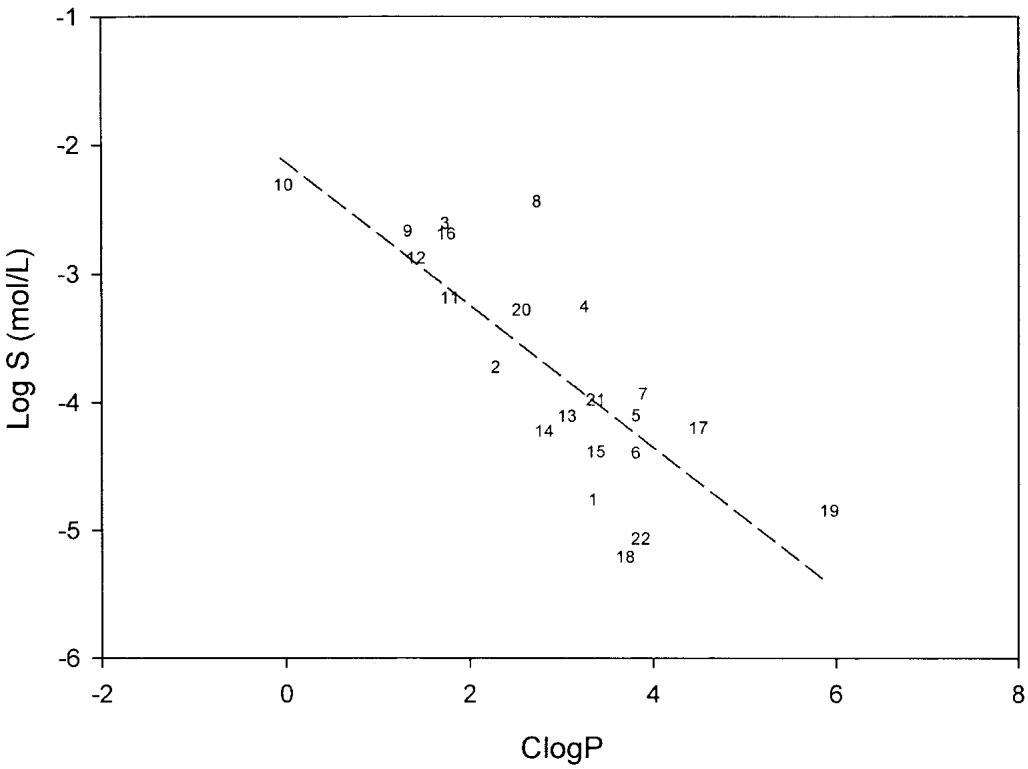


Fig. 3. Aqueous solubility of steroids as function of ClogP. Regression line: Y = −0.55 * X − 2.14 (r^2 = 0.64). The numbers correspond to the compounds of Fig. 2.

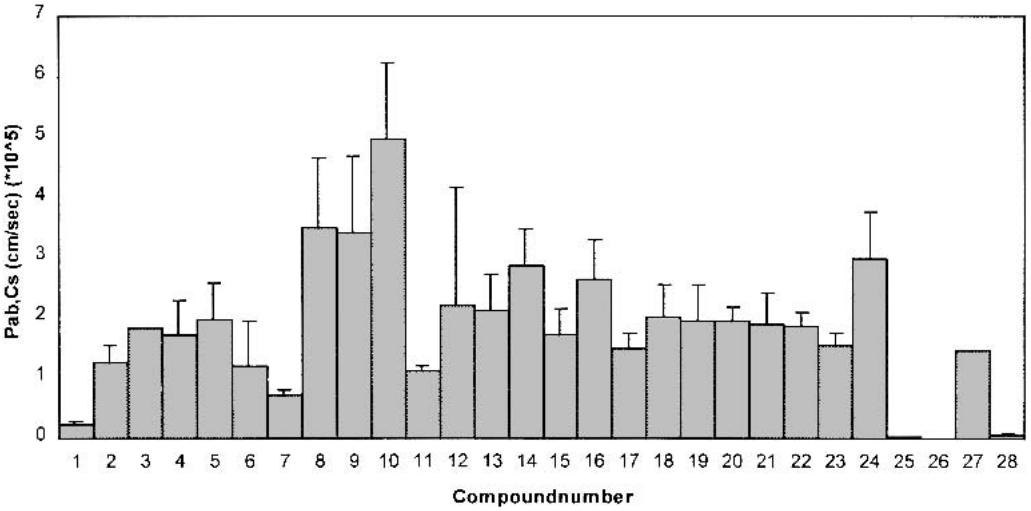


Fig. 4. Caco-2 P_{app} of the compounds tested. $P_{ab,Cs}$ = P_{app} from the A-to-B side, C_s = at saturation concentration. The numbers correspond to the compounds of Fig. 2. The error bars indicate the standard deviation (n = 3).

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 II); that is, because MW, hydrogen bonding capacity, and lipophilicity (ClogP) are often intercorrelated an apparent linear relationship is found between permeability and one of these three descriptors (31). The presented results clearly show that despite the hydrophobic character of steroids and low water solubility they 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. PSA 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 biologic membranes, as does cholesterol (32–34). Because cholesterol contains a steroid skeleton and is a "major" constituent of biologic membranes, the preference of steroids for biologic membranes might be explained. Therefore, one could argue that absorption might be facilitated because of an increased membrane fluidity. In the literature, no data are available concerning the absorption of steroids and mem-

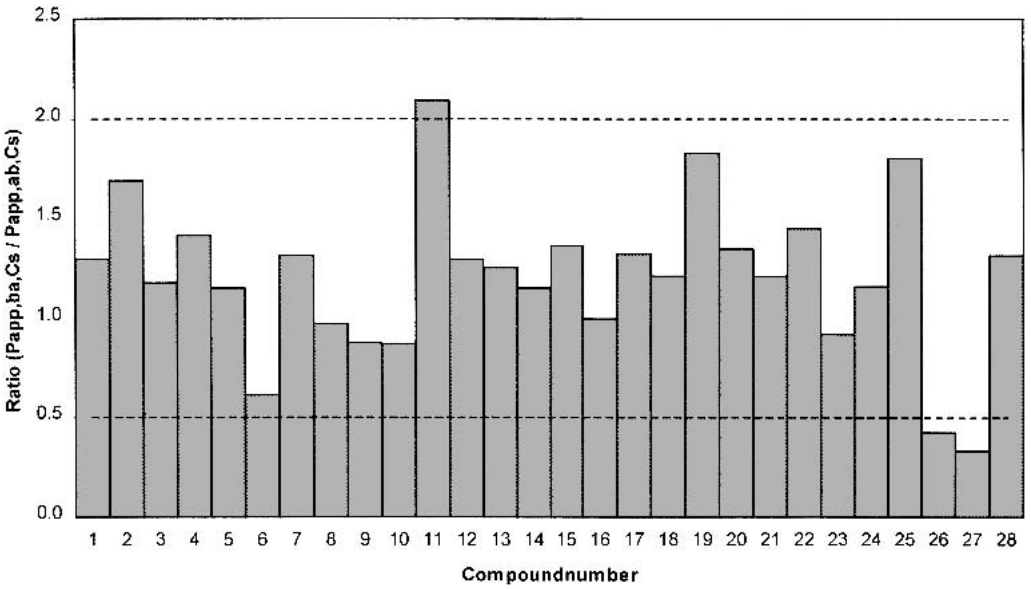


Fig. 5. Ratio of the B-to-A ($P_{ba,Cs}$) and A-to-B ($P_{ab,Cs}$) Caco-2 P_{app}. C_s = at saturation concentration. The dashed lines indicate the transitions toward influx (0.5) or efflux transport (2.0). The numbers correspond to the compounds of Fig. 2.

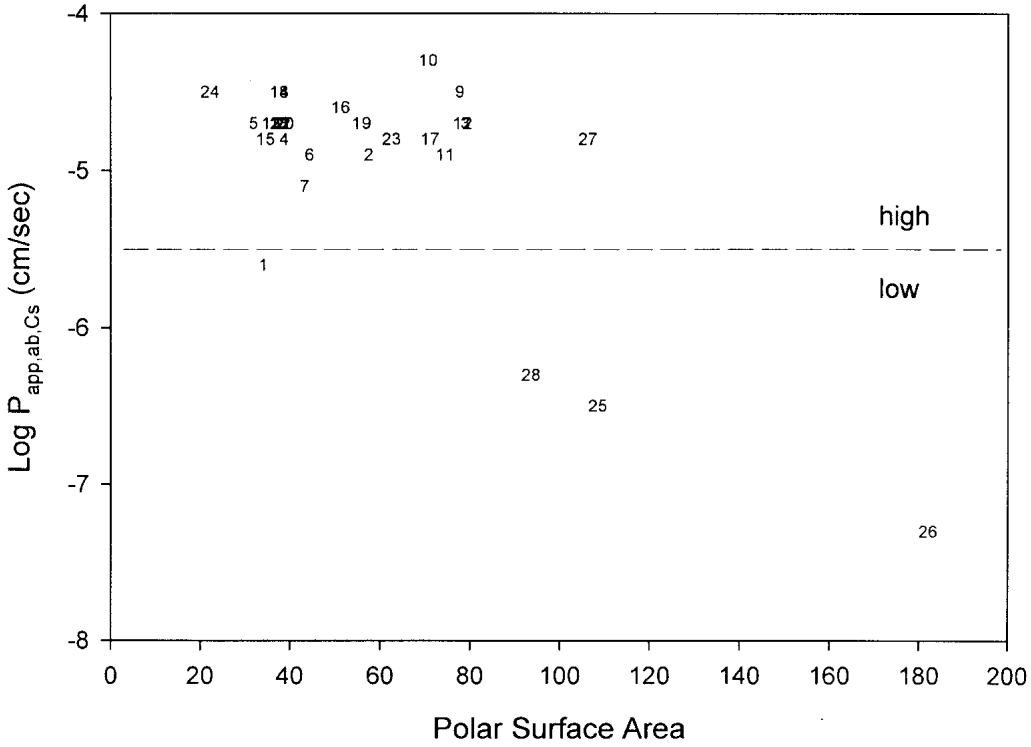


Fig. 6. Caco-2 P_{app} as function of the PSA. $P_{ab,Cs}$ = P_{app} A-to-B, Cs = at saturation concentration transport. The dashed line indicates the transition of a high to a low permeability. The numbers correspond to the compounds of Fig. 2.

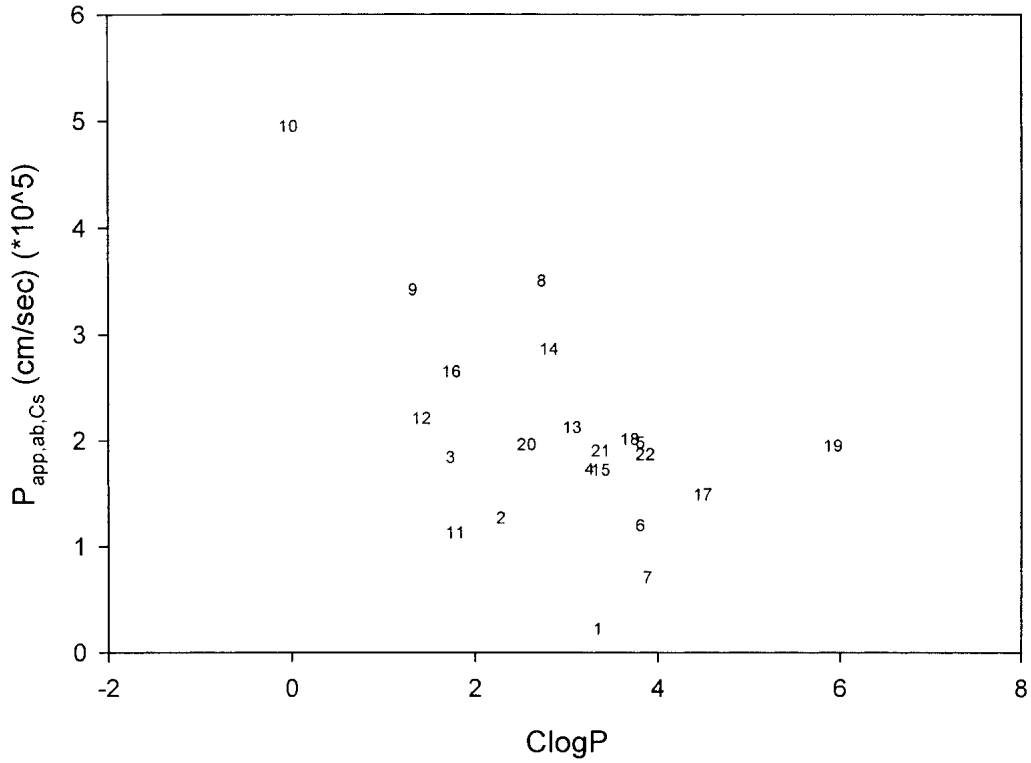


Fig. 7. Caco-2 P_{app} of steroids as function of the calculated ClogP. $P_{ab,Cs}$ = P_{app} A-to-B, C_s = at saturation concentration. The numbers correspond to the compounds of Fig. 2.

brane fluidity. However, pharmacological effects of steroids are reported to be related to a change in membrane fluidity (34–36). 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 (36). 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 biologic membrane, that is, 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).

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 PSA, MW, 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 that are comparable with 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 P_{app} of most steroids is in agreement with the expected behavior for compounds with small PSA (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 biologic membrane and the surrounding aqueous phase is one of the main mechanisms for absorption, indicating passive diffusion. This is supported by the observation that P_{app} was not influenced by the concentration. Because 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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