Pergamon

Bioorganic & Medicinal Chemistry 8 (2000) 909–916

BIOORGANIC & MEDICINAL CHEMISTRY

Ionization Behaviour and Tautomerism-Dependent Lipophilicity of Pyridine-2(1*H*)-one Cardiotonic Agents

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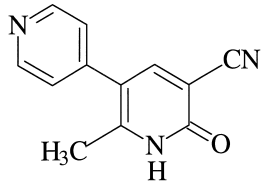
Received 7 October 1999; accepted 24 November 1999

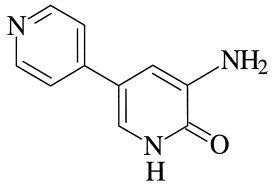
Abstract—The acid–base properties of pyridine-2(1*H*)-one derivatives, analogues of the cardiotonic agent milrinone, were studied by capillary zone electrophoresis (CZE). Electrophoretic mobility and pH data were fitted to equilibrium expressions and apparent dissociation constants (pK_a) calculated by non-linear regression. Compared with the ultraviolet (UV) spectrophotometric method and potentiometric titrations, the CZE technique showed advantages, such as rapidity and applicability to compounds that are sparingly soluble in water. Based on the pK_a values, intramolecular electronic interactions were assessed. The lipophilicity of a number of derivatives was also examined, by determining their *n*-octanol/water distribution coefficients over a wide pH range, and found to be significantly affected by 2-pyridone/2-hydroxypyridine tautomerism. As revealed by a comparison between experimental and calculated log P values, electron withdrawing substituents, especially at the C(6) position of 2-pyridone, favour the less polar hydroxypyridine tautomers both in water and octanol. Our results indicate that the positive inotropism of milrinone-related compounds could be explained taking ionization and tautomerism into account.

Introduction

Milrinone and amrinone are members of a relatively new class of bipyridine inotropic/vasodilator agents indicated for the short-term intravenous therapy of congestive heart failure.[1] Their mode of action is different from either the digitalis glycosides or catecholamines. At inotropic and vasorelaxant concentrations, they are inhibitors of PDE III isozyme (cyclic GMP-inhibited phosphodiesterase, cGMP–PDE) in cardiac and vascular muscle.[2]



Milrinone



Amrinone

The favourable therapeutic properties of these cardiotonic agents have stimulated synthesis of new analogues, structure–activity relationship (SAR) and molecular modelling studies.[3–8] In a continuous effort to characterize and better understand the mechanism of action of milrinone-related compounds, and to derive property-based SAR models, helpful in a rational design of more potent inotropic agents, we undertook a study aimed at determining their key parameters, such as dissociation constants and octanol/water partition coefficients, and to examine how the physicochemical properties resulting from their structure affect their pharmacodynamic behaviour. Thus, we selected a number of substituted pyridine-2(1*H*)-ones (from now on called 2-pyridones; structures in Table 1), among the numerous derivatives already synthesized by some of us,[9–16] and measured their acidity and lipophilicity, which both reflect tautomerism between 2-pyridone and 2-hydroxypyridine forms. Commercial compounds **1**–**3** and **18** were also included.

In this paper we report on the measurement of the apparent ionization constants (pK_a) by capillary zone

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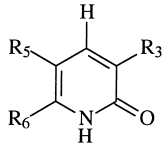
PII: S0968-0896(00)00025-0

Table 1. Apparent pK_a values of pyrid-2-one derivatives



Compound	Ref	R_3	R_5	R_6	pK_a (SEM) CZE	UV	pH metric	Σσ
1		H	H	H		11.62 (lit. value)		0
2		H	H	Cl	7.51 (0.04)	7.51 (0.08)	7.51 (0.04)	0.79
3		H	H	CH_3		[a]		−0.13
4	10	CN	H	CH_3	9.61 (0.06)	9.09 (0.29)	[e]	0.43
5	9	CN	$COCH_3$	CH_3	7.75 (0.07)	7.51 (0.11)	7.66 (0.03)	0.81
6	9	COOH	$COCH_3$	CH_3	4.55 (0.04)	4.68 (0.05)	5.19 (0.19)	
					11.19 (0.11)	10.87 (0.10)	[e]	0.15
7	10	CN	COOH	CH_3	3.49 (0.07)	3.51 (0.09)	4.30 (0.19)	
					9.30 (0.11)	8.97 (0.31)	[e]	0.15
8	9	H	$COCH_3$	CH_3	11.13 (0.04)	10.58 (0.05)	[e]	0.25
9	13	$COOCH_3$	$COCH_3$	CH_3	9.77 (0.07)	9.31 (0.07)	[e]	0.62
10	12	CN	H	4-Py	3.19 (0.12)	3.41 (0.24)	[e]	
					6.95 (0.04)	[b]	[e]	1.36
11	12	CN	H	3-Py	3.12 (0.18)	3.27 (0.22)	[e]	
					7.22 (0.04)	7.16 (0.05)	[e]	1.10
12	12	CN	H	2-Py	[c]	[b]	[e]	
					7.70 (0.07)	7.51 (0.10)	[e]	1.08
13	11	CN	$COOC_2H_5$	CF_3	3.27 (0.31)	2.86 (0.22)	3.92 (0.04)	1.99
14	14	CN	$COOCH_2CH(CH_3)_2$	CH_3	7.85 (0.06)	7.75 (0.13)	[e]	0.80
15	15	$COCH_3$	H	Ph	10.34 (0.05)	[b]	[e]	0.44
16	16	SO_2Ph	$-(CH_2)_4-$		10.57 (0.05)	[b]	[e]	0.42
17	16	COPh	$-(CH_2)_4-$		11.35 (0.14)	11.45 (0.20)	[e]	0.21
18		CN	4-Py	CH_3	5.10 (0.10)	4.5[d]	[e]	
(Milrinone)					9.30 (0.15)	8.5[d]	[e]	0.70

[a] pK_a value obtained from lipophilicity measurements at different pH: 12.30 (0.11).
[b] Not measurable because of low solubility and/or lack of significant difference between UV spectra of the molecular and ionized species.
[c] pK_a < 2.0.
[d] Data from Sanofi–Winthrop (Collegeville, PA).
[e] Not measurable by tritimetry because of both low acidity strength and solubility ($K_a \times c_a < 10^{-9}$).

electrophoresis (CZE). Recently, the CZE technique has been used to determine pK_a values of relevant classes of drugs,[17–19] and distinct advantages (e.g., need of small sample amounts, high solute purity not required) over the more common UV spectroscopy and potentiometry have been demonstrated. In our study, pK_a values determined by CZE were compared with those obtained with UV and potentiometric methods, and quantitative information was obtained on the substituent effects on 2-pyridone nucleus. The octanol/water partitioning behaviour of some derivatives over a wide pH range was also investigated, the final objective being the examination of the effects of tautomerism upon lipophilicity[20] and inotropic activity.

Results and Discussion

CZE determinations of pK_a

The selected compounds (structures in Table 1), all characterized by the 2-pyridone nucleus, include derivatives where the pyridyl moiety at C(5) of the reference compound milrinone (**18**) is substituted with an acetyl (**5**, **6**, **8**, **9**), a carboxylic (**7**) or an ester group (**13**, **14**), or is even absent (**1**–**4**, **10**–**12**, **15**); the cyano group at the C(3) position is absent (**1**–**3**, **8**) or substituted with a carboxylic (**6**), a carbomethoxy (**9**), an acetyl (**15**), a phenylsulphonyl (**16**) or a benzoyl (**17**) group. Derivatives bearing a chlorine (**2**), a pyridyl (**10**–**12**), a trifluoromethyl (**13**) and a phenyl (**15**) substituent in place of the methyl group at the C(6) position were also included in the data set. The apparent (non-thermodynamic) pK_a values of milrinone (**18**), 14 2-pyridone derivatives substituted at the positions 3, 5 and 6 (**4**–**17**), and three commercial products (**1**–**3**) were determined by capillary zone electrophoresis (CZE). The effective mobilities were calculated from the measured apparent mobilities at different pH values (employed buffers in Table 2), by using eq (4) (see Experimental). Mesityl oxide was used as the electro-osmotic flow (EOF) marker substance, since in a preliminary test it gave the best results (high absorbance and symmetrical peaks) compared with acetone, benzene and dimethyl sulphoxide.

In Table 3 μ_{EOF} values at various pH of samples containing the marker (1% m/v) are reported. The reproducibility of the CZE measurements was assessed by determining each μ_e value in triplicate from pH 2.6 to 12.1 at the temperature of 25 °C. The relative standard

Table 2. Employed buffers

pH range	Constituent	Stock solution	Ionic strength
2.6–3.5	Citrate	1 M $C_6H_8O_7 \cdot H_2O$ 1 M NaOH	0.03
3.6–5.5	Acetate	1 M CH_3COOH 1 M CH_3COONa	0.03
6.0–9.0	Phosphate	0.1 M NaH_2PO_4 0.1 M Na_2HPO_4	0.03
10.7–12.1	Borate	0.1 M $Na_2B_4O_7$ 0.1 M NaOH	0.03

Table 3. Electro-osmotic flow (10^{-5} cm^2 V^{-1} s^{-1}) as a function of electrolyte pH

pH	2.6	4.0	5.0	6.1	7.9	9.0	9.5	10.0
μ_{EOF}	12.9	13.2	44.9	51.4	73.3	75.5	76.1	77.9

deviation of the μ_e was less than 1% in the neutral and alkaline region and about 3% at acidic pHs.

The pK_a values were obtained by non-linear regression analysis using eqs (6), (8) or (10) (see Experimental). All pK_a measurements (Table 1) were made in triplicate at the same ionic strength and temperature.

Figure 1 shows the best fit of the non-linear regression for 6-chloro-pyridine-2(1*H*)-one (**2**) and the diacid derivative **6**, whereas the dependence of the effective mobilities on pH of 2-oxo-6-(4-pyridyl)-3-pyridincarbonitrile (**10**) is illustrated in Figure 2.

With the exceptions of the first dissociation constant of the bipyridine derivative **12**, whose pK_a value should be less than 2 (i.e., too low to be determined under the experimental conditions used), and compounds **1** and **3**, whose pK_a values are too high to be determined with accuracy, all pK_a values of 2-pyridone derivatives were determined by CZE with a satisfactory precision, as shown by the standard errors of means (SEM).

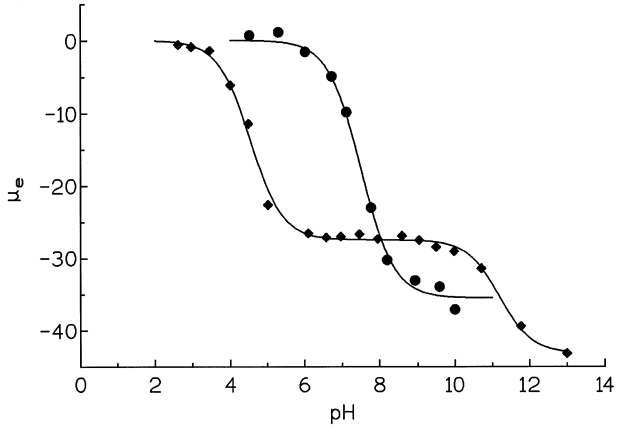


Figure 1. Dependence of effective mobilities of 2-pyridones **2** (●) and **6** (◆) on pH with best fits from non-linear regression analysis. Voltage: 25 kV, temperature: 25 °C, and UV detection at 214 nm. The curves for compounds **2** and **6** were obtained by fitting the experimental data to eqs (6) and (8), respectively.

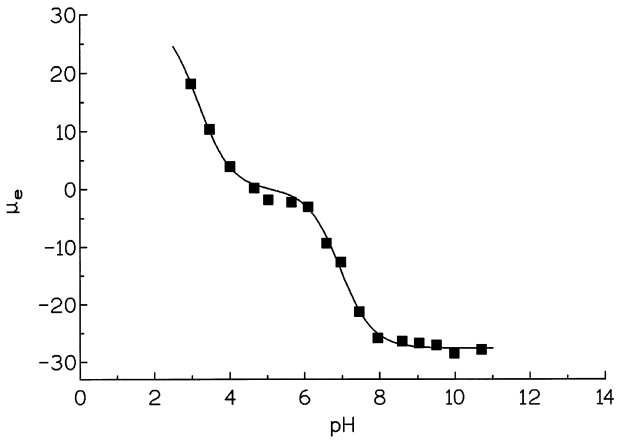


Figure 2. Dependence of effective mobilities of 2-pyridones **10** (■) on pH, other conditions as in Figure 1. The curve was obtained by fitting the experimental data to eq (10).

The CZE-determined ionization constants were compared with those determined by ultraviolet (UV) spectrophotometry and potentiometric titration methods (Fig. 3).

With few exceptions, pK_as obtained by CZE are more precise than those determined by the UV method, which in turn failed in the pK_a determination of some derivatives, because of the low solute solubility (**15** and **16**) and the lack of significant differences between the absorption spectra of the neutral and fully ionized species (**10**). On the contrary, the potentiometric method did not cope with the measurements of dissociation constants of most of our pyridones. Indeed, only five pK_as were measured, the others being undeterminable due to both low solute solubility and low acidity strength (i.e., $K_a \times c_a < 1 \times 10^{-9}$). Moreover, compared with the CZE and UV methods, the potentiometric method showed, for lower pK_as (compounds **6**, **7** and **13**), deviations ranging from ca. 0.5 to 1.0 pK units.

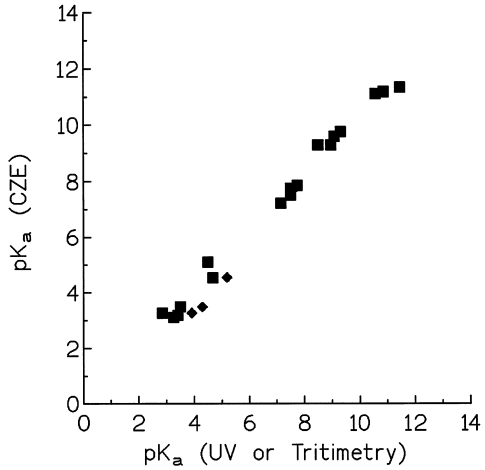


Figure 3. pK_a values as measured by CZE against pK_as determined by UV spectrophotometry (■) and potentiometry (◆).

As measured by CZE, pK_a values of the compounds examined are in the range 3.3–11.3, depending on the substituents on 1,2-dihydropyridine nucleus. As already shown,[21,22] electron withdrawing substituents, in particular at the C(6) position, displace the tautomeric equilibrium of pyridones toward the hydroxypyridine form, leading as a consequence to a decrease of pK_a. While the effect of the chlorine substituent had already been noticed,[21] our measurements revealed the strong acidity-increasing effect of the trifluoromethyl substituent (compound **13**) and the significant effect of pyridyl groups (compounds **10**–**12**) at the C(6) position. We expressed the effects of the substituents on pK_a in terms of Hammett electronic constants, by using standard σ_{meta} for substituents in positions 3 and 5 and the apparent σ_{ortho} constants from experimental pK_a values of pyridine derivatives.[23] If not available (CF_3, 4-Py, 3-Py and 2-Py in compounds **10**–**13**), σ_{ortho} values were calculated according to Williams and Norrington,[24] and corrected, multiplying them by a factor of 1.74 (i.e., the slope value obtained by regression of experimental pyridine's σ_{ortho} values for electron withdrawing substituents against calculated σ_{ortho} constants, the intercept being zero), using the following equation:

$$\sigma_{ortho}\ (\text{pyridine}) = 1.74\ (1.25F + 0.86R) \quad (1)$$

where F and R represent the field and resonance parameters of Swain and Lupton, taken from standard compilations.[25] The sum of σ constants ($\Sigma\sigma$) is reported in Table 1. Hammett's equation (2) is well verified for the whole series of compounds:

$$pK_a\ (\text{CZE}) = 11.65\ (\pm 0.25) - 4.02\ (\pm 0.31)\ \Sigma\sigma$$
$$n = 18 \quad r^2 = 0.9120 \quad s = 0.6828 \quad (2)$$

where n is the number of data, r^2 the squared correlation coefficient, and s the standard deviation of the regression line. The slope of eq (2) is within the 95% confidence interval of the ρ value (4.28) of the general equation for pK_a calculations of 2-pyridones.[26]

We re-computed the regression between the constants of tautomeric equilibrium K_T, defined as the ratios of concentrations of the pyridone ('oxo') and hydroxypyridine ('hydroxy') species, of substituted congeners taken from the literature[22] and Hammett σ values of the substituents and derived the following linear equation:

$$\log K_T = 3.08\ (\pm 0.11) - 4.43\ (\pm 0.23)\ \sigma$$
$$n = 19 \quad r^2 = 0.9349 \quad s = 0.4728 \quad (3)$$

whose ρ value is close to that of eq (2). Eqs (2) and (3) show that intramolecular electronic interactions affect protolysis (K_a) and tautomeric (K_T) equilibria in a similar way, and, therefore, that the acidity of 2-pyridones can be important in understanding their tautomeric behaviour.[27–29] Actually, three congeners (i.e., 3-NO_2, 5-NO_2, 5-NH_2) were omitted as outliers in the regression analysis of log K_T against σ values. However, their inclusion in the data set only made the statistics of eq (3) worse ($r^2 = 0.8510$, $s = 0.734$), whereas they did not significantly change its physicochemical content (i.e., similar slope and intercept values).

Partitioning behaviour

The role of lipophilicity in modulating the activity of 2-pyridone-containing inotropic agents has been discussed in the literature, and, at least within specific series, a direct relation between positive inotropic activity and lipophilicity has been found.[30] Fragment-based calculated lipophilicity data were given to support such a relationship, whereas it is known that a correct calculation of partition coefficients cannot neglect pyridone/hydroxypyridine tautomerism,[20] since 'hydroxy' tautomers are predicted to be at least 20-fold more lipophilic than the respective 'oxo' tautomer (see the CLogP values in Table 4). To examine how tautomerism affects lipophilicity of 2-pyridone-containing compounds, we investigated their octanol/water partitioning over a wide pH range.

The pH-dependent lipophilicity profiles of a number of derivatives (**1**–**5**, **13**, **18**) were determined by measuring their distribution coefficients (log D) at various pH in the range 2–12, using the so-called 'shake-flask' technique.[31] Experimental log D values at physiological pH (log D pH 7.4), partition coefficients of the neutral forms (log P), and calculated log P (CLogP) values for the 'oxo' and the 'hydroxy' tautomers of the neutral species are reported in Table 4, whereas Figure 4 shows two representative pH-dependent distribution profiles (compounds **4** and **13**).

Data in Table 4 show that, with the remarkable exceptions of compounds **2** and **13**, both bearing electron withdrawing substituents at the C(6) position (apparent σ_{ortho} constants of pyridine derivatives are 0.79 and 1.09 for Cl and CF_3, respectively), all the other compounds have experimental log P values not significantly different from those calculated for the respective 'oxo' forms. In contrast, the log P values of 6-chloro-pyridine-2(1*H*)-one (**2**) lies about midway between the CLogP values of the respective 'oxo' and 'hydroxy' tautomers, and the experimental log P of **13** (Fig. 4b) is superimposable to

Table 4. Experimental and calculated partition data in the *n*-octanol/water system

Compound	Log D pH 7.4[a]	Log P[a]	CLogP	
			'Oxo' form	'Hydroxy' form
1	−0.56	−0.56 (−0.58)	−0.57	0.93
2	0.78	0.91 (0.93)	0.34	1.71
3	−0.12	−0.04	−0.07	1.43
4	−0.37	−0.41	−0.19	1.13
5	−0.42	−0.30	−0.55	0.88
13	−0.14	2.08	0.84	2.09
18	0.00[b]	0.42	0.26	1.61

[a]Mean of at least five measurements at four different solute concentrations; SD < 0.03. The Pomona database value is given in parentheses.
[b]Value from Sanofi–Winthrop (Collegeville, PA) is 0.08.

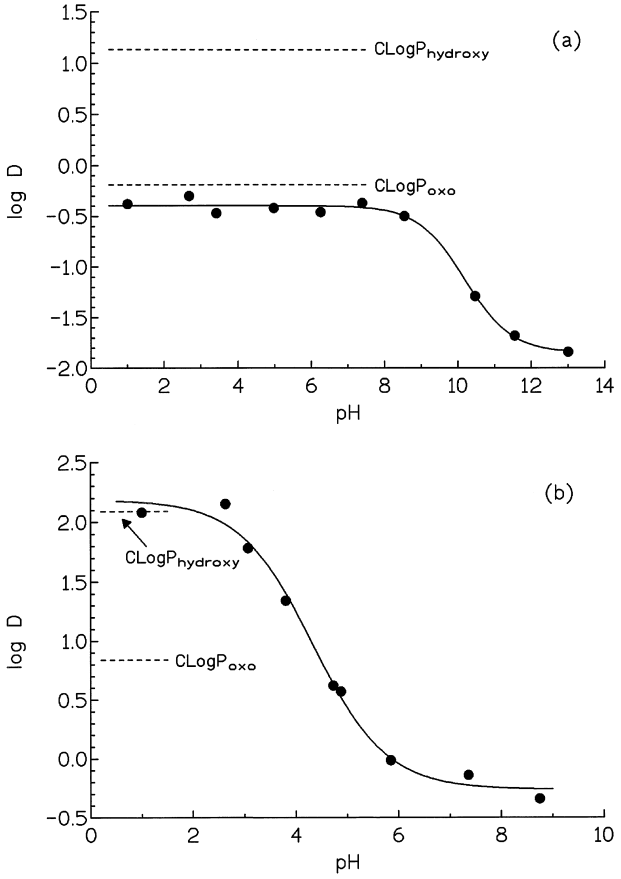


Figure 4. pH-dependent distribution profiles in the octanol/water system of 2-pyridone derivatives: **4** (curve a), **13** (curve b). Dashed lines represent the calculated log P values of 'oxo' and 'hydroxy' tautomers of the neutral species (i.e., pH $\leqslant$ pK_a–2).

the value calculated for the hydroxypyridine tautomer. Actually, these results were not completely unexpected. The different partitioning behaviour of some model monosubstituted 2-pyridones has been, in fact, already noticed by others, and interpreted taking the 'oxo'/'hydoxy' tautomerism into account.[20] The very high values of tautomeric ratios in water[22] and in less polar solvents[32–35] of the unsubstituted 2-pyridone **1** ($K_T = 912$), as well as its 6-methyl congener **3** ($K_T = 3980$), means that the 'oxo' forms are in very large excess over the 'hydroxy' forms of the neutral species, leading, as indeed observed, to log P values close to the calculated ones for the 'oxo' tautomers. As mentioned above, electron withdrawing substituents, in particular at the C(6) position, favour the 'hydroxy' tautomer, and this should lead to log P values intermediate between those calculated for the two forms or close to the CLogP value of the hydoxypyridine tautomer. Besides the already known behaviour of compound **2**,[20] our data brought further experimental evidence that, more than the global electronic effect exerted by the substituents on the 1,2-dihydropyridine ring, accounted for by $\Sigma\sigma$, it is the susbstituent at C(6) which mainly affects tautomerism and consequently lipophilicity of the neutral species. This is exemplified on one hand by comparing among them the lipophilicity data of compounds **2**, **4** and **18** (despite their close $\Sigma\sigma$ values, only the 6-chloro congener **2** has a log P value significantly higher than log P of the 'oxo' tautomer), and on the other hand by the behaviour of compound **13**, bearing a strong electron withdrawing substituent such as CF_3 at the C(6) position, whose log P value is identical to the CLogP of the respective 2-hydroxypyridine form, indicating the latter as the tautomer wholly existing both in water and in wet octanol. K_T of **13** in water should in fact be very low ($< 1\times10^{-5}$), as deduced by eq (3).

Our pH-dependent octanol/water distribution measurements, although not as definitive, gave information on pyridone/hydroxypyridine tautomerism in a biomimetic biphasic system, like the *n*-octanol/water system. Without doubt, spectroscopic measurements give the most definitive evidence for determining tautomeric ratios in various solvents. However, interpretation of spectroscopic results is not always straightforward, and sometimes inconsistent with lipophilicity behaviour.[20] In the case of our 2-pyridone derivatives, data from partitioning measurements yielded helpful information and suggested further investigation.

In summary, our data show that lipophilicity of the neutral forms (i.e., those existing almost exclusively at pH $\leqslant$ pK_a–2) is significantly affected by the pyridone/hyroxypyridine tautomerism, and that electron withdrawing substituents at C(6), which markedly increase acidity, push up log P values towards the values of the respective 2-hydroxypyridine tautomers. These findings could be of importance in the improvement of log P calculation of pyridones and related compounds.

Implications in inotropic activity

An assessment of cardiotonic activity of the milrinone analogues has been accomplished by measuring their effects on contractile force and frequency rate in spontaneously beating atria from reserpine-treated guinea pigs.[36] Previous structure–activity relationship studies showed that 6-methyl-3-cyano-pyridine-2(1*H*)-ones bearing an acyl group (e.g., $COCH_3$, compound **5** in Table 1) or a carboalkoxy group (e.g., $COOC_2H_5$)[37,38] at the C(5) position induced positive inotropic effects comparable or even more marked than those induced by milrinone. A relation has been suggested between positive inotropic activity and a flat topography containing the dipolar moiety (i.e., COR or COOR) at C(5) and the hydrogen-bonding pyridone moiety.[3] The importance of the substituent at C(6) has also been demonstrated. Its size should not exceed the receptor essential volume, and should allow COR or COOR groups at C(5) to be coplanar with the 1,2-dihydropyridine ring.[36–38] Even more recently, a chemometric study has demonstrated volume descriptors to be the independent variables mainly related to the decrease of positive inotropic activity.[39]

However, the implications of acid/base properties and tautomerism in the cardiotonic activity of milrinone-related agents have not received enough attention so far. The data reported here may help in explaining the inotropic activity in physicochemical terms. Thus, by

comparing compound **5**, a positive inotropic agent with an activity close to that of milrinone,[36] with compound **13**, a weak positive inotropic agent,[11] it could be inferred that compounds with high pK_a values are more effective in increasing inotropism. This suggests the inotropic activity being dependent mainly upon the molar fraction of the neutral species at physiological pH, which is 0.99, 0.69 and close to 0 for milrinone, **5** and **13**, respectively.

The positive inotropism of the 6-methyl congener of compound **13**, whose activity has been shown to be even higher than milrinone itself,[10] would support such a relation. Indeed, difference in van der Waals volume between a methyl ($V_W = 1.01$) and a trifluoromethyl group ($V_w = 1.11$) at C(6) is too low to explain the observed difference in activity, whereas the strong acidity-increasing effect exerted by the CF_3 substituent at the C(6) position of the derivative **13** could cause the lowering of activity compared with the corresponding 6-methyl congener. This trend is somehow confirmed by the developed tension data of the monosubstituted 2-pyridones, the 6-methyl derivative **3**[39] (p$K_a = 12.30$) being more active than the more acidic 6-chloro congener **2**[40] (p$K_a = 7.51$).

The rank order of inotropic activity, i.e., **13** < **18**≈**5** (but also **2** < **3**), is not directly reflected either in the log P scale or in the apparent distribution coefficient values (log D) at physiological pH. Neverthless, the partitioning data significantly encode for pyridone/hydroxypyridine tautomerism in a biologically relevant manner. The partition coefficients of the neutral species of the most active compounds (**18**, **5** and **3**), close to those calculated for the respective pyridone forms, indicated that they exist almost exclusively as 'oxo' tautomers both in polar and apolar media. This experimental evidence led us to put forward the hypothesis that the more polar[41] and hydrogen-bonding pyridone tautomers should be the 'active' forms of milrinone-related inotropic agents.

Conclusions

The present study reports the ionization and partitioning behaviour of a number of inotropic pyridine-2(1*H*)-one derivatives, and their relation with the pharmacological activity. Apparent dissociation constants were measured by the CZE technique, which, compared with conventional methods (e.g., UV spectrophotometry, potentiometric titration), showed advantages, such as rapidity and applicability to compounds that are sparingly soluble in water. Based on the CZE-determined pK_a values, intramolecular electronic interactions were precisely assessed, revealing the importance of the acidity in understanding pyridone/hydroxypyridine tautomerism. Octanol/water partition coefficients encode for tautomerism as well, and a comparison between experimental and calculated partition data allowed us to detect the 'oxo' tautomers as the most relevant forms from a pharmacodynamics point of view. While deserving further investigation, this physicochemical study carried out on a set of milrinone-related compounds, with a different degree of cardiotonic activity, brings evidence that a high fraction of the neutral species at physiological pH, predominantly in the more polar pyridone tautomer, is required for a good positive inotropism, so offering further insights into the structure–activity relationships of this class of cardiotonic agents.

Experimental

Materials

2-Pyridone derivatives **1**–**3** and Milrinone (**18**) were purchased from Sigma–Aldrich S.r.L. (Milan, Italy). All the other compounds used in this study were synthesized according to procedures already described.[9–16] Their purity was checked by HPLC, IR and NMR. Solvents and salts were of analytical grade.

pK_a measurements

Capillary electrophoresis. Capillary electrophoresis was performed using a Biofocus 3000 automated CE apparatus (Bio-Rad Laboratories, Hercules, CA, USA) equipped with a multiwavelength UV detector. The output detection wavelengths were 214, 254 and 280 nm. Uncoated fused-silica capillaries (Bio-Rad) of 50 μm i.d. ×41 cm (36.5 cm to detector), thermostatted at 25 °C by a liquid coolant, were used for the determinations.

Standard solutions of 2-pyridone derivatives were prepared at a concentration of 1 mg/mL in methanol, and then diluted 1:10 with the run buffer. The buffer solutions were filtered through a 0.2-μm syringe filter. Buffer solutions were prepared by mixing two stock solutions as shown in Table 2 and diluted to ionic strength $I = 0.03$. The pH of the buffer was measured at 25 °C by a PHM 82 Standard pH meter (Radiometer, Copenhagen, DK).

A new capillary was washed for 10 min with 1 M NaOH, followed by washing for 10 min with water and for 10 min with 1 M HCl. Before each injection, the capillary was flushed for 10 min with water, for 10 min with 1 M NaOH, for 10 min again with water, and finally with the actual buffer solution for 10 min. The temperature was kept at 25 °C. A constant separation potential of 25 kV was used. Mesityl oxide, as a marker of electroosmotic flow, was added to 10–100 μM of the sample solution. The samples were injected at 3–8 psi s (hydrodynamic injection).

Observed values of the migration times of the sample and mesityl oxide at different pHs were converted to the effective mobilities (μ_e) using the following equation:

$$\mu_e = \frac{L_d \cdot L_t}{V}\left(\frac{1}{t_m} - \frac{1}{t_o}\right) \quad (4)$$

where μ_e is the effective mobility ($cm^2\ V^{-1}\ s^{-1}$), L_d the length of the capillary to the detector (36.5 cm), L_t the total capillary length (41 cm), V the applied voltage, t_m

the migration time of the solute in s, and t_0 the migration time of the marker of electrosmotic flow in s.

The effective mobility can also be expressed as the sum of the products of mobility times fraction of the differents charged species. Thus, for most of the examined 2-pyridones, which within the explored pH range behave as weak monoprotic acids, the effective mobility is given by the following equation:

$$\mu_e = \alpha_{A^-} \cdot \mu_{A^-} \quad (5)$$

where α and μ are the fraction and the effective mobility, respectively, of the fully deprotonated species. By expressing the fraction as a function of pK_a and pH, eq (5) can be rewritten as

$$\mu_e = \left(\frac{1}{10^{pK_a - pH} + 1}\right) \cdot \mu_{A^-} \quad (6)$$

Compounds **6** and **7** are diprotic weak acids, and their effective mobility, μ_e, is given by:

$$\mu_e = \alpha_{HA^-} \cdot \mu_{HA^-} + \alpha_{A^{--}} \cdot \mu_{A^{--}} \quad (7)$$

where α and μ are the degrees of dissociation and mobilities, respectively, of the species HA^- and A^{--}. The dependence of the effective mobility on pH can be described as follows:

$$\mu_e = \left(\frac{1}{10^{pK_{a1} - pH} + 10^{pH - pK_{a2}} + 1}\right) \cdot \mu_{HA^-} + \left(\frac{1}{10^{pK_{a1} + pK_{a2} - 2pH} + 10^{pK_{a2} - pH} + 1}\right) \cdot \mu_{A^{--}} \quad (8)$$

where K_{a1} and K_{a2} are the first and second dissociation constants, respectively.

Compounds **10–12** and milrinone **18** are ampholytes, their net mobilities being expressed as a function of fully protonated (H_2BA^+) and fully deprotonated (BA^-) species by the following equation:

$$\mu_e = \alpha_{H_2BA^+} \cdot \mu_{H_2BA^+} + \alpha_{BA^-} \cdot \mu_{BA^-} \quad (9)$$

The pH-dependent mobility profile of ampholytes can be described as follows:

$$\mu_e = \left(\frac{1}{10^{2pH - pK_{a1} - pK_{a2}} + 10^{pH - pK_{a2}} + 1}\right) \cdot \mu_{H_2BA^+} + \left(\frac{1}{10^{pK_{a1} + pK_{a2} - 2pH} + 10^{pK_{a2} - 2pH} + 1}\right) \cdot \mu_{BA^-} \quad (10)$$

where K_{a1} represents the dissociation constant of the conjugated acid of the basic group (i.e., the pyridinium ion) and K_{a2} the dissociation constant of the pyridone function.

Data pairs of μ_e and pH were substituted into eq (6), (8) or (10) and, according to the method of Ishihama et al.,[17] the pK_a values were obtained by non-linear regression calculations. Quality of the fit was estimated by standard deviation.

UV spectrophotometry

Solutions (ca. 1×10^{-4} M) were prepared in appropriate buffers ($\leqslant$3% v/v methanol to aid solubilization), and spectra over the range 210–400 nm were recorded using a Hewlett–Packard (HP 8452 Diode array) UV spectrophotometer. The pK_a values were calculated from the UV spectroscopic data and pH values based on the Henderson–Hasselbach equation.[42]

Potentiometric titrations

Potentiometric titrations were performed with a PHM 82 Standard pH meter (Radiometer, Copenhagen, DK) equipped with a G2040 Glass Electrode. Solutions (ca. 5×10^{-3} M) were titrated with standardized 1×10^{-2} M solutions of NaOH. The low aqueous solubility of compounds required the presence of methanol as co-solvent ($\leqslant$2%, v/v). The titrations were carried out under helium at 25.0 ± 0.1 °C. The pH of the solution was measured after each addition of NaOH solution. Results from initial titration were used to discern areas of interest. Thus, initially the pH was measured after addition of 0.2-mL volumes of titrant until approximately 1 mL before the estimated endpoint, and then 0.1-mL volumes of titrant within the range $\pm$1 mL over the endpoint. A graph was then made of pH against volume of NaOH added. Titration curves were determined in triplicate, and the pK_a values were calculated using a non-logarithmic linearization of the titration curve proposed by Benet and Goyan[43] and modified by Leeson and Brown.[44] Before and after each set of potentiometric measurements, the electrode system was standardized against aqueous buffer solutions.

Partition coefficients

The distribution coefficients (log D) between *n*-octanol and water were measured using the 'shake-flask' technique[31] at room temperature. Buffers reported in Table 2 were used as the aqueous phases for the pH range 2–12. For pH 0 and 1, 1 and 0.1 M HCl served as the aqueous phases, respectively. The organic and aqueous phases were mutually saturated. At equilibrium, concentrations were measured in the aqueous phase by UV after centrifugation. Each log D value is an average of at least five measurements, the standard deviation being less than 0.03 log units.

Calculated log P values were obtained by the fragmental method of Hansch and Leo[45] using the Maclog P 3.0 program (BioByte Corp., Claremont, CA).

Acknowledgements

The authors are grateful to MURST and CNR (Rome, Italy) for partial financial support. The expert assistance of Dr. Anna Vignola in CZE experiments is gratefully acknowledged.

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