Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1061 (2004) 217–223
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Determination of dissociation constants of pharmacologically active xanthones by capillary zone electrophoresis with diode array detection

Xiaomu Wu, Suxuan Gong, Tao Bo, Yiping Liao, Huwei Liu*

Key Laboratory of Bioorganic Chemistry and Molecular Engineering of Ministry of Education, Institute of Analytical Chemistry, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China

Received 5 April 2004; received in revised form 13 October 2004; accepted 18 October 2004

Abstract

In this article, the dissociation constants (pK_a) of 10 pharmacologically active xanthones isolated from herbal medicine *Securidaca inappendiculata* were determined by capillary zone electrophoresis with diode array detection. The pK_a values determined by the method based on the electrophoretic mobilities (calculated from migration times) have been proved by the method based on UV absorbance calculated from the online spectra corresponding peaks. No conspicuous difference was observed between the two methods with acceptable reproducibility. Two pK_a values (pK_{a1} and pK_{a2}) were found for four xanthones while generally the 10 compounds possess the pK_a values ranging from 6.4 to 9.2.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Dissociation constant; Xanthone; Capillary zone electrophoresis; Diode array detection; Herbal medicine

1. Introduction

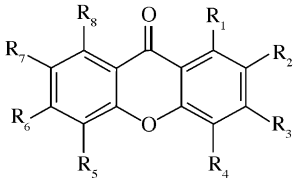
Xanthones, a series of biologically active compounds having similar structures, as shown in Fig. 1, are extracted from various herbaceous plants widely distributed around the world. They are generally used in medicines and food additives. According to extensive medical and pharmacological experiments, xanthones from these plants are reputed to possess digestive, stomachic, tonic, depurative, sedative, and anti-pyretic properties [1–3]. Recently, increasing attention has been given to xanthones due to their inhibitory effect on human immunodeficiency virus type I (HIV-I) replication and cytopathicity [4], monoamine oxidases (MAOs) [5], Epstein–Barr virus early antigen (EBV-EA) [6] as well as proliferation of human T-lymphocytes [7]. Consequently, these compounds have been considered to provide a potential source for the development of new drugs, e.g. anti-depressant, anti-tumor, anti-inflammatory, anti-oxidant, anti-microbial, anti-rheumatism and anti-malarial [4–10].

To develop xanthones as new therapeutic agents, the physicochemical parameters such as water solubility and chemical properties must be ascertained ahead. Particularly, their dissociation constants (pK_a) are of great importance because, from these constants, one can understand the passage of drugs into and across cell membranes, concentration of the medicament in blood, reaction rate, biological uptake and metabolism mechanism. However, the pK_a values of the xanthones have not been reported so far in the literature.

Capillary zone electrophoresis (CZE) method, which relies on measuring the mobility of the solute as a function of pH, has been proved to be an effective and convenient technique for determining the pK_a values, and a close agreement between pK_a values obtained by CZE and other methods has been observed [11–18]. Compared to potentiometry, spectroscopy, conductivity or other techniques, CZE has inimitable advantages, for instance, a lower solute concentration (between 10^{-4} and 10^{-5} M) can be used because of high sensitivity, which is very attractive when

* Corresponding author. Tel.: +86 10 62754976; fax: +86 10 62751708.
E-mail address: hwliu@pku.edu.cn (H. Liu).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.10.085



No.	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
1	OMe	OH	H	H	H	H	OMe	H
2	OH	OMe	OMe	H	H	H	OH	H
3	OH	H	H	OMe	H	H	OH	H
4	OH	OMe	OMe	H	H	OH	OH	H
5	OMe	OH	H	H	H	H	OH	H
6	OH	H	OH	H	H	H	OH	H
7	OMe	H	H	OMe	H	H	OH	H
8	OH	OMe	OH	H	H	H	OH	H
9	OH	H	OH	OMe	H	H	OH	H
10	H	H	OMe	OH	H	H	OMe	H

Fig. 1. Molecular structure of 10 xanthones.

sample amount is limited; the result is independent of solute purity since the disparate components could be effectively separated into individual peaks and calculated, respectively; it is applicable to samples that are weakly soluble in water; and the procedure does not require solute measurement or titrant concentrations, but only the migration times of solutes; it permits pK_a determination in aqueous solutions without difficulties, which is not the case for liquid chromatography (LC), in which the retention could be very strong (for reversed-phase LC) without addition of an organic modifier. In one word, this method is a universal technique for determining pK_a values in a wide pH range, presenting higher sensitivity and selectivity compared to other methods [11–19].

Another important advantage of CZE method, when online diode array detection (DAD) is used, is that we can determine the pK_a values of solutes based on both their migration times and UV absorbance measured from online spectra. Thus, it retains the advantage of CE, namely the possibility of analyzing with limited sample amounts, despite its purity, while the results can be proved to agree with each other [15].

Since the processes of separation, purification and enrichment of the natural products like xanthones are complicated and time-consuming, it is often difficult to obtain sufficient amount of standard samples for other analytical methods such as potential titration that requires relatively high quantity of samples. Therefore, CZE with online DAD should be a good choice for determination of the pK_a values of xanthones. In this paper, the pK_a values of 10 xanthones (Fig. 1) isolated from Chinese herbal medicine *Securidaca inappendiculata* were determined by CZE, based on both linear and nonlinear regressions, and the results were confirmed by UV absorbance from online DAD. The reproducibility of the method was also investigated

2. Theory

2.1. CE method

The CE method relies on the principle that a solute exhibits an electrophoretic mobility continuum versus pH and can be determined by regression analysis. It has its maximum mobility when in its fully charged state, and has no mobility in its neutral state.

The equations which relate eletrophoretic mobility to pK_a can be adequately derived as presented below.

The monoprotic acid HA ionizes; and the thermodynamic dissociation constant K_a is defined as:

$$K_a = \frac{a_{H^+} f_{A^-}[A^-]}{f_{HA}[HA]} = \frac{a_{H^+} f_{\pm}[A^-]}{r_{HA}[HA]} \quad (1)$$

where f_{A^-} can be approximated with the mean activity coefficient $f_{\pm}$. According to Debye–Hückel theory, for the monoprotic acids in water, when the standard molality of the solution (m^{Θ}) is 1 mol kg^{-1}, ionic strength (I) < 0.1 mol kg^{-1}, at 298 K, under p^{Θ}, then [20]

$$\log f_{\pm} = -0.5115\sqrt{\frac{I}{m^{\Theta}}} \quad (2)$$

From Eqs. (1) and (2) and the definition of the effective mobility (μ_e), the K_a values can be obtained by plotting $1/\mu_e$ versus a_{H^+} in the linear form based on Eq. (3) [11,13,19] below:

$$\frac{1}{\mu_e} = \frac{10^{-0.5115\sqrt{I/m^{\Theta}}}}{K_a \mu_{A^-}} a_{H^+} + \frac{1}{\mu_{A^-}} \quad (3)$$

or the nonlinear Eq. (4) [11–19] by setting its second derivative to zero. This was finished by a computer software named CurveExpert Version 1.3 (Daniel Hyams).

$$\mu_e = \frac{\mu_{A^-}}{1 + 10^{(pK_a - pH - 0.5115\sqrt{I/m^{\Theta}})}} \quad (4)$$

For polyprotic acids, which ionize in distinct steps and associate with separate acid ionization constants, the curve of Eq. (4) is generally the same as monoprotic acids except that there is more than one abrupt change in μ_e.

In this study, both the linear [Eq. (3)] and nonlinear [Eq. (4)] fits have been utilized to calculate pK_a values, and the two results were then compared and confirmed through UV spectra collected by online DAD. The migration time of electroosmotic flow (EOF) was calculated based on the negative peak of methanol that was used as sample solvent.

2.2. CZE–DAD method

The CZE–DAD method, similar to conventional spectrophotometry, hinges on the neutral and ionic species having different spectra. When this criterion is met, excellent precision is obtained [15]. Absorbance data were recorded and then processed using a nonlinear fit on the basis of Eq. (5) [11]:

$$pK_a = pH + \log\left(\frac{A - A_{HA}}{A_{HA} - A_{A^-}}\right) - 0.5115\sqrt{\frac{I}{m^{\Theta}}} \quad (5)$$

where A_{HA} and A_{A^-} represent the absorbance of the xanthones in the nonionized and ionized form respectively; and A is the absorbance at a certain pH of the buffer. Both A_{HA} and A_{A^-} are also determined by the regression.

3. Experimental

3.1. Chemicals

All chemicals, including NaH_2PO_4, Na_2HPO_4, HCl, $Na_2B_4O_7$, $NaHCO_3$, Na_2CO_3, $N(CH_2CH_2OH)_3$, $(CH_2)_6N_4$ (Beijing Chemical Factory, China) are of analytical grade and the solutions were prepared with pure water obtained from the DZG-303A water purification system (Aquapro, Chongqing, China) if not otherwise stated. The xanthone standards (purity ≥ 98.0%) were kindly presented by Dr. Yang at the Institute of Medicinal Plant Development [21]. All the xanthones were dissolved in methanol at a concentration of about 1 mg/mL as stock solutions.

3.2. Equipment

Capillary electrophoretic experiments were carried out with a HP ^{3D}CE system (Agilent, Waldbronn, Germany) equipped with a DAD system. Instrument control and data processing were performed with HP ChemStation software (ver. A.08.03). Three untreated fused-silica capillaries of 50 μm i.d. (375 μm o.d.) were purchased from Yongnian Optical Fiber Factory, Hebei, China, with the total length of 46.5 cm (38 cm to the detector), 48.5 cm (40 cm to the detector) and 53.5 cm (45 cm to the detector), respectively. An effective control of capillary temperature was achieved with a built-in air-cooling system, which maintained the temperature at 25 ± 0.1 °C. In order to reduce the analysis time, the applied voltage was set at 30 kV, and the sample was injected at 50 mbar for 5 s. The pH of each electrolyte solution was measured by a pHS-25 pH meter and an E-201-C composite electrode (Leici Instrumental Factory, Shanghai, China).

3.3. Sample preparation

The buffer solutions with different pH values were prepared by mixing appropriate volumes of the stock solutions listed in Table 1 and then diluting to ionic strength $I = 0.03$ [22], which were controlled by the calculation based on Eq. (2) and the acid–base equilibrium theory. The pH was measured at 25 °C by a pH meter, which had been calibrated in advance with standard buffers at the pH 4.01, 6.86, and 9.18.

Table 1
Preparation scheme of buffers with the ionic strength $I = 0.03$ and their pH range[a]

Buffer constituent	Stock solutions	pH range
$(CH_2)_6N_4$–HCl	1.0 M $(CH_2)_6N_4$ and 1.0 M HCl	4.5–6.0
Phosphate	0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4	6.4–7.9
$N(CH_2CH_2OH)_3$–HCl	1.0 M $N(CH_2CH_2OH)_3$ and 0.1 M HCl	8.0–8.5
Borate	0.1 M $Na_2B_4O_7$ and 0.1 M HCl	8.5–9.1
Carbonate	0.1 M $NaHCO_3$ and 0.1 M Na_2CO_3	9.4–11.2
Phosphate	0.1 M Na_2HPO_4 and 0.1 M NaOH	11.6–11.9

[a] The experimental conditions are described in Section 3.

3.4. Procedures

New capillary was flushed with 1.0 M NaOH for 30 min and then pure water for 10 min. In order to equilibrate the capillary and achieve repeatable migration time, a wash sequence with 1.0 M NaOH (1 min), water (2 min) and the running buffer (3 min) was carried out prior to each run, and pre-electrophoresis at 20 kV for 5 s was finally performed before each injection. All the electrolyte solutions were filtered through a disposable 0.45 μm membrane filter and degassed ultrasonically for 5 min before use.

The standard solutions of each xanthone were injected at least in triplicate and the mean value of the migration times was used in calculation. No neutral marker was used, and the EOF was measured by the migration time of solvent methanol appeared as a negative peak on the electropherogram. The migration times of the xanthones were collected at 254 nm and used in calculations by the CZE method. And the UV spectra in the maxima of the electrophoretic peaks were monitored from 190 to 600 nm with an online DAD for analysis in the CZE–DAD technique.

Three xanthones, which respectively possess one, two, and three hydroxyl groups, were chosen for CZE–DAD analysis due to their representatives. Moreover, to verify the experiment reproducibility, the pK_a values of six xanthones were determined by two or three independent series of analysis with disparate capillary length: 48.5 cm (40 cm to the detector), 53.5 cm (45 cm to the detector), 46.5 cm (38 cm to the detector) and disparate sets of buffers at different pH values. Due to the limit of sample amount, not all the compounds were analyzed in different ways.

4. Result and discussion

4.1. Choice of the buffer pH range

Ideally, to measure an unknown pK_a value, it is essential to cover a wide pH range. However, the 10 xanthones studied

Table 2
Measured current for xanthone 4 in used electrolyte solutions at different pH[a]

pH	5.14	5.41	5.64	5.94	6.15	6.45	6.75	7.06
Current (μA)	28.0	28.0	28.5	27.5	28.0	28.0	25.0	25.0
pH	7.36	7.66	8.09	8.39	8.60	8.88	9.10	9.26
Current (μA)	25.5	25.0	30.0	31.0	26.0	27.0	28.0	31.0
pH	9.51	9.80	10.05	10.34				
Current (μA)	30.0	30.0	27.0	27.0				

[a] The experimental conditions are described in Section 3.

constitute a homogenous chemical class and the mean pK_a values are expected to be around 9, according to the optimum buffer pH of separation in previous study [9]. For this reason, a pH range between 7 and 11 was firstly chosen to bracket the expected pK_a values. Notably, four of the xanthones exhibit secondary ionization process in the experiment, therefore the pH range in such cases was adjusted to 4–12.

4.2. Basic considerations on remaining constant ionic strength and viscosity of the background electrolytes

Since the ion–ion interactions may influence the mobility, it is crucial to remain constant ionic strength (I) throughout the buffer series when the pK_a value is determined. Based on literature, when the ionic strength varies from 0.045 to 0.055, the change of determined pK_a values is 0.02 pK_a units or less, which is at the same level as the S.D. of the mathematical nonlinear regression procedure [12]. Through calculation, when I is equal to 0.03, the concentration of the stock solutions are around 10–20 mM. This concentration is proper when taking two opposite effects into consideration together: the high concentration provides a high buffer capacity while a low one shows the advantage of a small joule heating [13,18].

Furthermore, it should be mentioned that there is an additional potential factor that may influence experimental results, namely the change of the viscosity with the electrolyte. Nevertheless, it has been proved that, in low concentration ranges, this effect is negligible [23]. As shown in Table 2, the low concentrations allow an acceptable current intensity between 25 and 35 μA (measured for xanthone 4), showing that the value of 0.03 is suitable for our experiments.

4.3. Effect of temperature and liquid junction potential

In addition to ionic strength, capillary temperature is another significant factor to be considered for pK_a determination. It has been proved in our previous study [19] that the built-in air-cooling system in the CE instrument can provide a satisfactory control of capillary temperature, and the fluctuation of temperature during the process of measuring buffer pH is negligible. Therefore, the experimental results are reliable.

The liquid junction potential will affect the determination of the buffer pH when using the pH meter and composite electrode. By computation, a liquid junction potential of 1 mV would cause a change of 0.02 pH unit [24] which may reduces the accuracy of the experimental results to very limited extent.

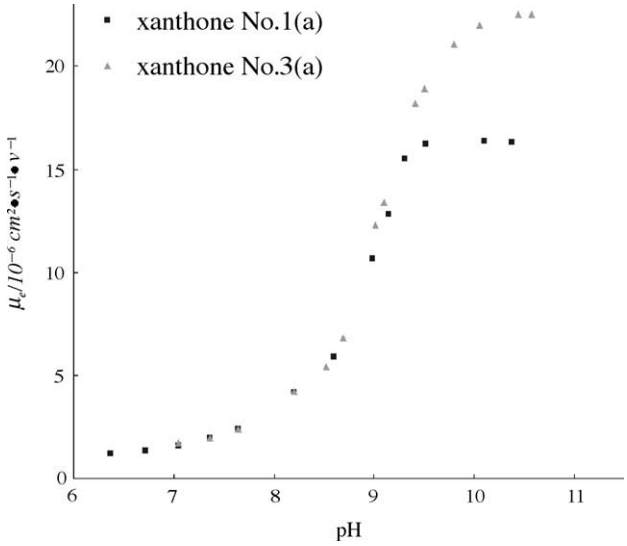


Fig. 2. Relationship between buffer pH and the effective mobilities of two xanthones.

4.4. Calculations of pK_a values

4.4.1. CZE method

Fig. 2 demonstrates an example of the plots of effective mobilities versus pH for the xanthones, from which the pK_a values (listed in Table 3) were obtained using CurveExpert software under the linear regression model Eq. (3) and the nonlinear model Eq. (4). It can be seen that there are no significant differences between the pK_a values determined by the two models, illustrating that both of the two fittings are applicable. In addition, the results obtained by using different capillaries are in concordance.

The curves in Fig. 3 show two inflection points that correspond to two ionization equilibriums. The secondary dissociation constant pK_{a2} is calculated in the same way as above. Here, the nonlinear fitting seems to be more suitable because it evaluates the whole data in one run and takes into account the dependence between the two ionization steps [11,17,18].

4.4.2. CZE–DAD method

Fig. 4 indicates the spectra obtained for xanthone 10 (as previously defined in Fig. 1) by CZE–DAD, demonstrating the relationship between the absorbance and pH values. Fig. 5 illustrates an example of the plots of absorbance versus pH for the xanthone 10, from which the pK_a values (listed in Table 3) were also calculated using the CurveExpert software under nonlinear model Eq. (5).

Once the pK_a values of the 10 xanthones are determined, respectively, their electrophoretic behaviors can be predicted and the optimum pH for their separation is understandable. It is beneficial to select a buffer pH around the mean pK_a

Table 3
pK_a determination of the 10 xanthones by means of the CZE and CZE–DAD[a]

No.	CZE								CZE–DAD	
	Linear fitting				Nonlinear fitting				pK_{a1}	pK_{a2}
	pK_{a1}	γ	pK_{a2}	γ	pK_{a1}	γ	pK_{a2}	γ		
1	9.15 ± 0.02[b]	0.999	–	–	9.15 ± 0.01[b]	0.999	–	–		
	9.19 ± 0.03[c]	0.999	–	–	9.18 ± 0.02[c]	0.999	–	–		
2	9.11 ± 0.02[b]	0.998	–	–	9.15 ± 0.02[b]	1.000	–	–		
	9.08 ± 0.01[c]	0.999	–	–	9.17 ± 0.02[c]	1.000	–	–		
3	9.19 ± 0.03[b]	0.997	–	–	9.16 ± 0.01[b]	0.999	–		9.20 ± 0.03	–
	9.07 ± 0.02[c]	1.000	–	–	9.09 ± 0.03[c]	0.998	–	–		
	9.12 ± 0.01[d]	0.996	–	–	9.15 ± 0.02[d]	0.998	–	–		
4	6.80 ± 0.01[b]	1.000	8.41 ± 0.04[b]	0.996	6.88 ± 0.02[b]	1.000	8.91 ± 0.03[b]	0.997		
5	8.78 ± 0.04[b]	0.996	–	–	9.05 ± 0.01[b]	0.998	–	–		
	8.98 ± 0.03[d]	0.997	–	–	9.10 ± 0.02[d]	0.999	–	–		
6	6.67 ± 0.03[b]	0.996	8.71 ± 0.04[b]	0.996	6.75 ± 0.03[b]	0.999	9.05 ± 0.01[b]	1.000		
7	9.28 ± 0.02[b]	0.995	–	–	9.18 ± 0.02[b]	0.998	–	–		
8	6.42 ± 0.01[b]	1.000	8.67 ± 0.05[b]	0.998	6.44 ± 0.03[b]	0.998	8.95 ± 0.02[b]	0.999		
9	6.40 ± 0.01[d]	0.995	8.66 ± 0.04[d]	0.996	6.68 ± 0.02[d]	0.999	9.02 ± 0.01[d]	1.000	6.71 ± 0.03	9.07 ± 0.02
			8.57 ± 0.04[b]	0.997			9.09 ± 0.01[b]	1.000		
			8.60 ± 0.02[c]	0.994			9.16 ± 0.02[c]	1.000		
10	9.20 ± 0.01[b]	0.997	–	–	9.21 ± 0.01[b]	1.000	–	–	9.26 ± 0.03	–
	9.25 ± 0.01[d]	0.997	–	–	9.23 ± 0.01[d]	0.999	–	–		

[a] The No. corresponds to the compound number in Fig. 1 and γ refers to the regression coefficient. See Section 3 for the conditions.
[b] Total length of the capillary: 48.5 cm (40 cm to the detector).
[c] Total length of the capillary: 53.5 cm (45 cm to the detector).
[d] Total length of the capillary: 46.5 cm (38 cm to the detector).

values of them (about 9) as the mobility differences between the analytes will exhibit a maximum. This has been confirmed by our work on the separation of these xanthones [9,25].

4.5. A brief understanding of the relationship between the structure and pK_a values

The acidity or basicity of a compound depends on the existence, within the molecule, of regions of electron deficiency or abundance. The number and kind of atoms in a molecule are no doubt influential, but the structure is an even more significant factor.

The 10 xanthones studied could be divided into three groups according to the number of hydroxyl they possessed. And a case-by-case analysis is indispensable for considering

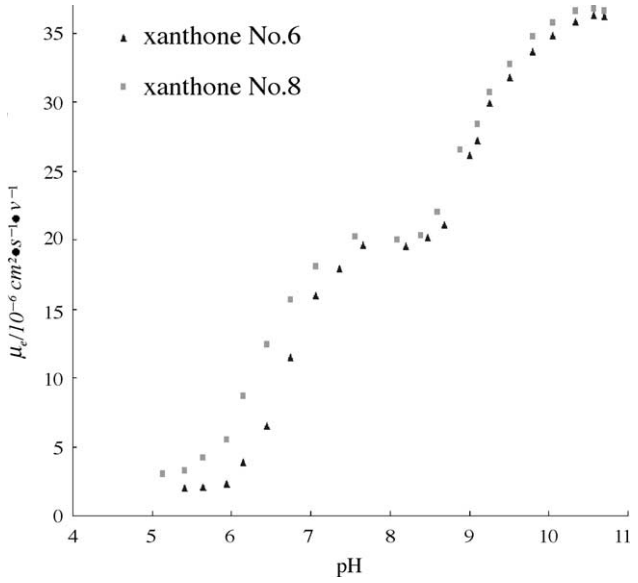


Fig. 3. Effect of pH on the effective mobility of two xanthones that display second ionization.

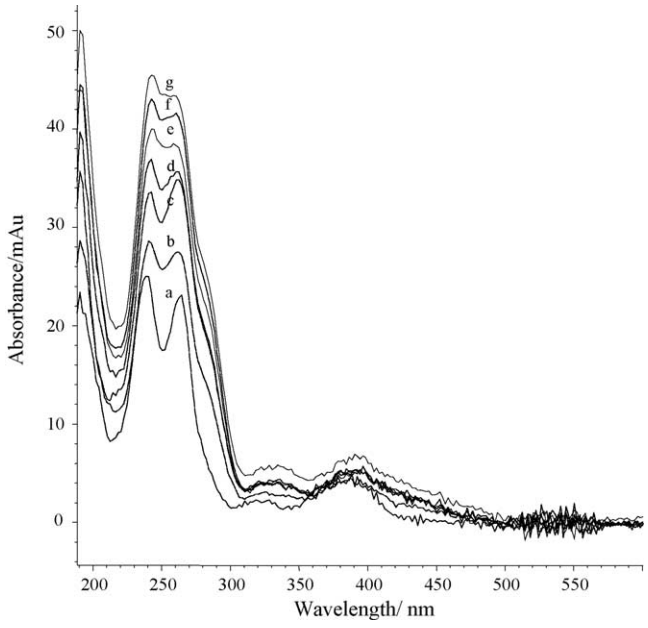


Fig. 4. Spectra collected for xanthone 10 from the maximum of CZE–DAD peaks at different pH values. (a) pH 7.45; (b) pH 8.61; (c) pH 9.04; (d) pH 9.33; (e) pH 9.57; (f) pH 9.86; (g) pH 10.39.

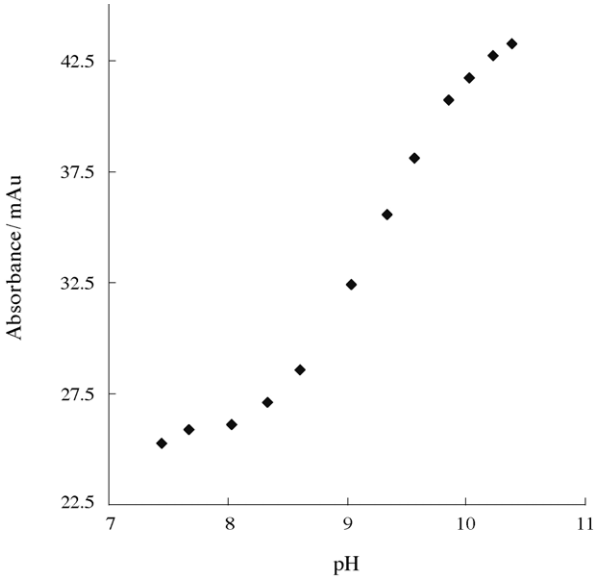


Fig. 5. Effect of pH on the absorbance for the xanthone 10.

the relationship between the structure and pK_a values. The results from nonlinear fit are chosen for their comparably high accuracy as stated above.

With respect to the xanthones 1, 7, and 10, which all have one hydroxyl, their pK_a values are similar (from 9.15 to 9.23, see Table 3), although the vicinity of hydroxyl is methoxy group, –OMe in xanthones 1 and 10. As a matter of fact, this substituent has the electron-releasing resonance as well as the electron-withdrawing inductive effect, and generally speaking, the former is stronger than the latter [26]. However, since the inductive effect falls off rapidly with distance and yet can be effectively transmitted through four or five bonds, when the methoxy group is in the immediate neighborhood, the inductive effect may become strong enough to counteract the influence of resonance. Thereby, xanthones 1 and 10 shows pK_a values alike with xanthone 7, whose adjacent group is hydrogen atom that does not have conspicuous electron-releasing or -withdrawing effect.

As for the two-hydroxyl compounds xanthones 2, 3 and 5, the primary problem that is worth inquiring is which of the two on earth ionizes at the first step. In xanthones 2 and 3, the hydroxyl in R1 might form the hydrogen bond with the oxygen atom in the carbonyl, thus hold it back from ionizing. Moreover, in xanthone 5, the situation about the hydroxyl in R2 and the oxygen atom in the adjacent methoxy group in R1 position is similar. Consequently, it stands a good chance that the hydroxyl in R7 in these three compounds ionizes first. Even though it is a weak acid, this process is comparatively easy. Whereas in the second ionization, the proton removal is opposed by the electrostatic force between the proton and the newly formed ion, which carries a double negative charge and makes the force considerably greater. Hence, this step is more difficult, and occurs at such high pH that the break in curve may be invisible [27]. So their pK_a values are parallel to those one-hydroxyl compounds discussed above.

When it comes to the three-hydroxy compounds, namely, xanthones 4, 6, 8, and 9, the systems turn to be more intricacy to analyze. Besides resonance and inductive effect, the size of ion and other complicated factors may exert influence on the dissociation constants.

Still, due to the existence of hydrogen bond, the hydroxyl in R1 might not be the first to ionize. Considering xanthone 4, once the hydroxyl in R6 or R7 ionizes, the formation of hydrogen bond would render the negative charge on the oxygen atom. The mechanism of shifting the electron density from a highly charged atom increases the stability of the ion and further, makes the ionization favorable. Therefore, the pK_{a1} value is noticeably much smaller than those of xanthones 2, 3, 5 and 7, which also possess a hydroxyl in R7. Moreover, it may be predicted that in xanthones 6, 8, and 9, the hydroxyl in R3, instead of the one in R7, ionizes in the first place. Otherwise, the pK_{a1} values of these three compounds would be higher than that of xanthone 4.

In view of xanthones 6 and 9, they have similar pK_a values (6.75 and 9.05; 6.68 and 9.09, respectively), indicating that the methoxyl group in R4 does not have much impact as presented above. Thereby xanthone 8 should be expected to show pK_a values similar to them; however, this is not the case. It may be ascribed to the disaccord between the theory and the actual situation, or the result of some complicated factors putting together.

5. Conclusion

In this study, the pK_a values of 10 pharmacologically active xanthones have been determined by CZE based on linear and nonlinear regression models; and the results were confirmed by UV absorbance from online DAD. The results showed that the precision of the two methods, expressed in terms of the acceptable repeatability and reproducibility of the migration time, mobility and pK_a values, is acceptable. Since the accuracy of pK_a values obtained from CZE has been confirmed to be in agreement with those from potentiometric methods and, in general, better than those from single chromatographic or spectrophotometric methodologies as described in earlier reports [16], the experimental results are reliable.

Besides the accuracy of the results, CZE and CZE–DAD offer several merits for it requires small amounts of sample, and the high purity or exact solute concentration is not necessary; it is suitable for analyzing substance in aqueous or nonaqueous solvents. And the total analysis time is relatively short.

Acknowledgements

The financial supports of the “President” Research Fund for Undergraduate Students of Peking University and the National Nature Science Foundation of China (NSFC) (grant

nos. 20275001, 20335010 and 90209056) are gratefully acknowledged.

References

[1] P. Valentão, P.B. Andrade, E. Silva, A. Vicente, H. Santos, M.L. Bastos, R.M. Seabra, J. Agric. Food Chem. 50 (2002) 460.
[2] W. Kobayashi, T. Miyase, S. Suzuki, H. Noguchi, X.M. Chen, J. Nat. Prod. 63 (2000) 1121.
[3] P.M. Pauletti, I. Castro-Gamboa, D.H. Siqueira Silva, M.C.M. Young, D.M. Tomazela, M.N. Eberlin, V. da Silva Bolzani, J. Nat. Prod. 66 (2003) 1384.
[4] H.R.W. Dharmaratne, W.M.A.P. Wanigasekera, Phytochemistry 42 (1996) 249.
[5] G. Rath, O. Potterat, S. Mavi, K. Hostettmann, Phytochemistry 43 (1996) 513.
[6] C. Ito, M. Itoigawa, H. Furukawa, K.S. Rao, F. Enjo, P. Bu, J. Takayasu, H. Tokuda, H. Nishino, Cancer Lett. 132 (1998) 113.
[7] M. Pedro, F. Cerqueira, M.E. Sousa, M.S.J. Nascimentoa, M. Pintoa, Bioorg. Med. Chem. 10 (2002) 3725.
[8] B. Madan, I. Singh, A. Kumar, A.K. Prasad, H.G. Raj, V.S. Parmarb, B. Ghosha, Bioorg. Med. Chem. 10 (2002) 3431.
[9] T. Bo, X.D. Yang, F. Liu, K.A. Li, L.Z. Xiu, H.W. Liu, Anal. Chim. Acta 474 (2002) 37.
[10] M.V. Ignatushchenko, R.W. Winter, H.P. Bächinger, D.J. Hinrichs, M.K. Riscoe, FEBS Lett. 409 (1997) 67.
[11] S.J. Gluck, J.A. Cleveland, J. Chromatogr. A 680 (1994) 43.
[12] E. Örnskov, A. Linusson, S. Folestad, J. Pharm. Biomed. Anal. 33 (2003) 379.
[13] M. Matoga, E. Laborde-Kummer, M.H. Langlois, P. Dallet, J.J. Bosc, C. Jarry, J.P. Dubost, J. Chromatogr. A 984 (2003) 253.
[14] Y. Mrestani, R. Neubert, A. Munk, M. Wiese, J. Chromatogr. A 803 (1998) 273.
[15] M. Pérez-Urquiza, J.L. Beltrán, J. Chromatogr. A 917 (2001) 331.
[16] J. Barbosa, D. Barrón, J. Cano, E. Jiménez-Lozano, V. Sanz-Nebot, I. Toro, J. Pharm. Biomed. Anal. 24 (2001) 1087.
[17] P. Bednář, P. Barták, P. Adamovský, A. Gavenda, J. Ševčík, Z. Stránský, J. Chromatogr. A 917 (2001) 319.
[18] J. Barbosa, D. Barrón, E. Jiménez-Lozano, J. Chromatogr. A 839 (1999) 183.
[19] S.X. Gong, X.D. Su, T. Bo, X. Zhang, H.W. Liu, K.A. Li, J. Sep. Sci. 26 (2003) 549.
[20] D.G. Han, Z.D. Gao, P.L. Gao, Physical Chemistry, Higher Education Press, Beijing, 2001, p. 603 (Chapter 21).
[21] X.D. Yang, Ph.D. Thesis, Peking Union College and Chinese Academy of Medical Sciences, 2001.
[22] J.A. Cleveland, M.H. Benko, S.J. Gluck, Y.M. Walbroehl, J. Chromatogr. A 652 (1993) 301.
[23] S.P. Porras, M.-L. Riekkola, E. Kenndler, J. Chromatogr. A 924 (2001) 31.
[24] D.G. Han, Z.D. Gao, P.L. Gao, Physical Chemistry, Higher Education Press, Beijing, 2001, p. 638 (Chapter 22).
[25] T. Bo, X.D. Yang, F. Gao, H.W. Liu, K.A. Li, L.Z. Xu, Chromatographia 55 (2002) 217.
[26] Q.Y. Xing, R.Q. Xu, Z. Zhou, W.W. Pei, Organic Chemistry, Higher Education Press, Beijing, 1993, p. 534 (Chapter 7), p. 273, 279 (Chapter 12).
[27] C.W. Hand, H.L. Blewitt, Acid–Base Chemistry, Macmillan, New York, 1986, p. 171 (Chapter 9).