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^{1}H and ^{13}C nuclear magnetic resonance studies of the sites of protonation in itraconazole and fluconazole

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Abstract

^{1}H and ^{13}C nuclear magnetic resonance spectra of itraconazole were measured in neutral solution, and upon addition of varying amounts of deuterated hydrochloride acid. The comparison of the chemical shift in the different solutions revealed the high proton affinity of the piperazine nitrogen N26. Upon addition of a surplus of acid, the triazole ring was protonated. Corresponding observations were made for fluconazole.

Keywords: Itraconazole; Sites of protonation; Nuclear magnetic resonance

1. Introduction

Itraconazole ((±)-2-sec-butyl-4-[4-(4-{4[(2*R**, 4*S**)-2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-ylmethoxy]phenyl} piperazin-1-yl)phenyl]-2,4-dihydro-1,2,4-triazol-3-one) is an orally active, azole-type antifungal agent, which can be used for the treatment of oropharyngeal and vulvovaginal candidiasis, as well as of dermatophytoses [1]. All azoles exert their activity through the specific inhibition of cytochrom P-450. Since the N-3 atom of the azole moiety (see Scheme 1) is believed to bind to the ferric ion in the heme prosthetic group and the N-1 substituents are able to enhance the affinity [2], it is important to know whether one of the nitrogens will be protonated under physiological conditions or in the presence of the target protein. Thus, it was the purpose of this study to find out the site of protonation by means of ^{1}H and ^{13}C nuclear magnetic resonance (NMR) measurements using varying amounts of acid. For sake of comparison, NMR data of fluconazole and ketoconazole (see Scheme 1) were taken into account.

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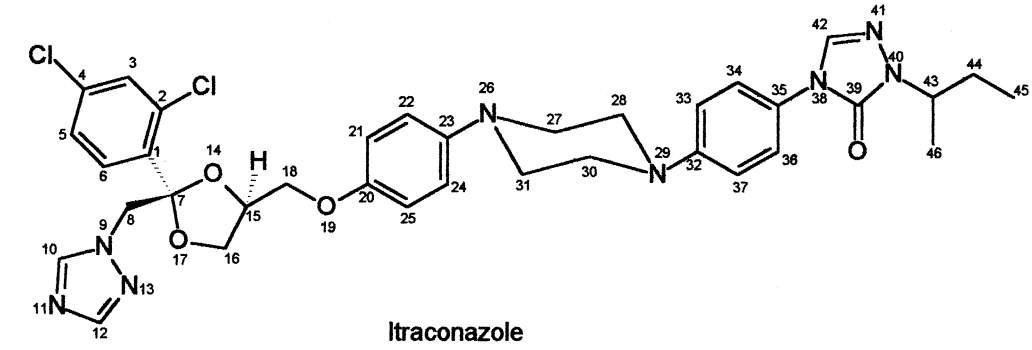
2. Materials and methods

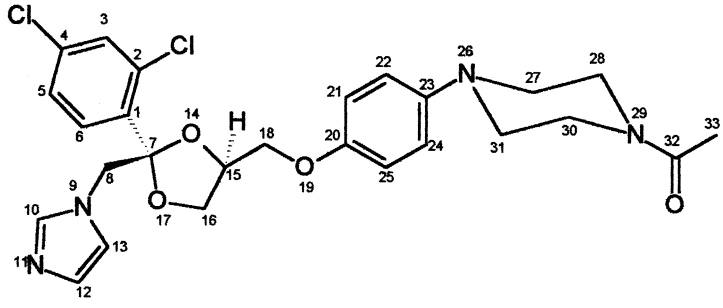
2.1. Sample preparation

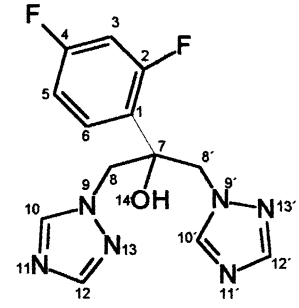
The samples for the high-resolution NMR experiments consist of about 10^{-4} M itraconazole and fluconazole dissolved in $CDCl_3$:CH_3OD:D_2O (or DCl) in a ratio of 16:8:1 (v/v/v) and DMSO-d_6:D_2O in a ratio of 4:1 (v/v), respectively. Defined equivalents of DCl (see Tables 2–4) were added while keeping the sample concentration constant. Itraconazole was a gratefully received gift from Prof. Dr M. Ertan, fluconazole was provided by Pfizer (Karlsruhe, Germany).

2.2. NMR spectroscopy

All spectra were recorded using a Varian XL 300 spectrometer operating at 299.958 MHz (^{1}H) and 75.433 MHz (^{13}C) with a probe temperature of 20°C. The ^{1}H chemical shifts were referenced to the CD_3OD signal at $\delta = 3.30$ ppm and to the DMSO-d_6 signal at $\delta = 2.49$ ppm, respectively. The ^{13}C chemical shifts were referenced to the centre of the solvent signal: $\delta = 49.0$ for CD_3OD and 39.5 for DMSO-d_6. One hundred and twenty-eight scans over a frequency width of 3999.9 MHz were collected into 32 K data points giving a digital resolution of 0.123 Hz/pt for ^{1}H NMR. ^{13}C NMR spectra were measured in the ^{1}H-broadband decoupled mode which is implemented in the VARIAN software package; homo- and heteronuclear shift correlations, i.e. COSY, HETCOR and COLOC, were also performed using the VARIAN software package. Abbreviations for data quoted are: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet.







Scheme 1.

Table 1
^{1}H NMR data: chemical shift δ (ppm), multiplicity, and J (Hz)

Itraconazole dissolved in $CDCl_3$:CD_3OD:D_2O (16:8:1, v/v/v)		Fluconazole dissolved in DMSO-d_6	
H-3	7.45, d, 2.1	H-3	6.86, m
H-5	7.25, dd, 8.5; 2.1	H-5	7.16, m
H-6	7.56, d, 8.5	H-6	7.18, m
H-8a	4.84, d, 14.8	H-8a/8a′	4.73, d, 14.5
H-8b	4.76, d, 14.8	H-8b/8b′	4.55, d, 14.5
H-10	8.30, s	H-10/10′	8.30, s
H-12	7.86, s	H-12/12′	7.78, s
H-15	4.35, m	OH	6.31, s
H-16a	3.91, dd, 8.4; 6.8		
H-16b	3,79, dd, 8.4; 5.4		
H-18a	3.75, dd, 10.0; 4.9		
H-18b	3.56, dd, 10.0; 5.7		
H-21/25	6.80, d, 9.2		
H-22/24	6.96, d, 9.2		
H-27/31	3.21, m		
H-28/30	3.35, m		
H-33/37	7.04, d, 9.1		
H-34/36	7.39, d, 9.1		
H-42	7.76, s		
H-43	4.23, m		
H-44a	1.81, m		
H-44b	1.70, m		
H-45	0.86, t, 7.3		
H-46	1.37, d, 6.8		

2.3. Molecular modelling

Itraconazole was modelled by means of standard geometries implemented in PC SPARTAN (Wavefunctions, Irvine, CA, USA, 1996). The geometry optimization was achieved using the force field program (MMFF for organic molecules) and the semiempirical AM1 method [3] in SPARTAN. The atomic charges were calculated using the 'Fits to electrostatic potential' option in SPARTAN.

3. Results and discussion

The structure and numbering scheme for itraconazole and fluconazole are given in Scheme 1. Due to solubility problems in aqueous systems, the NMR measurements were performed in mixtures of organic solvents with deuterated water. The ^{1}H NMR chemical shifts and coupling constants of itraconazole and fluconazole in neutral deuterated solvent are depicted in Table 1. ^{1}H NMR and ^{13}C NMR chemical shifts of itraconazole, measured in neutral solvent, and with one, two three, and four equivalents of DCl are given in Tables 2 and 3. Correlation spectra were run to assign both proton and carbon resonances. In addition, the long-range COLOC experiments helped to ensure the assignments.

The ^{1}H NMR chemical shifts of fluconazole, measured in neutral solvent, and with 0.5, 1, 1.5 and 2 equivalents of DCl, are given in Table 4.

3.1. Proton chemical shift in neutral solution

3.1.1. Itraconazole

The ^{1}H NMR spectrum of itraconazole (see Table 1 and Fig. 1) can be divided into three parts. The first part, from 0.8 to 1.9 ppm, is aliphatic showing, with the exception of the methine hydrogen, the signals of the isobutyl residue

on the triazole ring. The second part of the proton spectrum consists of all signals belonging to the piperazine hydrogens, and the dioxolane and neighbouring hydrogens: The piperazine hydrogens show the typical pattern of two narrow multiplets at $\delta = 3.21$ and 3.35 ppm, which can be more precisely assigned after protonation (see Section 3.3). The methylene group attached to the phenoxy and dioxolane rings (H-18a/b) gives two double doublets at $\delta = 3.56$ and 3.75 ppm, whereas the dioxolane hydrogen signals are characterized by two double doublets (H-16a/b) and a complicated multiplet system for the proton H-15. The methylene group connecting the triazole and dioxolane rings (H-8a/b) shows an AB system at $\delta = 4.76$ and 4.84 ppm. The third part of the spectrum, from $\delta = 6.7$ to 8.3 ppm, contains the signals of the aromatic protons. The dichlorobenzene and the phenoxy rings can be assigned analoguous to ketoconazole, studied by Dawson [4]. The dichlorobenzene hydrogens give the typical pattern of a 1,2,4-trisubstituted phenyl ring with a doublet ($J = 8.5$ Hz) at $\delta = 7.56$ ppm, a doublet at $\delta = 7.45$ ppm with a meta-coupling constant ($J =$ 2.1 Hz) and a double doublet at $\delta = 7.25$ ppm containing both constants. According to the para substitution, the aromatic ring between the piperazine and triazine is characterized by an AB system. Due to the effect of the piperazine, the hydrogens H33/37 are upfield shifted (7.04 ppm) in comparison to H34/36, which feel the electron-withdrawing effect of the amido moiety in the triazole. The hydrogens of the triazole ring systems show three singlets, which were assigned by means of the COLOC experiment, especially using the cross peaks between C12 and H10, and C10 and H12 in the left-hand triazole, and C39 and H42 in the right-hand triazolone ring (see Fig. 2).

Table 2
^{1}H NMR chemical shift δ (ppm) upon addition of no, one, two, three and four equivalents of DCl (eq. DCl)

	Itraconazole dissolved in $CDCl_3$:CD_3OD:D_2O (16:8:1, v/v/v)				
	0 Eq. DCl	1 Eq. DCl	2 Eq. DCl	3 Eq. DCl	4 Eq. DCl
H-3	7.45	7.47	7.48	7.49	7.49
H-5	7.25	7.29	7.32	7.33	7.33
H-6	7.56	7.61	7.64	7.65	7.66
H-8a	4.84	4.87	4.99	5.06	5.08
H-8b	4.76	4.79	4.91	4.99	5.01
H-10	8.30	8.40	9.20	9.66	9.84
H-12	7.86	7.90	8.33	8.56	8.65
H-15	4.35	4.41	4.42	4.44	4.45
H-16a	3.91	3.93	3.94	3.95	3.96
H-16b	3.79	3.82	3.82	3.83	3.84
H-18a	3.75	3.82	3.93	4.04	4.08
H-18b	3.56	3.69	3.79	3.83	3.84
H-21/25	6.80	6.98	7.00	7.01	7.02
H-22/24	6.96	7.66	7.76	7.79	7.81
H-27/31	3.21	3.70	3.79	3.83	3.89
H-28/30	3.35	3.74	3.79	3.83	3.89
H-33/37	7.04	7.13	7.16	7.21	7.29
H-34/36	7.39	7.46	7.47	7.50	7.54
H-42	7.76	7.85	7.86	7.88	7.90
H-43	4.23	4.23	4.23	4.22	4.22
H-44a	1.81	1.81	1.82	1.82	1.82
H-44b	1.70	1.71	1.71	1.71	1.71
H-45	0.86	0.86	0.87	0.87	0.87
H-46	1.37	1.37	1.37	1.37	1.37

Table 3
^{13}C NMR chemical shift δ (ppm) upon addition of no, one, two, three and four equivalents of DCl (eq. DCl)

	Itraconazole dissolved in $CDCl_3$:CD_3OD:D_2O (16:8:1, v/v/v)				
	0 Eq. DCl	1 Eq. DCl	2 Eq. DCl	3 Eq. DCl	4 Eq. DCl
C-1	136.54	136.69	136.93	137.07	137.10
C-2	133.59	133.61	133.64	133.70	133.70
C-3	131.83	131.91	131.97	132.02	132.02
C-4	134.47	134.29	133.67	133.40	133.28
C-5	127.76	127.86	127.97	128.04	128.06
C-6	130.21	130.26	130.34	130.42	130.43
C-7	108.05	108.13	107.59	107.34	107.22
C-8	54.14	54.07	54.81	55.30	55.46
C-10	145.58	145.59	144.41	143.81	143.56
C-12	151.15	150.73	146.69	144.51	143.63
C-15	75.29	75.03	75.11	75.21	75.23
C-16	67.66	67.24	66.89	66.78	66.72
C-18	68.30	68.10	68.09	68.15	68.17
C-20	153.41	159.15	159.61	159.66	159.69
C-21/25	115.88	116.58	116.69	116.77	116.82
C-22/24	119.20	122.69	123.22	123.36	123.40
C-23	146.39	136.28	135.22	135.19	135.05
C-27/31	51.22	55.44	55.77	55.65	55.36
C-28/30	49.57	47.70	47.60	47.91	48.44
C-32	151.26	149.51	149.07	148.54	147.54
C-33/37	117.15	118.15	118.36	118.66	119.16
C-34/36	124.44	124.50	124.46	124.44	124.39
C-35	125.99	127.51	127.81	128.34	129.19
C-39	152.86	152.78	152.72	152.70	152.66
C-42	135.31	135.21	135.13	135.10	134.97
C-43	53.54	53.62	53.59	53.62	53.63
C-44	28.82	28.80	28.78	28.79	28.77
C-45	11.02	11.01	11.01	11.01	11.01
C-46	19.52	19.50	19.50	19.49	19.49

Table 4
^{1}H NMR chemical shift δ (ppm) upon addition of 0, 0.5, 1, 1.5 and 2 equivalents of DCl (eq. DCl)

	Fluconazole dissolved in DMSO-d_6:D_2O (4:1, v/v)				
	0 Eq. DCl	0.5 Eq. DCl	1 Eq. DCl	1.5 Eq. DCl	2 Eq. DCl
H-3	6.78	6.79	6.80	6.80	6.81
H-5	7.07	7.07	7.08	7.08	7.08
H-6	7.07	7.07	7.08	7.08	7.08
H-8a/8a′	4.75	4.78	4.80	4.82	4.84
H-8b/8b′	4.53	4.58	4.62	4.65	4.69
H-10/10′	8.25	8.42	8.58	8.71	8.84
H-12/12′	7.74	7.84	7.95	8.03	8.11

3.1.2. Fluconazole

Even though the ^{1}H NMR spectrum of fluconazole was recorded in a different solvent, i.e. DMSO-d_6:D_2O (4:1, v/v), the chemical shifts of the triazole hydrogens as well as the methylene groups are almost in the same place as those found for itraconazole (see Table 1). The three hydrogens of the difluoro substituted phenyl ring were assigned using the coupling pattern caused by the fluorine atoms.

3.2. Carbon chemical shift in neutral solution

Having fully assigned the ^{1}H NMR spectrum of itraconazole in neutral solution, the assignment of the ^{13}C NMR spectrum can be done by means of

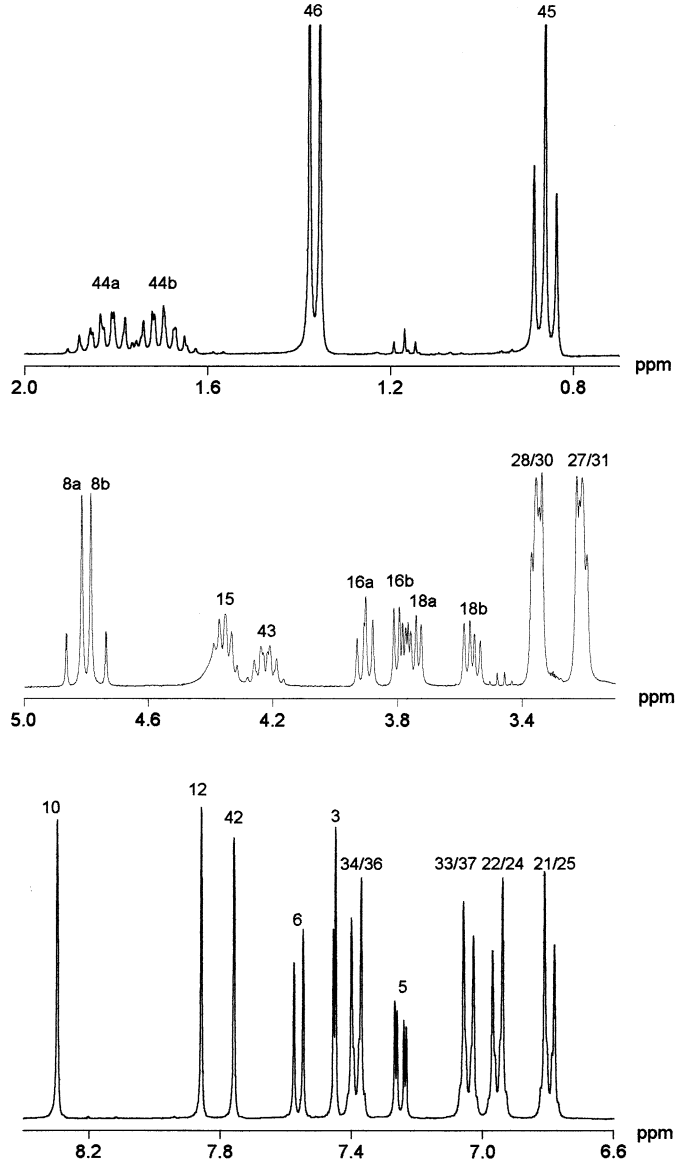


Fig. 1. ^{1}H NMR spectrum of itraconazole dissolved in $CDCl_3$:CH_3OD:D_2O (16:8:1, v/v/v).

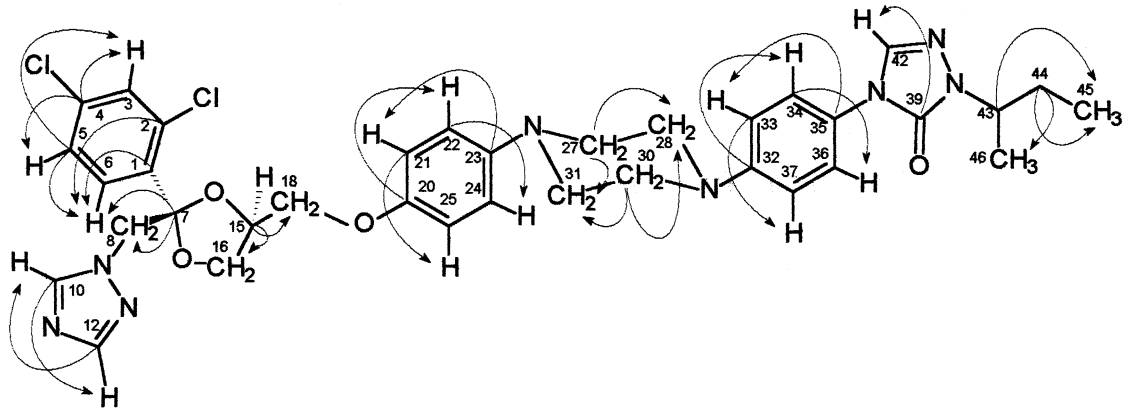


Fig. 2. Formula of itraconazole indicating the ^{2}J and ^{3}J couplings found in the COLOC experiment.

the HETCOR experiment. The chemical shifts of the carbon atoms of the left hand part of the molecules are in accordance with the δ-values of the corresponding carbons of ketoconazole reported by Dawson [4]. In order to ensure the assignment of the quaternary atoms, a COLOC experiment was exploited. The experiment enabled the resonances to be divided into isolated systems for either aromatic ring system. The chemical shifts of the phenoxy and dichlorophenyl carbon atoms are in agreement with findings in ketoconazole. All that remains is the assignment of three quaternary carbons, the phenyl C32 and C35, and the triazolone carbonyl carbon C39. The strong correlation of the signal at $\delta = 125.99$ ppm with the hydrogen at $\delta = 7.04$ ppm (i.e. H33/37) found by the COLOC experiment (Fig. 2) indicates that $\delta = 125.99$ ppm corresponds to C35. The carbon atom C32 can similarly be assigned to the signal at $\delta = 151.26$ ppm. The strong downfield shift of C32 is caused by the piperazine nitrogen. In comparison, the deshielding effect of the N-amido group of the triazolone on C35 is smaller, which is in agreement with increments reported in Ref. [5].

3.3. Chemical shifts in dependence on the addition of equivalents of DCl

3.3.1. Itraconazole

Fig. 3 impressively shows the downfield shift of the hydrogens which are mostly influenced upon the addition of the varying amounts of hydrochloride acid. The addition of one equivalent of acid resulted in a shift of $\Delta\delta + 0.49$ ppm for the H27/H31 hydrogens, whereas the neighbouring hydrogens at C28/C30 were less downfield shifted ($\Delta\delta = +0.39$ppm) (Fig. 3a). The difference in the influence on both attached phenyl rings is more significant: The ortho phenyl hydrogens attached to N26 (H22/24) exhibit a shift of $\Delta\delta = +0.70$ ppm, whereas the corresponding protons at the phenyl ring attached to N29 (H33/37) show a less pronounced shift of $\Delta\delta = +0.09$ ppm (Fig. 3b). This clearly indicates that the first protonation took place at N26. The differences in the carbon chemical shifts support this hypothesis. As expected for a protonation at N26, the carbon atoms C27/31 are downfield shifted, whereas the neighbouring atoms C28/30 are slightly upfield shifted. The carbon atoms of the attached phenyl ring are also influenced: the ipso carbon is strongly shielded, the ortho and para carbons are deshielded, and the meta carbons stay almost the same (see Fig. 4b), which is in agreement with observations reported in the literature. Interestingly, the hydrogens of the methylene group C18 still feel the influence of the positive charge located in the piperazine ring (see Fig. 3d). This may be explained by mesomeric effects.

Further addition of hydrochloride acid results in a strong downfield shift of the hydrogens H10/12 located in the triazole ring (e. g. $\Delta\delta$(H10) = 1.54 ppm, $\Delta\delta$(H12) = 0.79 ppm). Additionally,

the hydrogens of the neighbouring methylene groups also show a slight downfield shift. These findings indicate a protonation in this ring system. The carbon atoms C12, and to a lesser extent C10, are upfield shifted upon protonation, which is in line with similar observations reported for imidazole. Now the question arises at which of the three nitrogens the added hydrogen is located. Semiempirical calculations may help to answer this question. Since the chemical shifts of carbon atoms depend sensitively on the electronic charges [6], the charges were computed by means of the AM1 method, taking the protonation of the piperazine ring at N26 and every nitrogen in the triazole ring (N9, N11 and N13, respectively) into account. As can be seen in Fig. 5a, in case of the protonation at N26, the changes in the atomic charges of C20–C25 nicely mirror the changes in the chemical shifts of the carbon atoms upon addition of one equivalent of DCl (compare with Fig. 4b). This finding demonstrates that the semiempirical calculation will be an appropriate

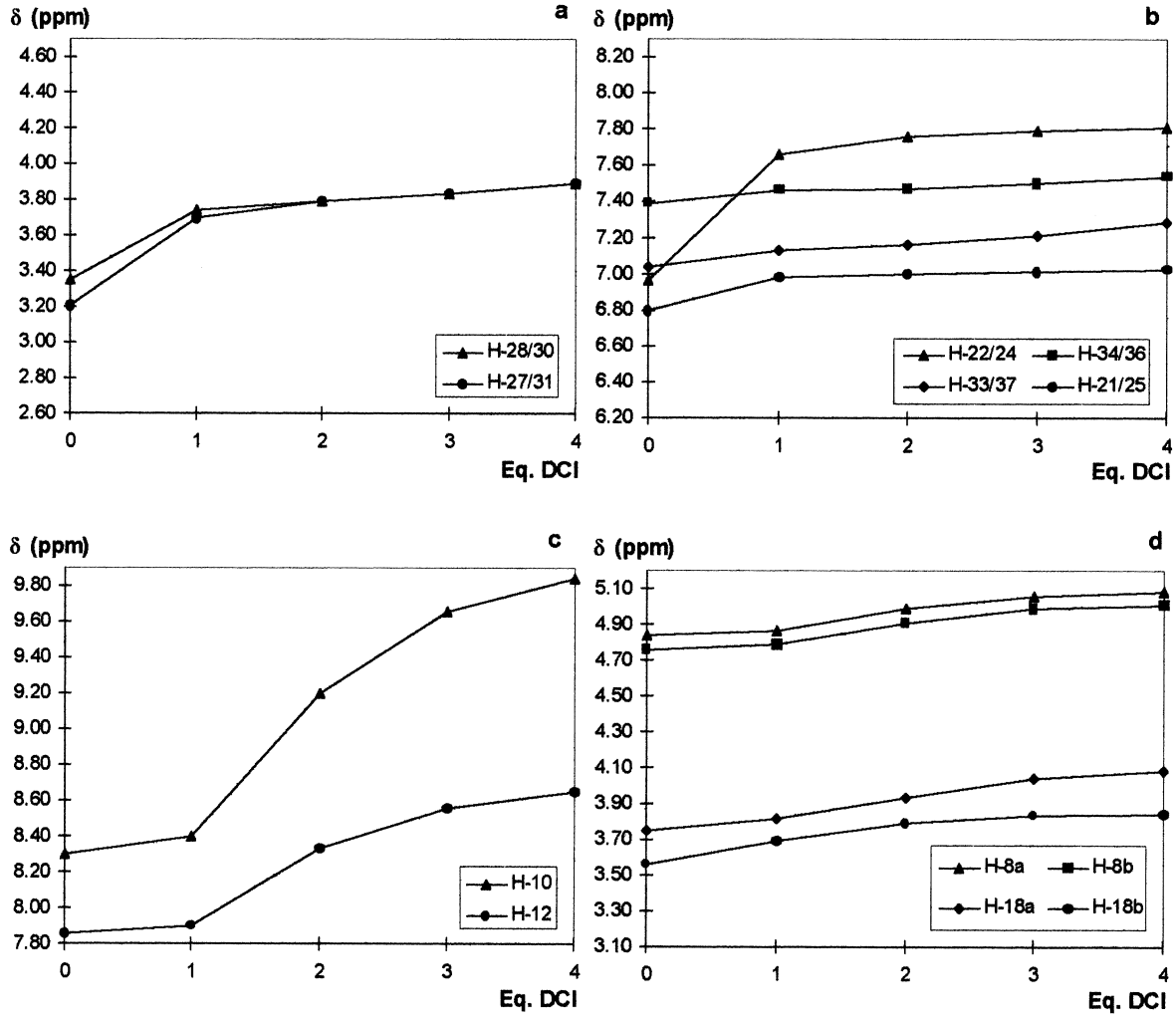


Fig. 3. Chemical shift of itraconazole hydrogens of the piperazine ring (a), the phenyl rings attached to the piperazine (b), the triazole ring (c) and methylene groups (d) in dependence of zero, one, two, three, and four equivalents of deuterated hydrochloric acid.

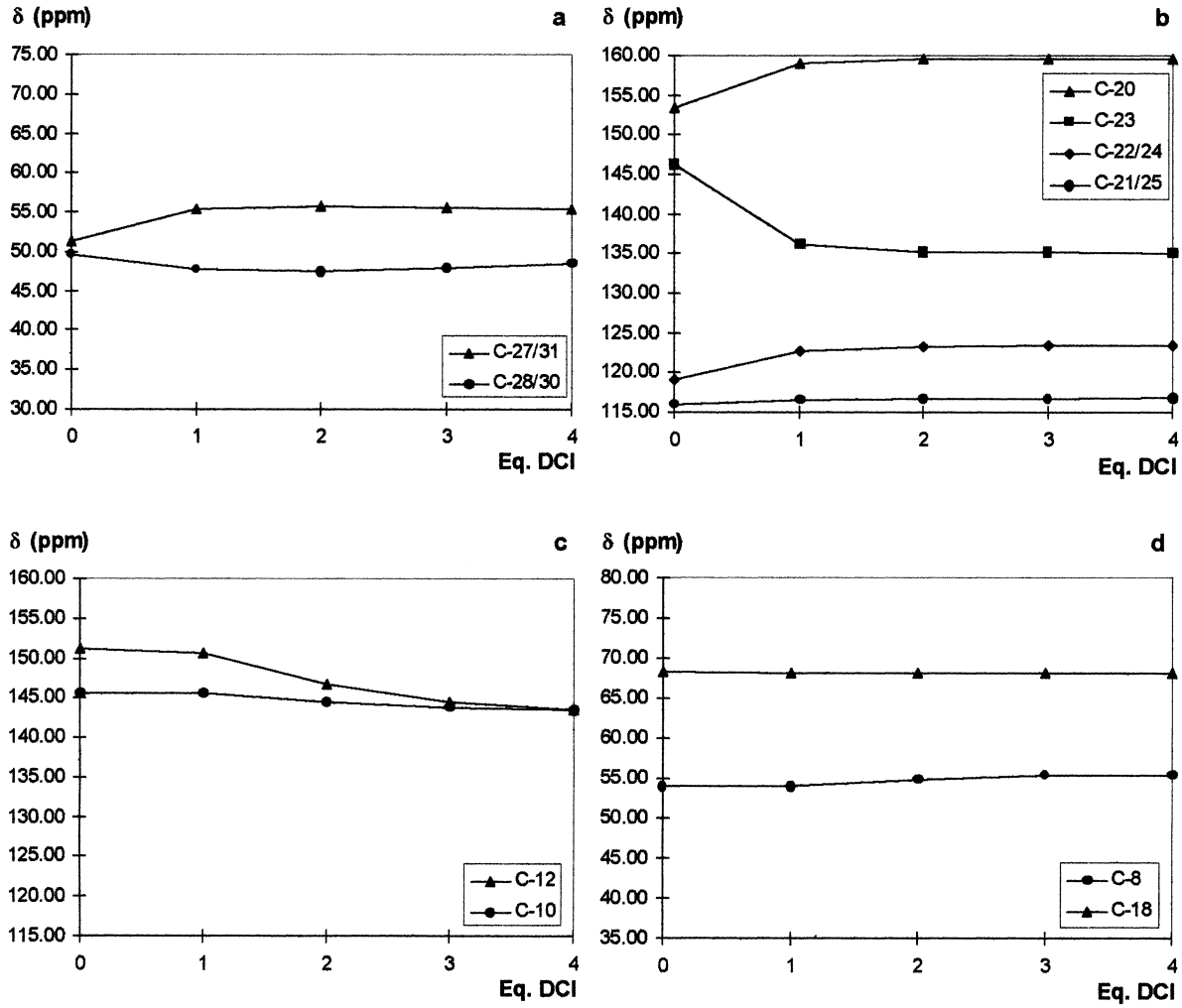


Fig. 4. Chemical shift of itraconazole carbon atoms of the piperazine ring (a), the phenyl ring attached to N26 (b), the triazole ring (c) and methylene groups (d) in dependence of zero, one, two, three, and four equivalents of deuterated hydrochloric acid.

method to find the locus of the second protonation in the triazole system. The atomic charges of the triazole carbon atoms C10 and C12 are differently influenced by the protonation at N9, N11, and N13. The changes to the charges on addition of a hydrogen at N11 (see Fig. 5c) are in line with the changes of the chemical shifts of the carbon atoms upon addition of two or more equivalents of acid. The calculation of the protonation at N9 or N13 (see Fig. 5b,d) predicts a downfield shift of the carbon atom C10 and C12, which is in contrast to the NMR measurements. Taken together, these findings indicate that the protonation of the triazole ring takes place at N11.

Since the differences in the heats of formation for each protonation type are rather high (higher than the error in the calculation of the heats of formation [7]), the theoretical, semiempirical calculation supports the hypothesis of N11 protonation derived from the NMR measurements: The heat of formation for the N26/N11 diprotonated itraconazole was found to be lower ($\Delta H = 429.8$ kcal/mol) than for the N26/N9 ($\Delta H = 463.3$kcal/mol) and N26/N13 diprotonated molecule ($\Delta H = 440.7$ kcal/mol).

3.3.2. Fluconazole

Since fluconazole does not have a piperazine ring, only two equivalents of DCl were added. As expected, the hydrogens of the triazole ring are mostly influenced upon addition of the acid (see Table 4). Again the H10 is more downfield shifted than H12, but the shifts are not as strong as observed for itraconazole. This may indicate that the triazole rings in fluconazole have a smaller proton affinity than the triazole in itraconazole.

4. Conclusion

The NMR spectroscopic measurements of itraconazole unambiguously showed that the piperazine nitrogen N26 had the highest proton affinity. In a second step, upon addition of a surplus of acid, the triazole ring was protonated. Whereas the piperazine nitrogen was fully protonated after addition of one equivalent of DCl (almost no further downfield shift of the corre-

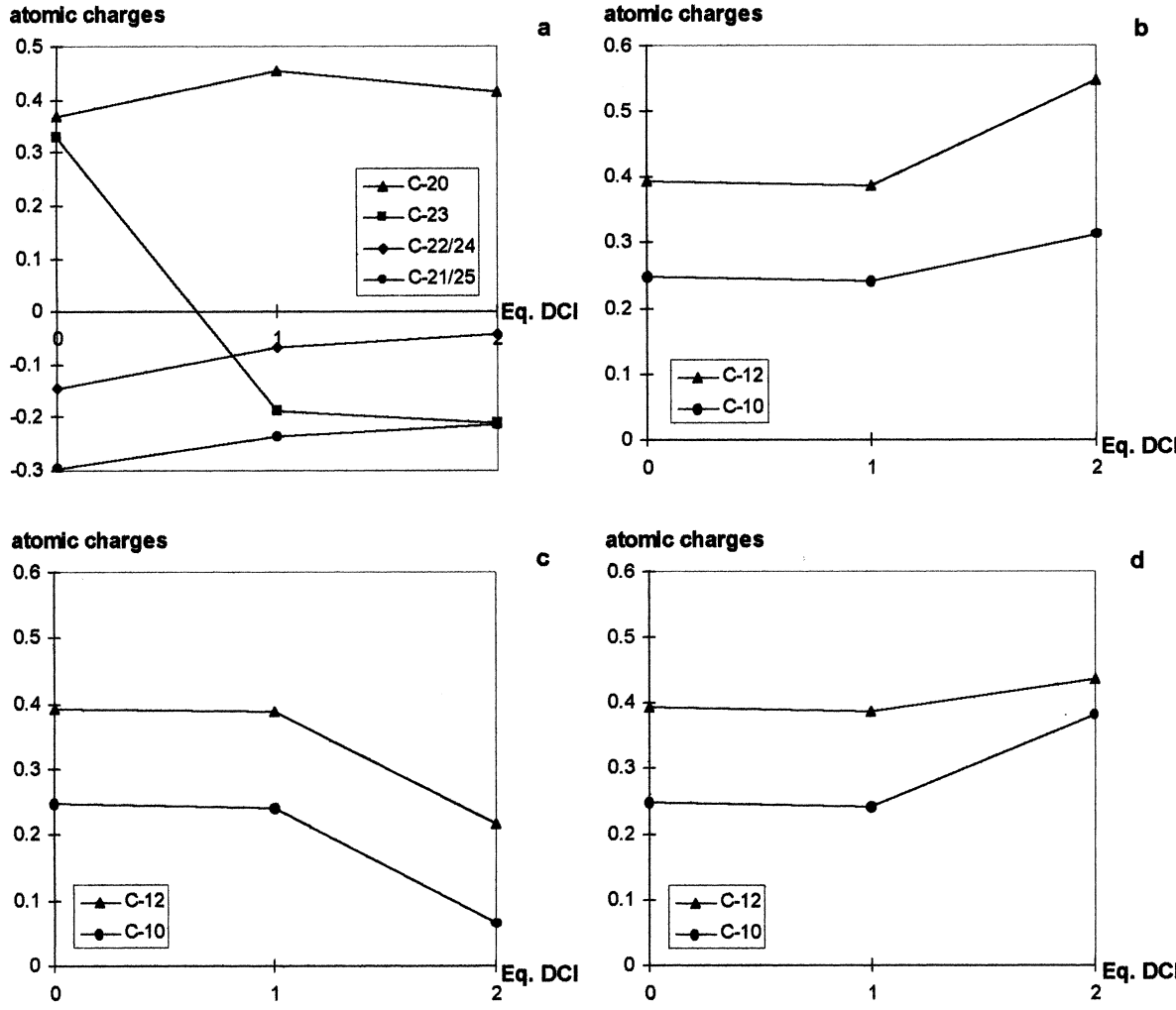


Fig. 5. Atomic charges of the carbon atoms of the phenoxy ring at neutral conditions, and after protonation of N26 and in addition of N11 (a), of the triazole ring at neutral conditions and after protonation of N26 and in addition of N9 (b), of N11 (c) and of N13 (d). Plot of the atomic charges versus equivalents of acid.

sponding hydrogens upon addition of the surplus of DCl), the quantitative protonation of the triazole nitrogen N11 affords a surplus of acid. Similar observations were made for fluconazole.

Even though the solvents of the NMR measurements do not imitate physiological conditions, which is due to the dissolution problems in aqueous medium, it is more likely that itraconazole can be protonated at N26 in physiological medium or in the presence of the target protein rather than at N11.

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