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DETERMINATION OF IONIZATION CONSTANTS (pK_a) OF β-HYDROXY-β-ARYLALKANOIC ACIDS USING HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT. pK_a values of five β-hydroxy-β-arylalkanoic acids and ibuprofen were determined using the RP-HPLC method. Stationary phase was octadecyl modified (C-18) silica gel, and mobile phase was a mixture of methanol and one of nine different buffers (60:40, v/v). ${}^{s}_{w}pH$ values were measured after mixing methanol with an appropriate buffer. The mean retention time of each compound was plotted against ${}^{s}_{w}pH$ of each mobile phase. The inflection point of each sigmoidal curve represented ${}^{s}_{w}pKa$ of the compound. Using ${}^{s}_{w}pKa$ in already known equations for the specific methanol/buffer mixture, ${}^{w}_{w}pKa$ values were calculated. Obtained pK_a values for synthesized compounds were in a narrow range from 3.34–3.81 and pK_a for ibuprofen was 4.45. Predicted pK_a values for these compounds in SPARC software showed good correlation with experimental pK_a values (R^2=0.8048).

Keywords: dissociation constant, anti-inflammatory compounds, ibuprofen, liquid chromatography.

INTRODUCTION

Inflammation is a defensive reaction of the organism to the nonspecific endogenous or exogenous damaging factors. It represents a part of the inborn immune system (ABBAS *et al.*, 2010). Nonsteroidal anti-inflammatory drugs (NSAID) are used for the treatment of the symptoms of inflammation, primarily pain and fever. NSAID represents a very numerous group of structurally different drugs that were introduced into therapy in the seventies of the last century, and today are among the most used drugs worldwide (RAINSFORD, 2007; BRUNE and HINZ, 2004). Substituted arylalkanoic acids represent one of the NSAID groups that include β-hydroxy-β-arylalkanoic acids. Some research showed that these acids have significant anti-inflammatory effect and possible selectivity towards COX-2 inhibition (DILBER *et al.*, 2008; SAVIĆ *et al.*, 2011; SAVIĆ *et al.*, 2017).

Most of the pharmacologically active compounds are weak acids or weak bases, so based on that in water solutions can be protonated or deprotonated. Ionization constant is a very important physico-chemical parameter for any pharmacologically active compound. A measure of acidity or alkalinity of a compound is pK_a. It is defined as a negative logarithm of ionization constant (pK_a=-logK_a). Ionization constant of the drug is an important data for as-

sessment of pharmacokinetic parameters: absorption, distribution, metabolism and elimination (MANALLACK, 2007). This constant also has a great impact on the drug-receptor binding process. There is the difference between interactions that can maintain the ionized form of drug (ion interaction, dipole-dipole interaction) and interactions of nonionized (hydrophobic, hydrophilic interactions) (ANDREWS *et al*., 1984). Besides the impact of pK_a value on drug behavior in biological environment, it also has a great impact on drug analysis process, for the choice of appropriate method conditions (e.g. pH of mobile phase in HPLC method). For all mentioned reasons, it is desirable to determine pK_a values of drug candidates as early as possible in the drug development process.

Drugs are poorly soluble in water, so different methods are used for pK_a determination and among them great significance have reversed phase high performance chromatographic (RP-HPLC) methods. RP-HPLC methods are simple, require only small amount of a compound and compound need not be of high purity which is a great advantage in the case of newly synthesized compounds that do not need much purification (MANDERSHEID and ESCHINGER, 2003; WICZLING *et al*., 2004; BARTOLINI *et al*., 2002). For pK_a determination, OUMADA *et al*. (2002) used HPLC method in which retention time of examined compound dissolved in different mixtures of organic solvent and buffer solution at different pH values was monitored. The retention times were further used in appropriate equations.

MATERIALS AND METHODS

Tested compounds

Structures of tested compounds (previously synthesized and fully characterized) (DILBER *et al.* 2007), as well as ibuprofen are presented in Figure 1.

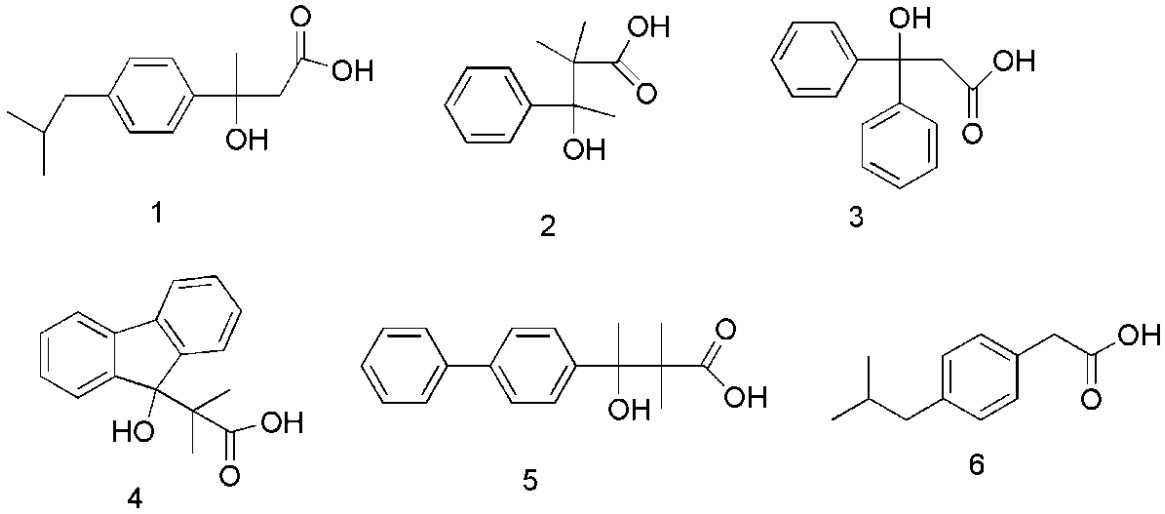

Figure 1. Structures of tested compounds (1-5) and ibuprofen (6).

Solvents

Methanol, CHROMASOLV, HPLC grade (J. T. Baker, Deventer, Netherlands), *ortho*-phosphoric acid 85%, (Merck, Darmstadt, Germany), acetic acid, glacial (Merck, Darmstadt, Germany), deionized water (TKA system for water purification, Niederelbert, Germany). Buffers for four-point pH-meter calibration: 1.679, 4.005, 7.000 and 9.180 (Radiometer, Analytical, France).

Solid compounds

Ibuprofen**,** 99% (Alfa Aesar, Karlsruhe, Germany), potassium dihydrogenphosphate, *p.a.* (Merck, Darmstadt, Germany), sodium acetate *p.a*. (Merck, Darmstadt, Germany), disodium phosphate *p.a.* (Merck, Darmstadt, Germany), potassium bromide, *p.a*. (Merck, Darmstadt, Germany).

Apparatus

pH values were adjusted using pH-meter Radiometer model PHM 240 pH/ION-meter (Radiometer, Copenhagen, Denmark).

It was used HPLC Hewlett Packard 1100 with a binary pump, manual injection and UV detection. It was used Eclipse XDB C18 3.5µm, 4.6 x 150mm chromatographic column.

Chromatographic conditions

Mobile phase flow was set to 1mL/min. The column temperature was maintained constant at 25˚C. Injection volume was 20µL. UV detection was performed at a wavelength of 254nm.

Experimental procedure

Marks used in this article:

- pH value of aqueous buffer solution: ${}_{w}^{w}pH$;
- pH value of mobile phase: ${}_{w}^{s}pH$;
- ionization constant in the mixture methanol/water: ${}_{w}^{s}pKa$;
- ionization constant in pure methanol: ${}_{s}^{s}pKa$;
- ionization constant in pure water: ${}_{w}^{w}pKa$.

Calibration of pH-meter combined glass electrode was performed using standard IUPAC buffers at four points: pH 1.679, pH 4.005, pH 7.000 and pH 9.180. pH values of aqueous buffer solutions (${}_{w}^{w}pH$) were measured, and after mixing those aqueous buffer solutions with methanol, pH values of mobile phases (${}_{w}^{s}pH$) were also measured. Each of nine used mobile phases was a mixture of aqueous solution of appropriate buffer (40%) and methanol (60%). Composition and pH values of used buffers are presented in Table 1.

Table 1. pH values of buffers (${}_{w}^{w}pH$) and corresponding mobile phases (${}_{w}^{s}pH$).

	Buffers	${}_{w}^{w}pH$	${}_{w}^{s}pH$
1	0.01 M H_3PO_4	2.350	3.165
2	0.005 M H_3PO_4 / 0.005 M KH_2PO_4	2.543	3.569
3	0.001 M H_3PO_4 / 0.009 M KH_2PO_4	3.423	4.682
4	0.01 M CH_3COOH	3.870	4.952
5	0.009 M CH_3COOH / 0.001 M CH_3COONa	4.393	5.600
6	0.005 M CH_3COOH / 0.005 M CH_3COONa	5.319	6.562
7	0.001 M CH_3COOH / 0.009 M CH_3COONa	5.674	6.870
8	0.009 M KH_2PO_4 / 0.001 M Na_2HPO_4	6.135	7.517
9	0.005 M KH_2PO_4 / 0.005 M Na_2HPO_4	7.084	8.569

Potassium bromide, synthesized compounds and ibuprofen were dissolved in the mobile phase in a concentration of about 600ppm. Potassium bromide is a compound that passes unretained throughout the chromatographic system, so it can be used for determination of holdup time. All tested solutions were injected in triplicate. Retention times of the tested

compounds in each mobile phase were determined from appropriate chromatogram, by subtracting of retention time of potassium bromide. Final retention time was expressed as the mean of triplicate determination ensuring that the difference between extreme values is less than 2%.

RESULTS AND DISCUSSION

Since tested compounds have small polarity, used RP-HPLC method is considered to be appropriate. Detection was carried out at 254 nm because ibuprofen and tested compounds show adequate absorption at that wavelength. Silica gel in chromatographic columns is sensitive to extreme pH values, so ${}^{s}_{w}pH$ values of used mobile phases must be compatible with selected column.

The first step was defining pH interval of mobile phases. It is necessary that upper pH value of mobile phase ensures that 99% of molecules of tested compounds are in ionized form, and lower pH value that 99% of molecules are in nonionized form. Those border pH values represent a first and last point on the sigmoidal Boltzmann curve which is used to determine pK_a value.

pK_a values of tested compounds predicted by computer program SPARC (Tab. 5) were used to determine the range of pH values of mobile phases considering pH values stated in the article of OUMADA *et al*., (2002). Chosen pH range of mobile phases was 3.2-8.6. The rest of pH values were within a defined interval.

The second step was to define the composition of the mobile phase. Methanol, acetonitrile and tetrahydrofuran are commonly used in RP-HPLC methods as organic solvents. Mobile phase choice depends on the nature of compounds that are tested. In accordance to this, methanol was chosen for several reasons. Methanol significantly increases the solubility of the most organic compounds. Acidic ionization occurs in methanol in an analog way as in water, so pK_a values determined in the mixtures methanol/water should be more reliable than ones determined in other organic solvents. There is the well-established linear dependence of pK_a value with the increase of methanol content in the mixture (RIVED *et al*., 2001), which is significant for the pK_a determination. Methanol is more acidic than water, so it is harder for the acids to ionize in it. This is the reason for increase of pK_a value of acids within the increase of methanol content in the methanol/water mixture.

Temperature and ionic strength also have an impact on pK_a. The temperature was set to 25˚C. It is known that pK_a values decrease within the ionic strength increase, but when the ionic strength is lower than 10-20mmol/L its impact can be neglected (OUMADA *et al*., 2002). The content of the buffer solution in the mobile phase was set to 40%, which eliminated the impact of ionic strength.

High methanol content (60%) is necessary to ensure reasonable retention times. pH values of chosen buffers are compatible with the most of the C18 columns. When mixing methanol with buffers, temperature slightly increases, therefore it was necessary to cool the mixture to 25˚C before pH adjustment.

Dependence of retention time of the tested compounds from the pH values of mobile phases is crucial for the experimental determination of pK_a values (Table 2). The stationary phase of the C18 column is made of highly hydrophobic chains and compound in its molecular form has a high affinity for these chains, while the ionized form of the substance has low affinity, resulting in faster elution. From the data given in Table 2, it can be seen that increase in pH value of mobile phase causes shortening of compound retention time. The increase of pH value increases the percent of the ionized form of the tested compounds, and decreases the affinity for the stationary phase of the used column.

Table 2. The impact of pH values of mobile phases on the retention time of the tested compounds.

Compound	Ibuprofen	1	2	3	4	5
pH			t_r (min)			
3.165	33.549	16.921	5.433	7.084	10.695	27.235
3.569	31.667	15.659	5.042	6.550	9.885	25.457
4.682	27.564	12.928	4.372	4.908	6.983	20.315
4.952	26.130	11.849	4.073	4.393	6.158	18.679
5.600	23.872	9.075	3.372	3.340	4.420	14.114
6.562	11.225	5.269	2.307	2.437	3.051	7.109
6.870	8.396	5.076	2.213	2.410	2.978	6.744
7.517	7.642	6.230	2.470	2.792	3.533	8.371
8.569	5.349	4.843	2.188	2.435	2.989	6.412

Chromatograms of ibuprofen for the whole range of pH values of the mobile phases are shown in Figure 2. Chromatograms are ordered by descending pH values of mobile phases. The x-axis represents retention time in minutes, while y axis represents absorbance.

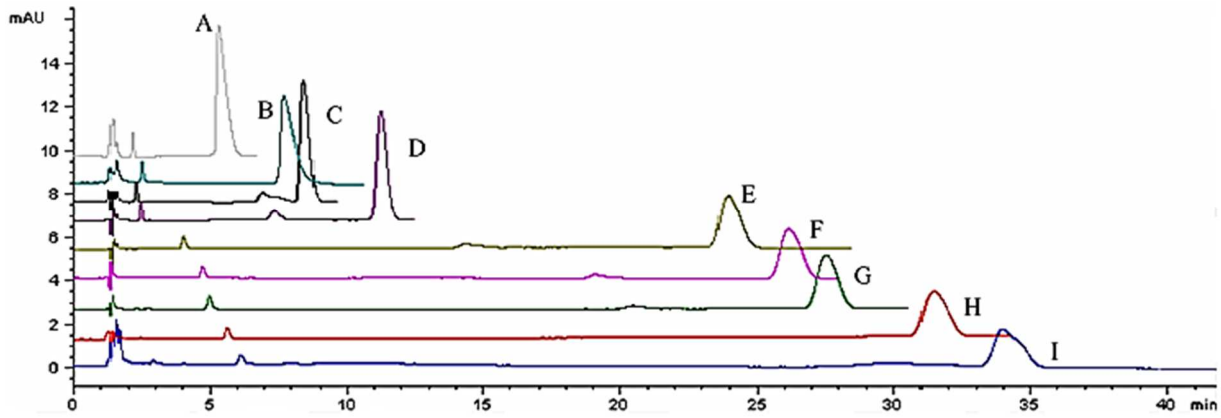

Figure 2. Chromatograms of ibuprofen.

Chromatogram A, closest to the Y-axis characterized by the shortest retention time was obtained using mobile phase with the highest pH value ($^{s}_{w}pH$ 8.569). This pH value caused that the greatest amount of the compound existed in an ionized form which had the lowest affinity towards C18 column, leading to peak occurrence in the shortest period of time. Last, chromatogram I, was obtained using mobile phase with the lowest pH value (pH 3.165) which caused that the greatest amount of the compound existed in molecular, nonionized form with high affinity for the stationary phase. This extended the retention time.

Graphic presentation of retention times in dependence of the mobile phase pH values forms sigmoidal function given by the Boltzmann equation (software Origin 7.0). pH values of mobile phases and retention time were included in the equation, sigmoidal curves were drawn, and pK_a values of synthesized compounds in the mixture methanol/buffer ($^{s}_{w}pKa$) were determined by extrapolation.

$$t_r = \frac{t_{r(HA)} + \left(t_{r(A)} - t_{r(HA)}\right)}{1 + exp\frac{x - x_0}{d_x}} \quad (1)$$

t_r → retention time (min);
x → $^{s}_{w}pH$;

t_{rHA} → the beginning points of the sigmoidal curve (min) or the retention time of the compound obtained using the mobile phase with the lowest ${}^{s}_{w}pH$ value when the most amount of the compound is in molecular form;

$t_{r(A)}$ → the final point of the sigmoidal curve (min) or the retention time of the compound obtained using the mobile phase with the highest ${}^{s}_{w}pH$ value when the most amount of the compound is in ionized form;

x_o → ${}^{s}_{w}pKa$ (value in the middle of the sigmoidal curve);

d_x → the slope of the sigmoidal curve.

Sigmoidal curves were drawn for ibuprofen and for each synthesized compound (Fig.3), and ${}^{s}_{w}pKa$ values in the methanol/buffer mixture were determined by extrapolation and are reported in Table 3 (standard deviations in parentheses).

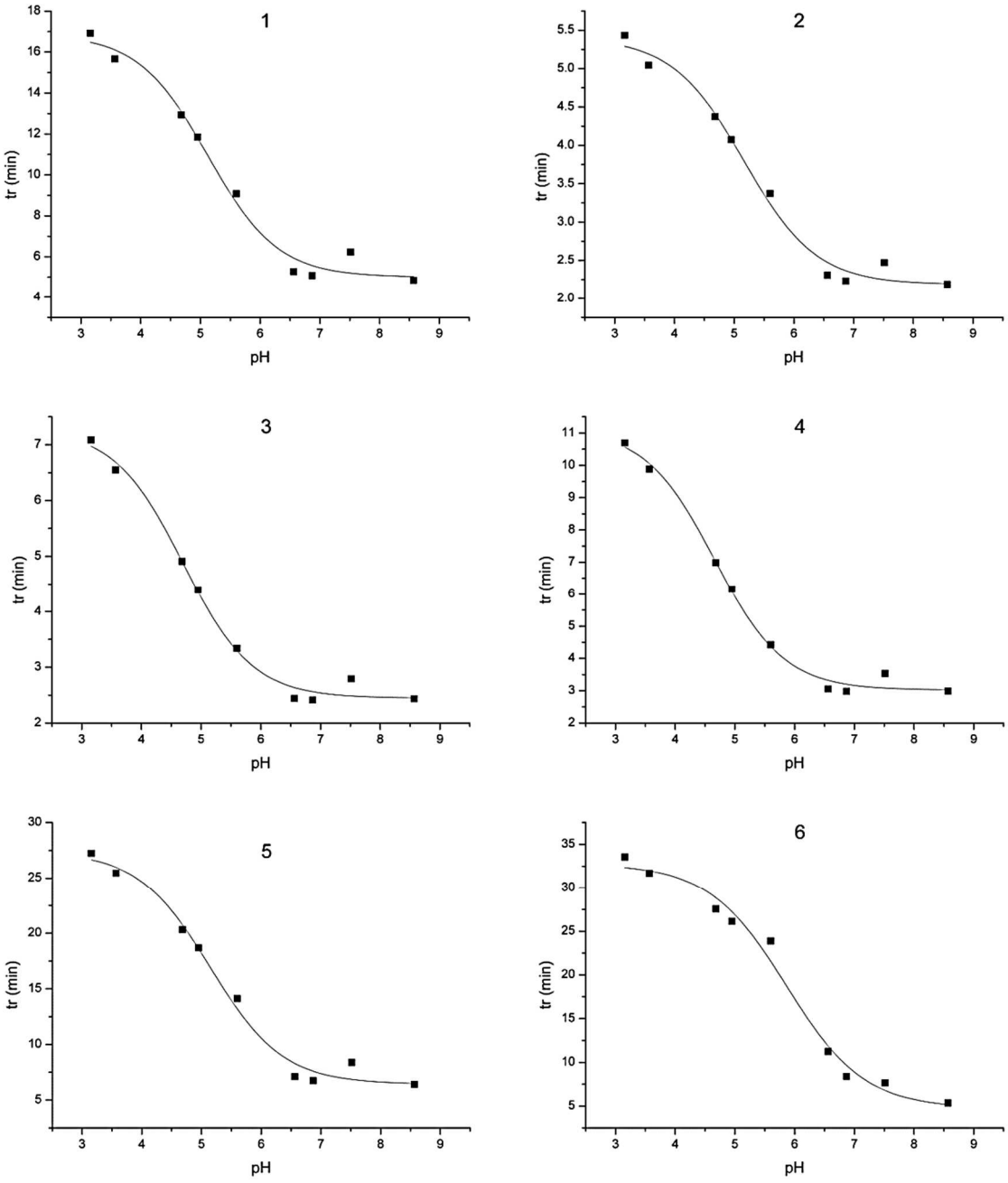

Figure 3. Sigmoidal curves of the tested compounds (1-5) and ibuprofen (6).

Table 3. Ionization constants for the studied compounds.

Compound	Ibuprofen	1	2	3	4	5
${}^{s}_{w}pKa$	5.87 (0.0038)	5.12 (0.0029)	5.17 (0.0031)	4.70 (0.0025)	4.66 (0.0034)	5.15 (0.0019)
${}^{s}_{s}pKa$	5.69	4.94	4.99	4.52	4.48	4.97
${}^{w}_{w}pKa$	4.45 (0.0035)	3.76 (0.0027)	3.81 (0.0029)	3.37 (0.0023)	3.34 (0.0031)	3.79 (0.0017)
${}^{w}_{w}pKa$ SPARC	4.53	4.25	4.10	4.07	4.01	4.07

Using ${}^{s}_{w}pKa$ values and appropriate δ constants, ${}^{s}_{s}pKa$ values can be calculated. Constant δ depends solely on the type and amount of used organic solvent. This value is well established for methanol, as well as following equations for 60% of methanol in the mobile phase (CANALS *et al*., 2000; CANALS *et al*., 2001; ESPINOSA *et al*., 2000; BATES, 2000).

$$ {}^{s}_{s}pKa = {}^{s}_{w}pKa - \delta \qquad (2)$$

$\delta = (0.09v\text{-}0.11v^2) / (1\text{-}3.15v\text{+}3.51v^2\text{-}1.35v^3) = 0.1756$
v = 60% CH_3OH

There are equations (RIVED *et al*., 2001; ROSES *et al*., 2000) which enable precise calculating ${}^{s}_{w}pKa$ values of phenols, aliphatic carboxylic acids, benzoic derivatives, amines and pyridine derivatives using their ${}^{w}_{w}pKa$ values in pure water.${}^{w}_{w}pKa$ ionization constant can be calculated from ${}^{s}_{s}pKa$ value.

When synthesized compounds are considered as aliphatic acids, ${}^{w}_{w}pKa$ ionization constant in pure water can be calculated using apparent ${}^{s}_{s}pKa$ values in pure methanol and appropriate values for slope and intercept.

Equation for aliphatic acids:

$$ {}^{w}_{w}pKa = ({}^{s}_{s}pKa - b)/a \qquad (3)$$

$a=(1\text{-}1.101v\text{+}0.103v^2)/(1\text{-}1.516v\text{+}0.518v^2)=1.090$
$b=(-0.178v\text{+}0.187v^2)/(1\text{-}1.699v\text{+}0.702v^2)=0.847$

Calculated ionization constants, ${}^{w}_{w}pKa$ obtained using RP-HPLC method and ionization constants, ${}^{w}_{w}pKa$ predicted using SPARC software are shown in Table 3. There is a good correlation between experimentally obtained and predicted ${}^{w}_{w}pKa$ values (R^2=0.8048).

Experimentally obtained values showed that synthesized compounds are stronger acids than ibuprofen which can be explained by the presence of tertiary alcoholic group on β carbon which enhances acidity of hydrogen in a carboxylic group of the investigated acids by inductive effect.

CONCLUSION

Ionization constant is a very important physico-chemical parameter of pharmacologically active compound from the aspect of analytics, receptor binding and pharmacokinetic profile. β-Hydroxy-β-arylalkanoic acids showed anti-inflammatory activity and good gastric

tolerability. The known chromatographic method which correlates retention time of tested compounds with mobile phase pH value was used to determine ${}^{w}_{w}pKa$ values in pure water. ${}^{s}_{w}pKa$ values of the tested compounds in the mixture methanol/buffer were calculated using Boltzmann equation from sigmoidal dependency of retention time on mobile phases` pH values.${}^{w}_{w}pKa$ values were calculated using known equations from obtained ${}^{s}_{w}pKa$ values. First, apparent ${}^{s}_{s}pKa$ values in pure methanol were calculated, and ${}^{w}_{w}pKa$ values in pure water were calculated from linear dependence with ${}^{s}_{w}pKa$ value in the mixture methanol/water. Obtained pK_a values for all tested compounds were lower than for ibuprofen which can be explained by the presence of the tertiary alcoholic group in their structure. Experimentally obtained results are in a good correlation with predicted values using SPARC software.

Acknowledgments

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