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INVESTIGATION OF THE COMBINED INJECTION SOLUTION 'NEURONUCLEOS' ACTIVE COMPONENTS STABILITY AND COMPATIBILITY

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Abstract

The article presents experimental data on the study of active components stability and compatibility in a combined injection solution. The described medicine contains three active ingredients: two nucleotides (uridine-5-monophosphate disodium salt (UMP), cytidine-5-monophosphate disodium salt (CMP) and vitamin B_6 (pyridoxine hydrochloride) (PHC). It was proved that the medicine components do not interact with each other. The research also showed that thermal sterilization leads to the decomposition of API and the growth of impurities quantity. Therefore, solution sterilizing filtration through membranes with 0.2 μm pore size and subsequent filling of sterile ampoules under aseptic conditions was proposed. As a result of the experimental studies the pH of the combined injection solution medium in the range from 4.0 to 5.0 was selected. Additionally the impurities of the medicine active substances were established and normalized.

Keywords:: *uridine-5-monophosphate disodium salt, cytidine-5-monophosphate disodium salt, pyridoxine hydrochloride, compatibility, injection medicine, liquid chromatography, buffer solutions*

Introduction

Nucleotides and vitamins of group B play a key role in the regeneration of a damaged peripheral nerve [1-3]. Previous preclinical trials showed that pyrimidine nucleotides uridine and cytidine possess positive effects in the treatment of various neuropathies and affect the acceleration of the nerves and muscle fibers regeneration [4-5]. They also increase the nerve conduction speed and the phospholipids level (phosphatidylcholine and phosphatidylethanolamine) of the neurons membranes [6].

The literature data describes the results of clinical trials for cytidine and uridine usage in combination with vitamins B in such conditions as pain in the lower back and cervix, diabetic neuropathy, traumatic-compression lesions and acute non-traumatic pain [7-10].

Medicines containing uridine and cytidine nucleotides have been used in the treatment of peripheral nervous system pathology for more than 40 years already, but interest in this area is not decreasing, new combined medicines appear all the time [11]. This can be explained by the important role of nucleotides in the restoration of damaged structures of the nerve fiber – axons and myelin sheath [12]. Clinical efficacy and a favorable safety profile of this combination have been noted in radiculopathies, diabetic and alcoholic polyneuropathy, neuropathy of the facial nerve. While taking nucleotides the regeneration of nerve tissue intensified, the processes of nerve impulse conduction were normalized, the severity of the pain syndrome weakened [13]. The further advantages of nucleotides as medicines should also include their good tolerability [14-15].

According to the above-mentioned information, the development of a new combined medicine, based on the uridine and cytidine nucleotides together with vitamin B, especially in an injection dosage form, appears to be a topical issue of modern medicine and pharmacy.

Such original injection medicine under the name 'Neuronucleos' has been developed in the laboratory of parenteral and oral liquid medicines of National University of Pharmacy. It was based on three active substances: two nucleotides (uridine-5-monophosphate disodium salt (UMP), cytidine-5-monophosphate disodium salt (CMP) and vitamin B_6 (pyridoxine hydrochloride – PHC). Complex experimental research was carried out, as a result of which the pH limits of the solution were determined and substantiated, the composition and technological parameters of an injection solution obtaining were clarified.

Since this injection medicine is original, one of the main issues at the stage of pharmaceutical development (in addition to studying the physicochemical properties of the dosage form components) was to determine the stability and compatibility of the solution components. Therefore the aim of our work at the stage of pharmaceutical development was to investigate the decomposition products profile while studying the stability of a new combined injection medicine.

Methods

In order to develop a methodology and to study stability and compatibility the standards of substances and possible impurities of synthesis and decomposition were used:

1. For uridine-5-monophosphate disodium salt: uridine-5-monophosphate disodium salt (Sigma Aldrich, Product # U6375, lot STBB2499V); uracil (Sigma Aldrich, Product # U0750, lot 080M0067V); uridine (Sigma Aldrich, Product # U3750, lot 071M1511V); uridine-5-monophosphate disodium salt hydrate (Sigma Aldrich, Product # 94300, lot BCBH2041V); uridine-5-triphosphate trisodium salt hydrate (Sigma Aldrich, Product # U6750, lot BCBD4670V)

2. For cytidine-5-monophosphate disodium salt: cytidine-5-monophosphate disodium salt (Sigma Aldrich, Product # C1006, lot 129K2161V); cytosine (Sigma Aldrich, Product # C3506, lot 069K1542); cytidine (Sigma Aldrich, Product # C122106, lot BCBG3699V); cytidine-5-diphosphate disodium salt hydrate (Sigma Aldrich, Product # C9755, lot 099K5164); cytidine-5-triphosphate sodium salt (Sigma Aldrich, Product # C1506, lot BCBG0248V)

3. For pyridoxine hydrochloride: pyridoxine hydrochloride (EP CRS, lot 2); pyridoxine hydrochloride impurity A (EP CRS, lot 2); pyridoxine

hydrochloride impurity B (Sigma Aldrich, Product # D0501, lot 071M1615).

Injection medicine (laboratory series 1610121, 1610122, 1010123). Water for injection according to the requirements of the Ph.Eur. and SPhU [16-17].

HPLC grade water was obtained from a water purifying system (Millipore, Bedford, MA, USA). Other chemicals and solvents were of analytical grade. All the solvents used for mobile phase were filtered through membrane (0.22 m pore size) and degassed before use.

HPLC

The analysis of decomposition products has been carried out on a ProStar liquid chromatograph, equipped with an autosampler 410 and photodiode array detector PDA 330, made by «Varian Chromatography System» (USA). A Hydrosphere C18 column (150 x 4.6 mm, particle size 3 µm) with a precolumn has also been used. The sodium phosphate dibasic solution, adjusted to pH 5.5, was used as the mobile phase. The separation has been carried out in an isocratic mode. The flow rate of the mobile phase was 1 ml / min, the injection volume was 20 µl, the detection has been carried out at a wavelength of 262 nm and a column temperature was 35 °C.

pH determination

The pH of the solution medium was measured by the potentiometric method on the 'Seven Easy' pH meter, manufactured by 'Mettler Toledo' (USA), in accordance with the Pharmacopoeia requirements (SPhU/ Ph.Eur., 2.2.3).

Results

We proposed to determine the content of impurities in the medicine by the high-performance liquid chromatography method, as the most sensitive and selective instrumental analysis method, which makes it possible to control concomitant impurities both in active pharmaceutical ingredients (API) and in the prepared medicine.

The literature data describes the spectrophotometric and HPLC methods for nucleotides and pyridoxine analysis in biological fluids and medicines. In the process of developing a method for analyzing the APIs decomposition products all available information was thoroughly analyzed [18-21].

In order to determine the main impurities of the APIs and active components of the finished dosage form (FDF) decomposition the method of 'artificial aging' was used. When API and FDF were kept under stress conditions, for example, at elevated temperatures, under the action of oxidizing and reducing agents, the main impurities arising under these conditions were determined [22].

The main impurities of the APIs decomposition that can be formed during the storage of the prepared medicine are presented in Table 1.

In Fig. 1 and 2 chromatograms, which show the complete separation of both APIs and their possible decomposition impurities under these chromatographic conditions, are depicted.

The standard conditions for the accelerated degradation of API in solution, used in the research, are presented in Table 2.

The analysis of the interaction in binary mixtures (CMP – UMP, UMP – PHC, CMP – PHC) was carried out according to the parameter – the total content of impurities. The results for each solution of the sample (each binary mixture) under the action of all the critical factors selected for the investigation were determined. The total content of impurities for each solution in the sum of impurities for all solutions was calculated.

As a result of the research the contribution of each factor to the instability of binary mixtures was analyzed with the following conclusions:

- there is a significant contribution of the heat treatment to the API decomposition;

- there is no increase in the amount of impurities with a decrease in pH, however, to establish relative pH, it is necessary to consider each API separately. A relative decrease in impurities at pH 9.18 was obtained, which is associated with the absence of UMP and CMP decomposition in this pH range;

- the API stability is influenced by oxidizing agents as well as by reducing agents.

The use of nitrogen or other inert gas in the process will reduce the likelihood of API oxidation by atmospheric oxygen. The introduction of catalytic oxidation inhibitors (chelating agents, for example, EDTA) into the composition will reduce the likelihood of API degradation due to redox reactions.

As a result of the research it was found that the effect of temperature at all stages of the technological process negatively affects the API stability. Air oxygen can lead to API oxidation in the dosage form.

All binary API mixtures showed similar values for the growth of impurities associated only with exposure factors. There were no impurities that would characterize the API interaction.

Discussion

The stability of the API in solution was also assessed at different pH values, since changes in pH can affect the stability of the API in aqueous solution. At different pH values each API molecule (PHC, UMP and CMP) exists in different ionic forms. This is typical for compounds with a nitrogen atom. If two substances are in equilibrium (ROH ↔ RO–), then the family of absorption curves corresponding to different solution pH will have one point at which its absorption capacity remains constant, independent of the ROH / RO– concentration ratio. The presence of an isobestic point indicates the formation of different ionic forms with different spectral characteristics, as well as, possibly, different physicochemical properties [23].

For UMP the isobestic point was absent. The stability of UMP aqueous solutions was tested at different pH values (in buffer solutions).

An UMP solution at pH 1.2 showed the growth of impurities, at pH 5.0 and 9.18 impurities were found within acceptable limits. The obtained data are presented in Table 3.

The main impurity in the decomposition of API UMP was uridine, which is formed when the uridine base ester bond with phosphoric acid is broken.

The stability of aqueous solutions of CMP was also tested at different pH levels (in buffer solutions).

A CMP solution at pH 1.2 gave insignificant formation of a hydrolysis product at the ester bond of cytidine; at pH 5.0 and 9.18 impurities within acceptable limits were found. The data are presented in Table 4.

The CMP is also characterized by the presence of an isobestic point. The second ionic form of CMP is formed above pH 4.5. However, this ionic form does not significantly affect the resistance of the molecule to hydrolysis.

The main impurity in the decomposition of API CMP was cytidine, which is formed when the ester bond of the cytidine base with phosphoric acid is broken.

The absence of significant decomposition in a strongly acidic medium suggests that with an increase in pH to a weakly acidic one (pH 4.0 – 5.0), the UMP and CMP substances will be relatively resistant to hydrolysis.

To test the pH effect on the lability of the FDF active components – CMP and UMP – additional studies of these solutions were carried out in a weakly acidic medium at pH from 3.5 to 5.3 under more severe conditions during thermal sterilization at 120°C for 15 min.

Solutions of UMP and CMP in buffer solutions at pH from 3.5 to 5.3 under exposure to temperature (120 °C for 15 min) showed an increase in hydrolysis impurities. Pronounced hydrolysis of both APIs at the ester bond at the sterilization temperature (120 °C for 15 min) was observed. At a pH of 4.2 or more this process slowed down and somewhat stabilized.

As a result of the investigation it was found that in a CMP solution in buffer media at pH from 3.5 to 5.3 a hydrolysis reaction occurs with the formation of a cytidine impurity. Similar results were obtained in a UMP solution; a hydrolysis reaction occurs under the same conditions with the formation of an uridine impurity. It is possible to reduce the formation of impurities to a predetermined maximum by replacing thermal sterilization with solution sterilizing filtration through membranes

with a 0.2 µm pore size and filling sterile ampoules under aseptic conditions.

According to the obtained results it can be concluded that the optimal pH value for CMP and UMP solutions should be in the range of 4.0-5.0 for the dosage form.

For PHC the isobestic point is at a wavelength of 308 nm. It was determined by comparing the absorption spectra of pyridoxine at different pH values (Fig. 3).

The obtained experimental data are in agreement with the literature data. As can be seen from Fig. 4 the PHC solution has different absorption spectra at different pH with an isobestic point at λ = 308 nm. PHC exists in different ionic forms at different pH (Fig. 4) [23].

As can be seen from Fig. 4, the absorption spectrum of PHC shifts to the visible region as it grows to pH 4.0. PHC exists in an acidic form, absorbs in the middle UV region and is colorless; another ionic form is formed at pH more than 4.0 and absorption is shifted towards the formation of solutions with a slightly yellow coloration. A third ionic form is formed above pH 8. The second and third forms are more labile and subject to oxidation processes.

This is confirmed by pKa calculations for various ionic forms of pyridoxine (Fig. 5) and spectral data (Fig. 4). So, at pH below 4.31, only a fully protonated form exists, in the pH ranges 4.31 – 8.7 and above 8.37 several ionic forms are possible.

The injection solution will acquire a yellowish tint when using the working range of FDF with PHC from pH 4.0. This is not associated with decomposition, but with the transition of the molecule to a different ionic form, which has an absorption spectrum covering the beginning of the spectrum visible region.

The formation of pyridoxine impurities at different pH of the PHC solution occurs in different ways (Table 5).

The main impurity in the decomposition of API PHC is an impurity B – an oxidation impurity.

APIs in FDF of three active substances (UMP, CMP and PHC) do not interact with each other. The use of the thermal sterilization method leads to the decomposition of the API and a significant increase in impurities. The process of hydrolytic destruction is most strongly dependent on temperature conditions. According to the experimental studies the pH of the medium for a combined injection solution containing active substances with different pH levels of the medium in the range from 4.0 to 5.0, and sterilizing filtration of the solution through membranes with a 0.2 µm pore size and filling sterile ampoules under aseptic conditions were selected. As a result of the experiment the impurities of active substances (UMP, CMP, PHC) were established and normalized. The injection solution will acquire a yellowish tint when using the working range of FDF with PHC from pH 4.0. This is not associated with decomposition, but with the transition of the molecule to a different ionic form, which has an absorption spectrum covering the beginning of the visible region.

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Table 1. The main impurities of the FDF API decomposition

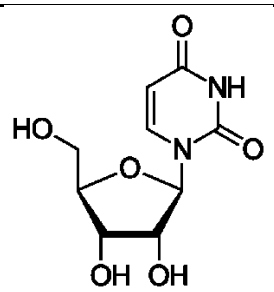
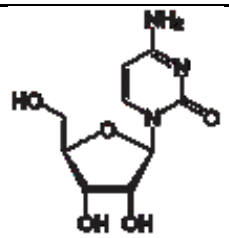
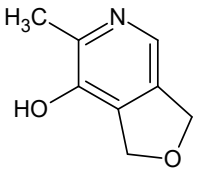
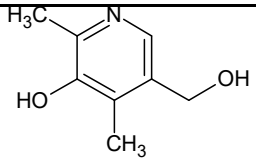
№	Impurity name	Structure
	Impurities of API uridine-5-monophosphate disodium salt	
1	Uridine (1-β-D- Ribofuranosyluracil) CAS 58-96-8	
	Impurities of API cytidine-5-monophosphate disodium salt	
2	Cytidine (1-β-D- Ribofuranosylcytosine) CAS 65-46-3	
	Impurities of API pyridoxine hydrochloride	
3	Impurity A (6-methyl-1,3-dihydrofuro[3,4-c]pyridine-7-ol) CAS 5196-20-3	
4	Impurity B (5-hydroxymethyl-2,4-dimethylpyridine-3-ol) CAS 61-67-6	

Table 2. Factors affecting API in solution

№	Factor code	Factor
1	A	Freshly prepared solution at room temperature (25°C)
2	B	Temperature (holding in a boiling water for 1 hour)
3	C	pH (1.2 in 0.1 M HCl) for 3 hours
4	D	pH (5.0 in buffer solution) for 3 hours
5	E	pH (9.18 in buffer solution) for 3 hours
6	F	oxidants (hydrogen peroxide – substance (1:2), m/m)
7	G	reducers (sodium borohydride – substance (1:2), m/m)

Table 3. Formation of impurities in an UMP solution at different pH

pH	Main decomposition impurities	Additional decomposition impurities
1.2	Slight uridine formation – at an identifiable limit of 0.15%	Another 2 unidentified decomposition impurities up to 0.2 and 0.4%
5.0	Is not formed	All decomposition impurities below the identifiable limit (less than 0.15%)
9.18	Is not formed	All decomposition impurities below the identifiable limit (less than 0.15%)

Table 4. Formation of impurities in a CMP solution at different pH

pH	Main decomposition impurities	Additional decomposition impurities
1.2	Slight cytidine formation (at an identifiable limit of 0.12%)	Another unidentified decomposition impurities up to 0.2%
5.0	Slight increase in cytidine impurity (up to 0.15%)	All decomposition impurities below the identifiable limit (less than 0.15%)
9.18	Is not formed	All decomposition impurities below the identifiable limit (less than 0.15%)

Table 5. Formation of impurities in a PHC solution at different pH

pH	Main decomposition impurities	Additional decomposition impurities
1.2	Slight formation of impurity B – oxidized form (less than the unidentified limit - 0.03%)	Other unidentified impurities: at the level of the unidentified limit (less than 0.1%)
5.0	Formation of impurity B – oxidized form (at the level of the unidentified limit – 0.1%)	All decomposition impurities are at the level of the unidentified limit, but their amount is increasing (about 0.1%)
9.18	Formation of impurity B – oxidized form (at the qualifying limit – 0.4%)	All decomposition impurities are at the level of the unidentified limit, but their amount has not increased relative to pH 5

Figure 1. Chromatogram of the solution for checking the suitability of the chromatographic system

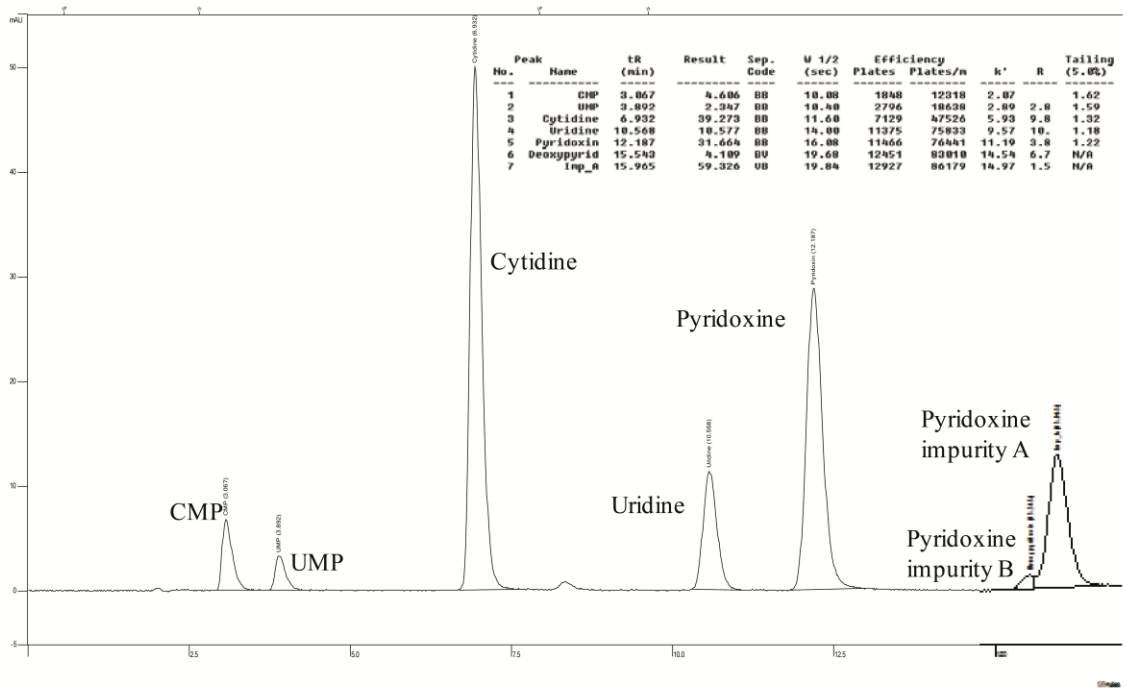


Figure 2. Chromatogram of the freshly prepared test solution

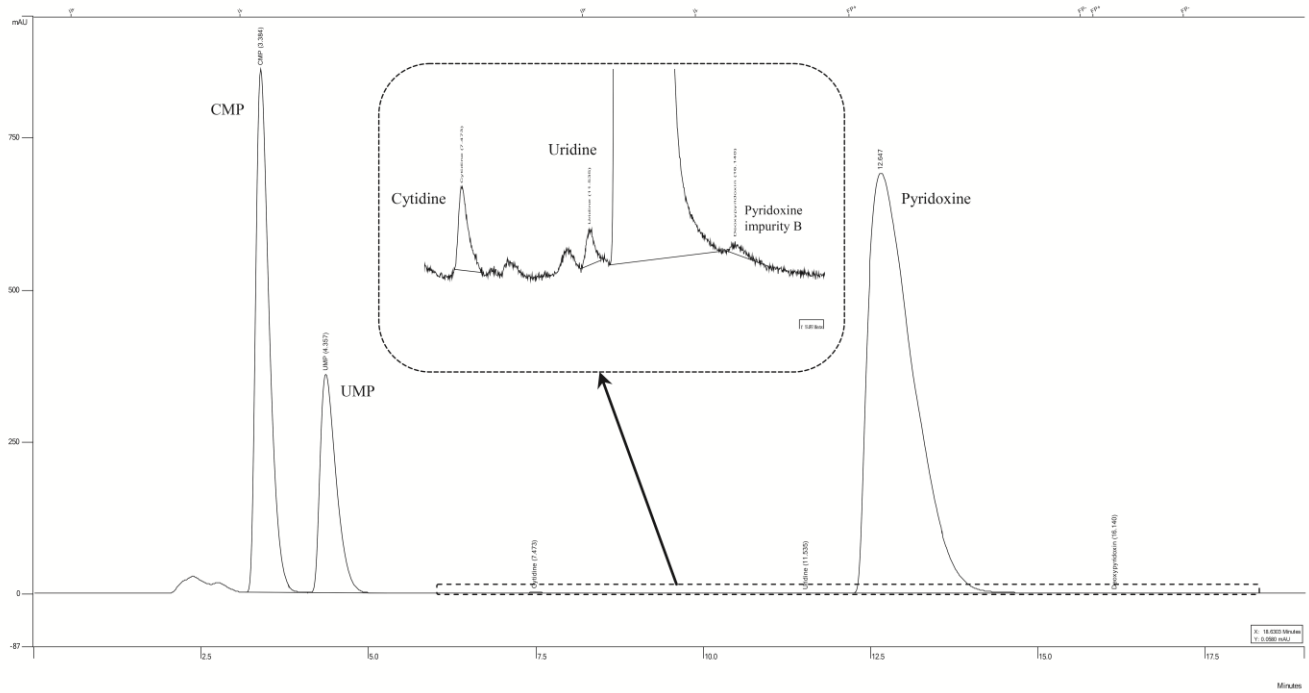


Figure 3. Isobestic point of pyridoxine

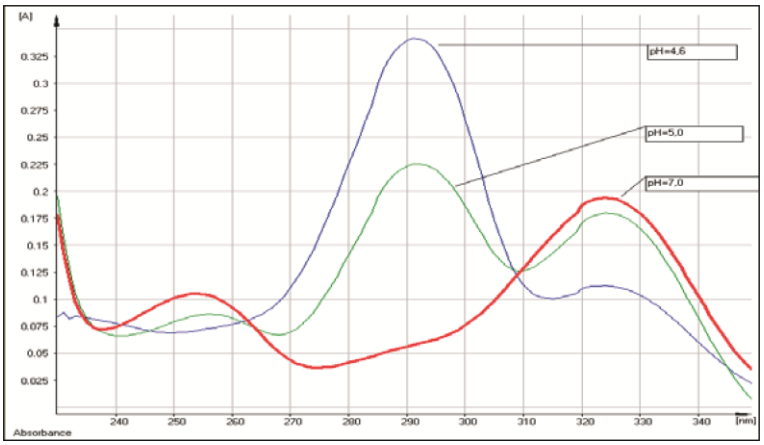


Figure 4. Titration curves of pyridoxine hydrochloride and determination of chemical equilibrium for PHC in aqueous solutions

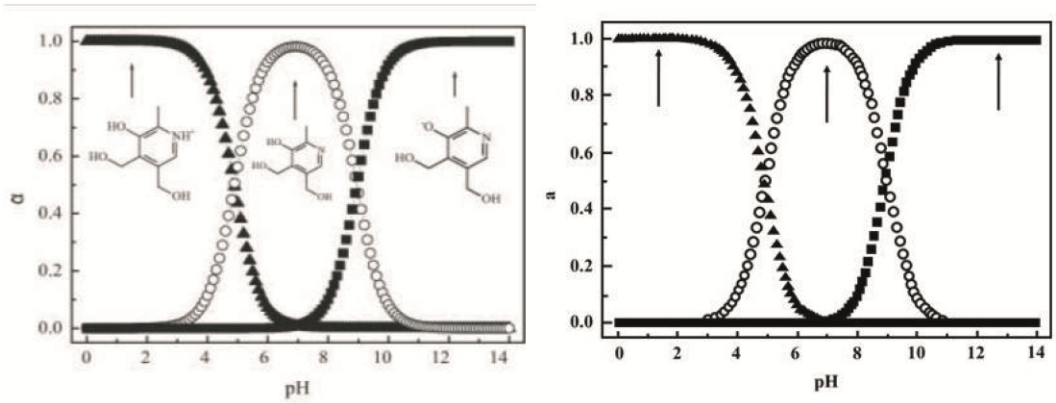
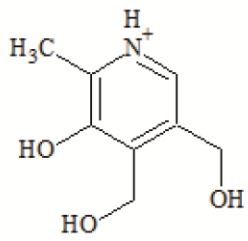


Figure 5. Results of calculating pKa for pyridoxine

H4L



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Approximated apparent pKa value(s):

1. pKa(HL/H+L)=14.18 ± 0.10
2. pKa(H2L/H+HL)=13.96 ± 0.10
3. pKa(H3L/H+H2L)=8.37 ± 0.20
4. pKa(H4L/H+H3L)=4.31 ± 0.10
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