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Suitability of skin-PAMPA and chromatographic systems to emulate skin permeation. Influence of pH on skin-PAMPA permeability

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ABSTRACT

The skin permeation, K_p, of a chemical compound is a relevant parameter in fields such as toxicology, exposure to pollutants, or dermal studies of pharmaceutical and cosmetic interest. Nonetheless, its experimental determination is not a trivial task, and for this reason alternative methods to estimate K_p have been developed.

This work evaluates the suitability of different methodologies to estimate skin permeation of neutral compounds. Three different approaches have been examined: estimation through the skin-PAMPA (Parallel Artificial Membrane Permeability Assay) permeability, P_e, estimation through the chromatographic retention factor combined with molecular volume, and finally estimation through a quantitative structure–property relationship (QSPR) based on the octanol–water partition coefficient, log $P_{o/w}$, and molecular volume as descriptors. The three approaches have been tested with the same set of compounds and it has been observed that all of them can be used to estimate K_p with similar results, although the chromatographic method presents slightly improved statistics in addition to the facility of measurement.

As many drugs are partially ionised at the pH of skin, the influence of pH in skin-PAMPA permeability has been also studied. To this end, the log P_e vs. pH profiles of a set of 25 compounds of different nature have been determined. As expected, the permeation of neutral forms is higher than the one of ionic forms, and permeation of neutral and ionic species are not governed by the same mechanisms.

1. Introduction

Skin permeation (K_p) is a rate constant that defines the transport of drugs from the external layer of the skin, the *stratum corneum*, into the inner layers and the systemic circulation [1]. The *stratum corneum* has a composition quite different in comparison to other membranes. On a weight basis, it contains about 70% of proteins, 15% of lipids and 15% of water; the lipid matrix, which mainly constitutes the *stratum corneum* barrier, is composed by ceramides (50%), free fatty acids (35%), and cholesterol (15%) [2,3]. The first step in the dermal absorption process is the permeation of compounds through the *stratum corneum* by passive diffusion, so it is often called the rate-determining barrier [4]. The knowledge of K_p values is of utmost importance in many fields such as dermal toxicology, exposure to environmental pollutants, and dermal studies of pharmaceutical and cosmetic interest [5].

The experimental difficulty and ethical implications in the determination of K_p values through *in vitro* and *in vivo* experiments has given rise to the development of alternative methodologies to estimate this parameter. Several models based on quantitative structure–property relationships (QSPRs) have been developed. The nature of the descriptors used to model K_p is diverse. However, among all the drug descriptors used, two of them have a very important role: the logarithm of the octanol–water partition coefficient (log $P_{o/w}$) and the molecular weight (or molecular volume) [6–13]. Besides in-silico models, other approaches based on experimental determinations have been developed to estimate K_p. A first approach is based on liquid chromatography [14–16]. This technique offers a fast, economic and highly automated way to estimate skin permeation, and it has been demonstrated that chromatographic retention factors in combination with molecular volume provide a very good approach to K_p values for neutral compounds

Abbreviations: PAMPA, Parallel Artificial Membrane Permeability Assay; QSPR, Quantitative Structure-Property Relationship; UWL, unstirred water layer.

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[15–16]. A second approach is based on PAMPA (Parallel Artificial Membrane Permeability Assay) measurements using membranes that emulate the *stratum corneum*. The skin-PAMPA system consists in a sandwich structure, with a donor compartment and an acceptor compartment separated by a membrane. Compounds permeate from the donor compartment to the acceptor compartment across the membrane, in a similar way as they would permeate in the skin, and then the PAMPA permeability coefficient (P_e) is obtained [17–18]. In this work a membrane composed by certramide, cholesterol, stearic acid, and silicon oil was used [18].

All the mentioned estimation methods work with more or less success for neutral compounds and defined ranges of K_p values. Nonetheless, few studies account for ionization when compounds with acid-base properties are tested. The permeation of ionizable drugs through skin membrane depends on the acid-base dissociation constant of the drug (pK_a) and the pH of the application medium. It is well known that skin permeability coefficients for neutral forms are much larger than that for ionic forms [19]. Roy and Flynn [20] studied the skin permeation of two basic pharmaceutical compounds, fentanyl and sufentanil, at nine different pH values (from pH 2.88 to 9.37). This data set shows that the permeability of the ionized forms of these drugs is very small compared to the permeability exhibited by the free base forms. Despite that, there was still measurable flux at the lowest pH studied, where drugs were in their ionic form, so it was suggested that the permeation of ions was through a transcellular route [2,20].

Like K_p, permeability in PAMPA is influenced by the pH of the donor compartment solution and pK_a of the drug. Sinkó et al. established the permeability – pH profiles of an acid (indomethacin) and a base (lidocaine) [18]. The works of Sinkó et al. and Roy et al. show some parallelism between K_p and P_e regarding pH dependence. In fact, the overall permeability of an acid-base compound can be expressed as the sum of the contributions of the neutral and ionic forms, according to the ionization degree. Thus, skin-PAMPA permeability for monoprotic acids and bases can be expressed as:

$$P_e = \alpha_{HA^z} P_{e,HA^z} + \alpha_{A^{z-1}} P_{e,A^{z-1}} \quad (1)$$

where z is 0 for monoprotic acids and +1 for monoprotic bases, and α is the ionization degree. P_e (in cm s^{-1}) can be also expressed as a function of the medium pH and the compound pK_a through equation (2):

$$P_e = \frac{P_{e,HA^z} + P_{e,A^{z-1}} 10^{(pH-pK_a)}}{1 + 10^{(pH-pK_a)}} \quad (2)$$

Despite it is a good approach for measuring permeability, not many studies have been published about skin-PAMPA permeability, especially for ionizable compounds [17,18,21–25]. In a previous work we performed a methodologic study to optimize the conditions in routine skin-PAMPA measurements [26]. We tested the stability of the skin-PAMPA membrane at different pH values and it was observed that the structure of the membrane is affected at pH values higher than 8. The best assay conditions for the measurements were 4 h of incubation time with continuous stirring. This incubation time allows the determination of permeability of an important number of compounds in a short time, while stirring diminishes the thickness of the unstirred water layer (UWL), which is formed at both sides of the lipophilic membrane [27].

The aim of the present work is, first, to evaluate some of the methods to estimate K_p of neutral compounds (QSPR, liquid chromatography and PAMPA) using a common set of nearly 50 compounds and, second, to perform a systematic study about the dependence of skin-PAMPA permeability with pH through the profiles of about 25 compounds of different acid-base nature.

2. Materials and methods

2.1. Instruments

pH measurements were done with a combined Crison 5202 electrode in a Crison 2001 pH meter (Hach Lange Spain, L'Hospitalet de Llobregat, Spain). The electrode system was calibrated with ordinary aqueous buffers of pH 4.01 and 7.00 (25 °C).

Permeability measurements were done with the PAMPA Explorer Permeability Assay instrument from Pion Inc (Billerica, MA, USA). This instrument is composed of the Gut-BoxTM and the TempPlate. The Gut-BoxTM is a mechanical device used for the PAMPA assay that reduces the unstirred water layer thickness that is always present by stirring. The TempPlate is used for the temperature control during plate incubation.

For the chromatographic model, measurements were performed with an Agilent Technologies (Santa Clara, CA, USA) 1200 Series instrument with a diode array detector, coupled to a UHD 6540 Accurate-Mass Q-TOF detector with electrospray ionization (ESI). The analytical column was a 100 mm, 4.6 mm i.d, 2.6 μm octadecylsilica Kinetex EVO C18 from Phenomenex (Torrance, CA, USA).

To obtain the Skin-PAMPA profiles, a Waters (Milford, MA, USA) I-Class UPLC with diode array detector was used. Instrument control and processing was performed through the software Empower. The column used for the determinations was an Acquity UPLC BEH C18 (50 × 2.1 mm, 1.7 μm).

2.2. Reagents

Acetonitrile LiChrosolv grade and 0.5 M sodium hydroxide solution were purchased from Merck (Darmstadt, Germany). Formic acid was obtained from Scharlau (Sentmenat, Spain). Dimethylsulphoxide was from Carlo Erba (Milano, Italy). Water was purified by a Milli-Q deionizing system from Millipore (Billerica, MA, USA) with a resistivity of 18.2 MΩ. Reagents used to prepare the buffer solutions used in chromatography were sodium phosphate monobasic monohydrate (Sigma-Aldrich, ≥99.0%), formic acid (Scharlau, eluent additive for LC-MS), acetic acid (Fluka Analytical, eluent additive for LC-MS), ethylendiamine (Fluka Analytical, ≥ 99.5%) and 25% w/w ammonia solution (Sharlau, extrapur). Most solutes employed were purchased from Sigma-Aldrich (Steinheim, Germany), Fluka Analytical VWR (West Chester, PA, USA), Riedel-de Haën (Seelze, Germany), Merck (Darmstadt, Germany), Carlo Erba (Milano, Italy) and Baker (Center Valley, PA, USA).

The concentrated PRISMA HT^{TM} solution was used to prepare the buffer solutions. This solution is a universal buffer designed by Pion Inc (Billerica, MA, USA) and is composed by several compounds with pK_a values evenly spaced to produce a constant buffer capacity in the range pH 3–10. The ionic strength of the PRISMA HTTM is about 10 mM. A hydration solution from Pion Inc. was used to rehydrate the artificial skin membrane. The skin-PAMPA plates were also obtained from Pion Inc.

2.3. Procedures

2.3.1. Chromatographic method

Chromatographic measurements were performed as described elsewhere [16]. Briefly, a 40% acetonitrile and 60% aqueous buffer isocratic mobile phase at a flow rate of 1 mL min^{-1} was used. Compounds were prepared at a concentration of 100 mg L^{-1} in an acetonitrile:water mixture (40:60), and the injection volume was 10 μL. The hold-up time was measured with KBr, detected at 200 nm. The aqueous buffers were prepared in a pH range between 2 and 11. All of them were MS compatible except the buffer at pH 2 (phosphoric acid), where detection was done with a DAD.

2.3.2. Skin-PAMPA method

Before permeation assays, the top part of the skin-PAMPA sandwich,

which contains the membrane, was hydrated overnight with the hydration solution. Different buffer solutions in the pH range 3 to 10 were prepared diluting 25 mL of concentrated PRISMA HTTM with water to a final volume of 1 L and then, adding 0.5 M NaOH up to the desired pH. Samples were dissolved in diluted PRISMA HTTM buffer solution at the different pH values. The concentration of the drug sample solutions was 50 μM (containing 0.5% v/v DMSO). Skin-PAMPA assays were carried out under gradient-pH conditions to mimic the pH change between the stratum corneum and the underlying epidermis and dermis. For this reason, the donor compartment pH was varied from 3 to 10 (in 1 pH unit increments) and the acceptor compartment pH was maintained at pH 7.4. The donor compartment (or bottom plate) was prefilled with 180 μL of sample solutions and the acceptor compartment (or top plate) was filled with 200 μL of PRISMA HTTM buffer solution at pH 7.4. The volume is lower in the donor compartment to avoid overflow, since the stirring bars have already a volume of 20 μL.

The skin-PAMPA sandwich was incubated at 32 °C for 4 h. After the permeation time was reached, the plates were separated and the compound concentration in acceptor, donor and reference (initial sample solution) was determined by UPLC-DAD. Chromatographic conditions were formic acid 0.1% and acetonitrile as mobile phases, flow rate of 0.8 mL/min, linear gradient elution from 2% to 98% of acetonitrile in 2.5 min, and injection volume of 5 μL. 3 to 5 replicate measurements were done per compound and pH, and every well-plate contained only one compound.

2.4. Data treatment

2.4.1. Chromatographic method

The retention factor (k) of the compounds is calculated through the following equation:

$$k = \frac{t_R - t_0}{t_0 - t_e} \tag{3}$$

where t_R is the retention time of the compound, t_0 is the column hold-up time, determined with KBr, and t_e is the extra column time, determined removing the column for the two different detection systems.

2.4.2. Skin-PAMPA method

The skin-PAMPA permeability was calculated through PAMPA equations. Taking into account the membrane retention (mole fraction of the sample that can be lost in the membrane) under gradient-pH conditions, these equations are the following ones [18]:

$$P_e = -\frac{2.303V_D}{A\cdot(t - t_{ss})\cdot\varepsilon_a}\cdot\left(\frac{1}{1+r_a}\right)\cdot\log_{10}\left[-r_a + \left(\frac{1+r_a}{1-R_M}\right)\cdot\frac{C_D(t)}{C_D(0)}\right] \tag{4}$$

$$R_M = 1 - \frac{C_D(t)}{C_D(0)}\cdot\frac{V_A}{V_D}\frac{C_A(t)}{C_D(0)} \tag{5}$$

where V_D and V_A are the volumes of solution in the donor side (0.18 cm^3) and acceptor side (0.2 cm^3), respectively, A is the membrane area (0.3 cm^2), t is the incubation time of the experiment (s), t_{ss} is the lag time (s) [t_{ss}=(54·R_M + 1)·60], ε_a is the apparent membrane porosity (0.76), $C_D(t)$ is the concentration (mol cm^{-3}) in the donor side at time t, $C_D(0)$ is the initial concentration (mol cm^{-3}) in the donor side, $C_A(t)$ is the concentration (mol cm^{-3}) in the acceptor side at time t, R_M is the membrane retention factor and r_a is the asymmetry ratio (gradient-pH-induced), defined as:

$$r_a = \left(\frac{V_D}{V_A}\right)\frac{P_{e(A\to D)}}{P_{e(D\to A)}} \tag{6}$$

When the pH is different in the two sides of the membrane, a gradient-pH is created and the permeation of ionizable molecules can be altered. This gradient-pH implies two different permeability coefficients, one denoted by the superscript (D → A), associated with donor to acceptor flux, and the other denoted by the superscript (A → D), corresponding to the reverse-direction flux. As equation (6) has two unknowns, $P_{e(A\to D)}$ and $P_{e(D\to A)}$, the following method is used to solve the equation: at least two assays are done, one with gradient-pH and the other with *iso*-pH, that is, the same pH at both compartments (7.4). For *iso*-pH, $P_{e(A\to D)} = P_{e(D\to A)}$. Therefore, $P_{e(A\to D)}$ can be solved directly using the *iso*-pH equation:

$$P_e = -\frac{2.303V_D}{A\cdot(t - t_{ss})\cdot\varepsilon_a}\cdot\left(\frac{1}{1+r_v}\right)\cdot\log_{10}\left[-r_v + \left(\frac{1+r_v}{1-R_M}\right)\cdot\frac{C_D(t)}{C_D(0)}\right] \tag{7}$$

where r_v is the aqueous compartment volume ratio, defined as:

$$r_v = \frac{V_D}{V_A} \tag{8}$$

Then, Eq. (6) is iteratively solved for $P_{e(D\to A)}$. Initially, r_a is assumed to be r_v, but with each iteration, the r_a estimation is improved by using the calculated $P_{e(D\to A)}$. The process continues until self-consistency is reached within the precision required (0.001). The Solver utility from Microsoft Excel was used for the iterative process.

3. Results and discussion

3.1. Evaluation of the different methods to estimate the skin permeability of neutral compounds

The K_p values of a set of 46 compounds covering a log K_p range from −4 to −8 (K_p in cm s^{-1}) have been selected from the compilation made by Zhang et al. in 2017 [28]. In there, K_p values of different sources (mainly a previous source from the same authors [19] and data from Baba et al. [29]) were evaluated and corrected for temperature and ionization, so K_p values of neutral and ionic species at 37 °C are provided. Apart from these 46 compounds, 8 additional compounds with no available K_p value were included for comparison between the skin-PAMPA and the chromatographic methods. Table 1 shows the compounds and the different parameters used for the evaluation: log K_p, log P_e, log k, log $P_{o/w}$ and McGowan's volume (V). log $P_{o/w}$ have been obtained from Bioloom (Biobyte, Covina, CA, USA) database [30] and McGowan volumes were calculated using the Percepta software (ACDLabs, Toronto, Canada) [31].

For acid-base compounds, the P_e values of the neutral form were obtained selecting an adequate pH in the donor compartment, according to the pK_a of the compounds. However, pH values higher than 7 could not be tested due to instability of the membrane at basic pH values [26]. For this reason, bases with a pK_a value higher than 5 were not included in the selection of 46 compounds. In the case of chromatographic measurements, log k was measured at a pH in which compounds were neutral, and the only limitation was the pH range established by the column manufacturer (pH 2–10).

Fig. 1A shows a direct correlation between the measured log P_e values and log K_p values from the literature. A good correlation is observed, much better than the one presented by Sinkó et al. when the membranes were initially developed [18]. There, skin-PAMPA log P_e values were compared to three different skin permeation log K_p datasets. Nonetheless, there was important variability in the data of the sets: different types of skin, different temperature, different degree of ionization of the compounds or use of excipients in the donor solution. Despite the high degree of heterogeneity in the K_p data good correlations were obtained, although not as good as the one obtained in the present work with a homogeneous set of compounds. The regression equation and statistics are the following:

$$\log K_p = 1.157\ \log P_e - 0.134 \tag{9}$$

N = 46; SD = 0.436; R^2 = 0.824; F = 206

Here and elsewhere, N is the number of compounds studied, SD is the

Table 1
Set of compounds used for the comparison of methods for K_p estimation. All parameters correspond to the neutral form of the compound. K_p and P_e values are expressed in cm s^{-1}, and McGowan volume in (cm^3 mol^{-1})/100.

Compounds	log K_p	log P_e	log k	log $P_{o/w}$	V
2,4-Dichlorophenol	−4.3	−3.92	0.446	3.06	1.0199
2-Isopropyl-5-methylphenol	−4.35	−4.01	0.743	3.3	1.3387
2-Nitro-p-phenylenediamine	−6.66	−5.25	−0.426	0.53	1.0902
2-Toluidine	–	−4.13	0.025	1.32	0.9571
3-Methylphenol	−4.89	−4.33	0.042	1.96	0.916
4-Amino-2-nitrophenol	−5.91	−4.59	−0.174	0.96	1.0491
4-Chlorophenol	−4.52	−4.27	0.177	2.39	0.8975
4-Ethylphenol	−4.53	−4.19	0.261	2.47	1.0569
4-Hydroxybenzyl alcohol	−6.26	−5.85	−0.839	0.25	0.9747
4-Hydroxy-methylphenylacetate	−5.26	−5.07	−0.368	0.45	1.2722
4-Hydroxyphenylacetamide	−6.89	−6.07	−0.891	−0.09	1.1724
4-Nitrophenol	−5.33	−4.91	−0.036	1.91	0.9493
5,5-Diethylbarbituric acid	−7.29	−5.75	−0.564	0.65	1.3739
5-Ethyl-5-phenylbarbituric acid	−6.68	−6.05	−0.198	1.47	1.6999
5-Fluorouracil	−6.82	−5.77	−1.595	−0.89	0.7693
8-Methoxypsoralen	−5.12	−4.3	0.253	2.07	1.4504
Aminopyrine	−6.55	−5.67	−0.23	0.8	1.8662
Aniline	−4.94	−4.55	−0.163	0.9	0.8162
Antipyrine	–	−5.63	−0.511	0.23	1.4846
Atrazine	–	−4.67	0.302	2.61	1.6196
Benzoic acid	−5.68	−4.82	−0.183	1.87	0.9317
Benzyl nicotinate	−4.87	−4.16	0.447	2.4	1.6393
Caffeine	−6.85	−5.45	−0.798	−0.07	1.3632
Capsaicine	–	−4.63	0.761	3.04	2.5971
Catechol	−5.87	−5.39	−0.472	0.88	0.8338
Cortexolone	−7.2	−5.45	0.147	2.52	2.7389
Corticosterone	−6.84	−5.59	0.097	1.94	2.7389
Cortisone	−7.38	−6.09	−0.162	1.47	2.7546
Dexamethasone	−7.27	−6.25	0.034	1.74	2.9132
Diclofenac	−5.3	−3.79	0.894	4.5	2.025
Digitoxin	−8.15	−6.38	0.53	2.83	5.6938
Estradiol	−5.61	−4.15	0.422	4.01	2.1988
Estriol	–	−6.07	−0.325	2.54	2.2575
Fluocinonide	−6.33	−5.38	0.797	3.19	3.4601
Flurbiprofen	−4.72	−3.69	0.784	4.16	1.8389
Griseofulvin	−6.44	−5.28	0.379	2.18	2.3947
Hydrocortisone	−7.22	−6.17	−0.222	1.61	2.7976
Hydroquinone	−6.31	−5.87	−0.83	0.59	0.8338
Hydroxyprogesterone	−6.3	−4.7	0.6	3.17	2.6802
Ibuprofen	−4.58	−3.61	0.906	3.5	1.7771
Indomethacin	−5.39	−4.4	0.9	4.27	2.5299
Isoquinoline	−5.11	−4.2	0.071	2.08	1.0443
Ketoprofen	−5.22	−4.68	0.434	3.12	1.9779
Methyl 4-hydroxybenzoate	−5.03	−4.88	−0.133	1.96	1.1313
Methylphenylether	−4.68	−4.34	0.427	2.11	0.916
N,N-Dimethylaniline	–	−3.95	0.587	2.31	1.098
Naproxen	−4.97	−4.19	0.454	3.34	1.7821
o-Phenylenediamine	−6.7	−5.42	−0.611	0.15	0.916
Piroxicam	−6.02	−4.67	0.191	1.78	2.25
Prednisolone	−7.91	−6.42	−0.261	1.42	2.7546
Progesterone	−4.9	−4.13	1.026	3.87	2.6215
Pyridine	–	−4.49	−0.441	0.65	0.6753
Testosterone	−5.54	−4.52	0.441	3.32	2.3827
Warfarin	–	−4.62	0.636	2.7	2.3077

standard deviation, R^2 is the determination coefficient, and F is the Fisher F-statistic. The good direct correlation between both parameters confirms the suitability of skin-PAMPA to estimate skin permeation.

However, other methods have been presented as important alternatives for log K_p estimation. Among QSPR studies, the correlation proposed by Potts and Guy [6] is the simplest approach. It only depends on two parameters, log $P_{o/w}$ and molecular weight (or molecular volume), which are very accessible descriptors. Other QSPR models appeared later with slightly better correlations [7], although the descriptors used can be more difficult to obtain. We have applied the model of Potts and Guy to the same set of compounds and Fig. 1B and equation (10) show the obtained correlation.

$$logK_p = 0.680\ logP_{o/w} - 0.914V - 5.652 \quad (10)$$

N = 45; SD = 0.425; R^2 = 0.840; F = 110

In this case 4-hydroxy-methylphenylacetate presented a standard error of 2.58. We excluded it as we set the limit to consider outliers in a standard error of 2.5, although the fact of leaving the compound in the correlation did not really change the obtained results. Statistics of the regression show that this method, like the direct regression with log P_e, seems to be also a good alternative to direct K_p determination. Eq. (10) is similar to the ones obtained by Potts and Guy [6] with different sets of data. log $P_{o/w}$ had a positive contribution to permeation, whereas molecular weight or volume had a negative effect. In fact, one would expect the volume contribution to be positive, since increase of drug volume increases permeation in lipophilic phases. Abraham correlated the skin permeation of large datasets of neutral and ionic solutes (log K_p) against different solute descriptors, including McGowan's volume, and obtained that drug dipolarity and hydrogen bonding decrease permeability, whereas drug polarizability and specially volume increase permeability [1,19,28]. The V coefficient was clearly positive and around 1.78 in the most recent correlation. The negative contribution of V in Eq. (10) is caused by the effect of the concomitant contribution of V to log $P_{o/w}$, which is positive and larger than for log K_p. Abraham correlated log $P_{o/w}$ to the same descriptors as log K_p and found similar results: dipolarity and hydrogen bonding decrease permeability, and drug polarizability and volume increase permeability; but V coefficient (3.81) was much larger for log $P_{o/w}$ than for log K_p [32,33]. A simple calculation of 0.680*3.81–1.78 gives 0.81 as excess contribution of V in log K_p vs. log $P_{o/w}$ correlation, which is quite close to our 0.91 fitting value.

The third approximation is the one based on liquid chromatography. For acid-base compounds, the retention factors were measured at adequate pH values, in order to get the retention of the neutral species. Then, following the method developed by Hidalgo-Rodríguez et al. [15], log K_p was correlated to log k and V. Fig. 1C shows the results obtained, and equation (11) the regression equation:

$$logK_p = 1.598\ logk - 0.963V - 4.270 \quad (11)$$

N = 46; SD = 0.383; R^2 = 0.867; F = 140

Similarly to Eq. (10), in Eq. (11) the contribution of the retention factor is positive and the contribution of the molecular volume negative, although the relative weight of each coefficient to the log K_p value is different. Again, the negative contribution of volume emerge from the larger contribution of volume to chromatographic retention (log k). An Abraham correlation analysis of log k for the column and mobile phase used [34] shows that solute dipolarity and hydrogen bonding decrease retention and polarizability and McGowan's volume increase it, as in log K_p and log $P_{o/w}$. The coefficient for V is 1.64 and the calculation 1.598*1.64–1.78 gives 0.84 as excess volume contribution from log k, close again to the 0.96 fitting coefficient of Eq. (11).

Statistics of Eq. (11) are slightly better than those of Eqs. (9) and (10). Standard deviation is the parameter with major improvement, most likely due to the lower dispersion in log k caused by the high reproducibility of chromatographic measurements.

After applying the 3 models to the same set of compounds, it can be concluded that all of them provide a good approximation to log K_p values, although the chromatographic method presents slightly improved statistics and facility of measurement. In fact, all three methods are related in some way.

Some studies have correlated Skin-PAMPA permeability to physicochemical descriptors and they point out the importance of the lipophilicity of the compounds in the permeation through skin-PAMPA membranes, although they also indicate this is not the only factor that governs the permeation through them [35,36]. Based on the good correlation between log K_p and log P_e (Eq. (10)), also a good correlation

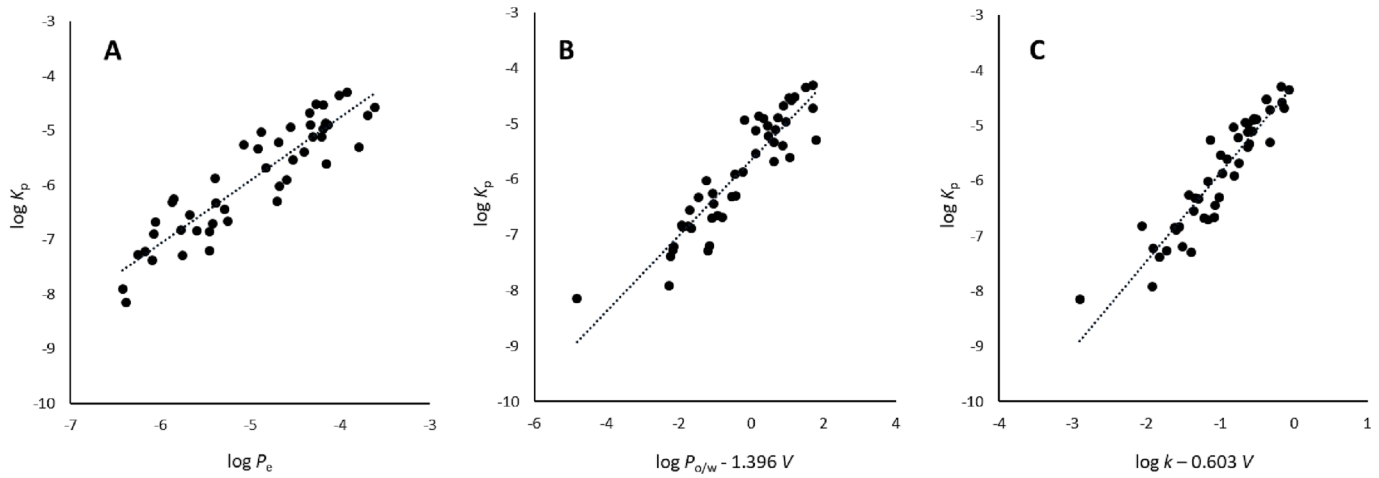

Fig. 1. Correlation between log K_p and log P_e (A), the combination of log $P_{o/w}$ and V (B), and the combination of log k and V (C) for the set of 46 neutral compounds.

between log P_e and the combinations of log $P_{o/w}$ or log k with V is expected. Fig. 2 and equations (12) and (13) show such correlations:

$$logP_e = 0.567\ logP_{o/w} - 0.619V - 4.950 \quad (12)$$

$$N = 52;\ SD = 0.342;\ R^2 = 0.813;\ F = 106$$

$$logP_e = 1.467\ logk - 0.656V - 3.898 \quad (13)$$

$$N = 52;\ SD = 0.262;\ R^2 = 0.892;\ F = 203$$

Phenobarbital was an outlier in both correlations. In addition, estriol was excluded in the correlation with log $P_{o/w}$ and V, and 5-fluorouracil in the correlation with log k and V. All these compounds have standard errors slightly higher than 2.5. Both parameters, combined with the molecular volume, provide a good estimation of log P_e, although a better correlation is observed when the estimation is done through chromatographic measurements. Coefficients of the equations show that, similarly to the estimation of log K_p, log k has a greater contribution than log $P_{o/w}$ in the log P_e value.

3.2. Influence of pH on skin-PAMPA permeability

The permeability values of a set of 25 ionizable compounds (8 monoprotic acids, 14 monoprotic bases, and 3 diprotic compounds) have been measured at 8 different pH values (from 3 to 10). Although 3 of the compounds are diprotic (2-hydroxybenzoic acid, piroxicam, and *o*-phenylenediamine), according to their pK_a values they behave as monoprotic acids or bases in the working pH range of the present work. Fig. 3 shows the experimental profiles obtained for the set of compounds and Table 2 their pK_a values.

As observed in a previous work [26], all permeability-pH profiles show an increase of the P_e values at pH 8 and especially at pH 9 and 10 (red points). This increase is more evident in acids than in bases since, according to their pK_a, most of the acids should present a constant or slightly descending log P_e value (corresponding to the ionic form) in this pH region. This fact confirms that the skin-PAMPA membrane is affected at basic pH values, most likely due to a change in the membrane packaging [37]. These values have been considered anomalous and discarded in the fit of Eq. (2) to experimental points. A direct consequence of working in a shorter pH range is that the log P_e of neutral forms of bases with pK_a values higher than 6 (tramadol, atropine, diethylcarbamazine, fentanyl, lidocaine, oxycodone, propranolol, and sufentanil) could not be experimentally determined. In order to obtain a better fit, these log P_e values have been estimated through Eq. (13), measuring the chromatographic retention factor of the compounds in the pH region where they are neutral. Table 2 shows the results and statistics of the fit.

The permeability of the neutral forms expressed as log P_e shows the highest value for ibuprofen (-3.6) and the lowest value for aminopyrine (-5.7). It must be noted that most of the acids are partially ionised at pH

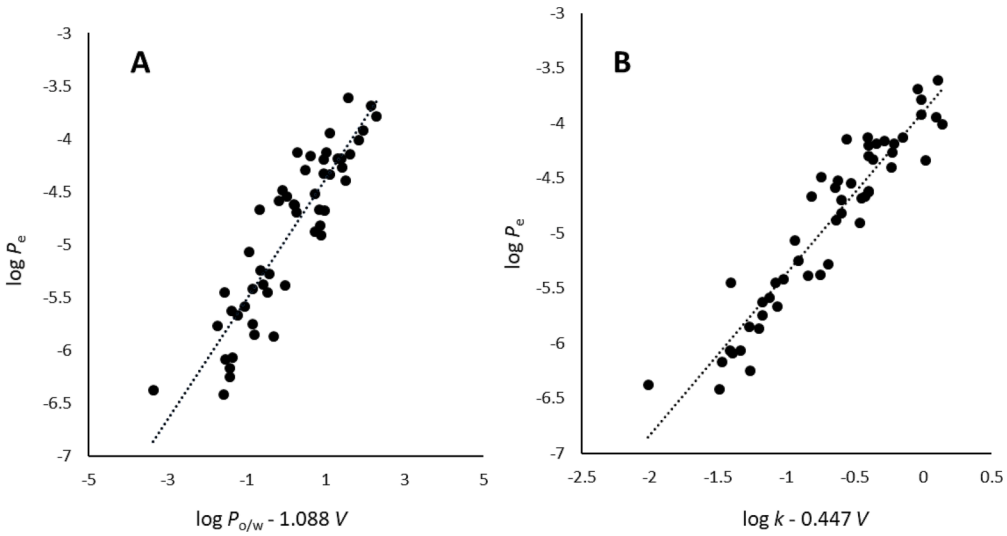

Fig. 2. Correlation between log P_e and the combination of log $P_{o/w}$ and V (A), and the combination of log k and V (B) for the set of 46 neutral compounds.

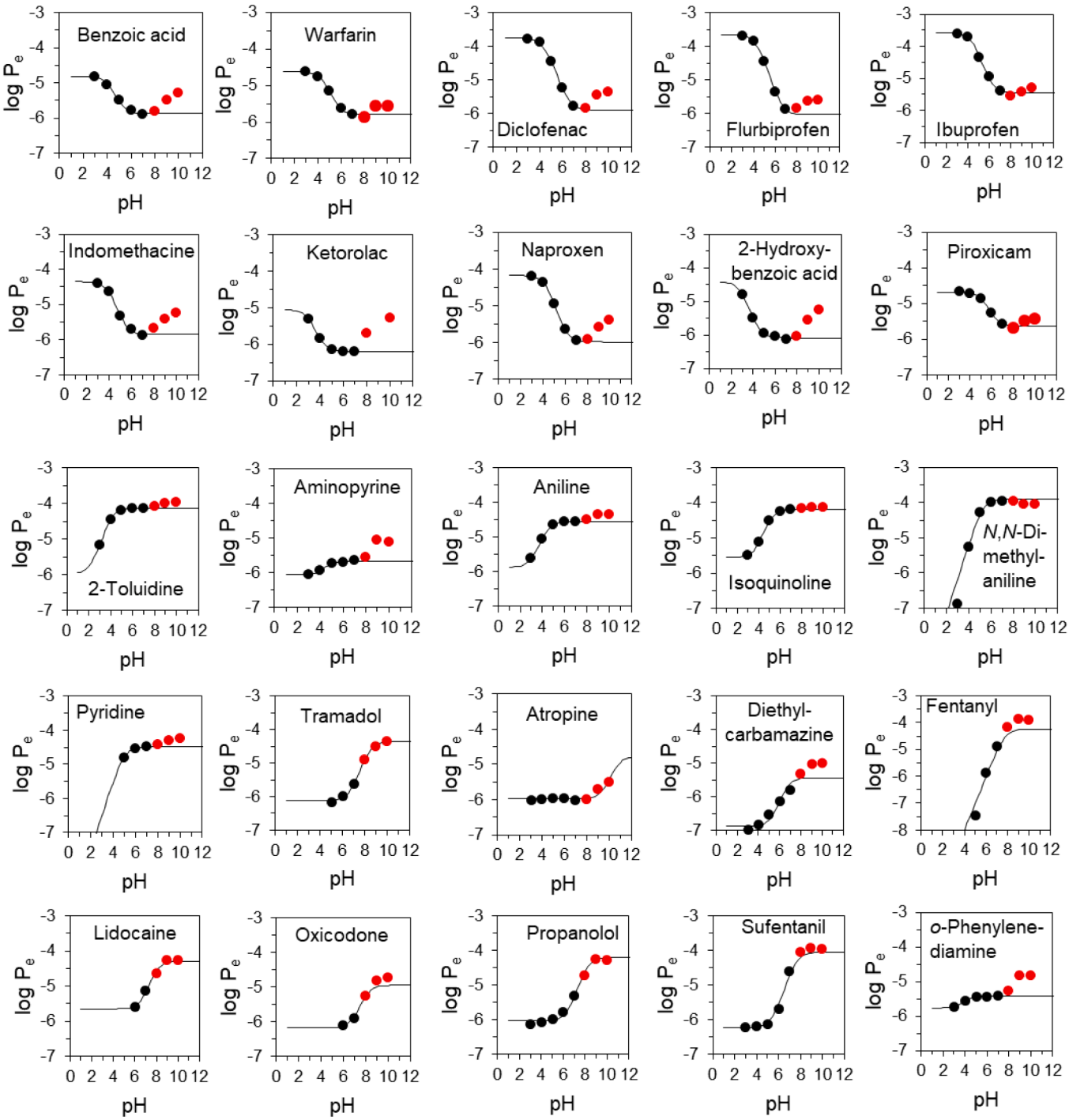

Fig. 3. log P_e vs. pH profiles. Dots are the experimental points, and the solid line is the fit of Eq. (2) to the experimental points. Red points have not been considered in the fit.

3 (the lowest experimentally tested pH), especially salicylic acid and ketorolac, and this implies certain extrapolation to obtain log $P_{e,neutral}$, which may be reflected in a higher standard deviation associated to its value.

Permeability of the ionic forms varies in a narrower range than the one of neutrals forms: from −5.5 (ibuprofen) to −6.9 (diethylcarbamazine). Now, acidic compounds are already in the ionic form in the last experimental point (pH 7), so no extrapolation is needed to get the log P_e values of anions. However, some of the bases were not 100% protonated at pH 3, and some extrapolation is done in the log P_e value of cations. This is critical in case of 2-toluidine, with an important extrapolation reflected in the standard deviation of the parameter, and also for *N*,*N*-dimethylaniline, pyridine, and fentanyl. For these latter three compounds the experimental points do not follow the sigmoidal shape, and the ionic permeability nor pK_a could be obtained. Some other compounds showed a very low concentration in the acceptor compartment at low pH, so the amount of drug in solution could not be quantified at pH values lower than 5. This the case for tramadol, pyridine, fentanyl, lidocaine, and oxycodone.

It is somehow surprising the narrow range of permeability values of the ionic forms of the compounds (log $P_{e,ionic}$). Fischer et al. measured the permeability in membranes that simulate the gastrointestinal tract

Table 2
Fit of Eq. (2) to the skin-PAMPA permeability experimental points. Standard deviations of the fitted parameters are shown in parenthesis.

	Fitting parameters			Statistics			$pK_{a,lit}$
	log $P_{e,neutral}$	log $P_{e,ionic}$	pK'_a	R^2	SD	F	
Benzoic acid	−4.81(0.04)	−5.87 (0.03)	4.13 (0.11)	0.996	0.04	253	4.22 [41]
Warfarin	−4.63 (0.02)	−5.80 (0.02)	4.54 (0.06)	0.999	0.03	778	5.17 [42]
Diclofenac	−3.76 (0.02)	−5.90 (0.03)	4.42 (0.03)	1.000	0.02	2866	3.99 [43]
Flurbiprofen	−3.65 (0.02)	−6.04 (0.04)	4.25 (0.04)	1.000	0.03	2660	3.91 [44]
Ibuprofen	−3.59 (0.06)	−5.45 (0.08)	4.38 (0.12)	0.996	0.07	253	4.49 [44]
Indomethacin	−4.35 (0.05)	−5.86 (0.04)	3.98 (0.10)	0.997	0.05	351	4.50 [45]
Ketorolac	−5.05 (0.04)	−6.20 (0.01)	3.05 (0.07)	0.999	0.01	1650	3.50 [45]
Naproxen	−4.18 (0.01)	−5.99 (0.02)	4.29 (0.02)	1.000	0.01	6087	4.43 [46]
2-Hydroxybenzoic acid	−4.42 (0.11)	−6.09 (0.02)	2.85 (0.15)	0.998	0.03	657	2.97; 13.60 [47]
Piroxicam	−4.69 (0.02)	−5.63 (0.03)	5.25 (0.06)	0.998	0.02	639	1.88; 5.29 [48]
2-Toluidine	−4.13 (0.01)	−5.96 (0.14)	4.07 (0.03)	1.000	0.01	4477	4.45 [49]
Aminopyrine	−5.66 (0.01)	−6.06 (0.02)	4.56 (0.10)	0.995	0.02	218	5.06 [50]
Aniline	−4.55 (0.01)	−5.86 (0.02)	4.38 (0.02)	1.000	0.01	4831	4.63 [41]
Isoquinoline	−4.19 (0.001)	−5.55 (0.001)	5.09 (0.002)	1.000	0.001	871,115	5.07 [51]
N,N-dimethylaniline	−3.91 (0.06)	–	–	0.989	0.08	185	5.15 [52]
Pyridine	−4.49 (0.01)	–	–	0.996	0.01	271	5.28 [41]
Tramadol	−4.35*	−6.12 (0.04)	8.44 (0.06)	0.996	0.05	1038	9.19 [53]
Atropine	−4.78*	−5.98 (0.03)	10.71 (0.12)	0.858	0.08	36	9.60 [54]
Diethylcarbamazine	−5.43*	−6.86 (0.19)	6.69 (0.36)	0.894	0.28	51	7.15 [31]
Fentanyl	−4.24*	–	–	0.939	0.35	77	8.43 [55]
Lidocaine	−4.30*	−5.65 (0.09)	7.98 (0.10)	0.989	0.07	281	7.97 [56]
Oxycodone	−4.95*	−6.17 (0.19)	8.09 (0.27)	0.946	0.17	52	8.60 [50]
Propanolol	−4.21*	−6.03 (0.04)	8.20 (0.08)	0.991	0.08	698	9.47 [41]
Sufentanil	−4.06*	−6.24 (0.08)	7.57 (0.11)	0.989	0.12	530	8.01 [55]
o-Fenylenediamine	−5.41 (0.01)	−5.77 (0.03)	4.02 (0.12)	0.993	0.02	138	0.72; 4.49 [31]

*Estimated by the chromatographic method through Eq. 17.

(GIT-PAMPA) of 20 quaternary amines, and obtained P_e values covering a range of 3–4 log units [38]. Instead, in the present study log $P_{e,ionic}$ has a range of only 1.4 log P_e units, and apparently no differences in the values of cations and anions can be observed. When log $P_{e,ionic}$ and log $P_{e,neutral}$ are represented one against each other (Fig. 4) a random distribution is observed. This can be expected by the almost null variability of log $P_{e,ionic}$ values. As a result, the equations previously described (Eq. (12) and (13)) to predict log $P_{e,neutral}$ cannot be applied to ionic species. Fisher et al. developed an *in-silico* model for predicting log $P_{e,ionic}$ in GIT-PAMPA for the same set of quaternary amines, and they concluded that molecular shape and electrostatic properties were the most relevant descriptors in this case [38]. However, this model has not been tested in Skin-PAMPA data, nor for a more varied set of compounds. The low and nearly constant permeability values of the ionic species confirm that all they move slowly and with a similar mechanism through the membrane. As pointed out by some authors, it is quite likely that small water channels are located inside the membrane, so that ions can permeate slowly through it [18,39,40].

As regards pK_a values, the ones obtained in the fit are similar to the values in water ($pK_{a,lit}$) [31,41–56]. This can be seen in Fig. 5, where the pK_a obtained in the fit is plotted against the bibliographic pK_a. *N,N*-dimethylaniline, pyridine, and fentanyl have not been included in the regression because their pK_as could not be accurately determined by the

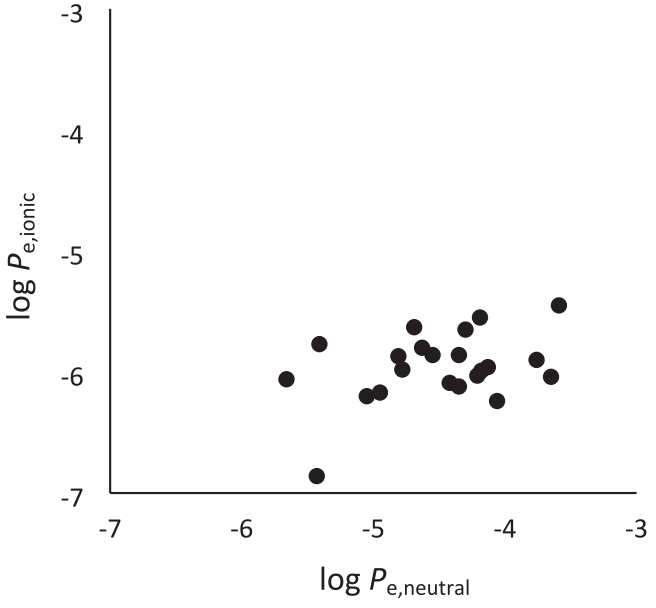

Fig. 4. Skin-PAMPA permeation of the ionic forms (log $P_{e,ionic}$) vs. permeation of the neutral forms (log $P_{e,neutral}$).

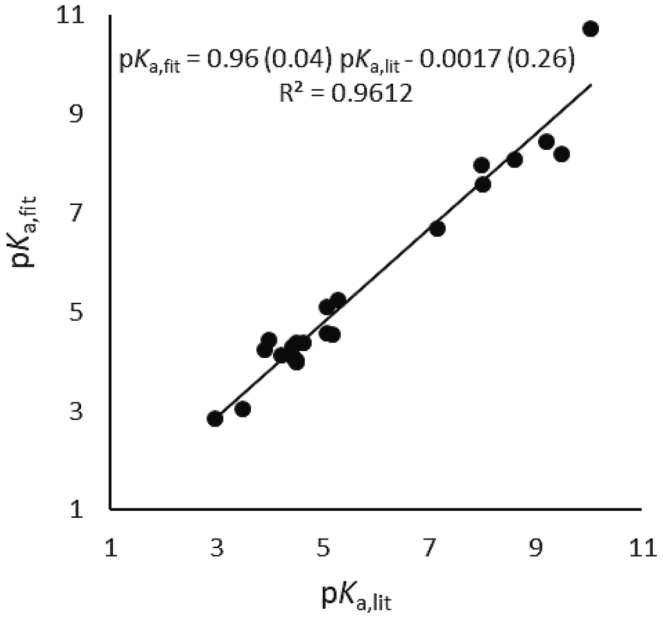

Fig. 5. pK_a obtained in the fit of Eq. (2) to experimental points ($pK_{a,fit}$) vs. literature pK_a ($pK_{a,lit}$). Slope and ordinate standard deviation are shown in parenthesis.

lack of experimental points, as seen in Fig. 3. The good correlation between both values, with a slope close to 1 and an ordinate close to 0, validates in some way the results of the fits.

All in all, results confirm that neutral compounds permeate faster across the membrane than ionic compounds, mostly due to the hydrophobic character of the membrane and that passive diffusion is the transport mechanism in PAMPA. As ionic species have low and more or less constant log P_e values and we demonstrated that for neutral species high lipophilicity is related to high permeability (Fig. 2A), the difference of permeability between non-ionized and ionized forms of the same chemical should decrease when the non-ionized species is hydrophilic and increase when it is lipophilic.

4. Conclusions

After the evaluation of three different approaches to estimate skin permeation with the same set of compounds it can be concluded that all of them perform similar from the statistics point of view. log P_e and log K_p have a direct correlation. This is explained because skin-PAMPA membrane directly emulates the skin through the use of similar components in similar proportions. Both, log k (in a system composed of a C18 column and a 40:60 acetonitrile:aqueous buffer as mobile phase) or log $P_{o/w}$ provide also a good estimation of K_p when combined to molecular volume. Whereas log k and log $P_{o/w}$ contribute positively, the molecular volume contribution is negative. According to other models, a positive contribution of volume would be expected. However, its contribution to log k and log $P_{o/w}$ is larger than its contribution to log K_p, so it emerges as a negative correction. The slightly improved statistics and the easiness and high throughput of chromatographic measurements make the chromatographic method a good choice for K_p estimation.

The analysis of the pH vs. log P_e profiles of a set of 25 acid-base compounds shows that neutral forms of drugs permeate faster than anions and cations and, similarly to log K_p, log P_e profiles also show a sigmoidal shape. Although small, ionic species have some permeability, which confirms their transport through water channels of the skin-PAMPA membrane. However, there is not direct correlation between the log P_e values of ionic and neutral forms.

CRediT authorship contribution statement

Sara Soriano-Meseguer: Methodology, Investigation, Visualization, Writing – original draft. **Elisabet Fuguet:** Conceptualization, Methodology, Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Supervision. **Adriana Port:** Conceptualization, Methodology, Formal analysis, Resources, Supervision, Writing – review & editing. **Martí Rosés:** Conceptualization, Formal analysis, Resources, Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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