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Acidity and Hydrophobicity of Several New Potential Antitubercular Drugs: Isoniazid and Benzimidazole Derivatives

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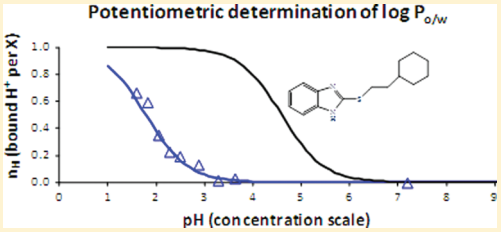
ABSTRACT: Hydrophobicity values (log $P_{o/w}$) determined both by potentiometry and by the conventional shake-flask method and aqueous pK_a values obtained by potentiometry for seven isoniazid and nine benzimidazole derivatives are presented. Several of these compounds have strong hydrophilic or hydrophobic character, and some of them show two or more ionizable sites, sometimes very acidic or very basic. These facts have compelled us to work near the limits of the potentiometric technique, and therefore, a critical evaluation of the results obtained in these extreme working conditions is presented. Particularly, details about the potentiometric determination of log $P_{o/w}$ values of the most hydrophilic and most hydrophobic compounds are clearly described.



INTRODUCTION

According to the World Health Organization (WHO) 2009 data, in 2008 there were 9.4 million new tuberculosis (TB) cases which killed 1.8 million people. One-third of the world's population is believed to be currently infected with the *Mycobacterium tuberculosis* (*M.tb*) bacillus.[1] Of all TB cases, 5 % were shown to be multidrug resistant (MDR-TB), which is defined as a form of TB resistant to isoniazid and rifampicin, the two most powerful first-line drugs. About 5.4 % of these cases are extensively drug-resistant TB (XDR-TB), that is, TB caused by bacteria that are resistant to isoniazid and rifampicin as well as to any fluoroquinolone and any of the second-line injectable drugs.[2] The growing mobility among populations, the early abandonment of TB lengthy and demanding treatments, and the unfortunate synergy with HIV/AIDS have contributed to this disease's dissemination and, particularly, to the emergence of severe drug resistant strains to known anti-TB drugs.

The economic and social burden against TB is widely recognized as being very high. With the creation in 2000 of The Global Alliance for TB Drug Development (TB Alliance), a considerable number of donors and partners are now seriously engaged in accelerating the discovery and development of new and more effective, but also affordable and available, TB drugs.[3]

Isoniazid (INH), introduced more than 50 years ago, is still one of the most powerful first-line drugs in multitherapeutic regimens. However, an increasing number of *M.tb* INH-resistant strains have been reported in the literature. INH derivatives, or derivatives of other families of active compounds such as benzimidazoles, with an antitubercular activity comparable with that of INH and that would retain their activity against a panel of INH resistant strains, would be of invaluable importance as lead compounds for further development as new anti-TB agents to be used as second line drugs to fight MDR-TB.

Due to the large costs involved in the development of new drugs, a reliable quantitative prediction of biological activity prior to the production phase is obviously of great interest to the industry. One way of achieving this is through the use of quantitative structure−activity relationships (QSARs). Additionally, QSARs may also shed some light into the complex interaction mechanisms of active compounds against *M.tb*, whose knowledge is still rather limited. It is generally accepted that any pharmacological process occurs in three steps: penetration, binding, and activation.[4] Hence, for an ideal drug to be effective, it is critical that it holds adequate hydrophilic/hydrophobic properties to be able to penetrate the biological membrane, that it exhibits specific structural, geometrical, and/or physicochemical characteristics that may facilitate its binding to the biological target, and last that the resulting adduct produces a response that will cause an observable effect.

In the process of screening and preformulation of potential new drugs, including TB drugs, it is therefore of the utmost importance that knowledge of certain physicochemical proper-

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ties is obtained, such as their lipophilicity (as measured, for instance, by its log $P_{o/w}$ value) which determines their ability to penetrate a biological membrane, and their acid dissociation constant (as measured by its pK_a value) which affects passive transport across the membrane, where only one of the forms of the drug—either the neutral or the ionized form—is active in the living organism. Evaluating these two properties, among others, and assessing their effect upon the activity of known compounds by using judicious QSAR analyses, seems therefore very important given their potential influence over ADME (absorption, distribution, metabolism, excretion) behaviors and may thus provide extremely useful information to anticipate the feasibility of new compounds, with higher predicted activities, as drug-like candidates.

In this work we present log $P_{o/w}$ values determined both by potentiometry and by the conventional shake-flask method (UV−vis detection) and aqueous pK_a values obtained by potentiometry for seven isoniazid and nine benzimidazole derivatives. Some of these compounds were designed and synthesized on the basis of robust QSAR studies.[5−7] Several of these compounds show two or more ionizable sites, sometimes very acidic or very basic, and some others show strong hydrophilic or hydrophobic character. These facts have forced us to work near the limits of the potentiometric technique, and therefore, a critical evaluation of the results obtained in these extreme working conditions is presented.

EXPERIMENTAL SECTION

Apparatus. For pK_a and log $P_{o/w}$ measurements an automatic titrator PCA 101 from Sirius Instruments Ltd. (UK) equipped with a Sirius 010604 combined electrode has been used. The potentiometric system was standardized at 298.15 K according to literature specifications.[8]

For log $P_{o/w}$ measurements by the shake-flask method, compounds were weighed in a Mettler Toledo AX 205 analytical balance (precision ± 10^{-5} g), and their complete dissolution was attained with a Elma Transsonic 420 ultrasounds bath. Their partition was performed in an in-house built tumbler.

Absorbance measurements were taken on a Thermo Unicam Nicolet Evolution 300 spectrophotometer, at 298.15 ± 0.1 K, using quartz analytical cells with a 10 mm path length and the apparatus software. Temperature control in the cells compartment was achieved through a Thermomix B. Braun 18BU recirculating bath associated to a Frigomix cooling unit and was monitored by reading the resistance of a copper thermistor immersed in an absorption cell filled with ethanol and connected to a Philips PM 2522A multimeter (precision ± 10^{-3} kΩ). The resistance−temperature relationship was previously established through a calibration curve. The solutions' pH values before and after partition were read on a Metrohm 632 equipment (precision ± 10^{-2} pH units).

Chemicals. INH and 1*H*-benzimidazole-2-thiol were supplied by Sigma-Aldrich (Portugal) and used without further purification. The remaining compounds were prepared as described elsewhere.[6,7] Briefly, hydrazide derivatives were synthesized by the acylation of isoniazid with the appropriated acid chloride using a *N*-methylmorpholine as catalyst; hydrazones were prepared by a reaction between isoniazid and the suitable ketone; benzimidazole derivatives were obtained by the alkylation of benzimidazol-2-thiol with the desired alkyl or benzyl chloride. Flash column chromatography over silica-gel and/or recrystallization afforded pure compounds. The purity of all compounds was assessed by GC or high-performance liquid chromatography (HPLC) analysis and was in all cases ≥ 98 %. All compounds were fully characterized by ^{1}H and ^{13}C NMR, IR, and HRMS analysis.

Procedures. Hereafter, we consider $P_{o/w}$ the true partition coefficient, taken as a constant value at a given temperature, resulting from the ratio of concentrations of the same molecular species in both solvents, in dilute solutions. We take $D_{o/w}$ as the distribution coefficient, sometimes also called the apparent partition coefficient, measured under conditions in which the solute is partially or completely ionized in the aqueous phase.

pK_a and log $P_{o/w}$ Values Determined by Potentiometry. pK_a values were determined from a series of samples of 0.01 g dissolved in water or in methanol/water mixtures (methanol content between 20 % and 50 % in weight). The ionic strength was kept constant at the physiological value with KCl (I = 0.15 m). Obtained values were converted into the thermodynamic ones (I = 0) by means of the Debye−Hückel correction. At least three titrations were performed for each compound.

The log $P_{o/w}$ values were determined from, at least, three sequential titrations. When the sample was dissolved in octanol (saturated with 0.15 *m* KCl aqueous solution) or in 0.15 *m* KCl aqueous solution (saturated with octanol) the appropriate volume of water or octanol was respectively added to achieve the working volume ratio between the organic and aqueous phases ($r_{o/w}$). All titrations were performed in 0.15 *m* KCl solution, under nitrogen atmosphere at 298.15 ± 0.1 K using standard 0.51 *m* HCl and 0.50 *m* KOH solutions. The samples were preacidified and alkalimetrically titrated to the appropriately high pH.

log $P_{o/w}$ Values Determined by the Shake-Flask (SF) Method. *INH Derivatives.* For compounds A, C, and D the sample was dissolved in octanol-saturated aqueous buffer prepared with KH_2PO_4/NaOH (pH of about 5.8), and water-saturated octanol was added to get two different partition systems. The volume ratios, $r_{o/w}$, were usually 30:5 and 30:10, except for compound D for which they were 0.3:45 and 0.15:45 or 0.25:45 and 0.125:45. For compound B the sample was also dissolved in octanol-saturated aqueous buffer, but since this compound does not exist in its neutral form at the working pH, log $P_{o/w}$ was calculated from the logarithm of the distribution coefficient (log $D_{o/w}$) determined at several pH values.[9] Compounds E to G were not stable in water and were therefore dissolved in water-saturated octanol to which octanol-saturated aqueous buffer was added. For compound E the volume ratios between the two phases were 30:10 and 30:5 and for compound F 12:15 and 6:15. For compound G the volume ratios used were 8:20 and 4:20 or 8:30 and 4:30 leading to no significant changes in log $P_{o/w}$.

Benzimidazole Derivatives. The sample was dissolved in water-saturated octanol, and octanol-saturated aqueous buffer was added. A buffer of $NaHCO_3$/NaOH (pH of about 9.5) was used for all derivatives, except for compound P for which the buffer used was KH_2PO_4/NaOH (pH of about 7.9) and for compound Q, which, like B, does not exist as a neutral species at the working pH and had therefore its log $P_{o/w}$ calculated from log $D_{o/w}$ determined at several pH values.[9] The volume ratios between the organic and aqueous phases, $r_{o/w,}$ varied between 2:48 and 1:48 and 10:30 and 5:30 with no significant changes in log $P_{o/w}$. For compounds L, N, and O it was not possible to obtain the corresponding log $P_{o/w}$ values by the shake-flask method due to their very high lipophilicity.

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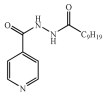
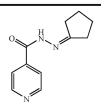
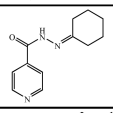
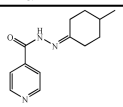
Table 1. INH Derivatives

Compound	Structure	M / g·mol^{-1}	Experimental			Calculated	
			pK_a (I=0)	Pontentiometric log $P_{o/w}$	Shake-Flask log $P_{o/w}$	SPARC pK_a	ChemDraw ClogP
isonicotinohydrazide (Isoniazid) **A**		137.14	pK_1= 3.53 ± 0.04 pK_2=11.14 ± 0.07	-0.65 ± 0.03	-0.85 ± 0.01 (N=4)[a]	pK_1= 4.09 pK_2=12.08	- 0.668
2-hydrazinoisonicotino-hydrazide **B**		167.17	pK_1= 2.06 ± 0.09 pK_2= 5.79 ± 0.02 pK_3=11.19 ± 0.12	-0.57 ± 0.02	-0.54 ± 0.05 (N = 10)	pK_1= 2.31 pK_2= 5.80 pK_3=10.88	-0.520
N′-acetylisonicotinoyl-hydrazine **C**		179.18	pK_1= 3.26 ± 0.01 pK_2= 8.65 ± 0.02	-0.87 ± 0.02	-0.91 ± 0.01 (N= 2)	pK_1= 3.25 pK_2= 8.37	-0.442
N′-decanoylisonicotinoyl-hydrazine **D**		291.39	pK_1= 3.24 ± 0.02 pK_2= 8.78 ± 0.01	3.66 ± 0.01	3.46 ± 0.11 (N= 4)	pK_1= 2.98 pK_2= 9.54	3.790
N′-cyclopentylidene-isonicotinohydrazide **E**		203.24	pK_1= 3.50 ± 0.03 pK_2=11.11 ± 0.03	-0.62 ± 0.01	0.22 ± 0.10 (N = 3)	pK_1= 4.64 pK_2=11.90	0.768
N′-cyclohexylidene-isonicotinohydrazide **F**		217.27	pK_1= 3.54 ± 0.03 pK_2=11.00 ± 0.03	0.27 ± 0.01	0.21 ± 0.04 (N=3)	pK_1= 4.64 pK_2=11.86	1.327
N′-(4-methylcyclohexylidene)-isonicotinohydrazide **G**		231.29	pK_1= 3.55 ± 0.04 pK_2=11.09 ± 0.05	0.63 ± 0.01	0.74 ± 0.01 (N=6)	pK_1= 4.64 pK_2=11.86	1.485

[a]*N* is the number of independent experiments.

For both compound series, the selected buffers ensure the presence of the neutral species only (except for compounds B and Q which are treated as referred in the literature[9]). All measurements were performed at 298.15 ± 0.1 K. Previously to any log $P_{o/w}$ determination, a spectrophotometer kinetic study of all compounds was carried out, at the same temperature, for at least 4 h in water, octanol, water-saturated octanol, and/or octanol-saturated water (depending on the compounds' solubility) to evaluate their stability in each of these solvents.

Before partition, and to guarantee total dissolution, solutions were sonicated for (10 to 25) min, depending on the compound, and only then partitioned in an in-house built tumbler until equilibrium was reached. Phase separation was facilitated by centrifugation during 3 min at 3000 rpm. Ionic strength was kept at 0.008 m for benzimidazole derivatives and 0.05 m for isoniazid derivatives, and the pH was measured before and after partition. Absorbance measurements were taken between (200 and 500) nm. When sampling was carried out in the aqueous phase, the relationships used for log $P_{o/w}$ calculations were the following (eq 1 can be shown to be equivalent to a similar expression in terms of concentrations):

$$P_{o/w} = \frac{A_i - A_f}{A_f} r_{w/o} \quad (1)$$

where

$$r_{w/o} = \frac{V_w}{V_o} \quad (2)$$

and A_i and A_f are the absorbance before and after partitioning, respectively, and $r_{w/o}$ is the volume ratio between aqueous and organic phases. When sampling was carried out in the organic phase, eq 1 was substituted by eq 3

$$P_{o/w} = \frac{A_f}{A_i - A_f} r_{w/o} \quad (3)$$

log $P_{o/w}$ values were determined, in average, from at least three independent experiments for isoniazid derivatives and from at least four experiments for benzimidazole derivatives.

RESULTS AND DISCUSSION

The studied compounds as well as their experimental acidity and hydrophobicity values are gathered in Tables 1 and 2. pK_a values and working pH values for log $P_{o/w}$ measurements were estimated through SPARC software.[10] Estimated log $P_{o/w}$ values were determined by means of ClogP through ChemDraw[11] which uses the algorithm developed by the Medicinal Chemistry Project and BioByte[12] on the basis of fragment-based methods.

Acidity Constants. INH and its studied derivatives (compounds A to G) show at least two pK_a values.[10] With the exception of compound B, the first pK_a is basic and is associated to the protonated heterocyclic nitrogen, and the second one is acidic and corresponds to the loss of a proton from the amidic nitrogen. Thus, the general deprotonation scheme involves cationic, neutral, and monoanionic forms for each compound. As expected from their chemical structure, all of them show very similar pK_a values, pK_{a1} around 3.4 and pK_{a2} about 11 except for compounds C and D, which show two amidic nitrogen atoms and consequently, their pK_{a2} values are more acidic and lower, around 8.7. Compound B is the only

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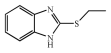
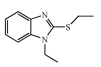
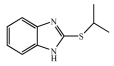
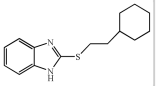
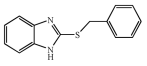
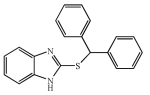
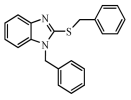
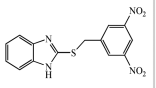
Table 2. Benzimidazole Derivatives

Compound		M / g·mol^{-1}	Experimental			Calculated	
			pK_a (I=0)	Pontentiometric log $P_{o/w}$	Shake-Flask log $P_{o/w}$	SPARC pK_a	ChemDraw ClogP
2-(ethylthio)-1*H*-benzimidazole **H**		178.25	pK_1= 4.51 ± 0.02 pK_2= 11.00 ± 0.06	2.67 ± 0.01	2.67 ± 0.03 (N=4)[b]	pK_1= 5.61 pK_2= 13.34	3.233
1-ethyl-2-(ethylthio)-1*H*-benzimidazole **I**		206.31	pK= 4.68 ± 0.05	3.36 ± 0.01	3.43 ± 0.14 (N=4)	pK= 5.52	3.784
2-(isopropylthio)-1*H*-benzimidazole **J**		192.28	pK= 4.67 ± 0.02	3.23 ± 0.01	3.25 ± 0.10 (N=4)	pK_1= 5.62 pK_2= 13.34	3.542
2-[(2-cyclohexylethyl)thio]-1*H*-benzimidazole **L**		260.4	pK= 4.56 ± 0.02	4.94 ± 0.02	n.d.	pK_1= 5.61 pK_2= 13.33	5.883
2-(benzylthio)-1*H*-benzimidazole **M**		240.32	pK= 4.28 ± 0.03	3.92 ± 0.03	4.07 ± 0.12 (N=6)	pK_1= 5.48 pK_2= 13.22	4.272
2-[(diphenylmethyl)thio]-1*H*-benzimidazole **N**		316.42	pK= 4.20 ± 0.04	4.90 ± 0.29	n.d.	pK_1= 5.31 pK_2= 13.07	5.620
1-benzyl-2-(benzylthio)-1*H*-benzimidazole **O**		330.45	pK= 3.32 ± 0.07	n.d.[a]	n.d.	pK= 5.09	6.062
2-[(3,5-dinitrobenzyl)thio]-1*H*-benzimidazole **P**		330.32	pK= 3.13 ± 0.03	2.56 ± 0.04	3.14 ± 0.10 (N=6)	pK_1= 4.50 pK_2= 12.43	3.758
1*H*-benzimidazole-2-thiol **Q**		150.2	pK= 7.26 ± 0.03	2.66 ± 0.01	2.65 ± 0.01 (N=8)	pK= 8.65	2.408

[a]n.d. is not determined. [b]*N* is the number of independent experiments.

member of the series with a hydrazine substituent in the 2-position in the pyridine moiety. This fact allows the stabilization of the cationic form by means of the "amidinium type" electron delocalization,[13] making this heterocyclic nitrogen a much stronger base than their unsubstituted analogues (pK_{a2} = 5.79). In addition, the primary amine of the amidic substituent becomes more basic (pK_{a1} = 2.06) than that of INH and, thus, can be determined potentiometrically. As expected, the acidic pK_{a3} of compound B agrees with pK_{a2} values of the other isoniazid derivatives. Thus, the complete deprotonation of compound B involves the dicationic, monocationic, neutral, and monoanionic main forms. Regarding the potentiometric procedure, it should be noticed that the high aqueous solubility of isoniazid compounds allowed the pK_a determination in aqueous solution except for the most hydrophobic one, compound D. In this instance pK_a values were determined in several methanol−water mixtures and the aqueous pK_a value estimated by extrapolation by means of the Yasuda−Shedlovsky approach as described elsewhere.[8,14,15] In all cases, thermodynamic pK_{a2} values (pK_{a3} and pK_{a1} for compound B) have been calculated through the Debye−Hückel equation despite the fact that experimental values were obtained at high ionic strength, 0.15 *m*, that is, beyond the recommended ionic strength range.[16] No ionic strength corrections are required for pK_{a1} (pK_{a2} for compound B). The obtained results show a high precision, even for the most acidic value determined.

The studied benzimidazole derivatives (compounds H to P) show a pK_a value between 3.1 and 4.7 attributable to a protonated heterocyclic nitrogen.[10] It should be pointed out that pK_a values of compounds O and P are about one unit lower than those of the other benzimidazole analogues. Compound O differs from compound M in a benzyl substituent, which is able to delocalize the electronic distribution and, hence, to stabilize the basic form increasing the acidic strength. This means that, despite the presence of a sp^3 hybridized carbon between the benzene ring and the heterocyclic nitrogen, a significant delocalization effect involving the free electron pair of the nitrogen atom occurs. A similar effect applies to compound P which differs from compound M in two nitro groups in the benzyl substituent of the sulfur atom. The known acid-strengthening character of nitro substituents is also transmitted through the free electron pairs of sulfur atom despite the intermediate sp^3 carbon, increasing in that way the acidity of the compound. Similar electronic effects were already described to explain the acidity

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of several arylacetic acids with anti-inflammatory properties.[17] The studied benzimidazole derivatives show a much higher hydrophobic character than the isoniazid compounds, and therefore, potentiometric pK_a determinations were performed in methanol−water mixtures and the aqueous values calculated by extrapolation as mentioned before.

The synthetic precursor for benzimidazole derivatives, compound Q, shows two tautomeric forms, thiol and thione.[18] Surprisingly, the pK_a value obtained in this work, 7.26, differs significantly from the value recently published, 5.8,[19] but it is consistent with that of 2-mercaptopyrimidine, 6.98, which shows a very similar acidic group.[20] On the other hand, Boraei et al. have published very high values, around 10.3, obtained in aqueous solutions which contain 0.1 mole fraction of organic solvent (methanol, ethanol, dimethyl sulfoxide, or dimethylformamide).[21] In all instances, only one pK_a value is attributed to this compound.

Tables 1 and 2 also include pK_a values estimated by the SPARC calculator. It should be remarked that estimated pK_{a1} for the isoniazid analogues (pK_{a2} for compound B) agree or are higher than the experimental ones, whereas calculated pK_{a2} values (pK_{a3} for compound B) match better the potentiometric values. The experimental pK_a values for benzimidazole derivatives are about one unit lower than those predicted by the SPARC software except for compounds O, P, and Q which show larger differences. Calculated values are very similar for compounds H to L, slightly lower for compounds M and N, and the lowest values are obtained for O and P derivatives, following the same trend as that observed for the experimental values. Estimated pK_{a2} values are higher than 12.4, that is to say, a working range not suitable for accurate potentiometric pK_a determination. In fact, no potentiometric jump associated to this acidity constant can be observed in the titration curves. In summary, the SPARC calculator is a useful tool to estimate relative acidities for isoniazid and benzimidazole derivatives, but calculated absolute values differ, in general, by about 0.3 to 1.1 pK_a units from the experimental ones, except for compounds O, P, and Q which show larger differences from 1.4 to 1.8.

Hydrophobicity Values. INH (compound A) and the derivative compounds B and C are low molecular weight water-soluble polar compounds and hence show a hydrophilic character. The lipophilicity increases for compounds E, F, and G with the addition of hydrophobic aliphatic hydrocarbon groups. Compound D was the only isoniazid derivative showing significant lipophilicity (log $P_{o/w}$ = 3.66) which is easily explained by the addition of the long-chain hydrocarbon group. Also, compound D was just slightly soluble in water and required pK_a measurements in methanol−water solutions.

On the other hand, all benzimidazole derivatives showed moderate to high log $P_{o/w}$ values with some of them being very water-insoluble. The addition of aliphatic hydrocarbon groups to the parent compound (compound H, log $P_{o/w}$ = 2.7) increased the lipophilicity proportionally with the number of additional $-CH_2-$ or $-CH_3$ groups (compounds I, J and L). Addition of aromatic groups to the parent compound (compounds M and N) also increased the overall lipophilicity although the effect of aromatic $=CH-$ groups is not as large as that of aliphatic $-CH_2-$ groups. Perhaps the most surprising result is that of compound P with the addition of two nitro ($-NO_2$) groups to the aromatic ring. There are examples in the literature of substituted benzoic acids and phenols where aromatic nitro groups, although containing polar atoms, generally do not dramatically decrease the overall lipophilicity of compounds due to strong delocalization of the electron density. Nevertheless, both the shake-flask and the potentiometric data show a large reduction in log $P_{o/w}$ for compound P when compared to compound M, disclosing a value of the same order of magnitude as that of compounds H and Q. It should be remarked the consistency between values obtained by both shake-flask and potentiometric methods with the exception of the most hydrolyzable compounds (A and E to G) and compound P.

Tables 1 and 2 also include ClogP values estimated by ChemDraw for both series of compounds. A comparison between experimental and theoretical log $P_{o/w}$ values is a usual procedure to check the presence or absence of intramolecular interactions.[22,23]

The comparison between experimental and estimated ClogP values for isoniazid derivatives (Figure 1) shows that in the case

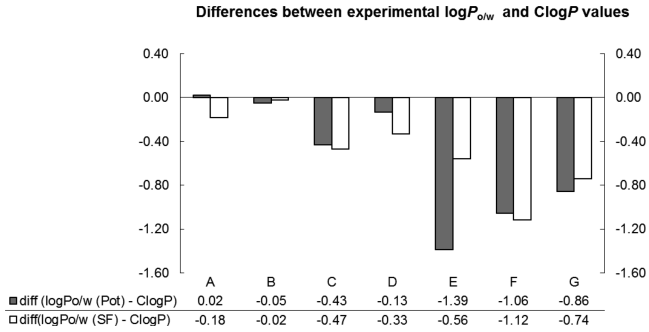

Figure 1. Comparison between experimental (gray, potentiometric values; white, SF values) and estimated (ClogP) log $P_{o/w}$ values for isoniazid derivatives.

of hydrazides and hydrazines (compounds A to D) the difference is more pronounced for compound C, followed by compound D and almost negligible for compounds A and B. Since for compounds C and D the difference (log $P_{o/w(exp)}$ − ClogP) is significant (experimental values are more hydrophilic than estimated values), this suggests the presence of intramolecular effects, perhaps due to the prevalence of the enol form in the diketo−enol tautomeric equilibria for these compounds. This hypothesis is supported by the fact that for both compounds the "enol" form is in fact more hydrophilic than the diketo form and its predicted ClogP is closer to the experimental value (compound C: ClogP (enol) = −0.604 and ClogP (keto) = −0.442; compound D: ClogP (enol) = 3.628 and ClogP (keto) = 3.790).

In the case of hydrazones (compounds E to G) the difference between log $P_{o/w(exp)}$ and ClogP is much more prominent than for hydrazides and hydrazines; that is, experimental values are systematically much more hydrophilic than those predicted by ClogP. This discrepancy might be explained by the fact that hydrazones hydrolyze very easily, giving rise to more hydrophilic products (Figure 2). For benzimidazole derivatives, the same type of comparison was performed. Figure 3 shows that these compounds, with the exception of 1*H*-benzimidazole-2-thiol (Q), are again more hydrophilic than expected on the basis of ClogP estimated values. This difference is particularly evident for compound P. As noticed earlier, compound P differs from M in the two nitro groups at the aromatic ring. The increased hydrophilicity of P when compared with M is reflected in a $\Delta(ClogP_{M-P})$ = 0.514. However, the difference between experimental and calculated

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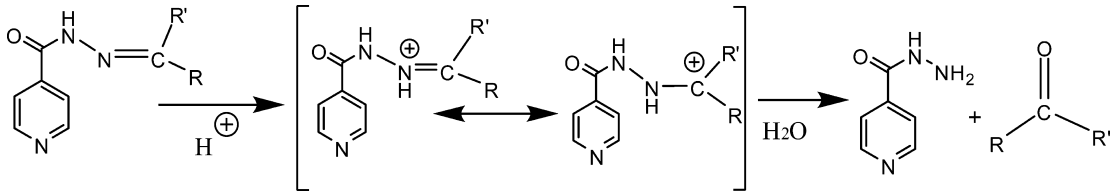

Figure 2. Scheme for the hydrolysis of hydrazones E to G.

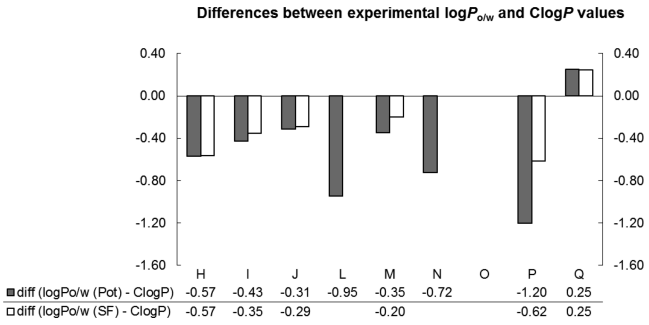

Figure 3. Comparison between experimental (gray, potentiometric values; white, SF values) and estimated (ClogP) log $P_{o/w}$ values for benzimidazole derivatives.

values doubles this amount. This might be due to a higher exposure of the two nitro groups, thus facilitating interactions with water (better water accessibility) and leading therefore to a decrease in lipophilicity. This rationale is in line with the strong increase in polarity resulting from the introduction of the nitro groups (μ(M) = 1.316 D, μ(P) = 33.3 D[24]).

Some Practical Considerations on Shake-Flask log $P_{o/w}$ Determination. In the shake-flask protocol the log $P_{o/w}$ determination was performed using UV−vis spectroscopy either on the aqueous or the organic phase, after separation of the octanol and water phases. There were two strong reasons to choose in some cases the organic phase for sampling purposes: (i) some compounds (e.g., compounds E to G) suffered hydrolysis and, therefore, could not be dissolved or sampled in water; (ii) very lipophilic compounds, such as benzimidazole derivatives, were quite insoluble in water, making it easier and more reliable to sample in the organic phase.

It is instructive to look at the chosen octanol−water ratios and the dynamic range of UV−vis spectroscopy to understand the limitations of the shake-flask protocol. A compound with log $P_{o/w}$ = 0 would be equally distributed between the octanol and the water phases. However, if an octanol/water ratio, for instance, of 30:5 was chosen, then six times as much compound would reside in the octanol layer compared to the aqueous layer. Thus the UV−vis absorbance of the compound in the aqueous layer would diminish 6-fold when compared to a solution prior to the addition of octanol. If an appropriate sample concentration was used such that the initial UV−vis absorbance in water was, for example, 1, then the UV−vis absorbance after octanol addition would be 0.2. This is easily within the range of most UV−vis spectrometers. However, for log $P_{o/w}$ = 1 at a ratio of 30:5 would lead to 60 times as much compound in the octanol phase compared to the aqueous phase. The UV−vis absorbance in the aqueous compartment would reduce to ∼0.02. This is now approaching the lower end of the UV−vis scale and higher log $P_{o/w}$ values would be difficult without adjusting the phase ratios.

Turning to the specific examples of the isoniazid derivatives shows that all of the log $P_{o/w}$ values (except compound D) were between −1 and +1. Hence, octanol/water ratios of 30:5 and 30:10 would provide UV−vis absorbance values within a suitably easy-to-measure range for the aqueous compartments.

For the benzimidazole derivatives let us assume that the octanol/water phase ratios were chosen, for instance, as 5:30 and 10:30. If log $P_{o/w}$ = 3, then 1000 times as much compound would reside in the octanol layer for equal volumes of octanol and water, and this would reduce by a factor of 6 to 167 times as much in the octanol for a phase ratio of 5:30. Using our chosen example of a compound with absorbance of 1 before octanol is added would then reduce to an absorbance of ∼0.006 in the aqueous layer after partitioning, again approaching the lower end of the UV−vis sensitivity. Thus anything that is much more lipophilic becomes increasingly difficult to measure. Indeed we see that compounds L, N. and O were too lipophilic and above the measurable range of the shake-flask methodology employed here.

Some Practical Considerations on Potentiometric log $P_{o/w}$ Determination. The potentiometric log $P_{o/w}$ methodology involves measuring the shift in the pK_a when a titration is performed in a two phase octanol−water system.[25,26] The apparent ionization constant compared to the aqueous ionization constant depends on the extent of partitioning of each species present into the octanol phase, and the following expression has been derived to show the relationship between the partition coefficients and ionization constants:

$$|{}^{p_o}K_a - pK_a| = \log((1 + rP_n)/(1 + rP_i)) \quad (4)$$

where pK_a is the ionization constant measured under aqueous conditions, P_n is the partition coefficient of the neutral species of the compound, P_i is the partition coefficient of the ionized form of the compound, r is the ratio of the volumes of octanol and water (i.e., $r = V_o/V_w$ where V_o = octanol volume and V_w = water volume) and the ${}^{p_o}K_a$ is the apparent ionization constant measured at that octanol/water volume ratio. The $|{}^{p_o}K_a - pK_a|$ indicates the absolute magnitude of the difference between the apparent ionization constant and aqueous pK_a since acids are shifted up to higher values and bases are shifted to lower values.

A plot of $|{}^{p_o}K_a - pK_a|$ vs log r (Figure 4) has a characteristic shape[27] provided that the difference P_n and P_i is sufficiently large. This happens to be the case for many monoprotic weak acids and bases where partitioning of the neutral compound in octanol is typically 3 to 4 orders of magnitude greater than partitioning of the ionized species.[28] It is instructive to apply shape analysis to such a curve to reveal the regions of interest. Figure 4 assumes a hypothetical weak acid with P_n = 10 000 (log P_n = 4) and a value of P_i = 10 (log P_i = 1). Also shown is the practical experimental range for the lowest octanol/water ratio and the highest octanol/water ratio that can typically be achieved during analysis. It is very difficult to experimentally achieve octanol/water ratios less than 0.01 mL octanol/20 mL

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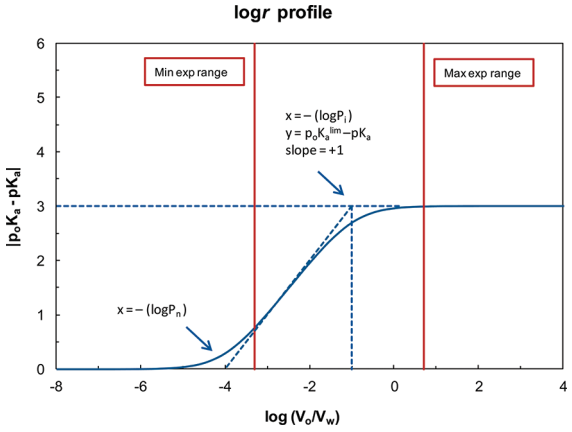

Figure 4. Theoretical log *r* profile.

water with any great precision, and hence log*r* ratios less than −3.3 cannot be attained. At the upper end it is not practical to use octanol/water ratios greater than about 20 mL octanol/4 mL water giving a log *r* value of 0.7. The region of the curve where $p_oK_a - pK_a$ is equal to zero occurs when the ratio of octanol to water is so low that the drug is only effective in the aqueous phase; that is, the presence of octanol causes no apparent shift in the aqueous pK_a. Such conditions are not experimentally attainable for the lipophilic compound used as an example here. As the octanol/water ratio is increased, the neutral species of the compound starts to partition, and this causes a shift in the observed apparent ionization (p_oK_a) which starts to change with a slope of 1; that is, for each logarithmic change in octanol/water ratio there is a one unit change in p_oK_a value. Eventually, as the octanol/water ratio is increased further then the ionized species also starts to partition into the octanol, and this is represented by the upper plateau in the log *r* plot. The plateau region occurs at high octanol/water ratios and is reached when the entire compound (both neutral and charged forms) is effectively in the octanol. The maximum shift is sometimes referred to as the limiting p_oK_a ($p_oK_a^{LIM}$) and represents the pK_a in pure octanol.[28] Two further features of interest are the intercepts between the lines of zero slope and the line that can be drawn for the intermediate region with a slope of unity.[27] At $p_oK_a - pK_a = 0$ the unit slope intercepts at a value equal to the negative logarithm of P_n; that is, in Figure 4 the compound has P_n = 10000 (log P_n = 4) and the curve intercepts the *x*-axis at −log P_n (= −4). Note that this log *r* value is smaller than the lowest ratio of octanol/water that can be used. The higher plateau is attained only when the limiting $p_oK_a^{LIM}$ is reached and the charged and neutral species are effectively only in the octanol under the experimental conditions. Hence, this depends on the value of P_i. The lower the value of P_i the greater the volume ratio of octanol/water required for the ion (as well as the neutral compound) to fully partition. The intercept of this plateau occurs with the unit slope at a value equal to the negative logarithm of P_i. In our example in Figure 4 where P_i = 10 (log P_i = 1) then the intercept on the *x*-axis occurs at value −1 (−log P_i). The relationship $\log P_n - \log P_i = p_oK_a^{LIM} - pK_a$ is also obvious and for the curve is 4 − (1) = 3. Thus, if the aqueous pK_a = 5 then for an acidic drug the octanol $p_oK_a^{LIM}$ = 8 (or for a basic drug the octanol $p_oK_a^{LIM}$ = 2), and the maximum shift during titration[28] is attained at octanol/water log *r* ratios > −1. Note that this log *r* value is easily within the experimental ratios of octanol/water that can be used (max log *r* = 0.7).

For compounds with low to moderate lipophilicity then partitioning of the ionized species may be outside the limit of measurement, and then the equation can be simplified and the magnitude of the shift in pK_a can be related to log $P_{o/w}$ by the following expression:

$$|p_oK_a - pK_a| = \log(1 + rP_n) \quad (5)$$

It is now instructive to look at some examples of measurements on the isoniazid and benzimidazole derivatives. This is first illustrated with respect to compound B with measured potentiometric log P_n = −0.57 (see Figure 5). At the lowest *r*

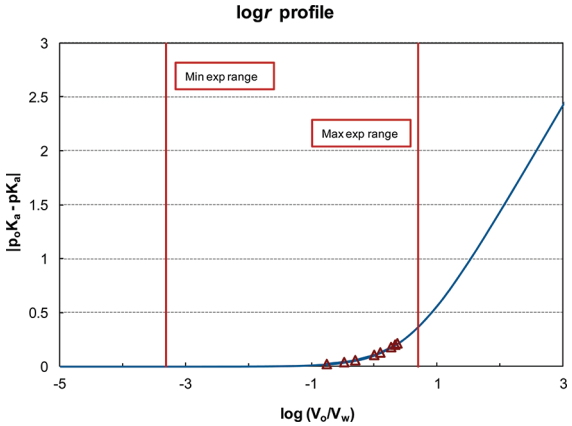

Figure 5. log *r* profile for compound B (log P_n = −0.57) with *r* between 0.15 and 2.0.

ratio of 0.15 (1.5 mL octanol/10 mL water) there is hardly a measurable shift in the apparent p_oK_a (5.77 versus the aqueous pK_a value of 5.79) and high-quality data is required to be certain of the result. At the highest used ratio of 2 (15 mL octanol/7.5 mL water) the shift is much more easily measured (p_oK_a = 5.60), and the result can be considered more reliable. Several additional titrations at different *r* ratios should also be measured to confirm the result. At a practical level, however, there is only so much octanol that can be added before the vessel would overflow (maximum vial contents = 25 mL).

When much more lipophilic compounds are studied, it is then necessary to also consider the partitioning of ionized species[29] (as ion-pairs) into the octanol layer as described in eq 4. Under physiological conditions of ionic strength (0.15 *m*) partitioning of monoprotonated species has often been shown to be around 3 orders of magnitude lower than for neutral species for many classes of compound (e.g., if log P_n = ∼3 then log P_i = ∼0). It is then possible that under certain experimental conditions that all forms of the compound (both neutral and charged species) will reside entirely in the octanol layer. The effect on how the p_oK_a values change in octanol is illustrated for the lipophilic compound L (log P_n = 4.94 and log P_i = 1.95) in Figure 6. At the lowest *r* ratio of 0.003 (0.05 mL octanol/15 mL water) there is a large shift in the apparent p_oK_a (to 2.21 versus the aqueous pK_a value of 4.56). At the highest used ratio of 0.3 (3 mL octanol/10 mL water) the shift is even larger (p_oK_a = 1.59), but virtually the entire compound resides in the octanol layer (100 % of the neutral species and 97 % of the ionized form). Consequently, the p_oK_a is almost the same as if the measurement were made in pure octanol, and performing titrations in higher volume ratios of octanol/water would not produce any further shift in the p_oK_a value. This result is the

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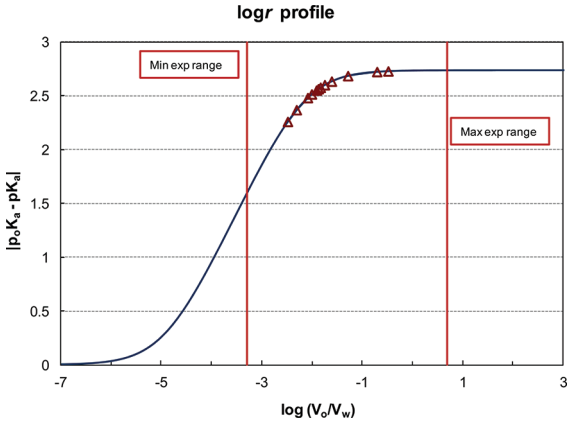

Figure 6. log *r* profile for compound L (log P_n = 4.94) with *r* between 0.003 and 0.3.

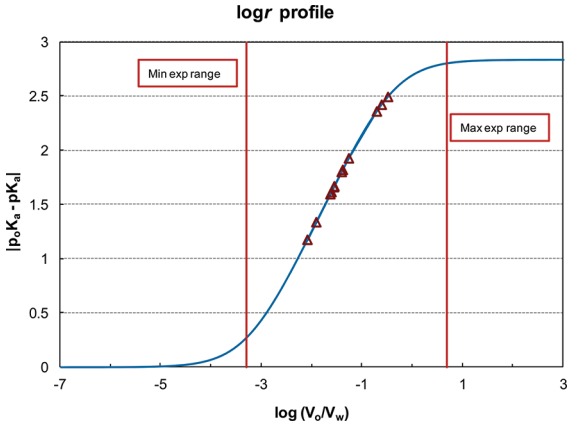

Figure 7. log *r* profile for compound J (log P_n = 3.23) with *r* between 0.01 and 0.3.

limiting $p_oK_a^{LIM}$ value and is the maximum shift that it is possible to attain. Also, if the entire compound resides in the octanol layer, this does not tell us anything about the levels of partitioning of each of the species. We must perform an experiment where there is differentiation between the amounts of each partitioned species. This was achieved with the experiment at 0.05 mL octanol/15 mL water where over 99 % of the neutral species partitions into octanol but only 25 % of the ionized form partitions into the octanol. Thus by performing experiments at different ratios of octanol and water it is possible to obtain enough differentiation between partitioning levels of each species such that it is possible to resolve and determine log $P_{o/w}$ for both the neutral and ionized species (log P_i = 1.95 for compound L). Note, that very quickly for a lipophilic compound, as octanol ratios are increased, the proportions of both species in the octanol increases, for example, for 0.5 mL octanol/10 mL water over 99.9 % of neutral species and over 80 % of ionized species is in the octanol layer. At a practical level, it is difficult to achieve octanol/water ratios below 0.01 mL octanol/20 mL water while maintaining two separate phases and avoiding solubility issues. The overall solubility of the compound often becomes a problem for such hydrophobic compounds as the sample must be soluble in the two-phase system. Nevertheless, such visualization tools as plotting the shifts in p_oK_a versus the octanol/water ratios, as shown in the figures, can easily help determine the experimental limits of the method and the overall confidence in the results.

As a final example, we will review the data on compound J (log P_n = 3.23 and log P_i = 0.40).This compound is ideally suited to potentiometric log $P_{o/w}$ measurements having good solubility in the two-phase system and easily selectable octanol/water ratios to obtain good differentiation in p_oK_a values between experiments. At the lowest used ratio of 0.01 (0.1 mL octanol/10 mL water) there is a large shift from the aqueous pK_a (4.67) of 1.2 units (p_oK_a = 3.43). At the highest used ratio of 0.3 (3 mL octanol/10 mL water) over 99 % of the neutral species is in the octanol, and there is a much larger shift of 2.5 units to p_oK_a = 2.2. Also, under these conditions the partitioning of the ionized form is evident, and log P_i can be determined from the data. Under these conditions around 40 % of the protonated base resides in the octanol, thereby making it possible to determine log P_i by comparing the shifts at different octanol/water ratios as shown in Figure 7.

Other points to consider: when p_oK_a are shifted to the extremes of pH, such as p_oK_a < 3 for the bases in this study, then adequately high sample concentrations must be used to overcome the buffering action of water and to provide a good signal-to-noise for p_oK_a measurement. The overall solubility of the compound in the two-phase system must also be considered to avoid precipitation during the titration.

CONCLUSIONS

This work presents and discusses the ionization and hydrophobicity of several isoniazid and benzimidazole derivatives with potential antitubercular activity and some molecular factors that influence each property. The shake-flask log $P_{o/w}$ methodology and the practical issues involved in such measurements are thoroughly discussed. Of particular importance is the volume phase ratios employed to perform the extraction, as this will dictate the relative amounts of the compound found in each layer. The use of UV−vis spectroscopy as the quantitation technique will only provide reasonable results when an adequate signal is found in the phase chosen for the analysis. In the particular cases of compounds L, N, and O the compounds were very lipophilic, and under the octanol/water ratios that were used most of the compound disappeared from the aqueous compartment, leaving a UV−vis signal too low to measure in this phase. However, even if the sampling was carried out in the organic phase (which was in fact the case for these three compounds, for solubility reasons) the difference in absorbance before and after partition was so meager that it turned it impossible to determine a $P_{o/w}$ value. Hence, these compounds were above the measurement limit for the operational conditions of the shake-flask method. Nevertheless, except for the most hydrolyzable compounds (isoniazid, A, and the hydrazones E to G) and the dinitro derivative of benzimidazole, compound P, log $P_{o/w}$ values obtained by both methods were very similar which makes the shake-flask method still an alternative in nonextreme conditions.

In the measurement of potentiometric log $P_{o/w}$ values the use of log *r* plots provides a useful indication of appropriate experimental conditions that should be employed to obtain reasonable results for a compound with given neutral and ionized log $P_{o/w}$ values. In other words, to determine the neutral log P_n, *r* ratios should be used that generate a measurable shift between the apparent p_oK_a and aqueous pK_a values, ideally in a region where the slope is changing with a

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value of 1. On the other hand, to determine the ionized log P_i, r ratios should be used that produce the largest shifts between the apparent p_oK_a and aqueous pK_a values, ideally in a region where the upper plateau and $p_oK_a^{LIM}$ is reached. In the particular case of the lipophilic compound O, which also had a low pK_a value, the octanol caused the p_oK_a value to shift outside the measurable range ($p_oK_a < 1.5$). The use of prediction tools can be complementary to measurement as it allows the analyst to assess the experimental conditions that should be used to perform the measurement before embarking upon the work.

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