**A Spectroscopic Study on p-Hydroxyphenylpyruvic Acid. Keto-Enol Tautomerism and Stability of Its Complex with Fe+3 Ions**

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**p-Hydroxyphenylpyruvic Acid (pHPPA) is a significant product of tyrosine metabolism in transitory and hereditary tyrosinemia [1]. Its determination in biological fluids contributes to obtain a precocious and valuable picture of the amino acid metabolic diseases. Actually, the colorimetric detection of pHPPA by the ferric chloride method is practically impossible: only a green-blue flash of colour can be observed [2]. Because pHPPA exhibits keto-enol tautomerism [3 – 5], this fact could be attributed to low amount in solution of the tautomer responsible for the coloured complex formation and to low chemical stability of this complex. It is, therefore, evident that a detailed investigation (i) of the keto-enol tautomerism of this substance and (ii) of the stability of its complex with Fe+3, in different organic solvents and in organic solvent-aqueous mixtures, imposes in order to identify and control the factors affecting the determination of pHPPA by FeCl3. For this purpose, NMR spectroscopy in concurrence with UV-Vis, IR, and MS spectroscopic techniques has been proved to be very suitable. Moreover, because pHPPA is a substrate or product in many enzyme reactions which are believed to involve either the keto or the enol form [4, 6] a better knowledge of its tautomeric properties may be useful to elucidate the mechanism of reactions in which it is involved.**

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**Experimental Section**

**Instruments and techniques**

The 1H-NMR spectra were recorded at 100 MHz on a Varian HA 100 spectrometer. Usually 0.5 m solutions were employed and a small amount of TMS was added to generate the lock and/or internal reference signal. For the spectra recorded in H2O-DMSO mixtures it was more convenient to lock the spectrometer on the DMSO signal. The solubility of pHPPA in water was too low to obtain CW 1H NMR spectra. The increase of solubility in buffered solutions (pH = 6) was enough for a satisfactory signal-to-noise ratio in a single scanned CW spectrum. In this case sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as internal reference and lock substance.

The FT 13C-NMR spectra, 1H noise decoupled and undecoupled, were recorded at 25.2 MHz on a Varian XL 100 spectrometer (1 m in DMSO-d6, 0.48 Hz per data point, number of transient: 1000 for...
The infrared spectra of pHPPA and its sodium salt in KBr pellets were recorded on a PerkinElmer 257. The UV-Vis measurements were carried out using a Zeiss PMQ II spectrophotometer fitted up with a thermostatted cell-holder. Mass spectra were obtained with a Perkin-Elmer 270 MS under the following experimental conditions: source pressure, 10−7 mm Hg; electron beam energy, 70 eV; ion-accelerating voltage, 2 kV.

The mole fraction of the H2O-DMSO mixtures was determined from the data of Cowie and Toporowski [7] by measuring the refractive index at 25 °C on an Abbé refractometer using the sodium D line. The volume percentages were calculated from the mole fraction data.

The keto-enol equilibrium in pure solvents and H2O-DMSO mixtures was investigated by UV spectrophotometry and 1H NMR spectroscopy. From optical measurements the enol fraction was obtained using the expression \( CE = A/(e \cdot 1) \). The absorbance, \( A \), was measured in correspondence to the maximum absorption of the enol assuming that absorption of the keto form was negligible [3, 5, 10].

For the 1H-NMR spectra of pHPPA recorded in pure solvents the enol fraction was obtained from the integrals of the signals of the −CH2− keto protons and of the −CH= enol proton. In H2O-DMSO solvent mixtures the integrals of the aromatic protons signals were used to calculate the enol fraction, because the intense signal due to H2O protons did not permit accurate integration of −CH2−signal. In order to obtain reliable values of the enol fraction at least ten integrals were averaged. All measurements concerning the keto-enol tautomersism were made 24 h after preparing the solutions so as to reach equilibrium.

The stability of the pHPPA-FeCl3 complex was studied at 25 °C by recording the decrease of the absorbance (\( \lambda_{max} = 680 \text{ nm} \)) of solutions, with different pHPPA concentrations and FeCl3/pHPPA mole ratios, in H2O-DMSO mixtures covering the range 20—100 vol.% DMSO.

Materials

Commercially available solvents were used. pHPPA was purchased from Fluka AG, Buchs SG, Switzerland.

Experimental Results

1H NMR spectra of pHPPA in organic deuterated solvents (DMSO, methanol, acetone and dioxane) display an intense singlet in the olefinic region (\( \delta = 6.3 \)), whereas either no signal (in methanol and dioxane solutions) or a small one at \( \delta = 3.9 \) (in DMSO and acetone solutions) is observed in the absorption range of methylene protons. On the contrary, in the spectrum recorded in aqueous solution buffered at pH=6 the methylene resonance (\( \delta = 4.1 \)) is very strong and the olefinic signal (\( \delta = 6.3 \)) small. Moreover, an additional small signal is found at \( \delta = 3.1 \) which can be assigned to the hydrated keto tautomer: −CH2−CH(OH)2−. In the aromatic region the chemical shifts (\( \delta \)) of the AA′BB′ systems can be attributed by considering the substituent effects on the chemical shift of benzene protons [8] (H−C(6): 6.85 (enol), 6.72 (keto); H−C(5): 7.64 (enol), 7.09 (keto)). The effects for −C=O(H)−COOH group (\( \alpha: +0.45; \ m: \sim0 \)); the plus sign refers to downfield shift) are derived from the PPA [9].

The percentages of the enol and keto forms in organic solvents have been calculated from the integrals of the olefinic and methylene proton signals (see Table I). In aqueous solution the proximity of the very intense H2O peak prevents integration of the methylene resonance. The use of deuterated buffer solutions allow us to only estimate the percentages of the tautomers because of the progressive deuteration of the −CH= and −CH2− signals. Exact values (Table I) of the tautomeric population in water have been obtained, at low pH values, from UV spectra by assuming that the UV spectrum of the enol form undergoes no significant variations in different solvents. UV data for pure enol form have been derived from methanol and dioxane solutions because 1H NMR spectra show no presence of the keto tautomer in these solvents. The following molar extinction coefficients, at 25 °C, are calculated: \( \varepsilon_{303 \text{ nm}} \) (methanol) = 25400 and \( \varepsilon_{307 \text{ nm}} \) (dioxane) =...
Table II. Thermodynamic quantities relative to enol-keto equilibrium for undissociated pHPPA in H₂O(HCl 1 m) and DMSO.

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>H₂O(HCl 1 m)</td>
<td>-900</td>
<td>-2000</td>
<td>-4.0</td>
</tr>
<tr>
<td>DMSO</td>
<td>2000</td>
<td>7200</td>
<td>17.2</td>
</tr>
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Table III. ¹³C NMR parameters of pHPPA in DMSO-d₆ (1 m).

<table>
<thead>
<tr>
<th>Carbon atom</th>
<th>Chemical a shift (δ)</th>
<th>Coupling b constant [Hz]</th>
</tr>
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<tbody>
<tr>
<td>C (1)</td>
<td>167.1</td>
<td>¹J(C₁H₃) = 3.7</td>
</tr>
<tr>
<td>C (2)</td>
<td>139.2</td>
<td>²J(C₂H₃) = 2.44</td>
</tr>
<tr>
<td>C (3)</td>
<td>111.1</td>
<td>²J(C₃H₆) = 157.2; ²J(C₁H₃) = 4.9</td>
</tr>
<tr>
<td>C (4)</td>
<td>126.3</td>
<td>²J(C₄H₆) = 7.8</td>
</tr>
<tr>
<td>C (5)</td>
<td>131.4</td>
<td>²J(C₅H₆) = 160</td>
</tr>
<tr>
<td>C (6)</td>
<td>115.6</td>
<td>²J(C₆H₆) = 159</td>
</tr>
<tr>
<td>C (7)</td>
<td>156.7</td>
<td>²J(C₇H₆) = 2; ²J(C₁H₃) = 9</td>
</tr>
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</table>

a Referred to TMS as internal standard.

IR spectra analysis

The infrared spectra of pHPPA and its sodium salt in the solid state show some remarkable differences as a consequence of the unusual behaviour of phenylpyruvic acids which are stable in the enol form as undissociated acid and in the keto one as anion.

In the IR spectrum of pHPPA two absorption band are present in the OH stretching vibration region: a band between 3500 and 3360 cm⁻¹ attributable to the overlap of the signals arising from the enol and the associated phenol hydroxyl groups, whereas a broad band between 3300 and 2500 cm⁻¹ originates from the OH-stretching of the bonded carboxylic group. A strong band at 1695 cm⁻¹ can be assigned to the νC=O of the carboxylic group. The decrease in frequency compared to aliphatic α keto acids could be ascribed to the decrease of the C=O bond force constant due to the conjugative effect of an α, β double bond. The νC=O vibration occurs at 1600 cm⁻¹. A band at 1200 cm⁻¹ could be assigned to the stretching vibration of the =C=OH group on the basis of the calculated value of 1205 cm⁻¹ for νC–OH for vinyl alcohols and the lack of this...
strong absorption in the spectrum of the sodium salt of pHPPA according to the presence of only the keto form [13]. In the spectrum of sodium $p$-hydroxyphenylpyruvate a strong band between 3550 – 3200 cm$^{-1}$ is assigned to the OH stretching vibration of the phenolic group. The carbonyl group gives rise to the characteristic band at 1720 cm$^{-1}$ due to the stretching vibration of the C–O bond. A very strong band at 1635 cm$^{-1}$ originates from the asymmetric stretching of the COO$^-$ group whereas a low intense band at 1400 cm$^{-1}$, absent in the IR spectrum of pHPPA, can be assigned to the symmetric stretching of the carboxylate anion and/or to the stretching band of the methylene group in the fragment $\text{–CH}_2\text{–C}$. 

**Mass spectra of pHPPA**

Tabular presentation of the spectrum is given in Table IV. The two main fragmentation sequences confirmed by metastable peaks are similar to those observed for PPA [11]. The presence of a $p$-hydroxyl group does not seem to influence the fragmentation process at least in the main cleavage and rearrangement modes. The circumstance that for the second fragmentation path only two fragmentation steps corresponding to those of PPA are present and the very prominent peak at $m/e = 107$ (an intense base peak is absent in the mass spectrum of PPA) indicate the presence of a stabilized $|\text{C}_7\text{H}_7\text{O}|^+$ ion which probably corresponds to the peak observed for cresols as a result of benzylic C – H cleavage.

**Properties of FeCl$_3$-pHPPA complexes**

Addition of FeCl$_3$ to pHPPA solutions in the considered organic solvents and water yields a “flash” of a green-blue colour in all the solvents except DMSO. As $^1$H NMR spectra show, the disappearing of the colour is due to decomposition of pHPPA. In DMSO the colour appears to be stable in time and $^1$H NMR spectra show that only the $Z$ enol tautomer does combine with the ferric cation to form a coloured complex ($\lambda_{\text{max}} = 680$ nm). From these findings it can be argued that a study on the stability of the complex in DMSO-H$_2$O mixtures would give useful information about the possibility to detect pHPPA in biological fluids simply by adding a solution of FeCl$_3$ in DMSO. Moreover, since the colour development depends on the enol content of H$_2$O-DMSO mixtures and on the enolization rate of the keto form, the keto-enol equilibrium of pHPPA has been studied throughout the composition range at 25 °C.

Fig. 1 shows that the enol fraction increases regularly with the volume percentage in DMSO. The trend of the plot is not linear and shows that it would be favourable to operate with solutions richer in DMSO.
The curves in Fig. 2 display the decrease in time of the absorbance ratio $A_t/A_0$ at 650 nm, of pHPPA-FeCl$_3$ complex in H$_2$O-DMSO mixtures. $A_t$ and $A_0$ are the absorbance of the sample at the time $t$ and $t = 0$, respectively. The quantity $A_t/A_0$ has been reported instead of $A_t$, because it allows to compare samples with different concentrations of pHPPA and FeCl$_3$. The decomposition products of the complex show no absorption at 680 nm; therefore, the $A_t/A_0$ ratio is proportional to the amount of the complex present in solution and its decrease has to be related to the chemical stability of the complex. The time dependence of $A_t/A_0$ indicates that the complex stability increases with the content of DMSO in the solvent mixtures. Solutions containing more than 80 vol.%% in DMSO show a satisfactory colour stability which appears to be independent of the initial concentration of pHPPA ($0.3 \div 6.3 \times 10^{-3}$ M) and of FeCl$_3$/pHPPA mole ratio ($4 \div 1$) in the limits of experimental error. Moreover, at this solvent composition the enol fraction falls between 0.90 and 0.94, thus allowing an instantaneous development of the green-blue colour and making the colorimetric analysis practically independent of the rather low enolization rate of pHPPA [3, 5]. Our results suggest the use of such solvent compositions for pHPPA determination by the FeCl$_3$ method. Improvement in the colour stability can also be achieved by keeping the temperature as low as possible. Indeed, the decomposition rate of the complex appears to depend firstly on the solvent composition and secondly on the temperature.