Chromatographic Estimation of Apparent Acid Dissociation Constants (pKa) in Physiological Resembling Conditions. A Case Study: Ionisable Non-Steroidal Anti-Inflammatory Drugs

Juan José Martínez-Pla, Laura Escuder-Gilabert, Salvador Sagrado, Rosa María Villanueva-Camañas and María José Medina-Hernández*

Departamento de Química Analítica, Facultat de Farmacia, Universitat de Valencia, C/Vicent Andrés Estellés s/n, E-46100 Burjassot, Valencia, Spain

Internet Electronic Conference of Molecular Design **2003**, November 23 – December 6

Abstract

Motivation. Biopartitioning micellar chromatography (BMC) is a mode of reversed phase micellar liquid chromatography that has proved to be useful in the description and prediction of several pharmacological properties of xenobiotics including oral drug absorption, ocular and skin drug permeability. The present paper deals with the application of BMC to modelling the acid dissociation constant (pKa values), consistent to physiological conditions, of eleven ionisable non-steroidal anti-inflammatory drugs (NSAIDs).

Method. The logarithm of the retention factor using 0.04 M Brij35 as mobile phase (logk4), of the set of compounds at two pH values, 5.5 and 7.4 and 36.5ºC, was measured. The difference between logk4 values at these pHs, dif_logk4 = $logk4_{(pH=5.5)}$ - $logk4_{(pH=7.4)}$, can be used to differentiate between ionisable (dif_logk4 > 0) and neutral NSAIDs (dif_logk4 = 0). A quantitative retention-pKa relationship, can be used to estimate the pKa values of future ionisable NSAIDs.

Results. The BMC based model in the form pKa = b_0 + b_1 dif_logk4 (n=11, r^2 = 0.85, SE = 0.26, F = 52.7, RMSEC = 0.23, RMSECV = 0.28) is made up of one descriptor.

Conclusions. A leave-one-out test procedure shows that the predictive ability of the model for NSAIDs is good. The estimated values should be more reliable for drug discovery studies than the currently used pKa values in water at 25ºC.

Keywords. Liquid chromatography, Non-steroidal anti-inflammatory drugs (NSAIDs), Acid Dissociation Constants (pKa), Physiological conditions

Abbreviations and notations

NSAIDs, non-steroidal anti-inflammatory drugs
BMC, biopartitioning micellar chromatography
$CI_{95\%}$, confidence interval at 95% confidence level
MLR, multiple linear regression
Brij35, polyoxyethylene(23) lauryl ether
r^2, correlation coefficient, $r^2 = 1 - \frac{\sum (y_{calc} - y_{act})^2}{\sum (y_{act} - y_{mean})^2}$

RMSEC, root mean square error in calibration, $RMSEC = \sqrt{\frac{\sum (y_{calc} - y_{act})^2}{n - d - 1}}$, n, number of molecules, d, number of descriptors

RMSECV, root mean square error in cross-validation, $RMSECV = \sqrt{\frac{\sum (y_{pred} - y_{act})^2}{n - d - 1}}$, n, number of molecules, d, number of descriptors

1 INTRODUCTION

Proton transfer is a vital part of many chemical processes and is determined by the acid dissociation constants (pKa) of the chemicals involved. The acid-base character of a xenobiotic is an important property in the study of drug action and in the development of new human and veterinary drugs, crop protecting agents, etc. [1]. This information is essential in the estimation of absorption, distribution, metabolism, and excretion of compounds (ADME properties) in biological systems and the environment since the pharmacokinetic and pharmacodynamic properties of different protonation/ionisation forms of the drug molecule may vary considerably [2]. Moreover, p*K*a values of ionisable drugs also affect their lipophilicity and permeability, which are important physio-chemical considerations to predict bio-availability.

pKa predictions serve as the basis of the more complex pH-dependent calculations of parameter used in quantitative structure-activity relationships, QSAR (i.e. drug solubility). However in most cases the pKa values of drugs used in QSAR are obtained in aqueous media, room temperature and low ionic strength, conditions very far from the physiological ones. Extracellular fluids are basically composed by water, salts, glucose, amino acids, cholesterol, phospholipids, triglycerides, fatty acids and proteins. Phospholipids, cholesterol, fatty acids and triglycerides form micellar complexes with proteins (lipoproteins) (cmc<10^{-6} M) [3]. In addition to the organized character of physiological fluids, it is necessary to consider the effect of temperature and ionic strength on the pKa values. It can be expected important shifts on the dissociation constants of ionizable drugs in physiological conditions with respect to those obtained in aqueous media. Therefore, there is a need to develop methods to estimate the apparent dissociation constant in physiological, or at least resembling, conditions.

Most of the aspects involving computational methods to estimate pKa values have been described: Software packages, QSARs, descriptors, etc. [4] and references therein. Well-defined experimental methods to estimate pKa values, such as potentiometric titration (the standard approach) and the spectrophotometric titrations (the main alternative approach) as well as new approaches such as capillary electrophoresis, the method of choice for the high-throughput determination of p*K*a values in industry, are described in [1] and references therein. However, there are only a few reports about the measurement of p*K*a values for other than completely aqueous electrolyte solutions [1]. The study of the acid–base behaviour in compartmentalized solutions, viz., micelles, reverse micelles, or water-in oil microemulsions, vesicles, etc., has attracted considerable interest [5].

Biopartitioning micellar chromatography (BMC) is a mode of reversed phase micellar liquid chromatography that has proved to be useful in the description and prediction of several pharmacological properties of xenobiotics including oral drug absorption, ocular and skin drug permeability [6]. The mobile phase in BMC composed by micellar solutions of polyoxyethylene (23) lauril ether (Brij35) adjusted to the physiological pH, ionic strength and temperature, resembles

the extracellular fluids. In this paper, the usefulness of BMC to evaluate the apparent pKa consistent to physiological conditions of ionisable drugs is investigated. As a case study, eleven non-steroidal anti-inflammatory drugs (NSAIDs) with available pKa in BMC conditions were used.

2 MATERIALS AND METHODS

2.1 Chemical Data

Table 1 shows available data for the set of eleven NSAIDs studied. Legend of this table indicates the source from which these data are obtained. The data have been ordered according to the experimental apparent pKa value found in BMC.

Table 1. Data for the set of NSAIDs

No	Molecule	logP [a]	pKa (water, 25ºC) [b]	pKa (BMC, 36.5ºC) [c]	logk4 (pH=5.5) [d]	logk4 (pH=7.4) [e]	dif_logk4 [f]
1	Acetamizin	4.13	4.00	4.26	1.242	1.236	0.005
2	Salicilic Acid	2.26	2.97	4.26	0.706	0.610	0.096
3	Tolmetin	2.79	3.50	5.04	1.033	0.849	0.184
4	Fentiazac	5.19	3.60	5.43	1.660	1.424	0.236
5	Ketoprofen	3.12	4.60	5.54	1.332	0.887	0.445
6	Indometacin	4.27	4.50	5.57	1.581	1.275	0.306
7	Diclofenac	4.4	4.50	5.60	1.586	1.285	0.301
8	Naxopren	3.18	4.20	5.85	1.395	0.909	0.486
9	Flurbiprofen	4.16	4.27	5.90	1.591	1.173	0.418
10	Fenbufen	3.62	4.51	5.97	1.381	1.012	0.369
11	Ibuprofen	3.5	5.20	6.14	1.758	1.250	0.508

[a] Logarithm of octanol-water partition coefficient. Data obtained from [7]
[b] Minus logarithm of acid dissociation constant in water at 25ºC. Data obtained from [8]
[c] Minus logarithm of experimental apparent acid dissociation constant in BMC mobile phase at 36.5ºC. Data obtained from [8]
[d] Logarithm of experimental retention factor in BMC conditions (0.04 M Brij35 and pH = 5.5)
[e] Logarithm of experimental retention factor in BMC conditions (0.04 M Brij35 and pH = 7.4)
[f] Difference between logk4 values at pH=5.5 and pH=7.4

2.1 Instruments and Measurements

A Hewlett Packard HP 1100 chromatograph with an isocratic pump, an autosampler an UV–vis detector, a column thermostat and an HP Vectra computer (Amsterdam, The Netherlands) equipped with HP-Chemstation software (A.07.01 [682], 1999) was used. The solutions were injected into the chromatograph by the autosampler with a 20 µl loop. Kromasil octadecylsilane C_{18} columns of 5 µm particle size (150 mm × 4.6 mm i.d.) and a guard column of similar characteristics (35 mm × 4.6 mm i.d.) (Scharlau, Barcelona, Spain) were used. The mobile phase flow rate was 1.0 $ml.min^{-1}$. The detection was performed in UV at 230 nm. The column was thermostated at 36.5ºC. The

retention factors (*k*) values were averages of triplicate measurements and were calculated taking as void volume the first perturbation in the chromatogram after injection. This value was always ranged between 0.93 and 0.96 ml. A Crison Micro pH 2000 pH meter from Crison Instruments (Alella, Barcelona, Spain) was employed to adjust the pH of the mobile phases.

2.3 Reagents and Standars

Mobile phases were prepared by aqueous solutions of polyoxyethylene(23) lauryl ether (Brij35, Acros Chimica, Geel, Belgium) 0.04 M. The pH was adjusted to the desired value (5.5 or 7.4) with 0.05 M citrate buffer prepared with sodium citrate (analytical reagent, Guinama, Valencia, Spain) and the appropriate amount of 2 M solution of hydrochloric acid (for analysis, Merck, Darmstadt, Germany). Some of the NSAIDs were kindly donated by several pharmaceutical laboratories: acemetacin was from Laboratorios Fher (Barcelona, Spain), diclofenac from Novartis (Barcelona, Spain), indomethacin from Laboratorios Llorens (Barcelona, Spain), ketoprofen from Rhône-Poulenc Rorer (Madrid, Spain), naproxen from Syntex Latino (Madrid, Spain), piketoprofen from Laboratorios Farmacéuticos Almirall (Barcelona, Spain), ibuproxam from Laboratorios Fher (Barcelona, Spain), and tolmetin from Laboratorio Estedi (Barcelona, Spain). The following compounds were acquired from different pharmaceuticals: ibuprofen from Nurofen 400 (Boots Healthcare, Madrid, Spain), fentiazac from Donorest 100 (Wyeth-Orfi, Barcelona, Spain), flurbiprofen from Froben 50 (Laboratorios Knoll, Madrid, Spain) and fenbufen from Cincopal (Cyanamid Ibérica, Madrid, Spain). Salicylic acid was from Panreac (Purissimum, Barcelona, Spain).

Stock standard solutions of compounds of 1000 mg l^{-1} were prepared using methanol (HPLC grade, Labscan, Dublin, Ireland) as solvent. Working solutions were obtained by dilution of the stock standard solutions with the mobile phase. Solutions were stored at 4°C. Water used to prepare solutions was purified through a Barnstead E-Pure (Sybron, Boston, MA, USA). Mobile phases were vacuum-filtered through 0.45 μm nylon membranes (Micron Separations, Westboro, MA, USA) and degassed in an ultrasonic bath. All solutions injected into the chromatograph were filtered through 0.45 μm pore size disposable nylon filters (Micron Separations, Westboro, MA, USA).

3 RESULTS AND DISCUSSION

The NSAIDs Ibuproxan and Piketoprofen were not included in Table 1 since the retention factor at pH 5.5 and 7.4 were equal, indicating the neutral character of these molecules. In the 5.5 – 7.4 pH range, which could be considered of therapeutic interest, the rest of NSAIDs give positive dif_logk4 values, which agree with its monoprotic acid nature. Since the mobile phase encountered by

molecules in BMC conditions, can be considered similar to that found around bio-membranes in the living organisms [6], the pKa value estimated in BMC conditions at 36.5ºC should be better than the currently employed pKa in water at 25ºC. Table I reveals that pKa values in water at 25ºC are systematically lower than those found in BMC at 36.5ºC (differences go from 0.26 to 1.83 pKa units), so different conclusion would be encountered with QSAR models using these pKa values rather than those in pure aqueous media.

In a previous work, we have demonstrated that the retention in BMC is related to the hydrophobicity (logP) and the molar total charge of the molecule (α), which can be calculated as a function of the concentration of the hydrogen ion and K, the acid dissociation constant; i.e. for monoprotic acid compounds $\alpha = (-1) / (1+ [H^+]/K)$ [9]. This suggests that a relationship between the retention in BMC and K should exist. Some models were investigated in order to establish such relationship. First, single-descriptor models using logk4 values are tested:

$$pKa_{(BMC)} = 3.1\ (\pm\ 1.4) + 1.6\ (\pm\ 1.0)\ logk4_{(pH=5.5)}$$
$$r^2 = 0.59,\ SE = 0.43,\ F = 13.0\ (p=0.0060) \qquad (1)$$

$$pKa_{(BMC)} = 4\ (\pm\ 2) + 0.9\ (\pm\ 1.8)\ logk4_{(pH=7.4)}$$
$$(p=0.29) \qquad (2)$$

Where errors associated to the coefficients are ± ½ $CI_{95\%}$. The model using logk4 at pH=5.5 (Eq. 1) to predict pKa in BMC is significant, but not very adequate according to its regression statistics, while the model using logk4 at pH=7.4 (Eq. 2) is not significant. This points out that a single logk4 value does not describe well the dissociation of the molecules. The fact that the results are better at pH = 5.5 can be explained in terms of larger differences in the degree of ionisation of NSAIDs at this pH value; while at pH = 7.4 the ionised form is predominant for all NSAIDs.

According to [9], logP would have some chance in improving the model quality. A two-descriptor models using logk4 (at pH = 5.5) and log P values are tested:

$$pKa_{(BMC)} = 3.6\ (\pm\ 1.1) + 2.7\ (\pm\ 1.1)\ logk4_{(pH=5.5)} - 0.6\ (\pm\ 0.4)\ logP$$
$$r^2 = 0.81,\ SE = 0.31,\ F = 17.0\ (p=0.0010) \qquad (3)$$

Eq. 3 shows that pKa (BMC) can be better described using logk4 (pH=5.5) together with logP, as independent variables than using the retention information alone. However, this implies the availability of log P values (i.e. for the new molecules to be estimated), the introduction of logP uncertainty to the relationships, which uses to be larger than that introduced by logk data [10], and finally, the statistical inconvenient of using a two-descriptor models (MLR) in contrast to the single-descriptor ones (Simple Linear Regression), particularly for series with low number of

molecules. To avoid these problems, we proposed the use of the difference between the retention data obtained at the two pH values as single descriptor:

$$pKa_{(BMC)} = 4.3\ (\pm\ 0.4) + 3.7\ (\pm\ 1.1)\ dif\_logk4$$
$$r^2 = 0.85,\ SE = 0.26,\ F = 52.7\ (p<0.0001) \qquad (4)$$

This model, besides more practical, is also more reliable and convenient than that of eq. 3, from a statistical point of view. Figure 1 shows the pKa (in BMC conditions) vs. dif_logk4 relationship and Figure 2 the validation plot including the fitted and cross-validated data.

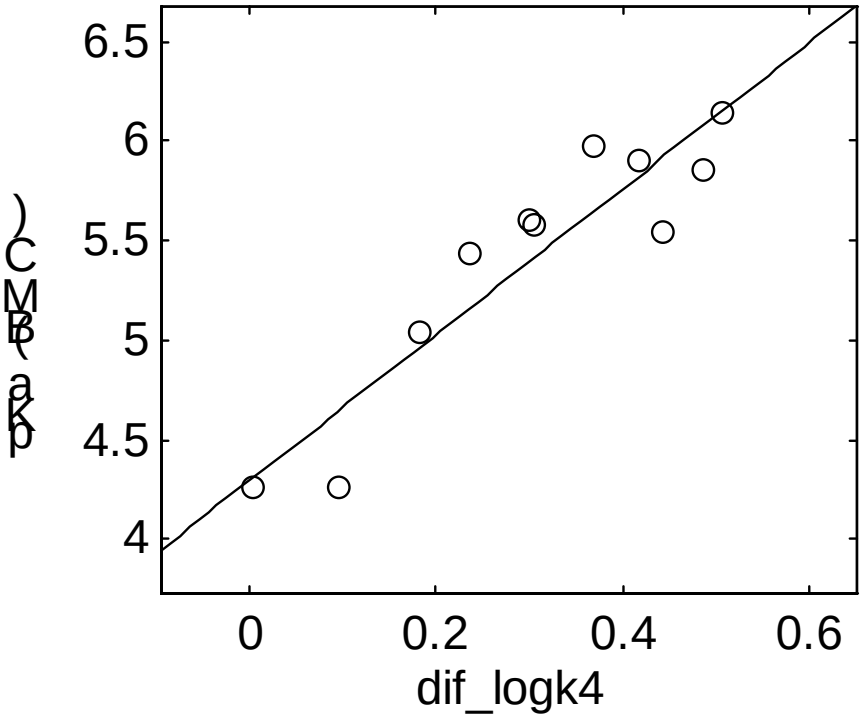

Figure 1. pKa (BMC)- dif_logk4 relationship

These plots suggest that the model is stable for the actual data set. The corresponding quality parameters for Eq. 4 are *RMSEC* = 0.23 and *RMSECV* = 0.28. All these results indicate that the model has an adequate predictive ability. pKa estimates of new NSAIDs are expected to be reasonably precise. For instance, for low and high dif_logk4 values (i.e 0.05 and 0.5), the confidence interval for pKa would be 4.5 ± 0.3 and 6.1 ± 0.3, respectively, while for intermediate results (i.e dif_logk4 = 0.25) the confidence interval for pKa would be 5.2 ± 0.2. In addition, these pKa estimations should be more consistent than those measured or estimated in water at 25ºC in

order to be used as descriptor for future QSAR studies involving physiologic drug bioactivity studies.

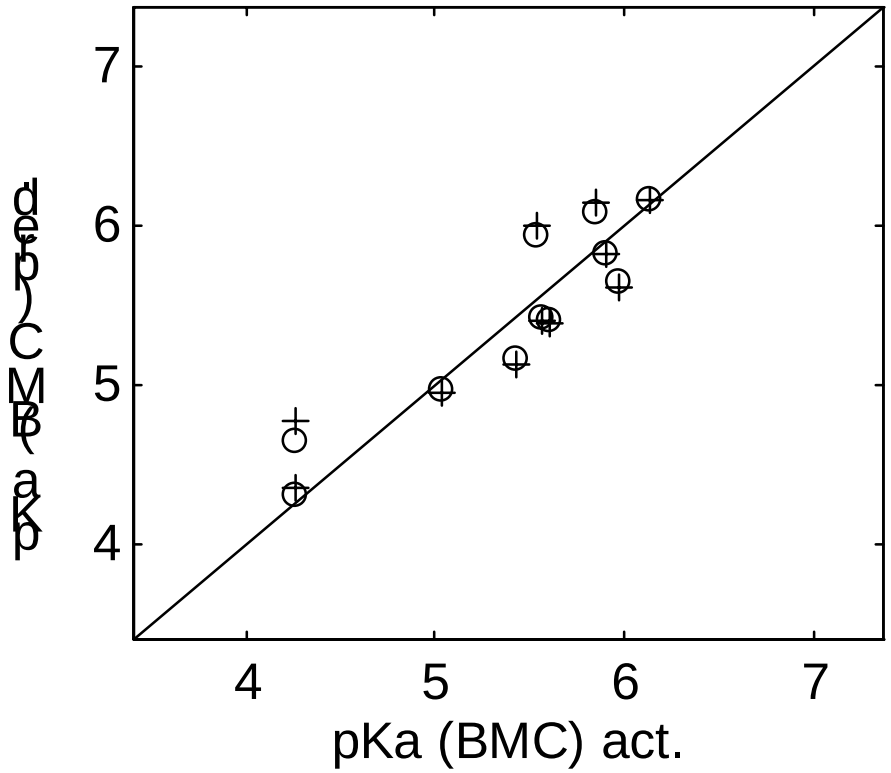

Figure 2. Predicted, fitted (o) and cross-validated (+), vs. actual pKa (BMC) values.

4 CONCLUSIONS

A single-descriptor model, $pKa_{(BMC)} = b_0 + b_1$ dif_logk4, is able to describe the relationships between the dissociation constant of NSAIDs and the retention of these molecules in BMC at two pHs in the therapeutic pH range of 5.5 to 7.4. This strategy should work also for other families of ionisable drugs showing a single pKa value in that pH range. For instance, for monoprotic basic molecules, negative values of the parameter dif_logk4 will be obtained, but they should probably be related to the pKa in BMC conditions. Finally, for new compounds with no previous information on its acid-base behaviour, the dif_logk4 values may determine the nature of the molecule, neutral, acid or basic, in the therapeutic pH range.

Acknowledgment

The authors acknowledge the Spanish Ministry of Science and Technology (MCYT) and the European Regional Development Fund (ERDF) for the financial support (Project SAF2002-01330). The authors also acknowledge the Generalitat Valenciana (Grupos 04/02) for the financial support.

5 REFERENCES

[1] S.K. Poole, S. Patel, K. Dehring , H. Workman and C.F. Poole, *J. Chromatograph. A*, **2004**, *1037*, 445–454
[2] P.J. Taylor, in: *Comprehensive Medicinal Chemistry,* Eds. C. Hansch, G. Sammer, J.B. Taylor, Vol. IV, Pergamon Press, Oxford, 1990 , pp. 241.
[3] D. Voet and J. Voet in *Bioquímica*, Ed. Omega. Barcelona 1992.
[4] A. Chaudry and P. L. A. Popelier, *J. Org. Chem.* **2004**, *69*, 233-241.
[5] M. Caselli, V. Daniele, A. Mangone, and P. Paolillo, *J. Colloid Interface Sci.* **2000,** *221***,** 173–180
[6] L. Escuder-Gilabert, J.J. Martinez-Plá, S. Sagrado, R.M. Villanueva-Camañas and M.J. Medina-Hernández, *J. Chromatogr. B* **2003***, 797,* 21-35.
[7] L. Escuder-Gilabert, S. Sagrado, R.M. Villanueva-Camañas and M.J. Medina-Hernández, *Bioomed. Chromatogr.* **2004***, 18 in press*.
[8] J.J. Martínez-Pla, Y. Martín-Biosca, S. Sagrado, R.M. Villanueva-Camañas and M.J. Medina-Hernández, *J. Chromatogr. A* **2004***, 1047*, 255-262.
[9] L. Escuder-Gilabert, J.M. Sanchis-Mallols, S. Sagrado, M.J. Medina-Hernández and R.M. Villanueva-Camañas, *J. Chromatogr. A* **1998***, 823,* 549-559.
[10] J.M. Bermúdez-Saldaña, L. Escuder-Gilabert, M.J. Medina-Hernández, R.M. Villanueva-Camañas and S. Sagrado, *J. Chromatogr. A* **1998***, 823,* 549-559.