Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
31 (2003) 679–684
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Determination of dissociation constants of anthrocycline by capillary zone electrophoresis with amperometric detection

Qin Hu [a], Gang Hu [a], Tianshu Zhou [b], Yuzhi Fang [b,*]

[a] *Department of Physiology and Pharmacology, Nanjing Medical University, Nanjing, Jiangsu 210029, People's Republic of China*
[b] *Department of Chemistry, East China Normal University, Shanghai 200062, People's Republic of China*

Received 16 May 2002; received in revised form 29 August 2002; accepted 21 October 2002

Abstract

Although daunorubicin, pharmorubicin and idarubicin are important drugs in medical and in pharmaceutical research, the dissociation constants (K_a) of these substances are frequently not known. A method based on a non-linear model enabling the efficient determination of the K_a of the three drugs by capillary zone electrophoresis with amperometric detection (CZE-AD) has been demonstrated. The pK_a values obtained through the method were in agreement with the values by typical spectrophotometric method.

Keywords: Capillary zone electrophoresis; Electrochemical detection; Anthrocycline; Dissociation constant

1. Introduction

Daunorubicin, pharmorubicin and idarubicin have similar structures (Fig. 1) and are a group of antitumoral antibiotics belonging to anthracycline, which are widely used in clinical cancer therapy [1].

Among the three drugs, idarubicin seems to be less cardiotoxic than daunorubicin and pharmorubicin, especially when given by mouth [2]. However, only idarubicin can be orally administered due to its lipophilic property. Therefore, there is a need to improve the oral bio-availability of the other drugs. For this purpose the physicochemical properties of the drugs must be determined. Water solubility, partition coefficient, and dissociation constant are usually measured for characterizing physicochemical properties. Especially, for understanding and quantifying chemical phenomenon such as reaction rates, biological activity, biological uptake, biological transport and environmental fate [3], dissociation constants (i.e. pK_a values) is a key parameter and has become of great importance because the passage of many drugs into cell and across other membranes is a function of the internal environment, of the physicochemical properties and of the pK_a of the drugs [4].

* Corresponding author. Tel./fax: +86-21-6245-1921.
E-mail address: yuzhi@online.sh.cn (Y. Fang).

0731-7085/03/$ - see front matter
doi:10.1016/S0731-7085(02)00681-7

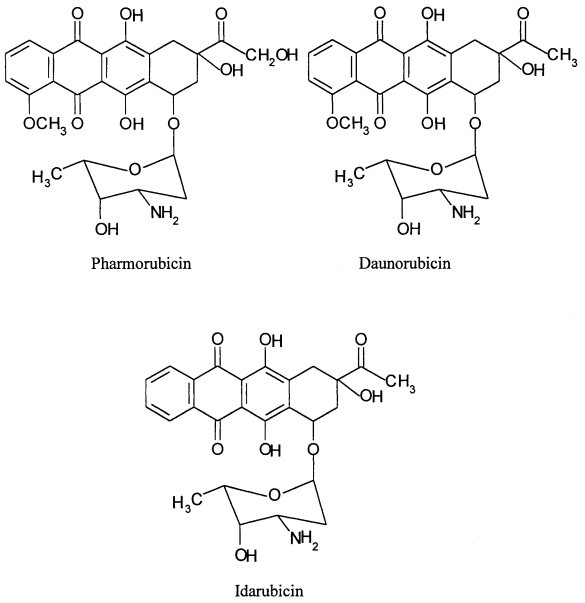

Fig. 1. Chemical structures of the investigated compounds.

To our knowledge, so far there is no report about the determination of pK_a values of the three drugs. Capillary zone electrophoresis (CZE) has been used as a newer method for convenient and precise aqueous pK_a determination [5–7]. The CZE technique has some advantages over the two most commonly used method for pK_a determination: potentiometric titration and ultraviolet spectroscopy [8–10]. Precise potentiometric titration at low concentrations requires time-consuming solvent preparation for carbonate-free solutions and the availability of fully automated. Determination of pK_a values by UV–Vis spectrometry hangs on the neutral and ionic species having different spectra. CE requires only small amounts of sample at low solute concentrations. The procedure does not require measurement of solute concentrations, only of migration times. Furthermore, the calculation of the pK_a is independent of solute purity.

In this paper, we first built the method to determine the dissociation constants of daunorubicin, pharmorubicin and idarubicin by using CZE with amperometric detection (CZE-AD). The spectrophotometric method was performed as a comparative method to CZE-AD for the determination of the dissociation constants.

2. Theory

The determination of dissociation constant by CZE is based on the principle that a solute has its maximum electrophoretic mobility when it is fully ionized, has no mobility in its neutral form, and has an intermediate, well modeled, mobility in the pH region surrounding its pK_a [8–10]. So, the thermodynamic pK_a is related in an expression similar to the Henderson–Hasselbach equation with electrophoretic mobility used to describe the state of solute ionization:

$$\mathrm{p}K_a = \mathrm{pH} - \log\left(\frac{\mu_e}{\mu_a - \mu_e}\right) + \frac{0.5085z^2\sqrt{I}}{1 + 0.3281a\sqrt{I}} \quad \text{(acids)} \tag{1}$$

$$\mathrm{p}K_a = \mathrm{pH} + \log\left(\frac{\mu_e}{\mu_b - \mu_e}\right) - \frac{0.5085z^2\sqrt{I}}{1 + 0.3281a\sqrt{I}} \quad \text{(bases)} \tag{2}$$

where μ_a is the electrophoretic mobility of the fully deprotonated acid, μ_b the electrophoretic mobility of the fully protonated base and μ_e is the electrophoretic mobility observed at the experimental pH. The third term in Eqs. (1) and (2) is the activity correction for buffer ionic strength, I. The variable z is the valency of the buffer and α is the ionic size parameter, 5 Å.

3. Experimental

3.1. Apparatus

The CZE system with wall-jet amperometric detection assembly was constructed in the laboratory and was similar to that described previously [11,12]. Electrophoresis in the capillary was driven by a ±30 kV high-voltage supplier (Shanghai

Institute of Nuclear Research, China). The cyclic voltammetry was carried out on CHI 630 Electrochemical System (CHI instruments, USA). The separations were proceeded in a 46 cm long, o.d. 360 μm, i.d. 25 μm, polyimide-coated fused silica capillary (Polymicro Technologies, Phoenix, AZ). The injector electrode was kept at high positive voltage, the electrochemical cell for detection was kept at ground and samples were all injected electro-kinetically, applying 14 kV for 10 s.

The electrochemical cell composed of a platinum auxiliary electrode, a carbon disk working electrode and an Ag/AgCl (3 mol/l KCl) reference electrode. A BAS LC-3D amperometric detector (Bioanalytical System, West Lafaytte, IN) provided potential control and current output.

The working electrode was a 500 μm carbon disk electrode which was prepared as described previously [13]. Prior to use, the surface of the carbon disk electrode was gradually polished with emery paper and 0.05 μm alumina powder, then ultrasonicated in de-ionized water, and finally positioned carefully opposite the capillary outlet with the aid of a micromanipulator to minimize the gap between the electrode tip and the capillary outlet. The potential applied to the working electrode was set at +1.00 V (against Ag/AgCl, 3 mol/l KCl).

3.2. Chemicals

Daunorubicin, pharmorubicin and idarubicin hydrochlorides were kindly offered by Shanghai Institute for the Control of Pharmaceutical and were used without further purification. All the other chemicals including carrier electrolytes were of analytical reagent grade and bought from local commercial sources, de-ionized water was used throughout. In addition, all experiments were performed at room temperature (25±0.5 °C).

3.3. Sample preparation

Individual samples at a concentration of 0.1 mmol/l were freshly diluted with the running buffer, filtered through a 0.2-μm syringe filter and degassed by ultrasonication prior to use.

3.4. Buffer preparation

A series of buffer solutions with pH from 4.20 to 8.20 were prepared by mixing phosphoric acid, disodium phosphate and monosodium phosphate stock solutions (0.1 mol/l) and diluting to ionic strength $I = 0.03$ [7]. The pH of the buffer was measured at 25±0.5 °C using a pHS-3C precise pH meter (Leici Instruments, Shanghai Precise Science Instrument Ltd. Co., Shanghai). The buffer solutions were filtered through a 0.2-μm syringe filter and degassed by ultrasonication prior to use.

3.5. Method

A new capillary was washed for 10 min with 0.5 mol/l NaOH, followed by washing for 10 min with 0.05 mol/l NaOH and for 10 min with running buffer solution with a laboratory-built high-pressure system. Before each injection, the capillary was flushed with the actual buffer solution for 5 min. Phenol, the pK_a of which is 9.1, was chosen as a neutral marker because it does not ionize at buffer pH < 9. The neutral marker was dissolved in deionized water (0.1 mol/l) and added to the sample solutions to measure the electro-osmotic flow (EOF). The concentration of neutral marker was adjusted to obtain a measurable reference peak. The temperature was kept at 25±0.5 °C, a separation potential of 14 kV was used. Samples were all injected electro-kinetically, applying 14 kV for 10 s.

Data pairs of pH and μ_e were imported into MATHCAD (MathSoft, Cambridge, MA, USA) where μ_e and pK_a were determined by performing a non-linear fit to Eqs. (1) and (2).

3.6. Spectrophotometric pK_a determination

Spectrophotometric pK_a values of the three drugs were determined by taking UV spectra with a Varian Model Cary 50 Probe spectrophotometer using 1-cm cuvettes at 25±0.5 °C. The experimental results showed that the three analytes were fully protonated and deprotonated at about pH 2.50 and 9.00, respectively. Moreover, they were stable in this pH range. So samples were

prepared in pH range of 2.50–9.00. Absorbance measurements were taken at a wavelength which showed a significant difference as a function of pH for each solute, 495 nm for daunorubicin, 485 nm for pharmorubicin and idarubicin.

4. Results

4.1. Buffer capacity

A consideration in creating the buffer series was the buffer capacity, β. Because the difference of pH between the analyte zone and the running solution is omitted when CZE method is used to determine pK_a. Too low of a buffer capacity would result in the inability of the buffer to maintain pH control in the analyte zone, then there would be a bias in the final pK_a determination. The β is defined as [14]

$$\beta = 2.3\left(\frac{k_w}{[H^+]} + [H^+] + \frac{k_a C_t [H_+]}{(k_a + [H^+])^2}\right) \quad (10)$$

where K_w is the equilibrium constant for the dissociation of water, K_a the equilibrium constant for the dissociation of the buffer acid, and C_t is the total concentration of the buffer. From the above equation, we know that β is dependent on C_t when the pH of the buffer is definite.

While dilute buffers are desirable from considerations of the run time, Joule heating and activity, concentrated buffers also offer certain advantages, such as buffering capacity. Concentrated buffers are less likely to exhibit pH shifts due to CO_2 dissolution. In the comprehensive consideration of above issues, 0.1 mol/l of phosphate buffer was used throughout the measurement.

4.2. Reproducibility of migration time

The reproducibility of migration time for the three drugs was determined by repeatedly ($n = 5$) injecting each drug and phenol in the running buffer of 0.1 mol/l phosphate (pH 8.00). The relative standard deviations (R.S.D.s) of the migration time were found to be between 0.09 and 0.26%. This high reproduction of migration time is suitable for the determination of the pK_a values.

4.3. Determination of pK_a values

At each of selected pH values, three replicate determinations were performed for daunorubicin, pharmorubicin and idarubicin, respectively, and the migration times of the analytes and the neutral marker, i.e. t_{app} and t_{eof} at the corresponding pH were recorded.

The pK_a values of the three drugs were determined with Eq. (2), not Eq. (1) for their alkaline bias. Several routines were written in the MATHCAD 4.0 (Mathsoft) program to do the non-linear regressions.

Plots of μ_e versus pH are given in Fig. 2 for daunorubicin, Fig. 3 for idarubicin, and Fig. 4 for pharmorubicin.

To verify the method built, we used spectrophotometric method to measure the pK_a values of the three analytes. pK_a values obtained by CZE-AD are compared with those by the typical method in Table 1. It was known that the CZE-

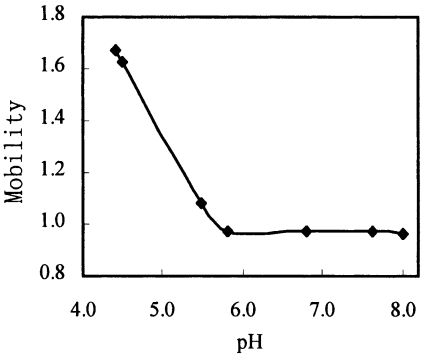

Fig. 2. Plot of mobility ($\mu_e \times 10^4$ cm^2/V s) vs. pH for daunorubicin. CZE-AD conditions: 46 cm × 25 μm i.d.; separation voltage: 14 kV; 500 μm carbon disk electrode; separation medium: 0.1 mol/l phosphate buffer; injection: 10 s at 14 kV; detection potential: +1.00 V (vs. Ag/AgCl, 3 mol/l KCl); temperature: 25±0.5 °C.

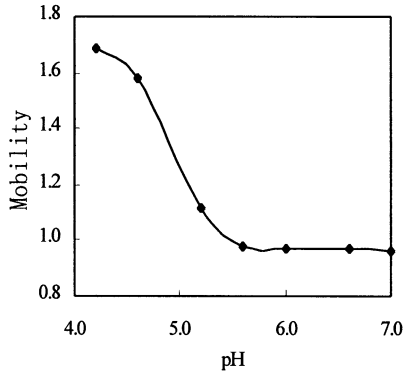

Fig. 3. Plot of mobility ($\mu_e \times 10^4$ cm^2/V s) vs. pH for idarubicin. CZE-AD conditions as in Fig. 2.

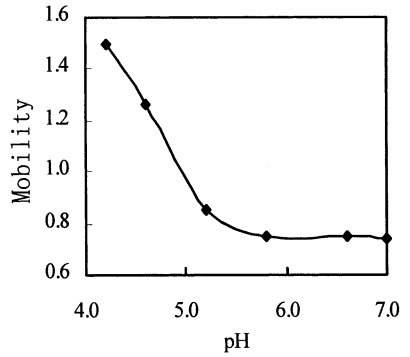

Fig. 4. Plot of mobility ($\mu_e \times 10^4$ cm^2/V s) vs. pH for pharmorubicin. CZE-AD conditions as in Fig. 2.

Table 1
CZE-AD determination of pK_a values compared with spectrophotometry

Sample	PK_a		
	CZE-AD method[a,b]	Spectrophotometry[b]	Difference
Daunorubicin	4.92±0.16	4.99±0.06	−0.07
Idarubicin	4.73±0.21	4.64±0.03	0.09
Pharmorubicin	4.81±0.13	4.92±0.03	−0.11

[a] CZE-AD conditions as in Fig. 2.
[b] The temperature at 25±0.5 °C.

AD determined values were in agreement with the values by spectrophotometric method.

4.4. Factors impacting precision and accuracy

For the determination of pK_a by CZE, some factors are important. Variation in temperature is un-doubt a source of error, since mobility varies by 2.7% per °C [15] and buffer pH is temperature-dependent. In our experiment, air-condition was used to control the room temperature at 25± 0.5 °C, so the largest factor impacting precision and accuracy appears to be the control of the temperature. Therefore, these pK_a values may contain some errors because the temperature inside the capillary was not exactly 25 °C due to the effects of Joule heating. The next factor to temperature control is the measurement of the buffer pH. In the experiments, a precise pH meter was used. Another important factor in the pK_a determination was the ionic strengths. All ionic strengths were set to 0.03, because K_a, μ_a or μ_b depend on the ionic strengths of the background electrolytes.

5. Conclusion

In this investigation, the determinations of dissociation constants of daunorubicin, idarubicin and pharmorubicin have been studied for the first time. So the data may be useful for the physical property studies of these drugs. The pK_a values obtained by CZE-AD were basically the same as those by spectrophotometry. The results of these studies showed that CZE-AD is suitable for the determination of pK_a values. In general, this technique has some advantages over other methods, especially compared with spectrophotometry we used. The procedure does not require measurement of solute concentrations, only migration times, so there is no need for samples in purity and stability and no time-consuming preparation of sample solutions, which is one of the most labor intensive operations in spectrophotometry. It requires only small amounts of sample at very low

solute concentrations due to the high sensitivity of AD method. CZE-AD is especially suitable for those cases where the analytes has a very weak or no chromophore and/or has sparingly low solubility in water. In addition, compared with 4 h per sample in spectrophotometry, the analysis time of the method built was very short, only less than 2 h per sample, which could be reduced by optimizing the column length, field strength, run time and wash cycle time.

References

[1] K. Olaf, H. Stefan, W. Michael, J. Chromatogr. B 728 (1999) 279–282.
[2] H. Georg, H. Stephan, S. Petra, M. Nora, B. Gottfried, B. Joachim, J. Chromatogr. B 698 (1997) 287–292.
[3] S.J. Gluck, J.A. Cleveland, Jr, J. Chromatogr. A 680 (1994) 43–48.
[4] M. Yahya, N. Reinhard, M. Angela, W. Michael, J. Chromatogr. A 803 (1998) 273–278.
[5] J.L. Beckers, F.M. Everaerts, M.T. Ackermans, J. Chromatogr. 537 (1991) 407.
[6] J. Cai, J.T. Smith, Z. Ei Rassi, J. High Resolut. Chromatogr. 15 (1992) 30.
[7] J.A. Cleveland, M.H. Benko, S.J. Gluck, Y.M. Walbroehl, J. Chromatogr. A 652 (1993) 301.
[8] R.F. Cookson, Chem. Rev. 74 (1974) 1.
[9] A. Albert, E.P. Serjeant, The Determination of Ionization Constants: A Laboratory Manual, third ed, Chapman & Hall, New York, 1984.
[10] E.J. King, Acid–Base Equilibria, Pergamon Press, Oxford, 1965.
[11] Y.Z. Fang, X. Fang, J. Ye, Chem. J. Chin. Univ. 10 (1995) 1514.
[12] C.G. Fu, L.N. Song, Y.Z. Fang, Anal. Chim. Acta 399 (1999) 259.
[13] Q. Hu, L. Zhang, T.S. Zhou, Y.Z. Fang, Anal. Chim. Acta 416 (2000) 15.
[14] C.J. Nyman, R.E. Hamm, Chemical Equilibrium, Raytheon Education Company, 1968.
[15] S. Hjerten, Electrophoresis 11 (1990) 665.