J. Pharm. Pharmacol. 1984, 36: 11–15
Received May 5, 1983

The ionization of β-adrenoceptor agonists: a method for unravelling ionization schemes

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To investigate the ionization schemes of β-adrenoceptor agonists, a combined electrochemical/uv-spectrophotometric method with computer-assisted data-analysis was developed, yielding the macroscopic and microscopic ionization constants. From the four species possible, cations and zwitterions were found the main species present at physiological pH, the formation of uncharged molecules and anions being less favourable or apparently negligible.

β-Adrenoceptor agonists and antagonists bear structural resemblance in the (oxy)alkanolamino side chain, whereas they differ in the (substitution pattern of the) aromatic moiety. Antagonists (like propranolol, Fig. 1a) often share apolar properties here (Evans et al 1979), while, in contrast, for both full (isoprenaline, Fig. 1b) and partial (prenalterol, Fig. 1c) agonists the substituents have a more or less acidic function (Brittain et al 1976; Comer 1980).

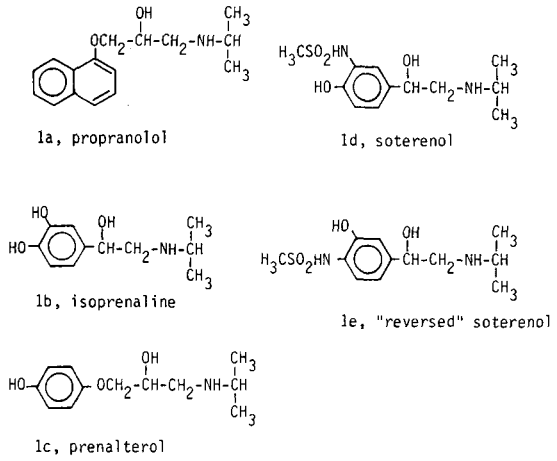


FIG. 1

On the basis of a series of isomeric hydroxysulfonamidophenethanolamines (Fig. 1d, e) as analogues of catecholamines, Larsen et al (1967) suggested that for agonistic activity the substituent *meta* to the side chain should be more acidic than the *para*-substituent. Though several more recently developed β-adrenoceptor agonists seem to support this proposal (Kaiser et al 1974), the *m*-substituent in several well-known agonists like salbutamol (Brittain et al 1968) and clenbuterol (Engelhardt 1972) does not possess an acidic proton.

Recently, we have found evidence indicating the formation of 1:1 complexes of mono- and dihydroxyphenylethanolamines with phosphate and carboxylate anions in which two hydrogen bonds participate (De Vente et al 1981). It was speculated that such a complex formation could be of relevance in the interaction with the β-adrenoceptor.

Obviously, the hydrogen bond forming capacity of a substituent increases with the acidity of the proton involved, and since a quantitative description relating ionization of the aromatic substituents with β-adrenoceptor affinity and/or intrinsic activity has not been reported thus far, it was considered worthwhile to investigate the ionization schemes of a number of arylethanolamines. Therefore, a method was developed for the determination of the macroscopic and microscopic ionization constants, and is described in the present paper.

MATERIALS AND METHODS

Materials

AH 3021 and salbutamol (free bases, Allenburys), Th 1206 and orciprenaline (sulphates, Boehringer Ingelheim), terbutaline (sulphate, Astra), Cp 22352-1 and pirbuterol (dihydrochlorides, Pfizer) were gifts. Isoprenaline (sulphate, ACF), noradrenaline and adrenaline (bitartrates, Sigma) were purchased. The structures are given in Table 1. Buffers, used to calibrate the pH-meters, were pH = 7·00 ± 0·02 (25 °C) and pH = 9·94 ± 0·05 (25 °C) (Merck). All other chemicals were of reagent grade.

* Correspondence.

Methods

The ionization scheme can be represented as follows:

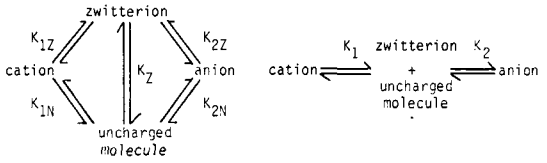


FIG. 2. Ionization schemes of dibasic β-adrenoceptor agonists.

K_{1Z}, K_{2Z}, K_{1N} and K_{2N} are the four microscopic ionization constants, K_1 and K_2 are the two macroscopic ionization constants, whereas K_Z is the 'zwitterion constant', which determines the ratio zwitterions/uncharged molecules.

Ultraviolet-spectrophotometric measurements: determination of the microscopic ionization constant, K_{1Z}

The compounds (0·1 mM in 0·1 M KCl) were analysed at 25·00 ± 0·05 °C, under N_2, catecholamines also with exclusion of light. pK_{1Z} was determined according to Sinistri & Villa (1962) whose method was the absorbance at any pH as a measure for phenolic deprotonation which are modified as follows:

(i) absorbance was measured at 245 and 295 nm (CP 22352-1 and pirbuterol: 245 and 305 nm), for both wavelengths yielding identical values for pK_{1Z};

(ii) instead of using glycine buffer solutions, the pH was adjusted by minimal amounts (1–5 μl) of 0·1 – 1·0 M HCl and 0·1 – 10 M NaOH, to obtain conditions comparable to the potentiometric titrations.

(iii) pK_{1Z} was calculated according to

$$pK_s = -\log K_{1Z} \cdot \frac{[H^+] + K_{2Z}}{[H^+] + K_{1N}}$$

in which $pK_s = pH - \log [(A - A_{min})/(A_{max} - A)]$ (A: uv-absorption).

The pH-range in which pK_s was determined was between pK_1 −0·6 and pK_1 +0·6. Outside this range a large scattering in pK_s-values was observed, due to the existing minimal difference in either numerator or denominator in $\log [(A - A_{min})/(A_{max} - A)]$. Within this limitation a good approximation of pK_{1Z} can be achieved by a computer-assisted curve-fitting procedure in which minimalization was analogous to the algorithm described in the following section.

pH limits were pH 5 in the acidic region, yielding A_{min}, thus avoiding protonation of the pyridine-nucleus in Cp 22352 and pirbuterol, and pH 11–12, yielding A_{max}, at which pH value maximal absorption occurs with minimal or no deviation in the isosbestic point. The absorbance was scanned with an Aminco DW-2A UV/VIS spectrophotometer using 1 cm cells; the pH was measured using a digital Radiometer PHM 84 Research pH-meter. The ionic strength was assumed to be that of 0·1 M KCl, and the activity coefficient was taken as 0·775. All measurements were made in triplicate, the 95% confidence interval being less than 0·04.

Electrochemical titrations: determination of the macroscopic ionization constants, K_1 *and* K_2

The compounds (0·6 mM in 0·1 M KCl) were potentiometrically titrated with 0·1 M KOH from a calibrated Mettler DV 10 micropipettor at 25·00 ± 0·05 °C under N_2 (catecholamines also with exclusion of light), using a digital Philips PW 9414 ion activity meter. The volume of solution was 25–30 ml, and as for the spectroscopic measurements, the ionic strength was assumed to be that of 0·1 M KCl, the activity coefficient was taken as 0·775. The low concentrations of the compounds were chosen to obtain results comparable to the uv-spectrophotometric ones. Data (values of pH and added KOH, ca 40 per titration) were analysed with the SCOGS computer program (Sayce 1968, 1971; Sayce & Sharma 1972), modified as follows:

(i) use of the Marquardt algorithm for minimalizations (Marquardt 1963),

(ii) iteration till minimum is obtained,

(iii) calculation including the 95% confidence intervals.

SCOGS (Stability Constants Of Generalized Species) is used to analyse appropriate pH titration data to yield acid association constants (and hence pK_a's), stability constants of simple complexes etc., making allowance for temperature, activity coefficients, volume of solution, normality of the titrant, added acid or base, following a non-linear least-squares minimalization of the added volumes of alkali.

All titrations were in triplicate.

Calculations

The remaining microscopic ionization constants were calculated, according to

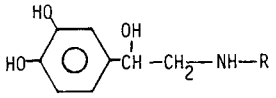
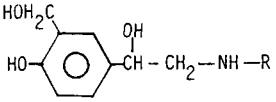
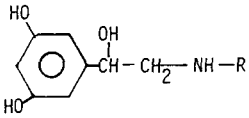
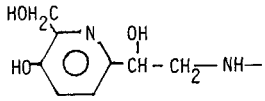
$$K_{1N} = K_1 - K_{1Z}$$
$$K_{2Z} = K_1 . K_2 . K_{1Z}^{-1}$$
$$K_{2N}^{-1} = K_2^{-1} - K_{2Z}^{-1}$$

and the zwitterion constant by $K_Z = K_{1Z} . K_{1N}^{-1}$.

RESULTS

The structures of the investigated compounds, together with the results of the potentiometric titrations are shown in Table 1, pirbuterol having the lowest value for pK_1, and the highest for pK_2. Within

Table 1. Macroscopic ionization constants of β-adrenoceptor agonists in 0·1 M KCl, 25·0 °C.

	R	Name	pK_1	95%*	pK_2	95%*
	H	Noradrenaline	8·63	0·03	9·73	0·02
	CH_3	Adrenaline	8·73	0·03	10·14	0·02
	iPr[a]	Isoprenaline	8·65	0·02	10·07	0·01
	tBu[b]	Th 1206	8·72	0·04	10·31	0·02
	iPr[a]	AH 3021	9·01	0·02	10·15	0·01
	tBu[b]	Salbutamol	9·07	0·02	10·37	0·01
	iPr[a]	Orciprenaline	8·70	0·03	9·92	0·02
	tBu[b]	Terbutaline	8·70	0·01	10·09	0·01
	iPr[a]	Cp 22352-1	8·08	0·05	10·25	0·03
	tBu[b]	Pirbuterol	8·01	0·07	10·64	0·04

* 95%: 95% confidence interval.
[a] iPr: *N*-isopropyl.
[b] tBu: *N*-(t-butyl).

the pairs, consistently higher values for pK_2 are found for the *N*-(t-butyl) substituted derivative than for the *N*-isopropyl analogue, whereas the respective pK_1 values are approximately identical. Except for pirbuterol, a considerable overlap in pK_1- and pK_2-values exists, a priori giving rise to a complex ionization scheme, as depicted under 'Methods'. Table 2 summarizes the microscopic ionization constants, together with the zwitterion constants, showing the formation of zwitterions is preferred as a result of monodeprotonation. Indeed, for most compounds where $pK_1 - pK_{1Z} = 0$ within experimental error, the formation of uncharged molecules seems to be negligible (referred to as (K_Z = [b]). pK_{2Z} for noradrenaline, the only primary amine, is markedly lower than for the other compounds.

Table 2. Microscopic ionization constants and zwitterion-constants of β-adrenoceptor agonists in 0·1 M KCl, 25·0 °C.

Name	pK_{1Z}	pK_{2Z}	pK_{1N}	pK_{2N}	K_Z
Noradrenaline	8·68[a]	9·68	—	—	[b]
Adrenaline	8·69[a]	10·14	—	—	[b]
Isoprenaline	8·69[a]	10·03	—	—	[b]
Th 1206	8·68[a]	10·31	—	—	[b]
AH 3021	9·23	9·93	9·41	9·75	1·5
Salbutamol	9·22	10·22	9·60	9·84	2·4
Orciprenaline[a]	8·67	9·92	—	—	[b]
Terbutaline	8·73[a]	10·06	—	—	[b]
Cp 22352-1	8·02[a]	10·25	—	—	[b]
Pirbuterol	7·95[a]	10·64	—	—	[b]

[a] $pK_1 - pK_{1Z} = 0$ within experimental error.
[b] Formation of neutral molecules is negligible.

DISCUSSION

The macroscopic ionization constants for noradrenaline, adrenaline and isoprenaline are in good agreement with the values obtained by others (Andrews et al 1962; Sinistri & Villa 1962; Jameson & Neillie 1965; Rajan et al 1972; Antikainen & Witikainen 1973; Armstrong & Barlow 1976; Mack & Bönisch 1979) although Sinistri & Villa (1962a) have presented slightly higher values. Thus, in our opinion, it is allowable to use very low concentrations (as much as 100 times less than usual) for the determination of the macroscopic ionization constants. Moreover, this method offers the advantage that concentration-dependent effects on the pK-values, as described by Armstrong & Barlow (1976), are presumably negligible.

The overlap in macroscopic ionization constants has often confused the interpretation of results. Among others, Jameson & Neillie (1965) and Rajan et al (1972) related pK_1 with the dissociation of the protonated amino function, whereas Antikainen & Witikainen (1973) and Granot (1976) assigned the phenolic function as the more acidic group in accordance with some previous interpretations (Lewis 1954; Kappe & Armstrong 1965). Table 2 substantiates the latter view: the red shift in the uv-spectra of all amines, caused by the transition

phenol-phenolate, yields pK_{1Z}, which value is in all cases very close to pK_1. Moreover, in the series of catecholamines, Table 1 shows a more extensive variation in pK_2 than in pK_1, suggesting pK_1 should be assigned predominantly to the common property of the catecholamines: the catechol moiety.

However, due to the overlap in both macroscopic constants, it certainly is not correct to identify pK_1 merely with the dissociation of the phenolic hydroxyl group, for this dissociation process is governed by pK_{1Z} and pK_{2N}. From the values for pK_{1Z} and pK_{2N} listed in Table 2, it is seen that this dissociation process is favoured in case of the protonated amino function.

Interestingly, the results obtained with pirbuterol validate the method followed: due to the difference between pK_1 and pK_2 ($\simeq$2·7), there will be no significant overlap in the macroscopic ionization constants (Albert & Serjeant 1971), implying that pK_{1Z} should be equal to pK_1, which is obviously the case.

Barlow (1982) has evaluated a method for the calculation of K_Z, the zwitterion constant, based exclusively on spectroscopic measurements. An equation relating the absorbance with K_1, K_2 and K_Z was derived in which K_Z is in the denominator. For most of the compounds used in our study, the formation of uncharged molecules is negligible (and hence K_Z is infinite, cf. Table 2), which renders a comparison with the method of Barlow impossible.

From the values for pK_{2Z} it is clear, that the formation of anions is relatively favoured for the primary amine noradrenaline. For the secondary amines there is a consistent difference in pK_{2Z} for the pairs of *N*-(isopropyl) and *N*-(*t*-butyl) substituted derivatives. As the Charton σ_I-parameter, which is supposed to be predominantly of an electronic nature (Charton 1977), approximates to zero for these alkyl substituents, it is difficult to see how electronic effects would explain the observed trend. Maybe an additional steric effect plays a role.

The microscopic ionization constants permit the fractions of the different species at pH = 7·4 to be calculated, as is represented in Table 3. Cations are in the majority (between 78·0% for pirbuterol and 97·9% for salbutamol) the remainder predominantly being zwitterions. This raises the question, which species is (are) involved in the affinity towards β-adrenoceptors and in the intrinsic activity. Armstrong & Barlow (1976) consider the occurrence of zwitterions probably not to be associated with activity at β-adrenoceptors. Ganellin (1977) presumes some proton transfer to be involved in the interaction with the β-adrenoceptor, as a reflection of different K_Z-values, derived from data by Sinistri & Villa (1962). Their results, however, differe from ours, due (as has been mentioned) to their relatively high values for pK_1, pK_2 and pK_{1Z}.

Table 3. Relative fractions of the different species present at pH = 7·4 (T = 25 °C), expressed as % of total drug concentration.

	Cation	Zwitterion	Uncharged molecule	Anion
Noradrenaline	95·0	5·0	—	0·026
Adrenaline	95·5	4·5	—	0·008
Isoprenaline	94·8	5·2	—	0·011
Th 1206	95·4	4·6	—	0·006
AH 3021	97·6	1·4	1·0	0·004
Salbutamol	97·9	1·5	0·6	0·002
Orciprenaline	95·5	4·5	—	0·013
Terbutaline	95·6	4·4	—	0·009
Cp 22352-1	80·6	19·4	—	0·027
Pirbuterol	78·0	22·0	—	0·013

In our opinion, it is not possible to derive conclusive evidence from the data presented arguing for the involvement of one of the four species in biological activity, or for the importance of K_Z. Whereas the relative fractions of the species with respect to the isomers isoprenaline and orciprenaline are practically identical, both compounds differ in affinity, selectivity and intrinsic activity on several β-adrenoceptor preparations, both intact organs and membrane fractions (O'Donnell & Wanstall 1974; Bilezikian et al 1978: Kaumann 1981). Obviously, moving the 4-hydroxy group to position 5 of the phenyl nucleus has a dramatic influence on biological activity, due to factors other than differences in deprotonization.

Some suggestions relating to the ionization schemes can be made. In the first place, uncharged molecules, because of lack of formation in most compounds, do not seem to be associated with biological activity, although experimental conditions differ from the physiological situation. This immediately raises the question, which of the other three species (i.e. cation, zwitterion, anion) is/are involved. Hardman & Reynolds (1965) and Reynolds & Hardman (1972) studied the pH-dependence of the effects of adrenaline on turtle heart and rabbit ileum. With increasing pH (up to pH 9·5) the sensitivity for adrenaline decreased in parallel with the fraction of adrenaline-cations present. Wagner et al (1975) and Camilión de Hurtado et al (1981), however, reported significant pH-dependent pD_2-changes on rabbit aortic strip and isolated rat atria respectively, for several β-adrenoceptor agonists in a pH-region where the concentration of cations hardly changes.

Since detailed studies of the pH-influence on agonist-induced displacement of radioligands from membrane fractions, as a more direct approach to measure receptor-affinity, have not been reported thus far, it remains questionable which species is/are responsible for β-adrenoceptor affinity and intrinsic activity. Finally, it should be realized, that with pD_2-values or other parameters based on 'total drug concentration', the relative importance of the different species is de facto neglected (cf. pirbuterol), which might reflect on the quantification of structure-activity relationships of β-adrenoceptor agonists.

Acknowledgements

We wish to thank Mrs Bea Fontijn-Brokmann for excellent secretarial assistance. The generous gifts by Allenburys, Astra, Boehringer Ingelheim and Pfizer of the compounds mentioned are gratefully acknowledged.

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