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Original scientific paper

A modified RP-HPLC method for the determination of the pK_a values of synthesized *β*-hydroxy-*β*-arylalkanoic acids

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Abstract: The pK_a values of twelve *β*-hydroxy-*β*-arylalkanoic acids and ibuprofen were determined using a modified RP-HPLC method. The stationary phase was octadecyl modified (C-18) silica gel, and the mobile phases were mixtures of methanol and one of ten different buffers (60:40 volume ratio). The mean retention time of each compound was plotted against the pH of each of the ten used mobile phases. The inflection point of the obtained sigmoidal curve represents the ${}^{s}_{w}pK_a$ of a compound. Using ${}^{s}_{w}pK_a$ in previously established equations for the specific methanol/buffer mixture, the ${}^{w}_{w}pK_a$ values (in pure water) were calculated. The obtained ${}^{w}_{w}pK_a$ values for the synthesized compounds were in a range from 3.40 to 3.74, and the ${}^{w}_{w}pK_a$ for ibuprofen was 4.27. The Predicted pK_a values for this type of compounds in the MarvinSketch 5.11.5. Program were in poor correlation with the experimental results, while in ACD/I-Labs pK_a values were calculated as a wide range.

Keywords: dissociation constant; anti-inflammatory compounds; ibuprofen; liquid chromatography; carboxylic acids.

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been used for decades to treat fever, pain and inflammation.[1] The search for new NSAIDs is still a challenge because of non-selective gastric side effects[2] and cardiovascular side effects of selective NSAIDs.[3] The aqueous dissociation constant (K_a) is a physicochemical parameter that has a great impact on the biopharmaceutical parameters of each drug candidate.[4] The negative logarithm of the aqueous dissociation constant, pK_a, affects the solubility, permeability through biological membranes,[5] receptor binding process of a compound[6] and the choice of optimal conditions in drug analysis.

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Determination of pK_a values at the early stage of screening of any newly synthesized, potentially biologically active compound is highly desirable. The prediction of pK_a values using computational methods is very attractive but not always applicable. Two frequently used programs for estimating pK_a values are MarvinSketch 5.11.5[7] and ACD/I-Labs.[8] These programs have a fragment-based approach and they are inadequate if the fragments present in a studied molecule are absent from the database, and hence the estimated values can only be considered as approximate.

Different approaches could be used for pK_a determination, such as potentiometric titration,[9] spectrophotometric titration,[10,11] capillary electrophoresis,[12,13] reversed-phase chromatography[14,15] and nuclear magnetic resonance.[16,17] Reversed phase high performance liquid chromatography (RP-HPLC) methods are widespread and their advantages are that they are simple, only a small amount of compound is needed and the examined compounds need not to be of high purity. The latter is very convenient when the pK_a for synthesized compounds should be determined.[18]

Since NSAIDs are poorly soluble in water, the pK_a values are often determined in mixtures of organic solvent (S, dimethyl sulfoxide, methanol, ethanol, propan-2-ol, acetone or tetrahydrofuran) and water. Methanol shows a solvation effect closest to that of water and it is often used as a solvent of choice for pK_a determination.

β-Hydroxy-*β*-arylalkanoic acids were previously synthesized and their anti-inflammatory activity was evaluated.[19–21] The general aim of the previous studies was to examine the impact of some structural modifications on selectivity towards COX-2 inhibition. The aim of this study was to determine pK_a values of previously synthesized *β*-hydroxy-*β*-arylalkanoic acids. Considering that the synthesized compounds are weak acids sparingly soluble in water and having the same phenylpropanoic moiety as ibuprofen, the modified chromatographic method reported by Oumada *et al*. was used.[22]

EXPERIMENTAL

The chemical structures of tested compounds, which were previously synthesized using modification of the Reformatsky reaction and fully characterized, are shown in Table I.

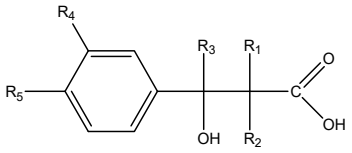
Solvents. Methanol, HPLC grade (J. T. Baker, Deventer, Netherlands), orthophosphoric acid 85 %, (Merck, Darmstadt, Germany), acetic acid, glacial (Merck, Darmstadt, Germany), deionized water (TKA system for water purification, Niederelbert, Germany).

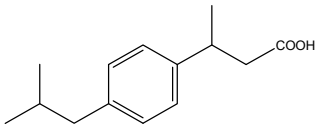
Solid substances. Ibuprofen 99 % (Alfa Aesar, Karlsruhe, Germany), and potassium dihydrogen phosphate *p.a*., sodium acetate *p.a.*, disodium phosphate *p.a*. and potassium bromide *p.a*. (Merck, Darmstadt, Germany).

Apparatus. The ${}^{s}_{w}pK_a$ values of the used mobile phases were measured on a Radiometer model PHM 240 pH/ion-meter (Radiometer, Copenhagen, Denmark). The HPLC analyses were conducted on Agilent HPLC instrument, equipped with a binary pump, a 20-µL loop

valve and a DAD detector. The obtained results were processed with ChemStation software (Agilent Technologies, USA).

TABLE I. Structures of the tested β-hydroxy-β-arylalkanoic acids and ibuprofen





Ibuprofen

Compound	R_1	R_2	R_3	R_4	R_5
1A	–H	–H	$-CH_3$	–H	$-C_6H_5$
1C	$-CH_3$	$-CH_3$	$-CH_3$	–H	$-C_6H_5$
2A	$-CH_3$	$-CH_3$	$-C_6H_5$	–H	–H
2B	–H	–H	$-C_6H_5$	–H	–H
2C	–H	$-CH_3$	$-C_6H_5$	–H	–H
2APN	$-CH_3$	$-CH_3$	$-C_6H_5$	–H	$-NO_2$
2APTF	–H	–H	$-C_6H_5$	–H	$-CF_3$
2APH	–H	–H	$-C_6H_5$	–H	–Cl
2APM	–H	–H	$-C_6H_5$	–H	$-CH_3$
2AMTF	–H	–H	$-C_6H_5$	$-CF_3$	–H
2AMH	–H	–H	$-C_6H_5$	–Cl	–H
2AMM	–H	–H	$-C_6H_5$	$-CH_3$	–H

Procedure. The potentiometric system was standardized with ordinary aqueous buffers, pH 1.679, 4.005, 7.006 and 9.18 (Radiometer Analytical, Villeurbanne Cedex, France). The ${}^{w}_{w}pK_a$ values of the aqueous buffers were measured, as well as the ${}^{s}_{w}pK_a$ values of the prepared mobile phases. The compounds were dissolved in the mobile phase at a concentration of approximately 600 ppm. The HPLC analyses were performed on a Zorbax Eclipse XDB C18 4.6 mm×150 mm, 3.5-µm column at a temperature of 25 °C at a flow rate of 1 mL min^{-1}. Detection was realized at 254 nm. The mobile phase was a mixture of methanol and the appropriate buffer (60:40 volume ratio). The ten buffer solutions listed in Table II were prepared so that the final strength was 0.01 mol m^{-3}. The holdup time was determined with potassium bromide. All measurements were performed in triplicate.

TABLE II. Used buffers, their ${}^{w}_{w}pH$ values and the ${}^{s}_{w}pH$ values of the mobile phases

Buffer	${}^{w}_{w}pH$	${}^{s}_{w}pH$
0.01 mol/L H_3PO_4	2.339	3.126
0.007 mol L^{-1} CH_3COOH + 0.003 mol L^{-1} CH_3COONa	2.555	3.456
0.005 mol L^{-1} H_3PO_4+ 0.005 mol L^{-1} KH_2PO_4	2.712	3.699
0.001 mol L^{-1} H_3PO_4 + 0.009 mol L^{-1} KH_2PO_4	3.338	4.537
0.01 mol L^{-1} CH_3COOH	3.433	4.207
0.009 mol L^{-1} CH_3COOH + 0.001 mol L^{-1} CH_3COONa	4.199	5.293
0.005 mol L^{-1} CH_3COOH + 0.005 mol L^{-1} CH_3COONa	4.835	5.801
0.001 mol L^{-1} CH_3COOH + 0.009 mol L^{-1} CH_3COONa	5.537	6.827
0.007 mol L^{-1} KH_2PO_4 + 0.003 mol L^{-1} Na_2HPO_4	5.585	7.258
0.005 mol L^{-1} KH_2PO_4 + 0.005 mol L^{-1} Na_2HPO_4	7.046	8.679

RESULTS AND DISCUSSION

The mean of triplicate retention times was calculated for each compound and plotted against the pH value of each of the ten mobile phases (Figs. 1–4). According to the used method, the ${}^{\mathrm{s}}_{\mathrm{w}}\mathrm{p}K_{\mathrm{a}}$ values that represent the true thermodynamic constant in a hydro-organic medium can be calculated from retention times of tested compounds in the chromatographic system (according to IUPAC nomenclature, the superscript "s" indicates organic solvent, while the subscript "w" indicates the solvent). The inflection point of the plotted sigmoidal curve represents the ${}^{\mathrm{s}}_{\mathrm{w}}\mathrm{p}K_{\mathrm{a}}$ value. The maximum and minimum retention times are given in Table III. The ${}^{\mathrm{s}}_{\mathrm{w}}\mathrm{p}K_{\mathrm{a}}$ and ${}^{\mathrm{s}}_{\mathrm{s}}\mathrm{p}K_{\mathrm{a}}$ values differ by a constant value δ, which depends solely on the hydro-organic solvent. The δ values for a number of water–methanol mixtures are well established.[23]

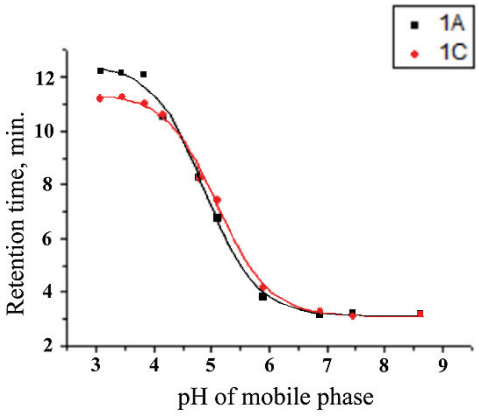


Fig. 1. Variation of retention time of compounds 1A and 1C with the pH of the mobile phase.

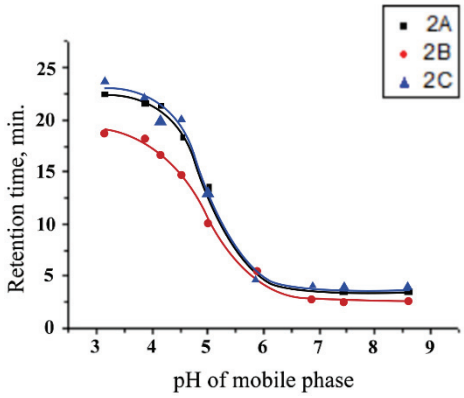


Fig. 2. Variation of retention time of compounds 2A, 2B and 2C with the pH of the mobile phase.

The linear relationship between ${}^{\mathrm{s}}_{\mathrm{s}}\mathrm{p}K_{\mathrm{a}}$ and ${}^{\mathrm{w}}_{\mathrm{w}}\mathrm{p}K_{\mathrm{a}}$ was predicted by Izmailov[24,25] and Rived *et al.*:[26]

$$ {}^{\mathrm{s}}_{\mathrm{s}}\mathrm{p}K_{\mathrm{a}} = a\ {}^{\mathrm{w}}_{\mathrm{w}}\mathrm{p}K_{\mathrm{a}} + b \qquad (1) $$

The constants a and b depend on the composition of the methanol/water mixture, and their values can easily be calculated from the percentage methanol using already established equations. Their value for the used mixture of methanol/buffer (60:40 volume ratio) is known.[27]

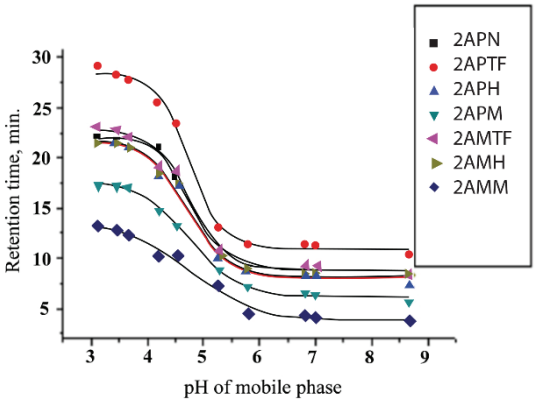


Fig. 3. Variation of retention time of compounds 2APN, 2APTF, 2APH, 2APM, 2AMTF, 2AMH and 2AMM with the pH of the mobile phase.

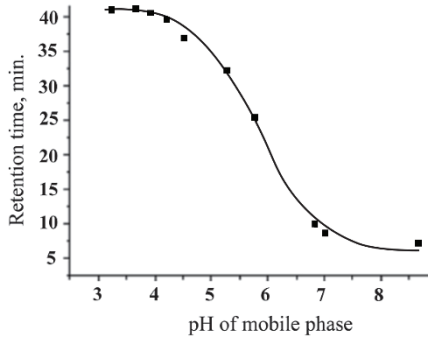


Fig. 4. Variation of retention time of ibuprofen with the pH of the mobile phase.

The mentioned method could be used under specific terms: a methanol/buffer mixture for which constants a and b are known must be used and the tested compounds must be from the same family (in the present case, aromatic carboxylic acids).

The ${}^{\mathrm{s}}_{\mathrm{s}}\mathrm{p}K_{\mathrm{a}}$ and ${}^{\mathrm{w}}_{\mathrm{w}}\mathrm{p}K_{\mathrm{a}}$ values were calculated using Eqs. (2) and (3):

$$ {}^{\mathrm{s}}_{\mathrm{s}}\mathrm{p}K_{\mathrm{a}} = {}^{\mathrm{s}}_{\mathrm{w}}\mathrm{p}K_{\mathrm{a}} - \delta \tag{2} $$

$$ {}^{\mathrm{w}}_{\mathrm{w}}\mathrm{p}K_{\mathrm{a}} = \frac{{}^{\mathrm{s}}_{\mathrm{s}}\mathrm{p}K_{\mathrm{a}} - b}{a} \tag{3} $$

where δ = 0.1756 (value taken from the literature for 60 vol. % of methanol); a = 1.364; b = 0.18.[22]

The slope a in Eq. (3) depends only on the specific solvation term determined by the solvent and family of the compounds studied.[27] Chantooni and

Kolthoff[28] showed that the slope value measures the resolution of acid strength of the family of the compounds in the solvent s in reference to water. This approach has been well established for the pK_a values of families of compounds in different solvents in reference to pK_a values in water.[26,29–31] It was shown that more precise pK_a values for diclofenac, flurbiprofen, naproxen, ibuprofen, butibufen and fenbufen are obtained using *a* and *b* constants for non-*ortho*-substituted aromatic carboxylic acids.[22] Due to certain structural similarity between ibuprofen and the tested *β*-hydroxy-*β*-arylalkanoic acids, the same values of *a* and *b* were used.

Calculated results for synthesized compounds and for ibuprofen are given in Table III.

TABLE III. Experimental and predicted ${}^{w}_{w}\mathrm{p}K_a$ values for the tested compounds

Compound	$t_{R(HA)}$ min	$t_{R(A)}$ min	${}^{s}_{w}\mathrm{p}K_a$	${}^{s}_{s}\mathrm{p}K_a$	${}^{w}_{w}\mathrm{p}K_a$	${}^{w}_{w}\mathrm{p}K_a$ MarvinSketch	${}^{w}_{w}\mathrm{p}K_a$ ACD/I-Labs
1A	12.17	3.18	4.90	4.73	3.60	4.53	4.40±0.40
1C	11.17	3.15	5.10	4.93	3.74	4.54	4.40±0.40
2A	22.44	3.33	5.04	4.87	3.70	4.73	4.10±0.40
2B	18.62	2.53	4.96	4.79	3.64	4.73	4.10±0.40
2C	24.01	3.79	5.00	4.83	3.67	4.72	4.10±0.40
2APN	22.02	8.12	4.80	4.63	3.52	3.53	3.90
2APTF	29.01	10.37	4.75	4.58	3.49	4.17	4.00±0.50
2APH	21.76	7.40	4.72	4.54	3.46	4.08	3.90±0.50
2APM	17.28	5.64	4.74	4.56	3.48	4.52	4.10±0.50
2AMTF	23.06	8.20	4.73	4.55	3.47	4.17	4.00±0.50
2AMH	21.60	8.26	4.72	4.54	3.46	4.08	3.90±0.50
2AMM	13.15	3.69	4.64	4.46	3.40	4.52	4.10±0.50
Ibuprofen	42.06	7.02	5.82	5.65	4.27	4.85	4.41±0.10

The original procedure was modified in that four of the original mobile phases were replaced with three new ones (0.005 mol L^{-1} H_3PO_4 + 0.005 mol L^{-1} KH_2PO_4, 0.007 mol L^{-1} CH_3COOH + 0.003 mol L^{-1} CH_3COONa, 0.007 mol L^{-1} KH_2PO_4 + 0.003 mol L^{-1} Na_2HPO_4). The four replaced mobile phases in the original method consisted of citric acid, potassium dihydrogen citrate, potassium sodium hydrogen citrate and sodium citrate. Instead of them, amounts of phosphate and acetic buffers (which were also used in the original method) were adjusted to ensure they had the same pH values as the replaced ones. The mobile phase preparation was simplified by using fewer substances in the buffers while appropriate pH range was achieved (3.126 to 8.679, Table II). It was taken into account that these pH values were compatible with the used column. The ionic strength of used mobile phases was low (10 mol m^{-3}) and had negligible effect on pK_a values.

Methanol content in the mobile phase was high in order to obtain reasonable retention times. From the solubility aspect, the use of methanol is desirable, because it increases the solubility of most organic compounds. In addition, its relatively high dielectric constant allows the solvation of ionic solutes and the prevention of ion-pair formation, at least at not very high solute concentrations.

The obtained pK_a value for ibuprofen (4.27) was well reproduced concerning the value already reported (4.30). All the tested compounds, including ibuprofen, have an acidic carboxylic group in their structure. All the synthesized compounds could be considered as derivatives of hydroxypropionic acid, which explains the lower pK_a values in comparison to that of ibuprofen, a derivative of propionic acid.

All the compounds have similar pK_a values in range from 3.40 to 3.74. Comparing the pairs of positional isomers: 2APTF and 2AMTF, 2APH and 2AMH, and 2APM and 2AMM, it could be concluded that the position of the substituent (*meta* or *para*) had no significant impact on dissociation constants, which was expected. The type of substituent (nitro, trifluoromethyl, chloro or methyl) also had no impact on protonation because the carboxylic group is not directly attached to the benzene ring. The influence of substitution of side branch with methyl groups had negligible effect on dissociation constant, and hence, there was no significant difference in pK_a values between compounds 1A and 1C, which are derivatives of β-hydroxy-β-biphenylylbutanoic acid, nor among 2A, 2B and 2C, which are derivatives of β-hydroxy-β,β-diphenylpropanoic acid. Compound 2A has the highest pK_a value.

The pK_a values of the tested compounds and ibuprofen were predicted using standard software MarvinSketch and ACD/I-Labs program and the results are presented in Table III. It could be seen that there is poor correlation between the pK_a values predicted by the MarvinSketch program both for the synthesized compounds and ibuprofen (R^2 = 0.3126). The experimental results fall into the range predicted by the ACD/I-Labs program, but the range is very wide and hence, neither of these programs is suitable for pK_a prediction for these compounds.

On the molecular level, acidic compounds with carboxylic groups inhibit both COX-1 and COX-2 by maintaining one of the most important interactions – salt bridges with Arg120. It is desirable for these compounds to have low pK_a values, which ensure their existence in the ionized form at the physiological pH (7.4). In inflamed tissue, the pH level is decreased compared to the physiological value[32] and thus, it could be concluded that it is ensured that the examined β-hydroxy-β-arylalkanoic acids will be almost completely ionized at lower pH values compared to ibuprofen, which will promote salt bridge formation.

CONCLUSIONS

The pK_a values of β-hydroxy-β-arylalkanoic acids, which exhibit anti-inflammatory activity, and ibuprofen were determined using the RP-HPLC method

by Oumada *et al*. It is shown that this method could be used in pK_a determination of compounds that are derivatives of hydroxypropionic acids. The pK_a values of all the synthesized compounds were lower than the pK_a of ibuprofen, which is a derivative of propionic acid. Correlation between the results calculated using the MarvinSketch program and the experimental results is very poor and this program cannot be used for the prediction of the pK_a values of this type of compound. The ACD/I-Lab program is also inadequate because it gives only a wide range of pK_a values. The lower pH values of the synthesized compounds would promote inhibition of COX because of the formation of salt bridges between Arg120 with the ionized form of the compound in comparison to ibuprofen.

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ИЗВОД

ОДРЕЂИВАЊЕ pK_a ВРЕДНОСТИ СИНТЕТИСАНИХ *β*-ХИДРОКСИ-*β*-АРИЛАЛКАНСКИХ КИСЕЛИНА МОДИФИКОВАНОМ RP-HPLC МЕТОДОМ

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pK_a вредности су одређене за дванаест *β*-хидрокси-*β*-арилалканских киселина и ибупрофен применом модификоване RP-HPLC методе. Стационарна фаза је била октадецил-модификовани (C-18) силикагел, а мобилне фазе су се састојале од смеше метанола и једног од десет пуфера (запремински однос 60:40). За свако једињење је израчуната средња вредност ретенционих времена и конструисан је график зависности добијене вредности од pH вредности мобилне фазе. Превојна тачка на сигмоидалној кривој представља ${}^{s}_{w}pK_a$ за свако тестирано једињење. Кад се вредност ${}^{s}_{w}pK_a$ за свако тестирано једињење уврсти у претходно утврђене једначине за специфичну смешу метанола и воде израчунава се вредност ${}^{w}_{w}pK_a$ (у чистој води). Добијене вредности су у опсегу од 3,40 до 3,74, а за ибупрофен је добијена вредност 4,27. Корелација између експериментално одређених pK_a вредности и вредности предвиђених помоћу програма MarvinSketch 5.11.5. је незадовољавајућа, док су у програму ACD/I-Labs pK_a вредности израчунате као веома широк опсег.

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