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## INTRODUCTION

Pyridoxamine (PM) is a vitamin B<sub>6</sub> derivative involved in a number of catalytic processes by virtue of its properties as enzyme cofactor<sup>1</sup>. Furthermore, it has been showed PM to be an inhibitor in non-enzymatic protein glycation<sup>2</sup> (Maillard reaction). The first step in a non-enzymatic glycation process is a reversible reaction between the terminal amino residues in the protein and the carbonyl group of the sugar, in its extended form, that leads to the formation of a Schiff base. The Schiff base is stable for a limited time after which it undergoes rearrangement to the Amadori compound. Subsequently, the Amadori compound can undergo various complex processes that result in the irreversible formation of advanced glycosilation end products (AGEs). It has been reported that changes in protein structure by the effect of the formation of AGEs are behind the development of various pathologies associated with hyperglycaemia<sup>3</sup>.

The mechanisms of inhibitory action of PM involves the scavenging of carbonyl compounds capable of modifying proteins. PM forms Schiff bases with such compounds<sup>2</sup>; therefore, an accurate knowledge of the mechanism of formation involved can be useful with a view to elucidating their pharmacological action. Nevertheless, kinetic studies on this subject are still scant<sup>4</sup>.

In this work, we study the reaction of PM with formaldehyde (FA), a very reactive carbonyl compound. The reaction was studied by using <sup>1</sup>H-NMR spectroscopy from pD 6 to pD 13 at 25 °C. Some of the reaction products were characterized by LC-MSD and NMR. Based on the results, a mechanism was proposed and the equilibrium constants were determined.

## MATERIALS AND METHODS

**Materials.** Pyridoxamine (PM), formaldehyde (FA, 37 % in water) and D<sub>2</sub>O (99.9 % D) were purchased from Sigma – Aldrich. The buffering material was reagent grade.

**NMR spectroscopy.** The NMR spectra were obtained on a Bruker AMX-300 spectrometer.

**LC-MS system.** Mass analyses were performed on a Agilent 1100 Series LC-MS instrument.

**Kinetics.** Equilibrium constants were determined by using the kinetic data processing Dynafit software<sup>5</sup> which performs numerical integration of the differential equations corresponding to a given kinetic scheme.

## RESULTS AND DISCUSSION

Figure 1 shows the <sup>1</sup>H NMR spectra for the reaction between PM and FA at different FA concentrations. It is observed that the peak areas associated to PM signals decreased when FA concentration was increased, and also, it exhibited new signals corresponding to the reaction products: a Schiff base, a carbinolamine compound (CA), and an hemiaminal isomer (HE)<sup>6</sup>. The Schiff base could be detected only at pD 12 and 13.

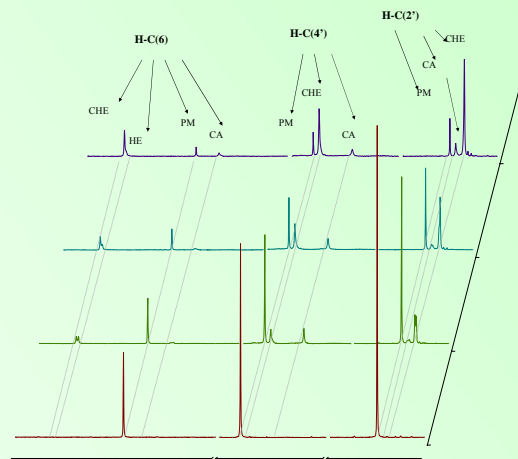
Scheme 1 shows the mechanism for the reaction between PM and FA. Initially, the nucleophilic attack of the amino group in PM with the carbonyl group in FA gives a carbinolamine (CA). Despite their high stability, carbinolamines are in equilibrium with their Schiff bases<sup>7</sup>; this facilitates the attack of the phenolate ion on the imine carbon to form its hemiaminal isomer (HE), product characterized by mass spectroscopy. This compound has been suggested in previous works between PM and glucose<sup>8</sup> and between PM and glyoxal/glycolaldehyde<sup>9</sup>. The excess of FA in the reaction mixture makes possible a new nucleophilic attack of the amino group in HE on another FA molecule to form a tertiary amine (CHE). The addition of a second FA molecule to HE was already described in reactions between primary<sup>10</sup> or aromatic<sup>11</sup> amines and FA.

The quantitative analysis of the NMR spectra allowed us to determine the equilibrium constants shown in scheme 1. Figure 2 shows the experimental concentration of the reaction products *versus* FA concentration. These values were obtained by integrating the signals of the compounds present in each <sup>1</sup>H-NMR spectrum for the different reaction mixtures of PM with FA. The equilibrium constants were obtained by fitting the experimental concentration changes for each compound to Scheme 1, by using Dynafit software<sup>5</sup>. The values obtained for the constants at different pH's are shown in table 1.  $K_2$  and  $K_3$  couldn't be calculated because Schiff base concentration was negligible. In spite of this fact, we determined an apparent constant  $K_{HE}$  ( $=K_2K_3$ ). Furthermore,  $K_1$  and  $K_4$  were also apparent constants because we didn't consider the acid base equilibrium of PM nor the hydration equilibrium of FA.

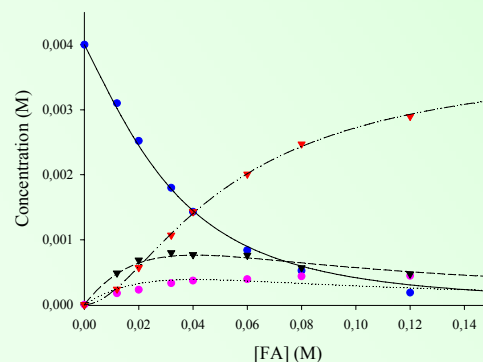
The values for  $K_1(ap)$  increases as pD becomes more basic as a consequence of the major proportion of deprotonated amine ( $pK_a = 10.7$ ). In the other hand,  $K_{HE}$  and  $K_4(ap)$  values are kept relatively constant up to pD 10 and then they diminish their values. These results are consistent with previous of Schiff base formation between amines and carbonyl compounds<sup>12</sup>.

## CONCLUSIONS

The reaction between PM and FA leads to the formation of CA, HE and CHE compounds. CA is the major product at pD higher than 10. The hemiaminal compound (HE) is the result of the attack of the phenolate ion at position 3 of the pyridine ring in PM on the imine carbon in the Schiff base. This reaction should be considered in studying reactions involving amine derivatives of vitamin B<sub>6</sub>. The formation of HE and CHE (addition of a FA molecule to HE) is favoured over that of the Schiff base around physiological pH.



**Figure 1.** <sup>1</sup>H-NMR spectra for the reaction between PM and FA at pD 7.0 (25 °C). PM concentration: 4 mM. FA concentrations: 0 mM (1), 20 mM (2), 40 mM (3) and 80 mM (4).



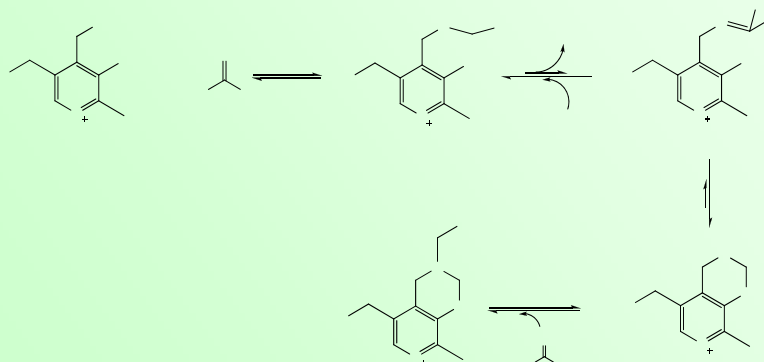
**Figure 2.** Graphical representation of the experimental concentrations of PM (●), CA (●), HE (▼) and CHE (▼) in front of FA concentration at pD 7 (25 °C). These values were obtained by integrating the signals at 7.63, 7.52, 7.95 and 7.96 ppm, respectively. Lines indicate the best theoretical fits.

**Table 1.** Equilibrium constants for the reaction between PM and FA (see scheme 1).

	Signals	$K_1(ap)$ (M <sup>-1</sup> )	$K_{HE}(ap)$	$K_4(ap)$ (M <sup>-1</sup> )
pD 6.0	H-C(6)	5.9 ± 0.3	1.9 ± 0.1	26 ± 1
	H-C(6)	7.4 ± 0.4	2.0 ± 0.1	51 ± 2
pD 7.0	H-C(2')	8.1 ± 0.4	1.5 ± 0.1	70 ± 3
	H-C(6)	36 ± 3	2.1 ± 0.2	40 ± 3
pD 8.0	H-C(6)	59 ± 6	1.7 ± 0.2	35 ± 2
	H-C(2')	52 ± 7	1.8 ± 0.2	37 ± 3
pD 10.0	H-C(6)	250 ± 38	0.77 ± 0.1	27 ± 3
	H-C(6)	1100 ± 150	0.20 ± 0.02	12 ± 1
pD 11.0	H-C(6)	4700 ± 390	-	-
	H-C(2')	3000 ± 400	-	-
pD 12.0	H-C(6)	2000 ± 150	-	-
	H-C(2')	1300 ± 57	-	-

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**SCHEME 1.** Proposed mechanism for the reaction between PM and FA.