SPECTROPHOTOMETRIC DETERMINATION OF ACIDITY CONSTANTS OF SOME NOVEL METHOTREXATE-LIKE COMPOUNDS IN WATER AND ACETONITRILE-WATER BINARY MIXTURES

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ABSTRACT

The acidity constants of four novel methotrexate like compounds (NP-ALA ; (N-[4-[[2-hydroxy -1-naphtyl)methylene]amino]benzoyl-β-alanin) TP-ALA; (N-[4-(2-thiophene-aldiminobenzoyl)-β-alanin), SAL-ALA; N-[4-[[2-hydroxyphenyl)methylene]amino]benzoyl-β-alanin], MeO-SAL-ALA; (N-[4-[[(2-hydroxy-4-methoxyphenyl)metylene]amino]benzoyl-β-alanin)) have been determined in water and acetonitrile-water binary mixtures (10%, and 20% (v/v)) by spectrophotometric method. The effect of the acetonitrile concentration on the ionization constant was also studied.

Keywords: Methotrexate; Acidity constants; Acetonitrile-water mixtures; Spectroscopic method.

1. INTRODUCTION

Methotrexate (*MTX*; 2,4-diamino-N10-methylpteroylglutamic acid) is a small molecule inhibitor of the ubiquitous enzyme dihydrofolate reductase (*DHFR*), which is a requisite component of the biosynthesis pathway for

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purine and pyrimidine. *MTX* is a classical type of inhibitor of dihydrofolate reductase and is known to be metabolized to polyglutamyl forms after its incorporation into cells /1/. *MTX* is also a potent folic acid antagonist which inhibits the reduction of folic acid to tetrahydrofolic acid, thereby leading to the suppression of the nucleic acid biosynthesis pathway, and cell death. It has been used in the treatment of neoplastic disorders since the late 1940s /2, 3/. As a consequence, *MTX* is used as a drug for the treatment of a wide range of diseases, e.g., for cancer treatment since its development and the identification of its biological activities /4/. At the same time *MTX* is widely utilized in the treatment of acute leukemia, carcinoma of the breast, osteosarcoma, ovarian cancer, and other malignant diseases /5/.

The dissociation constant (pK_a) of a drug molecule is a key parameter in absorption, distribution, metabolism, excretion and toxicity researches because it governs solubility, absorption, distribution, and elimination of substances /6/. Also, the pK_a values constitute important data for thorough understanding of certain chemical phenomena such as biological uptake, and the binding of these molecules to environmental matrices and forming chelates with metallic cations. The drugs' pK_a data are applied to estimate the major species of pharmaceuticals present in the environment (usually in neutral pH range) and dosage-form development.

There are large spectra of pK determination techniques such as CE /7/, liquid-liquid partitioning /8-10/, ultraviolet-visible (UV-vis) absorption, and potentiometry /8, 11-13/. Among these, potentiometry and spectrophotometry are methods of choice due to their simplicity, low cost, ease of application, and so on. Very often, the main difficulty in the determination of dissociation constants of drugs is their aqueous insolubility that forces the use of spectroscopic techniques. This technique requires very low analyte concentrations and allows suitable absorbance measurement in aqueous solution even for products with low aqueous solubility /14, 15/.

MTX is known for its severe serious side effects although it is used in a wide range in medicines. Because of these shortcomings the synthesis of *MTX*-like compounds is pursued. Despite the importance of *MTX*-like compounds as inhibitor of the ubiquitous enzyme *DHFR*, the pK_a values of studied compounds are either not known accurately or not available at all. The purpose of the present study is the determination of the dissociation constants of four *MTX*-like compounds [Fig. 1], namely, (NP-ALA ; (N-[4-[[2-hydroxy -1-naphtyl)methylene]amino]benzoyl-β-alanin) TP-ALA; (N-[4-

(2-thiophene-aldiminobenzoyl)-β-alanin), SAL-ALA; N-[4-[[2-hydroxy-phenyl)methylene]amino]benzoyl-β-alanin], MeO-SAL-ALA; (N-[4-[[(2-hydroxy-4-methoxyphenyl)metylene]amino]benzoyl-β-alanin)), in water and acetonitrile (MeCN)-water medium (10 %, 20 %, (v/v)), in order to overcome the lack of information related with the acid-base equilibria of this kind of compounds by means of spectrophotometric measurements.

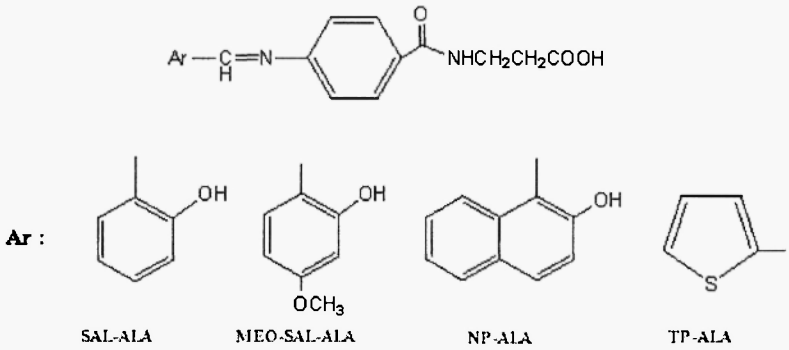

Fig. 1: Structure of studied methotrexate-like compounds

2. EXPERIMENTAL

2.1. Chemical and reagents

The studied novel Methotrexate-like compounds [Table 1] were synthesized, and the procedures of synthesis are described elsewhere /16/. Analytical reagent grade chemicals were used, unless otherwise indicated. MeCN (Sigma, HPLC grade) were used without further purification.

Potassium hydroxide (Titrisol), sodium hydroxide, hydrochloride acid (Titrisol), potassium hydrogen phthalate (dried at 110 °C before use), and potassium chloride (ionic strength adjuster; 0.1 mol L^{-1}) were supplied from Merck. All stock solutions of hydrochloride acid, potassium chloride and potassium hydrogen phthalate were prepared by water. Water, with conductivity lower than 0.05 μScm^{-1} was obtained with a Zeneer Power I (Human Corp.).

Table 1

Nomenclature and Formulas of the Studied Molecules

Molecule	IUPAC name
SAL-ALA	N-[4-[[2-hydroxyphenyl)methylene]amino]benzoyl-β-alanin]
NP-ALA	(N-[4-[[2-hydroxy-1-naphtyl)methylene]amino]benzoyl-β-alanin)
MeO-SAL-ALA	(N-[4-[[(2-hydroxy-4-methoxyphenyl)metylene]amino]benzoyl-β-alanin))
TP-ALA	(N-[4-(2-thiophene-aldiminobenzoyl)-β-alanin)

2.2. Apparatus

Potentiometric measurements were performed with Mettler-Toledo MA 235 pH/ion (resolution ±0.1 mV) analyzer system. All titrations were carried out under N_2 and at 25.0±0.1°C, which was maintained by circulating water from a constant-temperature thermostat (Heto CBN 8-30 and temperature control unit Heto HMT 200) through the double-wall *Pyrex* titration cell of 80-mL capacity.

The UV-Vis absorbance spectra were recorded at each pH using Perkin Elmer LAMBDA 25 spectrophotometer, equipped with 1.0 cm path length quartz cell with a fixed slit width (2 nm), controlled by personal computer. A peristaltic pump equipped with the spectrometer was used to circulate the solution from the titration vessel to the spectrophotometer cell, and vice versa, through Teflon or Tygon tubes in a closed loop circuit with continuous flow.

2.3. Procedure

Before the spectrophotometric titration, carbonate-free potassium hydroxide solutions were prepared under a nitrogen atmosphere. The ionic strength of KOH solution was adjusted to 0.10 mol L^{-1} by the addition of KCl. The alkali titre and absence of carbonate were periodically checked by pH-metry, using the appropriate Gran function /17/ against primary standard oven-dried potassium hydrogen phthalate.

The pK_a values of the methotrexates were determined by means of the data obtained from spectrometric titrations in water, 10% and 20% (v/v)

acetonitrile–water mixtures at 25.0±0.1°C and in 0.1 mol L^{-1} ionic strength (KCl). The spectrophotometric multiple-wavelength pH-titration was carried out as follows: in a first step, the standard emf values, E°, of the potentiometric cell were evaluated from titrations of a measured amount of an acidic solution, at the same conditions of temperature, ionic strength and solvent composition to be used in later experiments using KOH solutions in the same solvent and ionic strength as the titrant, and checking the calibration parameters from the Gran plots /17-18/ The standard emf of the cell, E°, is the average of at least 5 standardizations. The standardization of the electrode system was carried out, each time solvent media was changed and the constancy of E° values ensured by continual surveillance by means of periodic calibrations.

In a second step, a solution of fully protonated methotrexates (30.0 mL containing 1.10^{-5} mol.L^{-1} drug) by HCl at the required conditions of temperature, ionic strength and solvent composition were titrated using KOH solutions in the same solvent and ionic strength in the pH range of 2.0-11.0. After each addition, the potential was allowed to stabilize, its value used, in combination with E° calculated in calibration step, to calculate the pH solution.

In the UV-Vis spectrometric titrations, the test solution was pumped to a spectrometric flow-cell by means of a peristaltic pump. After each addition of titrant, and after waiting for the emf reading to be stable, a spectrum, UV-Vis spectra were recorded with 1 nm resolution at 210-400 nm intervals in order to obtain different spectra around the maximum λ for each methotrexates.

2.4. Data Treatment

Spectrometric titrations data were processed using the program STAR (Stability Constants by Absorbance Readings) which calculates stability constant and molar absorptivities of the pure species by multilinear regression /19/. The program STAR requires a previous model of the chemical equilibria, based upon the existence of certain chemical species, to be postulated. The refinement of equilibrium constants is done using the Gauss-Newton non-linear least squares algorithm by numerical differentiation, until a minimum in the sum of the squares residual (U) is attained. Wavelength (U_{abs}) is obtained:

$$U_{abs} = \sum_{i=1}^{ns} \sum_{j=1}^{nw} (A_{i,j,\text{exp.}} - A_{i,j,calc.})^2 \quad (1)$$

where ns and nw indicate the number of spectra and wavelengths, respectively. $A_{i,j,exp}$ and $A_{i,j,calc}$ are the experimental and calculated absorbance values for the wavelength j in the spectrum i. The calculated absorbances are obtained in three steps: the program first solves the mass balances for each spectrum according to the guessed equilibrium constants and experimental conditions; then, a multiple linear regression procedure is applied in order to determine the molar absorbances of each unknown species, and finally the individual absorbance values are re-calculated from the guessed species concentration and the corresponding molar absorbances. The optimization is performed by means of a non-linear least-squares procedure. The minimization process is repeated until the relative change of U between two iterations is $\leq 0.01\%$.

3. RESULTS AND DISCUSSION

The obtained pK_a values of MTX derivatives in different mediums, together with those predicted by the program ACD/pK_a DB /20/ are given in Table 2. ACD/pK_a DB is a software program that calculates accurate acid-base ionization constants under 25°C and zero ionic strength in aqueous solutions for almost any organic structure. This program uses fragment methods to build a large number of equations with experimental or calculated electronic constants to predict aqueous pK_a values. The first dissociation constant pK_{a1} of the studied compounds is generally attributed to carboxyl group and nearly between 3.0-3.5 in water. The second dissociation constant pK_{a1} is due to the phenolic system.

As can be seen in Table 2, the pK_a values for NP-ALA in water media could not be calculated due to the aqueous insolubility. It is known that one of the most important factors in determining pK_a is the reaction medium. The pK_{a1} values of studied compounds obtained in MeCN-water binary mixtures increase and pK_{a2} values decrease with percentage of MeCN. These variations could be explained by the fact that there is preferential solvation in these media that is related to the structural features of these binary mixtures.

Table 2

The pK_a values determined in this study at 0.10 mol L^{-1} ionic strength and 25°C.

Compounds	Water		10 % (v/v) MeCN		20 % (v/v) MeCN		ACD LAB	
	pK_{a1}	pK_{a2}	pK_{a1}	pK_{a2}	pK_{a1}	pK_{a2}	pK_{a1}	pK_{a2}
SAL-ALA	3.28[δ] (0.05)*	8.99 (0.04)	3.37(0.05)	8.81 (0.03)	3.48(0.02)	8.75(0.01)	4.35	8.62
MeO-SAL-ALA	3.00(0.04)	8.55 (0.05)	3.12(0.08)	8.38 (0.03)	3.24(0.03)	8.13(0.02)	4.34	8.16
NP-ALA	ND	ND	3.03(0.03)	8.57 (0.02)	3.35(0.08)	8.50(0.04)	4.33	8.20
TP-ALA	2.98 (0.04)	-	3.20(0.02)	-	3.41(0.08)	-	4.34	-

[δ] The experiments are repeated at least three times

* The values between parentheses are the standard deviations

ND not determined

UV-vis absorption spectrum and molar absorbances of species of SAL-ALA and MeO-SAL-ALA over the (220 to 400) nm interval and various pH values in pure water are shown in Figure 2 and Figure 3 respectively. The data were processed using the STAR program /19/ to obtain the pK_a values for substances using an iterative procedure.

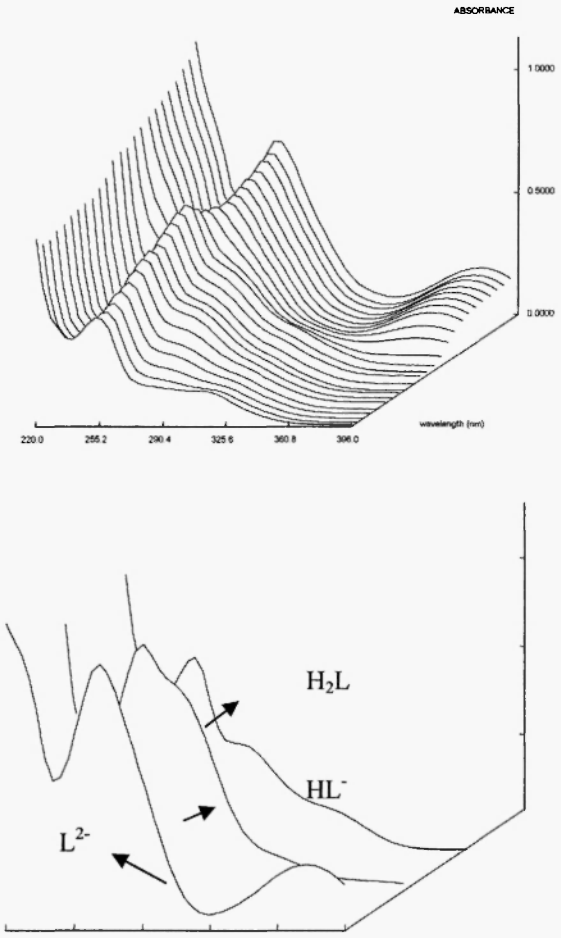

Fig. 2: Wavelength (nm) absorbance graphic and molar absorbances of species for 1.10^{-5} $mol.L^{-1}$ for SAL-ALA as a function of pH in water.

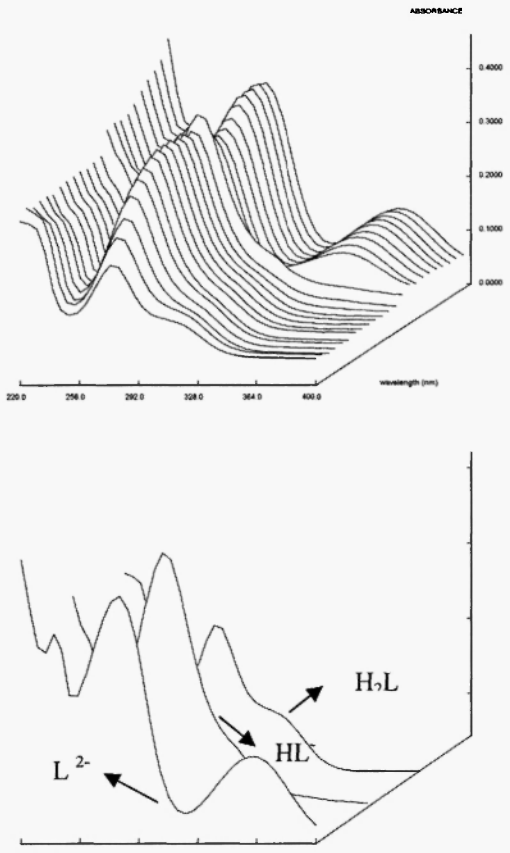

Fig. 3: Wavelength (nm) absorbance graphic and molar absorbances of species for 1.10^{-5} mol.L^{-1}for MeO-SAL-ALA as a function of pH in 10 % (v/v) MeCN-water.

It is known that one of the most important factors determining the equilibrium constants is the reaction medium. The variation of the pK_a values of compounds versus the mole fraction of MeCN, X_{MeCN}, in MeCN-water mixtures is presented in Fig. 4. The variation of pK_a values with the mole fraction of MeCN is different for each substance although, in general, pK_a values decrease with the mole fraction of MeCN. The different ways in which pK_a values change might be explained by the fact that the dissociation process is ruled by electrostatic interaction as well as by specific solute-solvent interactions. The linear relationships between experimental pK_{a1} and

pK_{a2} values for these analytes and the mole fraction of MeCN are given in Table 3.

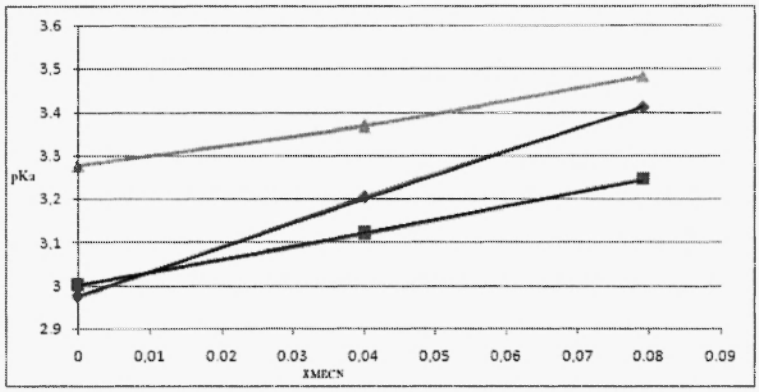

Fig. 4: pK_a values versus mole fraction of MeCN. (♦)MeO-SAL-ALA, (▲)SAL-ALA; (■)TP-ALA; (pK_1).

Table 3

The linear equation between experimental pK_{a1} and pK_{a2} values and the mole fraction of the organic modifier; x represents the mole fraction of organic modifier (MeCN).

Compounds	Equation	Regression Coefficient
SAL-ALA pK_{a1}	$y = 2.593\ x^a + 3.272$	R = 0.997
pK_{a2}	$y = -3.081\ x + 8.969$	R = 0.966
MeO-SAL-ALA pK_{a1}	$y = 3.062\ x + 3.000$	R = 0.999
pK_{a2}	$y = -5.311\ x + 8.565$	R = 0.992
TP-ALA pK_{a1}	$y = 5.494\ x + 2.977$	R = 0.999

This paper presents the first study dealing with the determination of pK_a values of MTX derivatives by spectroscopic methods in water and MeCN-water binary mixtures and gives a possibility for deeper analysis of the processes which take place during analysis of MTX-like compounds. Also,

the important data extracted from this exploration can be used for other pharmacokinetic, pharmacological or technological studies concerning these compounds.

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