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Determination of Dissociation Constants of Loop Diuretics in Acetonitrile–Water Mixtures

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The dissociation pK values of the representative loop diuretics furosemide, bumetanide and ethacrynic acid in 10, 30, 40, 50 and 70% (w/w) acetonitrile–water mixtures at 298.15 K were determined, according to the rules and procedures endorsed by IUPAC. The variation in pK values over the whole composition range studied can be explained by tacking into account the preferential solvation of ionizable substances in acetonitrile–water mixtures. With a view to determining the pK values of the loop diuretics studied in any of the binary solvent acetonitrile–water mixtures, correlations of pK values and different bulk properties of the solvent were examined, and the linear solvation energy relationships method, LSER, has been applied. The pK values were then correlated with the π^*, α and β solvatochromic parameters of acetonitrile–water mixtures. The resulting equations allowed us to calculate pK values for the loop diuretics in any acetonitrile–water mixture up to 70% (w/w) acetonitrile.

Optimising the separation is an important part of any methods development project involving LC. In this technique hydro-organic mobile phases are used, and obviously the understanding and prediction of retention in LC are of fundamental importance to chromatographers. Most researchers have focused attention on mobile phase optimization, since this is the easiest way to control retention and selectivity in LC. Recently, the linear solvation energy relationship (LSER) based on the Kamlet–Taft multiparameter scales[1,2] has been used to predict the retention of series of quinolones,[3] peptides[4] and anabolic steroids[5,6] in LC. However, this approach only allows for the prediction of retention in mobile solvent mixtures of different compositions, but provides no information about the pH of the mobile phase, which is important in understanding the retention process.[7]

The standardization of pH measurements in acetonitrile–water mixtures has not been solved in the past.[8] In a previous study[9] reference pH-values have been assigned to primary standard buffer solutions for the standardization of potentiometric sensors in acetonitrile–water mixtures, according to the NIST multiprimary standard scale.[10] Thus, pH measurements in these media can be performed in a manner similar to that in water.[11]

For pH measurements in acetonitrile–water mixtures, the use of the NIST standard scale with a multiple-point calibration procedure[12] is recommended. Multiple-point calibration with linear regression yields high-precision pH values with maximum possible thermodynamic meaning if applied on the basis of the multiprimary standard scale according to NIST.[12] Linear regression of several, instead of two, E emf(s)/ pH(s) standard couples eliminates the problem of overdefinition caused by the NIST scale as well as the bracketing procedure in which the unknown pH(X) is bracketed by a higher and lower standard pH(s) value according to IUPAC.[13]

Although only a few pK values can be obtained from literature in acetonitrile–water mixtures,[14–17] these values imply some important pK differences from the values observed in water. Thus, the study of acid–base behaviour of analytes in acetonitrile–water mixtures could be very important to predict the influence of pH on retention and selectivity in LC.

Many procedures for the separations, detection and quantitative measurement of individual diuretic agents are based on high-performance liquid chromatography methodologies.[18,19] These drugs are generally excreted in unchanged form to high extent.[18]

Diuretics are widely used in the treatment of congestive heart failure and hypertension.[20] Most increase urinary potassium excretion and can cause hypokalemia in patients taking them for a prolonged period, either covertly or on prescription. Thus, when reliable information is not available from the patient or patient's history,

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the investigation of electrolyte imbalances may be helped by screening the urine for the presence of diuretics. Diuretics are also used to lose weight quickly to enable inclusion in lower weight categories in certain sports (e.g. weightlifting, judo or taekwondo) and to deliberately dilute a urine specimen in an attempt to avoid detection during a drug test.[21] For this reason, the Medical Commission of the International Olympic Committee has banned diuretics. During the Olympic games in Seoul, four athletes tested positively for the loop diuretic furosemide.

The most representative diuretics belonging to the loop diuretics group are considered: furosemide, bumetanide and ethacrynic acid. Loop diuretic activity is mostly the result of the inhibition of the luminal Na^+–$2Cl^-$ – K^+ co-transport system present in the thick ascending limb of the loop of Henle.[20] Ethacrynic acid is a high-ceiling loop diuretic, primarily used in the treatment of pulmonary oedema when a rapid and potent diuretic action is required. Bumetanide is a potent loop diuretic which has an efficiency 40 or 60 times greater than furosemide.[22] These two compounds belongs to the sulfonamide family, although their structures differ considerably.

The aim of this work is the determination of pK values of the representative loop diuretics furosemide, bumetanide and ethacrynic acid in 10, 30, 40, 50 and 70% (w/w) acetonitrile–water mixtures in accordance with IUPAC procedures.[23] The variation in pK values over the whole composition range studied can be explained by the presence of preferential solvation of ionizable substances in acetonitrile–water mixtures. With a view to determining the pK values of the loop diuretics studied in any of the binary solvent acetonitrile–water mixtures, correlations of pK values and different bulk properties of the solvent were examined, and relationships of pK values with the solvatochromic parameters π^*, α and β for the LSER method[1] were studied. The equations obtained allowed us to calculate pK values of the loop diuretics in any acetonitrile–water mixture up to 70% (w/w) acetonitrile.

Experimental

Apparatus. The EMF values were measured (± 0.1 mV) with a model 2002 potentiometer (Crison Instruments, Barcelona, Spain), using a Crison-Ingold 102 623 015 glass electrode and an Ag/AgCl reference electrode prepared according to the electrolytic method.[24] The glass electrode was stored in water when not in use and soaked for 15–20 min in an acetonitrile–water mixture before potentiometric measurements were made.

In all instances the electrode system gave stable and reproducible potentials within 4 min.

The reference electrode was stable for 3 months, during which time the standard potential in each solvent, E°, remained essentially constant (standard deviation, $s < 0.6$ mV). The standardization of the electrode system was carried out each time the solvent medium was changed, and the constancy of E°-values was ensured by periodic calibrations. The cell was thermostatted externally at 25 ± 0.1 °C. The titrant was added from a Metrohm Dosimat 665 autoburette. The potentiometric assembly was automatically controlled with a PC microcomputer.

Reagents. Analytical reagent grade chemicals were used unless indicated otherwise.

All solutions were prepared by mixing double-distilled freshly boiled water whose conductivity did not exceed 0.05 $\mu S\ cm^{-1}$ and acetonitrile (Merck, chromatography grade). 0.1 M potassium hydroxide working solutions were obtained by diluting a concentrated solution 1 M (Merck), and were standardized volumetrically against potassium hydrogen phthalate. Because of the low solubility of potassium hydroxide when using 70% (w/w) acetonitrile–water, the concentration of KOH solution in this medium was 0.02 M. HCl 0.05 M solutions were prepared by diluting the commercial reagent (Merck, 25%).

Ethacrynic acid, furosemide and bumetanide were supplied by Sigma.

Procedures. The pK-values of the diuretics were determined from titration of appropriate solutions of acid species in 10, 30, 40, 50 and 70% (w/w) acetonitrile–water mixtures using potassium hydroxide solutions in the same mixture as the titrant and ca. 7×10^{-3} M KCl solution for the correct response of the electrode system.

pK-values were obtained from systematic measurements of the EMF of the cell:

Pt|Ag|AgCl|HA + A + KCl in acetonitrile–water|GE

where HA and A are the acidic and basic species involved in the dissociation equilibrium studied. The EMF, E, of this cell is directly related to the activities of the hydrogen and chloride ions in solution:

$$E = E^\circ + g \log(a_{H^+} a_{Cl^-}) \quad (1)$$

where E°, the standard EMF of the cell, was determined by the method of Gran from titrations of diluted HCl solutions in the desired solvent using KOH solutions in the same solvent as the titrant, and evaluation of the calibration parameters using a multiparametric data-fitting procedure or Gran plot, as in a previous study.[24]

The dissociation constant for these species could be expressed as:

$$K = \frac{c_A \gamma_A c_{H^+} \gamma_{H^+}}{c_{HA} \gamma_{HA}} \quad (2)$$

Thus the equation which allows pK-calculation is:

$$pK = \frac{E^\circ - E}{g} + \log \frac{c_{HA} \gamma_{HA} c_{Cl^-} \gamma_{Cl^-}}{c_A \gamma_A} \quad (3)$$

where c_{HA} and c_A are the molar concentrations of acidic and basic species, c_{Cl^-} is the molar concentration of the mixed electrolyte KCl and γ_X the molar activity coefficient of species X.

Table 1. pK values of diuretics in acetonitrile–water mixtures up to 70% (w/w) at 298.15 K (values in parentheses are standard deviation, $30 < n < 60$).

Diuretic		Acetonitrile (% w/w) 0	10	30	40	50	70
Ethacrynic acid	pK_1	3.5	3.75(0.02)	4.10(0.01)	4.45(0.01)	4.83(0.02)	5.79(0.02)
Furosemide	pK_1	3.9	4.52(0.04)	4.87(0.02)	5.25(0.03)	5.58(0.01)	6.41(0.02)
	pK_2	—	10.1(0.09)	10.29(0.1)	11.01(0.07)	11.27(0.1)	12.29(0.05)
Bumetanide	pK_1	—	—	5.12(0.01)	5.50(0.01)	5.86(0.03)	6.83(0.01)
	pK_2	—	—	10.75(0.1)	10.91(0.2)	11.11(0.2)	11.75(0.3)

For ethacrynic acid, furosemide and bumetanide p$K_1 < 5$, and computation of c_{HA} and c_A values required knowledge of c_{H^+}, which is a function of the molar activity coefficient γ, which could be calculated using the Debye–Hückel equation:

$$-\log \gamma_{Cl^-} = \frac{AI^{1/2}}{(l + a_0 BI^{1/2})} \quad (4)$$

where A and B are the Debye–Hückel constants, a_0 is the ion size parameter in the solvent mixture and I is the ionic strength. Values of A and a_0B at 25 °C at different percentages of acetonitrile in all mixtures with water have been reported in previous studies.[24,25]

Calculation of $-\log \gamma_{Cl^-}$ requires knowledge of the ionic strength I of the HA + A + KCl mixed electrolyte solution. I is a function of c_{H^+}, which is expressed by:

$$-\log c_{H^+} = \frac{E^\circ - E}{g} + \log c_{Cl^-} + \log(\gamma_{H^+}\gamma_{Cl^-}) \quad (5)$$

Thus, determination of pK values requires an iterative cycle for each point of the potentiometric titration at which E is measured. The calculation begins with $I = c_A + c_{Cl^-}$ and γ_{Cl^-} values are obtained using the Debye–Hückel equation for their subsequent use in obtaining $-\log c_{H^+}$ values and a better value of I and so on, until the constancy of I values is obtained.

The second ionization equilibrium of furosemide and bumetanide took place in very alkaline conditions. Thus the calculations were carried out making use of the program written in PASCAL, PKPOT.[26] The PKPOT program allows the determination of thermodynamic acid–base constants, in aqueous and non-aqueous media, taking into account the activity coefficients of the species. These mathematical procedures also permit the determination of pK values in overlapping ranges ($pK_i - pK_j < 2$) and dissociation constants in very alkaline conditions. The procedures are based on the postulation of a chemical model, i.e. of an initial set of species defined by their stoichiometric coefficients and formation constants, which are then refined by least-squares minimization. The minimized function (U) can be defined as the unweighted sum of squares residuals in the EMF. It takes the form:

$$U = \sum_{i=1}^{n_{tit}} \sum_{j=1}^{n_p} (E_{ij\,exp} - E_{ij\,calc})^2 \quad (6)$$

where n_{tit} is the number of titrations, and n_p the corresponding number of experimental points in each titration. $E_{ij\,exp}$ indicates the measured EMF, and $E_{ij\,calc}$ the calculated EMF.

Results and discussion

EMF measurements for the cell were taken at different concentrations of acidic, HA, and basic, A, species of loop diuretics in 10, 30, 40, 50 and 70% (w/w) acetonitrile–water solvent. For each diuretic in each solvent mixture studied, from four to six series of measurements were performed for a total of 1250 independent measurements over the solvent interval explored. The calculations were made using the program written in PASCAL, the PKPOT previously reported.[26]

Table 1 shows the ionization constant values determined for the loop diuretics, ethacrynic acid, furosemide and bumetanide in 10, 30, 40, 50 and 70% (w/w) acetonitrile–water mixtures and the respective standard deviation, s. Values of bumetanide in 10% (w/w) mixtures was not determined because of the non-solubility of this substance in these media.

The few pK values of diuretics in the literature correspond to their values in water and are shown in Table 2.[27] The value of pK for bumetanide are omitted in Table 1, because values found in the literature, such as $pK_1 = 3.6$ and $pK_1 = 5.2$,[27] differ considerably and the methods used for their determination are not adequately described (temperature, solvent media, ionic strength ...).

As is shown in Table 1, ethacrynic acid has only one pK value corresponding to the dissociation of the carboxylic acid; whereas furosemide and bumetanide have two acid–base functional groups. Values obtained for loop diuretics such as furosemide and bumetanide are

Table 2. Linear solvation energy relationships for pK_1 and pK_2 values of diuretics.

Diuretic	Multiparametric equation	r
Ethacrynic acid	$pK_1 = 36.25 - 5.57\alpha - 37.01\beta - 4.25\pi^*$	0.9998
Furosemide	$pK_1 = 26.15 - 1.93\alpha - 22.11\beta - 5.85\pi^*$	0.9994
	$pK_2 = -9.48 + 22.94\alpha + 32.45\beta - 21.19\pi^*$	0.98

consistent with the protolytic equilibrium of carboxylic acid which takes place in acid media and the dissociation of sulfonamide group under alkaline conditions,[28] as is shown in Fig. 1.

Consideration of the data in Table 1 shows that the extrapolation of pK-values in water to acetonitrile–water mixtures is not linear. This table shows that it is difficult to interpret the variations of pK_1 and pK_2 of loop diuretics according to the percentage of acetonitrile in the mixtures.

Dissociation of a compound in a solvent S is governed by electrostatic interaction, as well as by specific solute–solvent interactions (solvation effects). In the dissociation of neutral or anions acids, charges are created ($HA \rightleftharpoons H^+ + A^-$ or $HA^- \rightleftharpoons H^+ + A^{2-}$), and the dissociation process is disturbed when the dielectric constant of the medium changes with the changes in acetonitrile content. In these cases the electrostatic interaction overwhelms the specific solvation: and for a series of solvents with similar acidity, the change in the dissociation constant can mainly be attributed to the change in dielectric constant. Thus, the following expression can be written:[14,16]

$$pK = A + B/\varepsilon$$

Hence, the correlations between pK values, corresponding to the dissociation of carboxylic and sulfonamide groups, with the reciprocal of the dielectric constant, $1/\varepsilon$, of the acetonitrile–water mixtures, should be linear with good correlation coefficients.

The variation of the pK_1 and pK_2 values of loop diuretics with the reciprocal of dielectric constant is presented in Fig. 2. The variation is different for each substance, although in general the pK values increase when the acetonitrile content increases. Plots of pK_1 of loop diuretics vs. the reciprocal of the dielectric constant show, as expected, good correlation coefficients, greater than 0.996. Plots of pK_2 vs. $1/\varepsilon$ show values of correlation coefficients greater than 0.99. These values are good, considering that the pK_2 values are obtained in very alkaline solutions.

In acetonitrile–water mixtures up to 70% (w/w), plots of ε^{-1} vs. x (the molar fraction of acetonitrile) are related by the expression $\varepsilon^{-1} = 1.26 \times 10^{-2} + 1.73 \times 10^{-2} x$, where 0.9999 is the standard deviation of this equation. Thus, the equation $pK = A + B/\varepsilon$ becomes $pK = A' + B'x$.

The variation of pK_1 and pK_2 values of diuretics vs. the mole fraction of acetonitrile, x, is given in Fig. 3. As

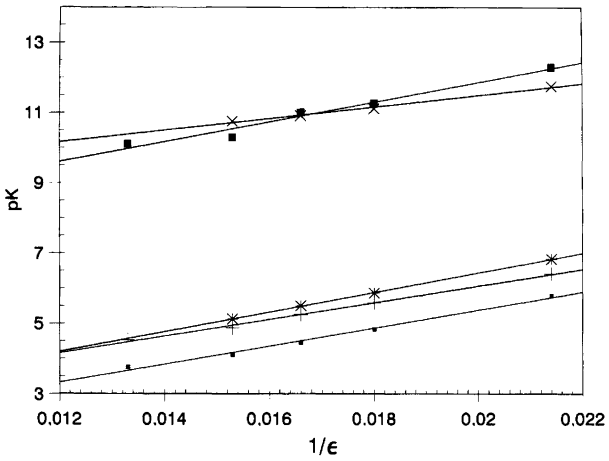

Fig. 2. pK_1 and pK_2 values of loop diuretics vs. $1/\varepsilon$ up to 70% (w/w). ■, Ethacrynic acid; +, pK_1 of furosemide; *, pK_1 of bumetanide; ■, pK_2 of furosemide; x, pK_2 of bumetanide.

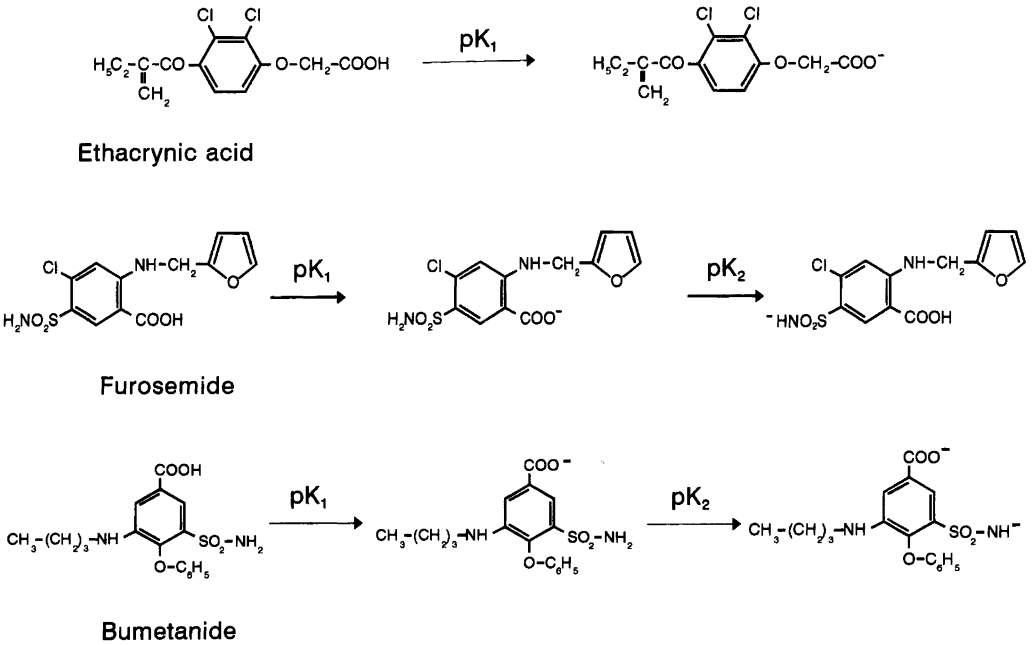

Fig. 1. Protolytic equilibria of loop diuretics.

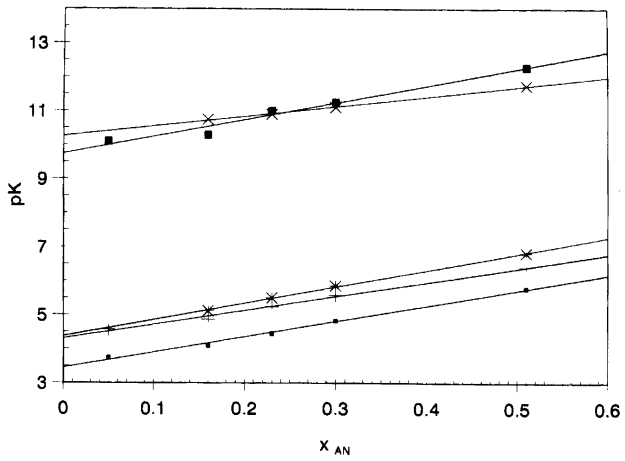

Fig. 3. pK_1 and pK_2 values of loop diuretics vs. x up to 70% (w/w). ■, Ethacrynic acid; +, pK_1 of furosemide; *, pK_1 of bumetanide; ■, pK_2 of furosemide; x, pK_2 of bumetanide.

expected, the pK_1 values of ethacrynic acid, furosemide and bumetanide are linearly correlated with x, with a correlation coefficient greater than 0.997, while the correlation coefficients for plots of pK_2 vs. x are greater than 0.98.

Although pK_1 and pK_2 values of diuretics obtained in acetonitrile–water mixtures increase with the percentage of acetonitrile, they are lower than expected values considering the high pK values expected in acetonitrile.[29] The change of pK values of diuretics in acetonitrile–water mixtures could be explained by the fact that preferential solvation by water exists in acetonitrile–water mixtures,[30] and is probably related to the structural features of these mixtures.[31] Preferential solvation in acetonitrile–water mixtures produces lower pK values than expected when the preferred solvent is water. The composition of the immediate surroundings of a solute may be different from the composition of the bulk mixture. Preferential solvation is attributable to an excess or a deficiency of molecules of one of the solvents in these surroundings.[32] If the solute displays no preference for the solvent molecules, the solvent composition in the cybotactic zone, in the immediate neighbourhood of the solute, is the same as in the bulk. For such cases:

$$pK_S = x_1 pK_{S_1} + x_2 pK_{S_2} \qquad (7)$$

where pK_S is the pK value in the mixture and pK_{S_1} and pK_{S_2} represent the pK values in acetonitrile and water, respectively. The deviation from the ideal dependence on the composition of the mixture indicates that the solvent composition in the neighbourhood of the solute may be different from that in the bulk. In acetonitrile–water mixtures there are three regions.[33] On the water-rich side there is a region in which the water structure remains more or less intact and the acetonitrile molecules gradually occupy the cavities between water molecules. In the range $0.15 \leqslant x_{AN} \leqslant 0.75$ there are clusters of molecules of the same kind surrounded by regions where molecules of both kinds are near each other. In these regions preferential solvation by water exists, which could explain the low increase of pK_1 and pK_2 values of loop diuretics when the percentage of acetonitrile increases. This is in accordance with the previously obtained values of preferential solvation, δ_W, of hydrogen ions by water in acetonitrile–water mixtures.[30] In these regions ($x_{AN} < 0.75$) the solutes are preferentially solvated by water and the variations of pK values are not great. At $x_{AN} \geqslant 0.75$ the number of water clusters is low, and water–acetonitrile interactions that could be discounted in the middle range now become important. This may be considered as a region in which preferential solvation by water decreases.[30]

pK values of the loop diuretics in acetonitrile neat solvent are not known, but pK values of citric acid have been determined in previous studies[14,16] over the whole composition range of acetonitrile–water mixtures. Figures 4 and 5 show these pK values as a function of x_W, the bulk mole fraction of water, where the dotted line represents the expected variation of the pK values between $x_{AN} \cong 0.5$ and pure acetonitrile solvent. Figure 4 also shows the obtained pK_1 values of loop diuretics, and Fig. 5 their pK_2 values. The pK_1 and pK_2 values obtained differ but are lower than the theoretical ones because of the preferential solvation by water; a concave variation of pK vs. x_W may be expected with an inflection point at $x_W = 0.25$, where preferential solvation by water is maximal.[30]

However, it remains unclear whether solvatochromic parameters would be valid to act as stand-ins for generalized solutes in binary solvent mixtures with regard to the properties they are supposed to measure. Preferential solvation in such mixtures may interfere more seriously with the ability of indicators to act as stand-ins for generalized solutes than in the case of single solvents. Progress has been made,[33,34] and although this problem

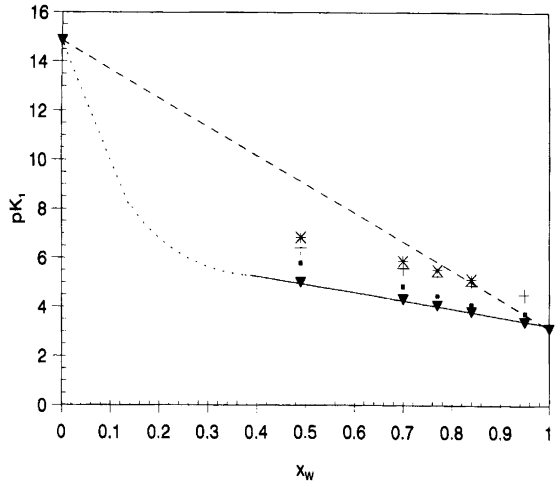

Fig. 4. pK_1 vs. mole fraction of water, x_W, in acetonitrile–water mixtures. ■, Ethacrynic acid; +, furosemide; *, bumetanide; ▼, citric acid. The dashed straight lines correspond to the ideal variation of the pK_1 value for the citric acid.

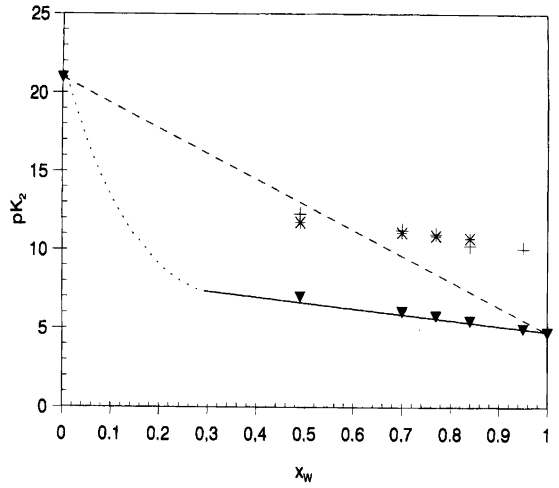

Fig. 5. pK_2 vs. mole fraction of water, x_W, in acetonitrile–water mixtures. +, Furosemide; *, bumetanide; ▼, citric acid. The dashed straight lines correspond to the ideal variation of the pK_2 values for the citric acid.

has not been solved unequivocally, these investigations provide significant evidence that the solvatochromic parameters seem to have general validity. It is therefore of interest to examine the linear solvation energy relationships (LSER) which explain any solute property varying with solvent composition as a linear combination of the microscopic parameters of solvent responsible. The Kamlet–Taft[35] expression states:

$$XYZ = (XYZ)_0 + a\alpha + b\beta + s\pi^* \qquad (8)$$

where α is the solvent hydrogen bond donating acidity, β is the solvent hydrogen accepting basicity and π^* is the solvent dipolarity/polarizability, XYZ is the solute property, $(XYZ)_0$ the value of this property for the same solute in a hypothetical solvent for which $\alpha=\beta=\pi^*=0$, and a, b and s are the susceptibilities of the solute property studied to changes in α, β and π^*, respectively. This equation can include additional terms or some of its terms can become equal to zero, depending on the property of the solute to be described.[35] Values of the Kamlet–Taft solvatochromic parameters π^*,[33,36] α[33,37] and β[33,38] for acetonitrile–water mixtures over the entire range of composition are known.[30]

Several attempts were made to find the best form of the Kamlet–Taft equation to describe the variation of pK_1 and pK_2 values of the loop diuretics in acetonitrile–water mixtures. Multiple regression analysis was applied to our pK data. All possible combinations of solvatochromic parameters, including the normalized parameter E_T^N of Dimroth and Reichardt, were checked. The best fit was obtained when the three solvatochromic parameters α, β and π^* were used, providing the general equations in Table 2 excepted for bumetanide because of the lack of experimental data due to the non-solubility of this substance in the 10% (w/w). The high coefficient in the β terms compared with the α and π^* terms confirmed the main dependence of the pK_1 and pK_2 values of diuretics on the hydrogen bond accepting the basicity of the solvent for the whole range of composition studied, up to 70% (w/w) of acetonitrile, in acetonitrile–water mixtures. The coefficient of the π^* terms is negative in all instances, which means that an increase in the polarity of the mixed solvent causes the pK values to fall. The linear solvation energy relationships obtained, Table 2, permit the pK values of ethacrynic acid and furosemide in any acetonitrile–water mixture up to 70% (w/w) acetonitrile to be known.

From a practical point of view it could be of great interest to apply multiple regression analysis to the whole set of pK values of diuretics and the usual concentration by volume % (v/v), v and weight % (w/w), was the intercept variables. In these cases the third-order polynomials shown in Table 3 were obtained for ethacrynic acid and furosemide, while second-order polynomials were obtained for bumetanide. The equations given in Table 3 enable us to determine the pK_1 and pK_2 values of the diuretics studied in any binary solvent acetonitrile–water mixture up to 70% (w/w) acetonitrile, and thus permit the interpretation of their acid–base behaviour in these widely used hydrorganic mixtures.

Table 3. Relationships between pK values of diuretics and weight, w, and volume, v, percentages of acetonitrile.

Diuretic		r
Ethacrynic acid	$pK_1 = 3.52 + 1.77e - 2w + 3.18e - 5w^2 + 2.57e - 6w^3$	0.999
	$pK_1 = 3.51 + 1.67e - 2v - 1.17e - 4v^2 + 3.81e - 6v^3$	0.999
Furosemide	$pK_1 = 3.95 + 5.33e - 2w - 8.96e - 4w^2 + 9.13e - 6w^3$	0.995
	$pK_1 = 3.93 + 4.83e - 2v - 8.33e - 4v^2 + 8.22e - 6v^3$	0.996
	$pK_2 = 10.43 - 5.52e - 2w + 2.26e - 3w^2 - 1.56e - 5w^3$	0.992
	$pK_2 = 10.61 - 6.50e - 2v + 2.05e - 3v^2 - 1.19e - 5v^3$	0.993
Bumetanide	$pK_1 = 4.38 + 1.72e - 2w + 2.52e - 4w^2$	0.9997
	$pK_1 = 4.60 + 1.23e - 3v + 3.65e - 4v^2$	0.9996
	$pK_2 = 10.71 - 8.74e - 3w + 3.36e - 4w^2$	0.9999
	$pK_2 = 10.96 - 2.02e - 2v + 4.00e - 4v^2$	0.9998

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