ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
26 (2001) 477–486

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

# p$K_a$ determination of angiotensin II receptor antagonists (ARA II) by spectrofluorimetry

E. Cagigal, L. González, R.M. Alonso, R.M. Jiménez *

*Dpto. Química Analítica, Facultad de Ciencias, Universidad del País Vasco, Apdo. 644, E-48080 Bilbao, Spain*

Received 24 November 2000; received in revised form 9 February 2001; accepted 24 February 2001

**Abstract**

The acid–base equilibrium constants of a new family of antihypertensive drugs, the angiotensin II receptor antagonists (ARA II), Losartan, Irbesartan, Valsartan, Candesartan cilexetil, its metabolite Candesartan M1 and Telmisartan were determined by spectrofluorimetry. Relative fluorescent intensity ($I_{F,rel}$)–pH data were treated by graphical (derivatives and curve-fitting) and numerical methods (LETAGROP SPEFO). The resultant p$K_a$ values at an ionic strength of 0.5 M were ($3.15 \pm 0.07$) for Losartan, ($4.70 \pm 0.06$) for Irbesartan, ($4.90 \pm 0.09$) for Valsartan, ($6.0 \pm 0.1$) for Candesartan cilexetil, ($3.9 \pm 0.1$) for Candesartan M1, and ($4.45 \pm 0.09$) for Telmisartan. © 2001 Elsevier Science B.V. All rights reserved.

*Keywords:* Acid–base equilibrium constants; Spectrofluorimetry; Antihypertensive drugs; Angiotensin II receptor antagonists

## 1. Introduction

The absolute risk of cardiovascular events is mainly determined by high blood pressure, although there are some other important contributors, such as age, race and presence of other cardiovascular risk factors. Hence, antihypertensive therapy enables to reduce considerably the risk of developing cardiovascular complications that cause a high mortality ratio in the industrialised countries [1].

The trend in cardiovascular drug research has been to develop new compounds acting on very specific targets such as cell surface receptors. Angiotensin II receptor antagonists (ARA II) represent a new pharmacological class of antihypertensive drugs [2–4]. They block selectively the $AT_1$ angiotensin II receptor, are long-acting and have a good tolerability profile.

Losartan, the prototype of this class of drugs, was approved in 1995 by the U.S. Food and Drug Administration. Some other antagonists such as Valsartan, Irbesartan, Candesartan cilexetil and Telmisartan have been launched recently. Thus, any advance in the development of analytical methods for their determination or in the knowledge of the properties of these compounds represents a great contribution.

* Corresponding author. Tel.: +34-94-6015504; fax: +34-94-4648500.

*E-mail address:* qapjisar@lg.ehu.es (R.M. Jiménez).

0731-7085/01/$ - see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0731-7085(01)00413-7

The knowledge of acid–base equilibria of these antihypertensive drugs has a great pharmacological importance, since it makes easy the study and understanding of the physiological mechanisms in which the angiotensin II receptor antagonists are involved. The role of these agents depends on their charge, and so does their liposolubility. The $pK_a$ values of these drugs can be of great relevance for the passage of the drug across membranes in processes such as body absorption, tissue distribution and elimination. So, knowing the structure and the different acid–base species, the best way of drug administration, the absorption rate, the distribution profile and the excretion percentage can be established [5].

The experimental techniques mainly used for the determination of acid–base constants are potentiometry, UV–vis spectrophotometry, liquid–liquid extraction and ionic exchange [6]. Potentiometry and UV–vis are the most adequate and versatile for the study of equilibrium constants in homogeneous phase [7].

Besides, the information about dissociation constants is also necessary to choose the optimal conditions for the extraction of these drugs from body fluids, which is an essential step to develop analytical methods for their determination [8].

However, different values for acid–base constants for Losartan (2.36 and 5.55 [9], 4.9 [10], 5.6 [11], 5.6 [12]), Irbesartan (4.5 [13]) and Valsartan (3.9 and 4.7 [14,15]) are collected in studies dealing with chromatographic determination methods or pharmacological studies. In these works the method used for the $pK_a$ determination is not mentioned.

$pK_a$ values of other antihypertensive drugs such as diuretics, beta-blockers and calcium antagonists have been determined by potentiometric titrations [16,17] and UV–vis spectrophotometry [18]. In our laboratory, $pK_a$ determination of ARA II was tried by means of UV–vis spectrophotometry, but it was not possible due to the slight variation of their absorption spectra with pH, with the exception of the pro-drug Candesartan cilexetil.

The existence of different fluorescent groups (fluorophores) in the molecular structure of ARA II such as biphenyl, imidazole and benzimidazole [19,20] together with studies dealing with the application of fluorescent detection coupled to HPLC for the determination of Losartan [21,22], Irbesartan [13], Valsartan [23,24] and Candesartan [25,26] led us to carry out a study of the fluorescent properties of this type of antihypertensive drugs as a function of pH.

The aim of this work is the application of spectrofluorimetry to the determination of the acidity constants of some ARA II. The compounds studied were *Losartan* (2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazol, potassium salt), *Irbesartan* (2-butyl-3-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl] 1,3diazaspiro[4,4]non-1-en-4-one), *Valsartan* ((*S*)-*N*-valeryl-*N*-{[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl}-valine), *Candesartan cilexetil* (( ± )-1-(cyclohexyloxycarbonyloxy)ethyl 2-ethoxy-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-benzimidazol-7-carboxylate), its metabolite *Candesartan M1* (2-ethoxy-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-benzimidazol-carboxylic acid), and *Telmisartan* (4′-[(2-*n*-propyl-4-methyl-6-(1-methylbenzimidazol-2-yl)-benzimidazol-1-yl)methyl)biphenyl-2-carboxylic acid).

## 2. Experimental

### 2.1. Apparatus

pH measurements were made using a Radiometer PHM 84 pHmeter (Bagsvaerd, Denmark), with a Crison 5209 combined glass electrode (Barcelona, Spain). An Ag/AgCl reference system was used with 3 M KCl saturated in AgCl as electrolyte.

Excitation and emission spectra and relative fluorescence intensity measurements of ARA II solutions were obtained using a Shimadzu RF-540 spectrofluorimeter (Kyoto, Japan) controlled by a Shimadzu DR-3 data recorder (Kyoto, Japan). A quartz cell of 1 cm of optic length was used. Data collection was made by means of FLUORIM software. This program allows the digital collection of the main types of scans that a commercial

spectrofluorimeter can perform: emission, excitation and synchronic; as well as the measurement of the relative fluorescent intensity as a function of time [27]. A Haake D8 thermostatic bath (Karlsruhe, Germany) was used to keep the temperature constant, $20 \pm 0.5$°C.

### 2.2. Reagents and solutions

ARA II studied were supplied by the pharmaceutical companies: Losartan (Merck, NJ, USA), Irbesartan (Sanofi, Montpellier, France), Valsartan (Novartis Pharma, Basel, Switzerland), Candesartan cilexetil and its metabolite Candesartan M1 (Astra, Mölndal, Sweden) and Telmisartan (Boehringer Ingelheim, Rhein, Germany).

Methanol was Lab-Scan HPLC grade (Dublin, Ireland). Water was obtained from a Milli-RO, Milli-Q Waters systems.

Stock solutions of the ARA II (1000 μg/ml) were prepared in methanol and were kept in dark and stored under refrigeration at 4°C to minimise decomposition.

Different buffer solutions at a concentration of 0.5 M were prepared: $H_3PO_4/KH_2PO_4$, $CH_3COOH/CH_3COONa$, $KH_2PO_4/K_2HPO_4$ and $H_3BO_3/NaH_2BO_3$. 1 M HCl and 1 M NaOH were added to these buffer solutions to get the desired pH value. Diluted solutions (5 μg/ml) were prepared daily in 5% methanol with the above mentioned buffers at a 0.05 M concentration and at a constant ionic strength of 0.5 M in KCl. The rest of reagents used were of analytical grade.

## 3. Results and discussion

A study of the fluorescent properties of ARA II was carried out in order to choose the optimum wavelengths for the measurement of fluorescent intensity for each compound. The procedure consisted of an iterative process where one of the wavelengths (excitation or emission) was fixed while the other was extracted from the maximum of the spectra. That operation was made alternatively for both wavelengths to obtain the optimum values. Excitation and emission spectra were obtained at an excitation and emission slit of 5 nm width and at the highest sensitivity of the instrument. In Table 1 the optimal wavelengths for each drug are collected.

### 3.1. Influence of pH on the relative fluorescent intensity

Solutions of ARA II at different pH values were prepared to study the influence of pH on relative fluorescent intensity, $I_{F,rel}$. The plots $I_{F,rel}$–pH (Fig. 1) showed increasing (Telmisartan) or decreasing (Losartan, Irbesartan, Valsartan, Candesartan cilexetil and Candesartan M1) sigmoidal curves, similar to those obtained in the studies of absorption variation with pH for organic compounds with acid–base properties.

### 3.2. Data treatment

Spectrofluorimetry is not a usual technique for the determination of acidity constants of molecules at their ground state. The treatment of $I_{F,rel}$–pH data simply considers that the sigmoidal curve is symmetric and the inflexion point corresponds to the p$K_a$ value.

In this work, graphical (derivatives and curve-fitting) and numerical (LETAGROP SPEFO) methods, normally used for the determination of p$K_a$ values from A–pH data, will be applied. From the curves obtained in the $I_{F,rel}$–pH plots for each ARA II, the existence of a unique acid–base equilibrium in the pH range 2–9 can be deduced (Fig. 1).

Table 1
Optimised values of the excitation and emission wavelengths for angiotensin II receptor antagonists

| | Excitation wavelength (nm) | Emission wavelength (nm) |
|---|---|---|
| Losartan | 247 | 387 |
| Irbesartan | 259 | 385 |
| Valsartan | 259 | 399 |
| Candesartan cilexetil | 272 | 384 |
| Candesartan M1 | 259 | 392 |
| Telmisartan | 304 | 371 |

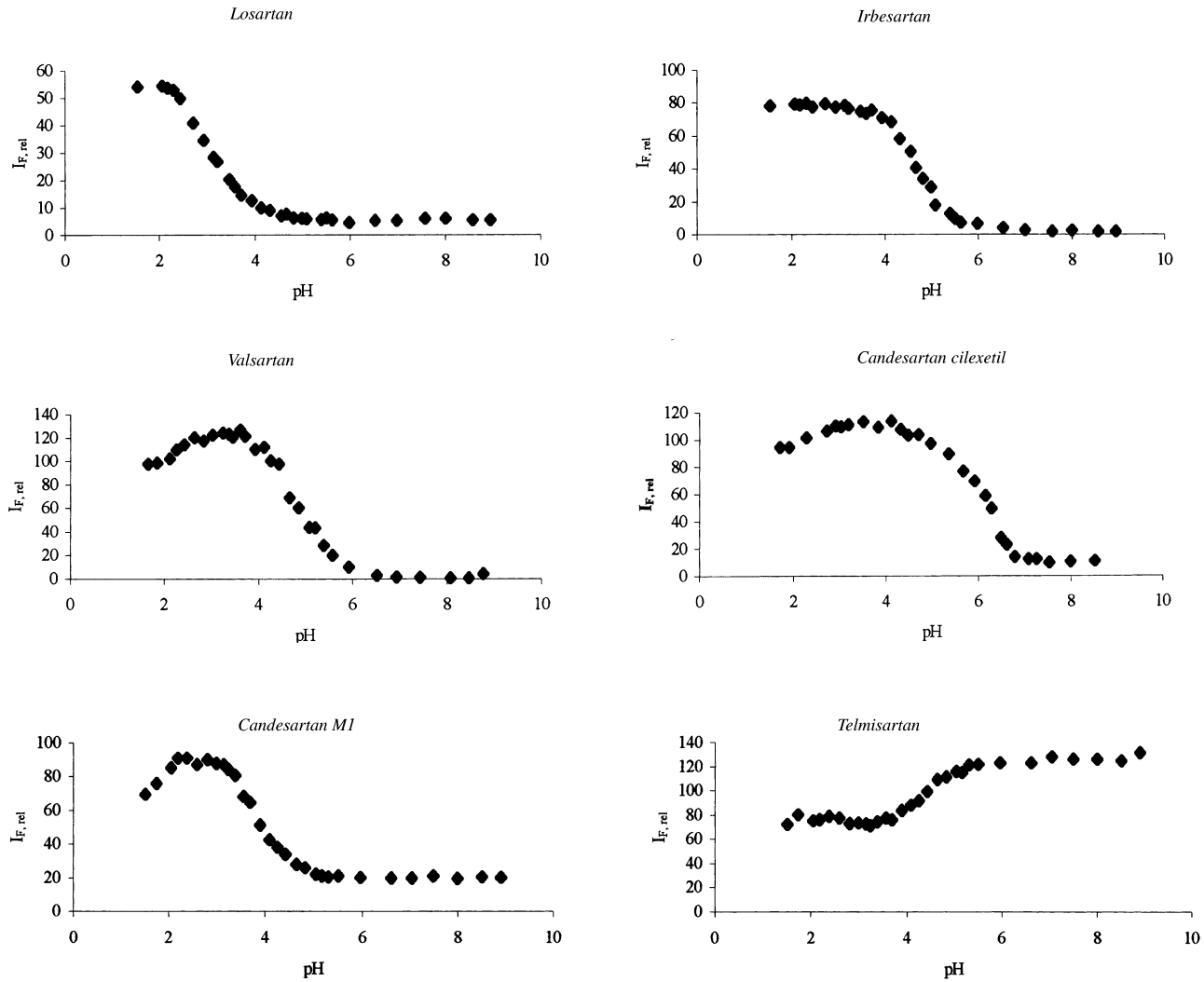



Fig. 1. Relative fluorescent intensity ($I_{F,rel}$)–pH data at 0.5 M ionic strength for ARA II studied.

Table 2
$pK_a$ values obtained with graphical (inflexion point, derivatives and curve-fitting) and numerical (LETAGROP SPEFO) methods for angiotensin II receptor antagonists at 0.5 M ionic strength

| | $pK_a$ | | | | |
|---|---|---|---|---|---|
| | Graphical methods | | | | Numerical methods |
| | Inflexion point | 1st Derivative | 2nd Derivative | Curve-fitting method (normalised variable) | LETAGROP SPEFO |
| Losartan | 3.0 | 2.7 | 2.7 | $3.1 \pm 0.1$ | $3.15 \pm 0.07$ |
| Irbesartan | 4.7 | 4.6 | 4.6 | $4.6 \pm 0.1$ | $4.70 \pm 0.06$ |
| Valsartan | 4.7 | 4.6 | 4.6 | $4.8 \pm 0.1$ | $4.90 \pm 0.09$ |
| Candesartan cilexetil | 6.3 | 6.3 | 6.3 | $5.9 \pm 0.1$ | $6.0 \pm 0.1$ |
| Candesartan M1 | 3.9 | 3.9 | 3.8 | $3.8 \pm 0.1$ | $3.9 \pm 0.1$ |
| Telmisartan | 4.4 | 4.4 | 4.4 | $4.4 \pm 0.1$ | $4.45 \pm 0.09$ |

*3.2.1. Derivatives*

This method was the easiest one to apply. It consisted of deriving graphically the relative fluorescent intensity versus pH data. $pK_a$ values were obtained from the maximum–minimum points for the first derivative and from the intersection of the function with the abscissa axis for the second derivative. Rough $pK_a$ values were calculated from these representations. $pK_a$ values obtained using this treatment are given in Table 2. Derivatives representations for Irbesartan are shown as an example in Fig. 2. These values were refined afterwards by numerical methods.

*3.2.2. Curve-fitting method*

The curve-fitting method with a normalised variable was applied to the $I_{F,rel}$–pH data obtained for the six ARA II using ApH software [28], designed for the determination of $pK_a$ values from A–pH data.

Graphical curve fitting methods have several advantages over linearization methods (Stenträm-Goldsmith [29], Sommer [30]) such as the treatment of the whole series of data, simultaneous determination of several dissociation constants and evaluation of errors [6,7].

ApH program needs the knowledge of relative fluorescent intensities of acid and basic species, which can be deduced from the plateaus of the experimental sigmoidal curves obtained. Moreover, an estimated $pK_a$ value is also necessary so as to calculate the refined $pK_a$ value. The $pK_a$ values obtained using the derivatives methods have been used as estimate values.

The program calculates the theoretical function $I_{F,rel} = f(pZ)$ and it is introduced in the experimental data plot, $I_{F,rel} = f(pH)$, allowing by means of a graphical interface to get the best fit between both functions. From the position of the best fit, the $pK_a$ value is obtained. For systems with only a $pK_a$ value (as for the ARA II assayed), the $I_{F,rel}$ may be expressed as

$$I_{F,rel} = \frac{I_{F,rel\ a} + I_{F,rel\ b} K_a^{-1}[H^+]}{1 + K_a^{-1}[H^+]}$$

$I_{F,rel\ a}$ and $I_{F,rel\ b}$ being the intensities of acid and basic species.

If a normalised variable, $Z$, is defined as

$$Z = K_a^{-1}[H^+]$$

This equation is then transformed in

$$I_{F,rel} = \frac{I_{F,rel\ a} + I_{F,rel\ b} Z}{1 + Z}$$

Both equations have thus the same shape and their position along the $x$-axis differs only in the constant term.

$$pH - pZ = pK_a$$

If the theoretical $I_{F,rel} = f(pZ)$ and the experimental $I_{F,rel} = f(pH)$ are superimposed, the $pK_a$ value can be calculated from the position of best fit.

Results obtained from the application of curve-fitting methods (normalised variable) are collected in Table 2. The position of the best fit for theoretical and experimental functions is shown as an example for Irbesartan in Fig. 2.

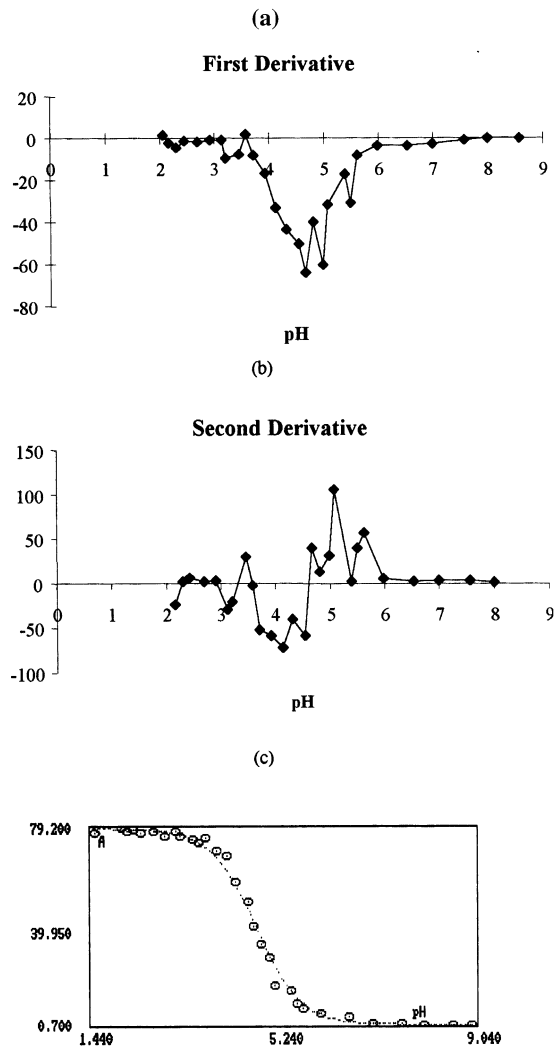



Fig. 2. $I_{F,rel}$–pH data treatment for Irbesartan by different graphical methods, (a) first derivative, (b) second derivative and (c) curve-fitting method (normalised variable) using APH software.

Table 3
$pK_a$ values calculated at 0.5 M ionic strength by spectrofluorimetry and found in literature for angiotensin II receptor antagonists

| | $pK_a$ | |
|---|---|---|
| | Calculated LETAGROP SPEFO | Bibliographic |
| Losartan | $3.15 \pm 0.07$ | 2.36, 5.55; 4.9; 5.6 |
| Irbesartan | $4.70 \pm 0.06$ | 4.5 |
| Valsartan | $4.90 \pm 0.09$ | 3.9,4.7; 3.9;4.73 |
| Candesartan cilexetil | $6.0 \pm 0.1$ | 5.95 |
| Candesartan M1 | $3.9 \pm 0.1$ | 5.3 |
| Telmisartan | $4.45 \pm 0.09$ | – |

### 3.2.3. Letagrop

The LETAGROP SPEFO program [31] allowed to refine the $pK_a$ values of the ARA II studied from the raw experimental data and the $pK_a$ values deduced from the graphical methods, which were used as initial values for the iterations of the mathematical algorithm used.

This refinement consisted of the minimization of the sum of the square deviations defined for the following function

$$U = \sum_{Np} (I_{F,rel\ cal} - I_{F,rel\ exp})^2$$

where $Np$ is the number of experimental points, $I_{F,rel\ exp}$ are the experimental values and $I_{F,rel\ cal}$ are the data calculated after the adjustment. From an initial $pK_a$ value, the LETAGROP SPEFO program makes a systematic variation of the constants to get a group of values which minimise the $U$ function and the total Standard Deviation of the data. In Table 2, the $pK_a$ values for ARA II obtained by LETAGROP are given. The function FEL(VAL), which corresponds to the sum of absolute squared deviations shows maximum deviation in the surroundings of the $pK_a$ value.

ARA II studied have in their molecular structure functional groups with acid–basic properties such as tetrazole and/or carboxy, the acidity of this last group being generally greater than the tetrazole one ($pK_a$ tetrazole = 4.89 [32]).

p$K_a$ values reported in the bibliography for some of these compounds (Table 3) are attributed to the above mentioned functional groups.

For Losartan, Irbesartan and Candesartan cilexetil, the p$K_a$ values calculated can be attributed to the deprotonation of the tetrazole group (Fig. 3).

The fluorescent behaviour of Telmisartan, which is the unique ARA II studied without the biphenyltetrazole group, is different from the rest of compounds. Its acid species has a lower fluorescence than the basic one. The p$K_a$ value obtained for this compound corresponds to the acid–base equilibrium in which the carboxylic

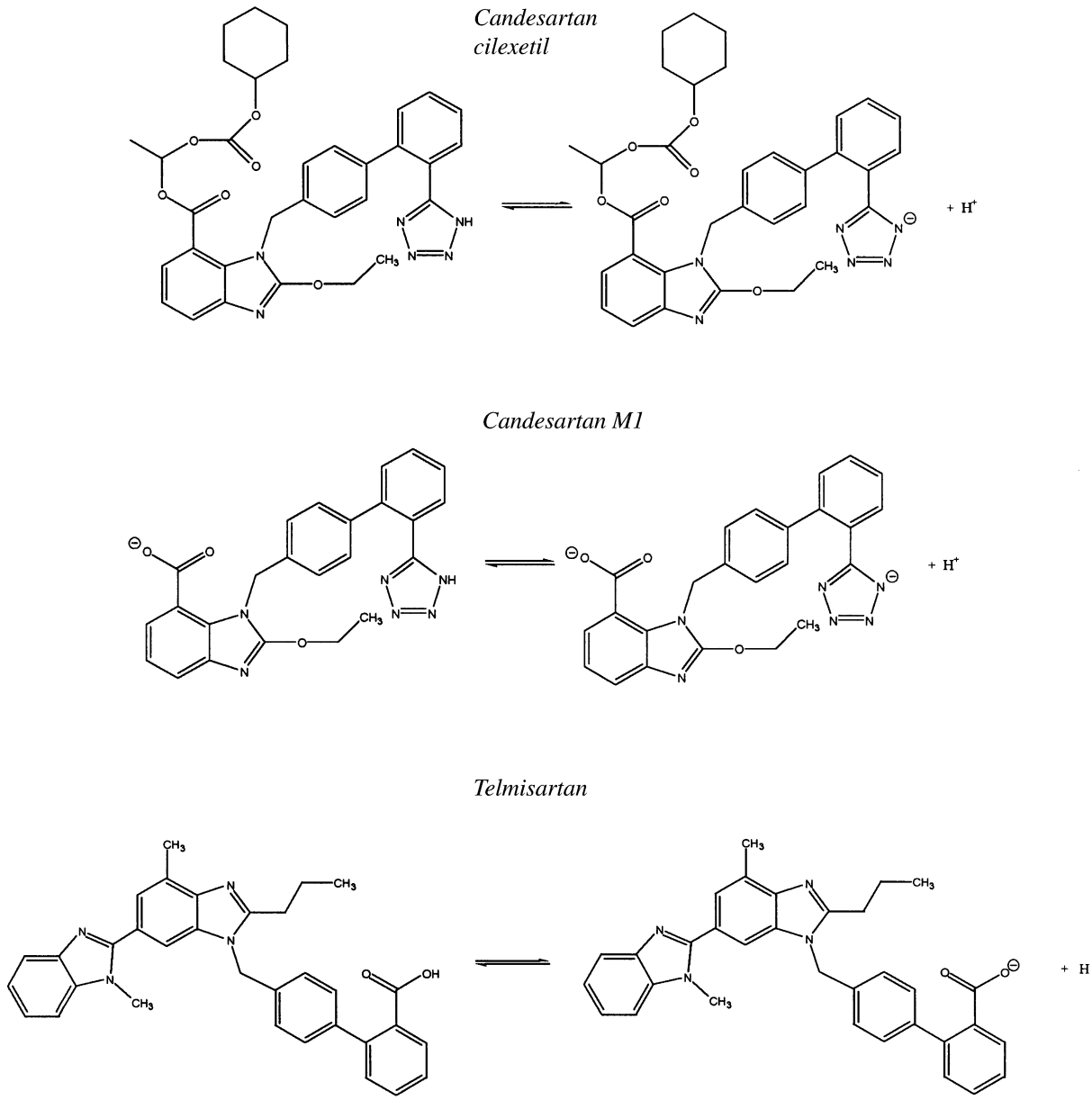
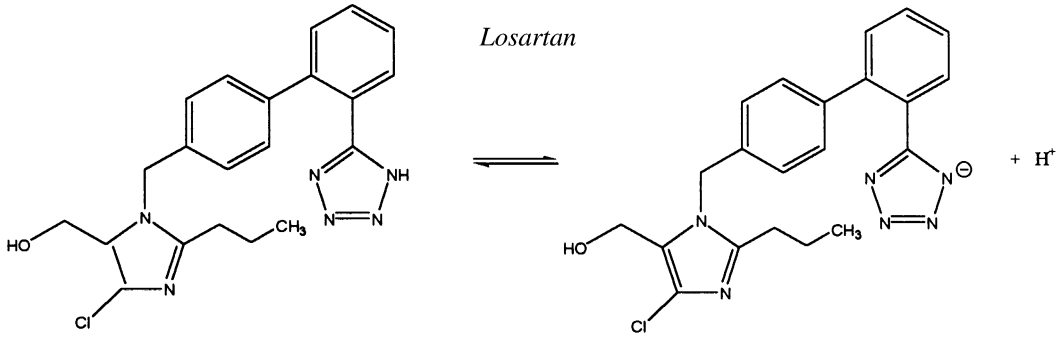
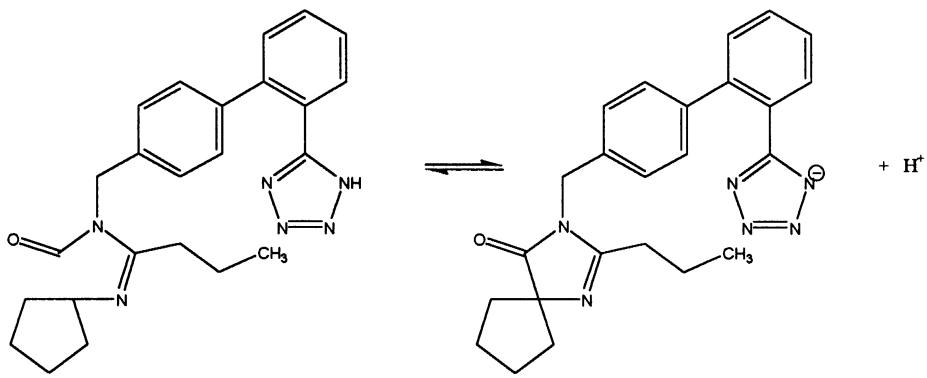



Fig. 3. Acid-base equilibria for ARA II studied.

*Losartan*



*Irbesartan*



*Valsartan*

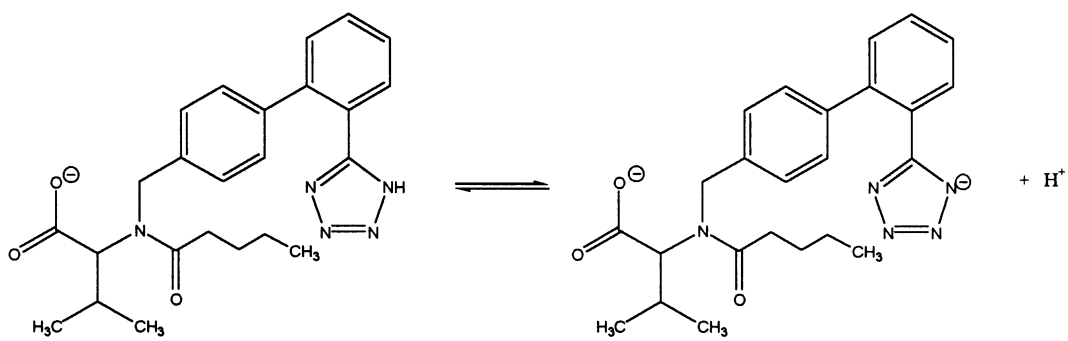


Fig. 3. (*Continued*).

group is involved (Fig. 3). For Valsartan and Candesartan M1, which both have tetrazole and carboxylic groups, only a $pK_a$ value could be calculated with reliability. As it can be seen in Fig. 1 at pH values lower than two, the $I_{F,rel}$ decreases probably due to the existence of other acid–base equilibrium, which could be attributed to the carboxylic group. The unique $pK_a$ determined corresponds to the equilibrium which involves the tetrazole group (Fig. 3).

$pK_a$ values determined by spectrofluorimetry are attributed to the acid–base equilibria of ARA II in their ground state, since there is a great similarity between the bibliographic data and the calculated ones. Besides, in the case of Candesartan cilexetil, the $pK_a$ value deduced by spectrofluorimetry is corroborated by the value obtained in our laboratory by UV–vis spectrophotometry ($pK_a = 6.0$).

The small difference in $pK_a$ values calculated and reported in the bibliography for Losartan is not enough to affirm that the species at the excited state is involved in the equilibrium, since in most of cases the differences observed ($pK_a$ at excited state and ground state) are ca. six units [33].

## 4. Conclusions

It is worth mentioning the importance of this work due to the lack of research dealing with the determination of $pK_a$ values for angiotensin II receptor antagonists (ARA II) because of their recent introduction in the market.

UV–vis spectrophotometry could not be successfully applied to the $pK_a$ determination of ARA II, except for Candesartan cilexetil whose absorption spectra does change significantly with pH, whereas spectrofluorimetry proved to be a good technique for the determination of acidity constants of these antagonists.

Although spectrofluorimetry is not a usual technique for the $pK_a$ determination of molecules in their ground state, the fluorescent properties of the ARA II, have allowed calculation of the $pK_a$ values of six members of this new family. $pK_a$ values obtained by means of the different graphical and numerical methods used are similar to those found in the literature (Valsartan 4.73 and Irbesartan 4.50) and obtained in our laboratory (Candesartan cilexetil 6.0), with the exception of the $pK_a$ value obtained for Losartan.

Potentiometric methods will be applied in a future study in order to corroborate the $pK_a$ values obtained by spectrofluorimetry.

## Acknowledgements

The authors thank the Interministerial Commission of Science and Technology (Project PB98-0231) for financial support and Merck Sharp and Dohme, Sanofi Recherche, Novartis Pharma, Astra Zeneca and Boehringer Ingelheim for their kind supply of these ARA II. L. González thanks the Basque Country Government for a FPI grant.

## References

[1] G.E. McVeigh, J. Flack, R. Grimm, Drugs 49 (2) (1995) 161–176.
[2] M. Burnier, H. Brunner, Kidney Int. 68 (1998) S107–S111.
[3] J. Ménard, G. Chatellier, M. Azizi, J. Hum. Hypertens. 11 (Suppl. 2) (1997) S1–S7.
[4] J.H. Bauer, G.P. Reams, Arch. Intern. Med. 155 (1995) 1361–1368.
[5] M. Litter, Farmacología Experimental y Clínica, El Ateneo, Buenos Aires, 1986, pp. 69–77.
[6] F.J.C. Rossotti, H. Rossotti, The Determination of Stability Constants, McGraw-Hill, New York, 1961.
[7] H. Rossotti, The Study of the Ionic Equilibria. An Introduction, Longman, London, 1978.
[8] J. Chamberlain, Analysis of Drugs in Biological Fluids, CRC Press, London, 1987.
[9] K.E. McCarthy, Q. Wang, E.W. Tsai, R.E. Gilbert, D.P. Ip, M.A. Brooks, J. Pharm. Biomed. Anal. 17 (1998) 671–677.
[10] R.C. Williams, M.S. Alasandro, V.L. Fasone, R.J. Boucher, J.F. Edwards, J. Pharm. Biomed. Anal. 14 (1996) 1539–1546.
[11] C.I. Furtek, M.W. Lo, J. Chromatogr. B 573 (2) (1992) 295–301.
[12] T. Iwasa, T. Takano, K.I. Hara, T. Kamei, J. Chromatogr. B 734 (1999) 325–330.
[13] S.Y. Chang, D.B. Whigan, N.N. Vachharajani, R. Patel, J. Chromatogr. B 702 (1997) 149–165.
[14] G. Flesch, M.P. Lloyd, Eur. J. Clin. Pharmacol. 52 (1997) 115–120.

[15] F. Waldmeier, G. Flesch, P. Müller, T. Wrinkler, H.P. Kriemler, P. Bühlmayer, M. de Gasparo, Xenobiotica 27 (1) (1997) 59–71.
[16] A. Fini, P. de Maria, A. Guarnieri, L. Varoli, J. Pharm. Sci. 76 (1) (1987) 48–52.
[17] V. Martínez, M.I. Maguregui, R.M. Jiménez, R.M. Alonso, J. Pharm. Biomed. Anal. 23 (2000) 459–468.
[18] B. de Castro, P. Gameiro, C. Guimaraeas, J.L. Lima, S. Reis, J. Pharm. Sci. 87 (3) (1998) 356–369.
[19] A.R. Katritzky, C.W. Rees, Comprehensive Heterocyclic Chemistry. The Structure, Reactions, Synthesis and Uses of Heterocyclic Compounds, Pergamon Press, London, 1984.
[20] G. Verdasco, M.A. Martín, B. del Castillo, P. López-Alvarado, C. Menéndez, Anal. Chim. Acta 303 (1995) 73–78.
[21] D. Farthing, D. Sica, I. Fakhry, A. Pedro, T.W.B. Gehr, J. Chromatogr. B 704 (1997) 374–378.
[22] M.A. Ritter, C.I. Furtek, M.W. Lo, J. Pharm. Biomed. Anal. 15 (1997) 1021–1029.
[23] L.A. Brunner, M.L. Powell, P. Degen, G. Flesch, Lab. Robotic Autom. 6 (1994) 171–179.
[24] A. Sioufi, F. Marfil, J. Godbillon, J. Liq. Chromatogr. 17 (10) (1994) 2179–2186.
[25] T. Miyabayashi, M. Motohashi, K. Izawa, T. Yashiki, J. Chromatogr. B 677 (1996) 123–132.
[26] H. Stenhoff, P.O. Langerstrom, C. Andersen, J. Chromatogr. B 731 (1999) 411–417.
[27] L.A. Berrueta, L.A. Fernández, F. Vicente, Comput. Chem. 15 (4) (1991) 307–312.
[28] M.J. Citores, R.M. Alonso, L.A. Fernández, Trends Anal. Chem. 11 (9) (1992) 310–314.
[29] W. Stentröm-Goldsmith, N. Goldsmith, J. Phys. Chem. 30 (1926) 1683–1692.
[30] L. Sommer, V. Kuban, J. Havel, in: Spectrophotometric Studies of the Complexation in Solution, Folia Fac. Sci. Nat., Univ. Purkynae Brunensis, Chemia 7, Brno, 1970.
[31] N. Ingri, L.C. Sillén, Acta Chem. Scand. 16 (1971) 173–182.
[32] J. March, Advanced Organic Chemistry: Reactions, Mechanisms and Structure, Wiley, New York, 1992.
[33] G.G. Guibault, Practical Fluorescence, Marcel Dekker, New York, 1990.