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Structural Elucidation of Warfarin, Application of the DP4+ Method to Equilibrium Systems †

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Abstract

Warfarin is an oral anticoagulant widely used in the treatment of cardiovascular diseases and remains a relevant model in pharmacological and structural studies. Its behavior in solution, particularly the equilibrium between different neutral species, is crucial for understanding its bioactivity and affinity for its molecular target, the VKOR enzyme. Nuclear magnetic resonance (NMR) studies and theoretical calculations based on density functional theory (DFT) have confirmed the existence of keto and hemiacetal forms in dynamic equilibrium. The DP4+ method, which compares experimental and calculated chemical shifts statistically, has been tested for the validation of structures of species in equilibrium.

Keywords: equilibrium; warfarin; DP4+; NMR

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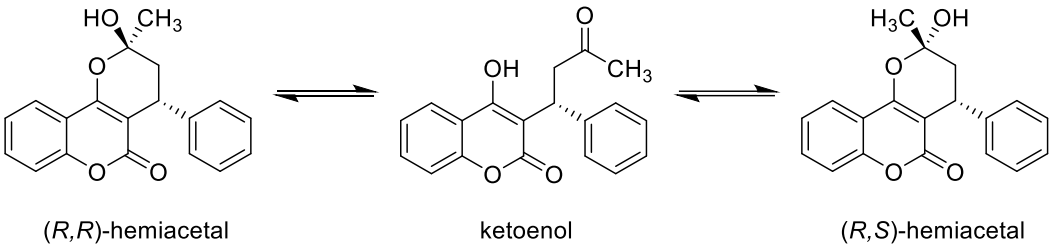
1. Introduction

Warfarin (2-hydroxy-3-(3-oxo-1-phenylbutyl)chromen-4-one) is a drug commonly used as anticoagulant to prevent thrombosis and thromboembolism. Warfarin action involves the decrease of blood coagulation by inhibiting the active site of VKOR, the vitamin K dependent epoxide reductase [1]. It is usually described in literature as an open tautomer (Scheme 1, center), although it has been shown that it exists as well as two diastereomeric hemiacetal forms (Scheme 1, left and right) [2].

The identification of the tautomers of drugs has important implications, since these molecules are capable of tautomerism and they can adapt their appearance to their environment [3]. Temperature, solvent and pH can influence the tautomeric equilibrium [4]. Among the several possible techniques used for studying tautomerism, NMR has become very useful [5]. In this sense, the tautomerism of warfarin has been studied by a variety of techniques [6], concluding that the hemiacetal forms are more stable than the open chain tautomer. However, the matter of which tautomer is more stable (*R*,*R* or *R*,*S*) is somehow controversial [2,3,7].



Scheme 1. Possible tautomers of (*R*)-warfarin.

In this communication, we compare NMR spectra of warfarin with theoretical calculations (carried out on the three possible structures depicted in Scheme 1) by means of the DP4+ method, which has recently proved efficient in comparing tautomers in equilibrium [8].

2. Materials and Methods

2.1. Materials

Warfarin as obtained from a racemic sodium salt commercialized under the name Circuvit 5 mg®. The solid was isolated by dissolution in distilled water and subsequent filtration under vacuum in order to remove insoluble residues. Neutral warfarin was precipitated from the aqueous solution by controlled acidification with conc. HCl. The solid was filtrated and dried in vacuum until constant weight.

DMSO-d_6 was purchased from Sigma Aldrich (99.9% purity).

2.2. NMR Spectra

The NMR spectroscopy analyses of warfarin were performed in DMSO-d_6 and recorded using a Bruker Avance Neo 500 (500 MHz for ^{1}HNMR and 125.7 MHz for ^{13}CNMR) using TMS as internal standard.

Typical spectral conditions for ^{1}HNMR experiments were as follows: acquisition time 3 s. 8–16 scans per spectrum. Spectral width 10,000 Hz. Digital resolution was 0.32 Hz per point. Spectra were measured at 25 °C. Sample concentrations were 0.05 M.

Typical spectral conditions for proton decoupled ^{13}CNMR were as follows: spectral width 27,000 Hz, acquisition times 1.3 s and 512–1000 scans per spectrum.

2.3. Theoretical Calculations

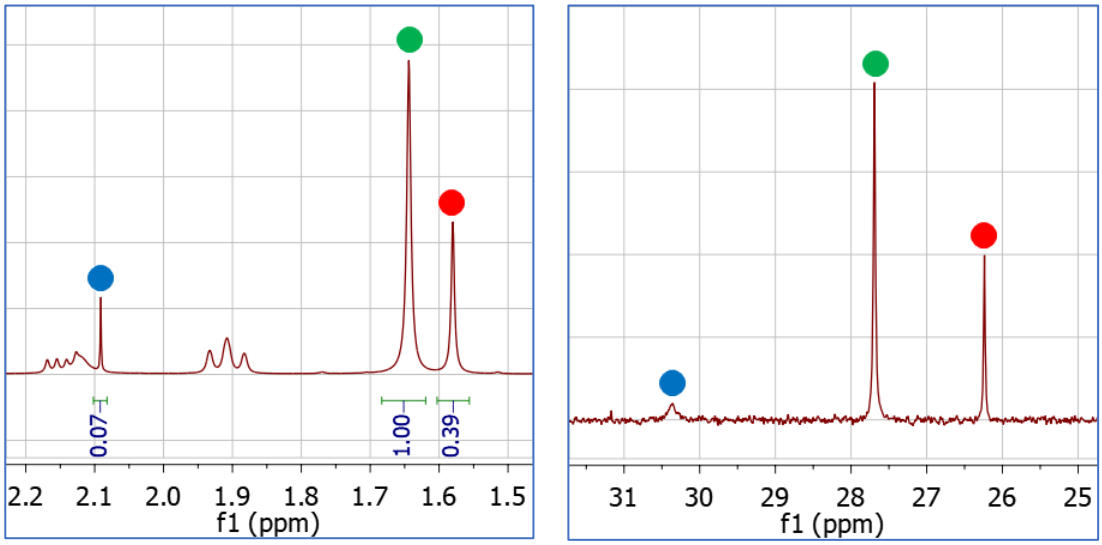
Theoretical calculations were carried out in two steps. Firstly, each one of the three isomers of warfarin (ketoenol, (*R*,*R*) and (*R*,*S*) hemiacetal was subjected to geometry optimization by means of the DFT method [9]. Gaussian 03 package was used to perform the B3LYP hybrid exchange-correlation functional [10] at the 6-311+G(d,p) level of theory in vacuum. The energies of different structures were evaluated by rotation of all unrestrained bonds.

Chemical shifts relative to TMS were then calculated for ^{1}H and ^{13}C nuclei in all isomers as follows. The isotropic magnetic shielding tensors were evaluated in DMSO by means of GIAO NMR calculations at the B3LYP/6-31+G(d,p) level of theory using the polarization continuum model, SCRF-PCM version [11]. Each scalar isotropic shielding value (σi) was turned into a chemical shift by subtracting it from the corresponding σ of ^{1}H or ^{13}C of TMS (calculated using the same method and basis set) [5].

DP4+, a method which compares experimental and calculated chemical shifts statistically, was used in order to estimate the accuracy of the calculated NMR data and to assign each set of signals to a specific structure. The DP4+ calculations were carried out by means of a free spreadsheet available online [12].

3. Results and Discussion

The ^{1}HNMR and ^{13}CNMR spectra of warfarin show many duplicated signals, which can be interpreted as the presence of two (or more) species in the system. As an example, Figure 1 shows the high field region of both spectra: where only one methyl signal was expected, there are three (1.58, 1.64 and 2.09 ppm in ^{1}HNMR and 26.2, 27.7 and 30.4 ppm in ^{13}CNMR, marked with color spots in Figure 1). HSQC permitted to match H and C atoms corresponding to the same methyl group (data not shown).

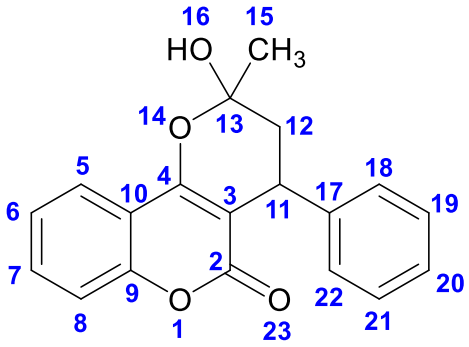


(**a**) (**b**)

Figure 1. High-field expansion of ^{1}HNMR (**a**) and ^{13}CNMR (**b**) spectra. The peaks marked with a colored spot correspond to methyl groups. Same-colored peaks correspond to attached C and H atoms (confirmed by HSQC).

The spectra show two major sets of signals (suggesting two major species), which can be grouped by means of integration as well as bidimensional NMR spectra (^{1}H-^{1}H COSY, HSQC and HMBC). A minor set of signals indicates a third molecule in low concentration (blue dots in Figure 1), whose peaks could not be fully identified and are not considered among the experimental data.

Given that the structures shown in Scheme 1 are quite similar (especially the two diastereomeric hemiacetals), theoretical calculations have been carried out in order to assign each set of peaks to each theoretical structure. Only the most abundant experimental peaks were considered for this assignment. Table 1 shows the experimental and calculated chemical shifts. The atom numbering follows Scheme 2.



Scheme 2. Atom numbering followed in NMR assignment.

Table 1. Experimental and calculated chemical shifts (according to atom numbering in Scheme 2).

Atom		Experimental		Calculated		
				Hemiacetal		Ketoenol
		Major	Minor	(*R*,*S*)	(*R*,*R*)	
coumarin	C2	160.69	161.14	160.5021	159.1478	159.5214
	C3	103.88	102.43	99.2314	106.8387	111.1335
	C4	159.26	159.86	160.8968	159.2033	157.1524
	C5	123.17	123.11	121.5922	123.3723	122.4145
	C6	124.46	124.54	121.703	121.3146	122.0967
	C7	132.41	132.49	129.994	129.7804	129.7285
	C8	116.65	116.73	114.86	114.0824	114.3775
	C9	152.78	152.82	153.1458	151.4476	151.7762
	C10	116.09	115.96	114.2663	117.0582	114.7053
hemiacetal ring	C11	35.65	36.54	39.1527	43.7114	40.2349
	C12	43.30	42.03	42.1031	44.543	45.2572
	C13	100.12	101.86	105.5357	104.1946	210.8019
CH3	C15	27.69	26.24	29.3291	29.0448	31.7659
phenyl	C17	144.43	144.32	140.431	145.1129	139.1335
	C18/22	126.39	126.11	126.0794	125.89265	127.70125
	C19/21	128.71	128.34	126.6593	126.1457	124.42465
	C20	127.55	127.77	125.5924	124.1089	124.2069
coumarin	H5	7.85	7.85	8.3166	8.2309	8.2687
	H6	7.41	7.41	7.6621	7.6421	7.6868
	H7	7.65	7.65	7.9130	7.9622	7.9564
	H8	7.38	7.38	7.5056	7.5622	7.4885
hemiacetal ring	H11	4.01	4.02	4.5643	4.1499	4.7918
	H12a	2.34	2.30	2.7109	2.0437	2.5537
	H12b	1.91	2.14	2.5505	2.4118	4.5528
CH3	H15	1.64	1.58	1.5917	1.7464	2.37303
phenyl	H18/22	7.16	7.16	7.4676	7.45905	7.33855
	H19/21	7.25	7.25	7.8196	7.6828	7.63995
	H20	7.20	7.20	7.5056	7.4899	7.3422
OH	H16	7.21	7.21	3.3314	2.9495	6.8029

The experimental and theoretical chemical shifts were then compared by means of DP4+ method, which was created for testing two or more candidates against one set of experimental data and assigning a probability to each one of them, allowing to decide "which one is the best" [12]. Results are shown in Table 2.

Table 2. DP4+ probability calculated for unscaled chemical shifts of warfarin tautomers.

Isomer	DP4+ Probability		Relative Energy (kcal/mol)
	Major	Minor	
Hemiacetal (*R*,*R*)	64.2%	14.0%	0.0
Hemiacetal (*R*,*S*)	35.8%	86.0%	1.4
Ketoenol	0.0%	0.0%	5.0

DP4+ analysis suggests that the two major species are the hemiacetals, being the (*R*,*R*) form slightly more stable than the (*R*,*S*) isomer. The open ketoenol form appears to be less abundant than the closed ring tautomers. This assignation is in accordance with the calculated energies listed in Table 2.

4. Conclusions

Experimental spectra together with theoretical calculations permitted the study of the structure of the drug warfarin. DP4+ method was used to compare theoretical and experimental data. The most stable species in DMSO are two diastereomeric hemiacetals, being (*R*,*R*) slightly more stable than (*R*,*S*). The open keto form is much less stable and so much less abundant.

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Abbreviations

The following abbreviations are used in this manuscript:

NMR	Nuclear magnetic resonance
DMSO	Dimethyl sulfoxide
TMS	Tetramethyl silane
DFT	Density functional theory
COSY	Correlation spectrocopy
HSQC	Heteronuclear single-quantum correlation
HMBC	Heteronuclear multiple bond correlation

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