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Combined effect of polarity and pH on the chromatographic behavior of some angiotensin II receptor antagonists and optimization of their determination in pharmaceutical dosage forms

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ABSTRACT

In the present study, the combined effect of mobile phase polarity and pH on retention behavior of some ARA-IIs (irbesartan, losartan, valsartan and telmisartan) is investigated. The linear relationships established between retention factors of the species and the polarity parameter of the mobile phase has proved to predict accurately retention in LC as a function of the acetonitrile content (50%, 55%, 60%, v/v). The suggested model uses the pH value in the acetonitrile–water mixture as mobile phase instead of pH value in water and takes into account the effect of activity coefficients. Moreover, correlation between retention and the mobile phase pH can be established allowing prediction of the retention behavior as a function of the mobile phase pH. The model can be used to estimate the pK_a in an acetonitrile percentage between 50% and 60%, at 30 °C. The developed method was successfully applied to both the simultaneous separation of these drug-active compounds and individual determination in their commercial pharmaceutical dosage forms.

1. Introduction

Inhibitors of renin–angiotensin–aldosteron system are successfully used in many cardiovascular diseases. Angiotensin II receptor antagonists (ARA-IIs) represent a relatively new pharmacological class [1,2]. They act mainly by selective blockade of AT_1 receptors thus reducing the pressor effects of angiotensin II. It is a potent vasoconstrictor, the primary vasoactive hormone of renin–angiotensin system and an important component of the pathophysiology of many cardiovascular diseases. The therapy with these drugs offers a good quality of life for hypertensive patients due to the absence of side effects [3]. They may be used alone or in combination with other antihypertensive or diuretic agents. The knowledge of acid–base equilibria of these drugs has a great pharmacological importance. The degree of ionization of the drug strongly affects solubility, permeability and drug disposition properties (ADME) [4]. Besides, the knowledge on dissociation constant of ionisable compounds at different solvent composition is also significant to determine the optimal separation conditions on reversed phase liquid chromatography (RP-LC).

Nowadays, RP-LC has been the major technique used to separate and quantify ARA-IIs. A number of high performance liquid chromatographic methods with UV detection has been reported for the determination of ARA-IIs in biological fluids [5–7] and pharmaceutical formulations [8,9], whereas there are a limited number systematic studies for the optimization of separation parameters. Although, many of RP-LC separation procedures are based on water-acetonitrile mobile phase, the pK_a values of ARA-IIs have not been determined in water-acetonitrile binary mixtures yet. The pK_a constants of ARA-IIs have been studied only in a scattered and incomplete manner, and frequently with confusing and conflicting results [10,11]. There are only a few studies for pK_a values of the simple tetrazole models [12] and ionization constants of the imidazole, 1H-benzimidazole and 2-ethoxy-1H-benzimidazole in literature [13].

The acid–base chemistry of pharmaceutically active compounds plays a pivotal role in the development of a new drug. Pharmaceuticals and the determination of useful dosage forms and regimes for drugs depend on an understanding of drug dissociation and the extent of dissociation that will occur in the body. Also, the solubility and permeability of drug compounds is pK_a dependent.

Chromatographic retention in LC can be described mathematically as a function of pH and solvent composition for determination of chromatographic pK_a. The retention behavior of an ionisable compound in RP-LC is different from the retention of a neutral one. The pH of the mobile phase has a strong influence on the chromatographic retention of compounds with acid–base properties. An ionisable compound presents equilibrium between its acidic

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(HA) and its basic (A^-) forms related by the dissociation constant. The observed retention factor (k) is an average of the retention factors of the acidic (k_{HA}) and basic forms (k_{A^-}). The theory for studying the pH dependence of chromatographic retention for ionisable solutes in liquid chromatography was proposed by Horváth et al. [14]. The expression:

$$k = \frac{k_{HA} + k_{A^-}(K_a/a_{H_m^+}\gamma_{A_m^-})}{1 + (K_a/a_{H_m^+}\gamma_{A_m^-})} \quad (1)$$

represents the variation of the retention factor for a weak monoprotic acid (HA) with the hydrogen ion activity in the mobile phase, ($a_{H_m^+}$). The dissociation constant in the acetonitrile–water mixture used as mobile phase is represented by K_a; $\gamma_{A_m^-}$ is the activity coefficient of the dissociated acid in the mobile phase [15,16]. A plot of retention factor as a function of pH of the mobile phase yields a sigmoidal curve, from which the inflection point corresponds to the pK_a value [17,18]. For a weak monoprotic base, BH^+, a similar equation can be written:

$$k = \frac{k_{BH^+} + k_B(K_a\gamma_{BH_m^+}/a_{H_m^+})}{1 + (K_a\gamma_{BH_m^+}/a_{H_m^+})} \quad (2)$$

where K_a is defined as the protonation constant of the protonated base (BH^+) in the hydro-organic mixture used as mobile phase, and k_{BH}^+ and k_B are the limiting retention factors of the protonated and undissociated base, respectively.

The appropriate pH measurement is crucial for model chromatographic retention as a function of pH and to get reliable predictions. Different authors have realized that better models are obtained when the pH in the mobile phase is considered instead of the aqueous pH of the buffer [19,20].

The retention factor k of a solute is a function of the volume fraction φ of the organic modifier in the mobile phase. In LC, the retention behavior is described by using quadratic equation [21].

$$\log k = C_0 + C_1\varphi + C_2\varphi^2 \quad (3)$$

In Eq. (3), C_i is regression coefficients with characteristic values for a given solute and separation system. Linear plots of $\log k$ *versus* the φ were also satisfactory for most solutes for narrow ranges of organic solvent. However, Johnson et al. [22] demonstrated linear relationships in a wide range of organic solvents between the solute $\log k$ and E_T^N [23]. A widely employed measure of solvent polarity is Dimroth and Reichardt's $E_T(30)$ [24]. This polarity index is often used in the normalized dimensionless form, E_T^N.

$$\log k = C + eE_T^N \quad (4)$$

The expressions:

$$\log k_{HA} = C_{HA} + e_{HA}E_T^N \quad (5)$$

$$\log k_{A^-} = C_{A^-} + e_{A^-}E_T^N \quad (6)$$

represent the variation of the retention factors of the neutral and ionic species with E_T^N.

Substituting Eqs. (5) and (6) into Eq. (1) the theoretical expression [25] describing the dependence of the retention factors for acidic solutes as a combined function of pH and polarity of the mobile phase may be expressed as follows:

$$k = \frac{10^{(C_{HA}\times e_{HA}\times E_T^N)} + 10^{(C_A\times e_A\times E_T^N)}(K_a/a_{H_m^+}\gamma_{A_m^-})}{1 + (K_a/a_{H_m^+}\gamma_{A_m^-})} \quad (7)$$

Taking into account that the variation of the retention factors of basic compounds with polarity of the mobile phase, we can write the following equations, which indicate the dependence of the retention factors of the neutral and protonated species with E_T^N.

$$\log k_{BH^+} = C_{BH^+} + e_{BH^+}E_T^N \quad (8)$$

$$\log k_B = C_B + e_BE_T^N \quad (9)$$

A similar equation can be obtained by substituting the expressions for variation of k_{BH^+} and k_B with E_T^N (analogous to Eqs. (8) and (9)) in Eq. (2).

$$k = \frac{10^{(C_{BH^+}\times e_{BH^+}\times E_T^N)} + 10^{(C_B\times e_B\times E_T^N)}(K_a\gamma_{BH_m^+}/a_{H_m^+})}{1 + (K_a\gamma_{BH_m^+}/a_{H_m^+})} \quad (10)$$

In the HPLC method optimization, the most important aim is to provide the best separation conditions. Several approaches have been investigated to estimate the solute retention behavior as a function of chromatographic conditions. The factors generally selected to optimize the chromatographic separation of ionisable compounds are the pH of the mobile phase and content of organic solvent of the eluent. The influence of these two parameters on the chromatographic behavior is somewhat more complex since the variation of the content of organic solvent induces a variation of the degree of ionization as well. Achievement of good resolution is crucial and frequently only possible at very specific pH values. The aim of this study is to describe the combined effect of acetonitrile content and pH of the mobile phase on the retention behavior of some ARA-IIs. The range of pH values used should be broad enough including the pK_a of the analyte. A complete description of the chromatographic retention of each ARA-II in the space by the pH of the mobile phase and E_T^N has been used for modeling of retention behavior and further for the separation adequation. The equations derived have also been used to calculate the pK_a value of these compounds, as well as the retention factors of the conjugate acid–base species. The chromatographic conditions estimated were applied for the determination of ARA-IIs in their dosage forms.

2. Experimental

2.1. Chemicals and reagents

All the chemicals were of analytical reagent grade and all the solvents were of HPLC grade. Analyte drugs (Fig. 1) were kindly provided by the following pharmaceutical companies: Irbesartan and Losartan by Nobel Pharm. Company (Istanbul, Turkey); Telmisartan by GlaxoSmithKline Pharm. (Istanbul, Turkey); Valsartan by Sanovel Pharm. Company (Istanbul, Turkey) and naproxen (I.S.) by Sigma (USA) (Fig. 1). HPLC grade acetonitrile (Merck, Darmstadt, Germany) was used as organic modifier. Ortho-phosphoric acid (Riedel-de Haen Germany; min. 85%) and sodium hydroxide (Merck, Darmstadt, Germany) was used for pH adjustment.

2.2. Apparatus

HPLC analyses were performed using an HP chromatographic system, consisting of a Model Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA), a pump (Quat Pump G1311A), a diode array detector (DAD G1315B), auto sampler (ALS G131A) with a 20 μL injection loop, column oven (GOLCOM G1316A) and a degasser system (G1379A). A Gemini C_{18} analytical column (150 mm × 4.6 mm I.D., 5 μm) provided by Phenomenex® (USA) was used for all the determinations. Results were acquired and processed with the Agilent ChemStation data system software (Agilent Technologies, Santa Clara, CA, USA).

pH measurements of the mobile phase were carried out with a Mettler Toledo MA 235 pH/ion analyser (GmbH; Schwerzenbach, Switzerland) using M–T combination pH electrode. Potassium hydrogen phthalate was used as reference value standard for the standardization of this apparatus in acetonitrile–water binary mixtures in accordance with IUPAC rules [26,27].

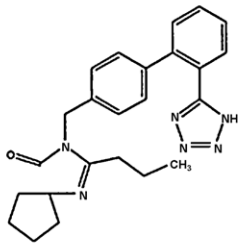
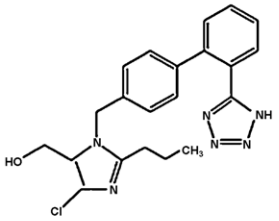
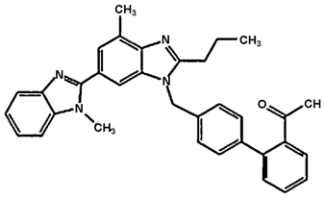
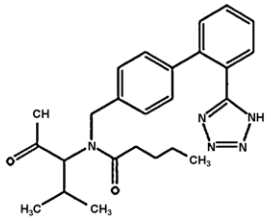
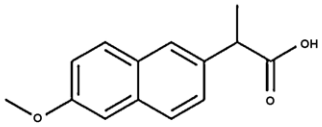
Compounds	Structure
Irbesartan	
Losartan	
Telmisartan	
Valsartan	
Naproxen	

Fig. 1. Chemical structures of ARA-II compounds and naproxen (I.S.).

2.3. Chromatographic procedure

Throughout this study, the effect of solvent composition on the chromatographic separation was investigated at three solvent levels (50, 55, 60, v/v). The pH of the mobile phase containing 50 mM phosphoric acid was adjusted between 2.75 and 8.0 by the addition of sodium hydroxide. The flow rate of the mobile phase was 1 mL/min. For each ARA-II, the retention time values (t_R) were determined from three separate injections for every mobile phase composition and pH value. Retention factors for each compound and mobile phase were calculated using the expression $k = (t_R - t_0)/t_0$. The dead time (t_0) was measured by injecting uracil solution (Sigma, USA, 0.1%, in water) which was established for each mobile phase composition and pH studied. The chromatographic conditions were as follows: the sample injection volume: 20 μL; the column temperature: 30 °C. In this study different wavelengths were used for each compound with photo diode array (PDA) detector [for irbesartan and losartan 206 nm, for valsartan 210 nm, for telmisartan 230 nm and for naproxen (I.S.) 240 nm].

2.4. Preparation of standard solutions

Stock solutions (100 μg/mL) of each AT_1 receptor antagonist and naproxen (I.S.) were prepared by dissolving it in the mobile phase. All the subsequent dilutions for working standards were prepared with the mobile phase. All the solutions were protected from light and were used within 24 h to avoid decomposition. The concentrations of irbesartan, losartan, telmisartan and valsartan were varied in the range of 50–400 μg/mL, 20–150 μg/mL, 50–400 μg/mL and 20–200 μg/mL, respectively and the concentration of I.S. was maintained at a constant level of 10.0 μg/mL. Separate standard calibration graphs were constructed for each component by plotting the ratio of the peak area of the drug to that of I.S. against the drug concentration.

2.5. Analysis of tablets

Ten tablets from the sample to be analyzed were accurately weighed and ground until just reduced to a fine powder. An accurately weighed amount of the powder equivalent to one tablet was transferred into a 100 mL volumetric flask. Approximately 50 mL of acetonitrile was added and the content of the flask was sonicated for 15 min. The solution in the flask was completed to volume with acetonitrile. After filtration, appropriate solutions were prepared by taking suitable aliquots of clear filtrate and adding the appropriate I.S. solution, diluting them with mobile phase in order to obtain the final solution. The amounts of irbesartan, losartan, telmisartan and valsartan were calculated from the corresponding regression equations.

2.6. Recovery studies from tablets

To keep an additional check on the accuracy of this developed method, recovery experiments were performed by adding the known amount of pure drug to pre-analyzed samples of tablets. Known amounts of the pure drug and a constant level of an internal standard were added to pre-analyzed tablet solution and the mixtures were analyzed. The percent recovery was calculated by comparing the concentration obtained from spiked samples with the actual added concentration. After five repeated experiments, the average recovery percentage of these compounds was calculated for each compound. Thus, the effect of common tablet formulation excipients on chromatograms (e.g., tail, broadening, etc.) was investigated. Recovery experiments also showed the reliability and suitability of the proposed method.

3. Results and discussion

A satisfactory knowledge of the acid–base behavior of substances in hydro-organic media is essential to predict the influence of pH on the selectivity and retention in LC and also to optimize analytical procedures for the separation of ionizable compounds. In this study, the retention behavior of ARA-IIs was investigated using different elution conditions on an RP column, in order to investigate the combined effect of pH and acetonitrile percentage on the retention in reversed phase liquid chromatography. The ARA-IIs studied here, namely losartan, irbesartan, telmisartan, valsartan contain different ionizable fragments such as acidic functions or basic heterocyclic moieties (Fig. 1).

The pK_a values of these compounds were determined in acetonitrile water binary mixtures at three solvent levels (50, 55, 60, v/v) at 30 °C by using LC methodology. For this purpose, the effect of mobile phase pH on the retention factors was investigated. pK_a,

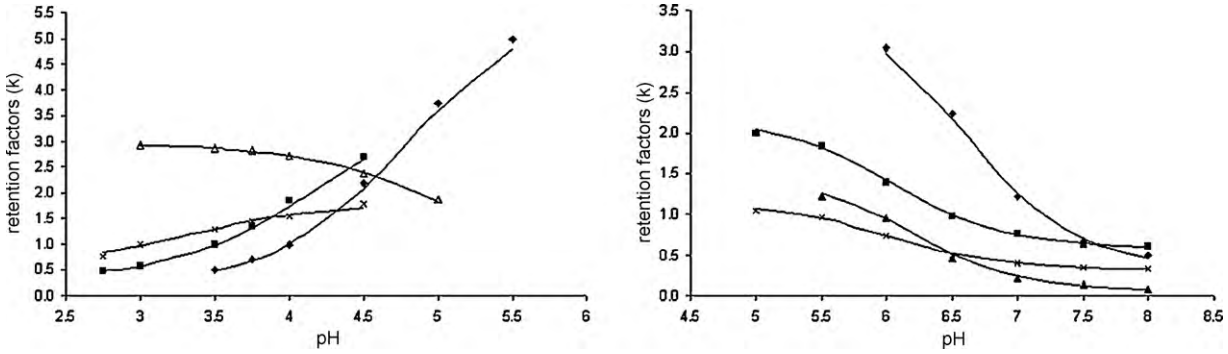


Fig. 2. Plots of the retention factors of ARA-IIs the pH of the mobile phase for 50% (v/v) ACN: (■) irbesartan, (×) losartan, (▲) valsartan, (♦) telmisartan. The solid lines indicate the retention factors predicted by Eqs. (1) and (2).

k_{HA} and k_{A^-} values of these compounds were calculated by using the software NLREG 4.0 from k/pH data [28]. In our case, the dissociation constants and the retention factors of the neutral and ionic species are treated as parameters to be optimized. The program refines the retention factors, until a minimum value in the sum of squared differences between the experimental and calculated retention factors for each data point. The examples of dependence of the retention factors on pH value in the mobile phase (50%, v/v) are given in Fig. 2. The curves calculated using Eqs. (1) and (2) are indicated as continuous lines and the plotted points are experimental data. The correlation between the experimental retention factors of the compounds studied over the whole experimental pH range was good as shown in Fig. 2.

Three of the ARA-II compounds studied have two ionizable functional groups. Both losartan and irbesartan contain one acidic center (tetrazole ring) and one basic center, the imidazole and the 4,5-dihydro-5-oxo-1H-imidazole ring, respectively. Valsartan contains two acidic centers (carboxyl and tetrazole). In contrast, telmisartan has three ionizable functional groups. Telmisartan contains one acidic center (carboxyl) and two basic centers (benzimidazole ring). Two pK_a values were determined for each of these compounds. Dissociation constants and the intrinsic retention for each species of the compounds in acetonitrile–water mixtures in the interval (R (v/v), 50–60%) are summarized in Table 1.

In this work, Eqs. (7) and (10) were evaluated statistically for ARA-IIs at different conditions. In this way, a complete description of the retention behavior of each solute in the space defined by the pH of the mobile phase and E_T^N variables was obtained. The coefficients described a retention behavior of these solutes using Eqs. (7) and (10) are listed in Table 2. The retention behavior of these compounds was accurately modeled as a function of pH and polarity of the mobile phase with the equations proposed. The results shown in Table 2 demonstrate a good performance of the liquid chromatographic method for determination of pK_a. Theoritical retention values (k_{theor}) and selectivity values (α_{theor}) values were calculated using Eqs. (7) and (10). The results illustrated an excellent agreement between k_{theor}/k_{exp} and $\alpha_{theor}/\alpha_{exp}$ for these ARA-IIs.

Our ultimate aim was not to describe retention, but to optimize separation. The efficiency, selectivity and retention terms were calculated in the usual way and summarized in Table 3 for optimum separation condition. It can be achieved when the acetonitrile content in the mobile phase is 50% (v/v) at pH 4.0, in which all solutes are well separated in an analysis time of about 7 min.

In this study, the quantitative determination of these compounds was also carried out under the optimized conditions. A widely used technique of quantitation involves the addition of an internal standard to compensate for errors in the analytical measurements. Hence, the quantitative determination of ARA-IIs was carried out using internal standard method. This method compensates for variations in physical parameters, especially inaccuracies in pipetting and injecting μL volume, requiring significant extraction, pretreatment or preparation steps. Generally, sample preparation steps that include reaction, filtration, precipitation, extraction and so on, may cause unexpected results because of unavoidable sample losses. For the precision, the selection of inter-

Table 1
The pK_a values and the retention factors of losartan, irbesartan, valsartan and telmisartan in acetonitrile–water mixtures.

Losartan	pK_{a1} (imidazole)	k_{H2A^+}	k_{HA}	pK_{a2} (tetrazole)	k_{HA}	k_{A^-}
60% ACN	3.326(0.102)	0.071(0.057)	0.954(0.031)	6.122(0.091)	0.711(0.023)	0.225(0.014)
55% ACN	3.345(0.088)	0.266(0.055)	1.266(0.031)	6.066(0.048)	0.908(0.017)	0.263(0.009)
50% ACN	3.367(0.141)	0.557(0.109)	1.830(0.067)	6.004(0.053)	1.139(0.024)	0.325(0.013)
Irbesartan	pK_{a1} (imidazole)	k_{H2A^+}	k_{HA}	pK_{a2} (tetrazole)	k_{HA}	k_{A^-}
60% ACN	4.058(0.043)	0.048(0.015)	1.341(0.045)	6.294(0.077)	1.030(0.024)	0.379(0.018)
55% ACN	4.075(0.026)	0.151(0.012)	1.880(0.037)	6.144(0.056)	1.555(0.033)	0.444(0.071)
50% ACN	4.089(0.007)	0.323(0.014)	3.612(0.019)	6.033(0.044)	2.155(0.038)	0.605(0.021)
Valsartan	pK_{a1} (carboxyl)	k_{H2A}	k_{HA^-}	pK_{a2} (tetrazole)	k_{HA^-}	k_{A^-}
60% ACN	5.321(0.028)	1.379(0.005)	0.531(0.017)	6.189(0.128)	1.070(0.093)	0.003(0.036)
55% ACN	5.143(0.113)	1.930(0.036)	0.717(0.082)	6.163(0.109)	1.365(0.102)	0.018(0.038)
50% ACN	4.982(0.055)	2.971(0.038)	0.749(0.068)	6.130(0.085)	1.524(0.088)	0.058(0.031)
Telmisartan	pK_{a2} (benzimidazole)	k_{H2A^+}	k_{HA}	pK_{a3} (carboxyl)	k_{HA}	k_{A^-}
60% ACN	4.688(0.044)	0.004(0.028)	1.961(0.048)	6.768(0.071)	1.978(0.074)	0.178(0.046)
55% ACN	4.745(0.036)	0.045(0.032)	2.972(0.063)	6.693(0.070)	2.431(0.094)	0.262(0.050)
50% ACN	4.789(0.027)	0.197(0.045)	5.933(0.095)	6.597(0.101)	3.776(0.238)	0.317(0.102)

Table 2
Results of the application of the retention model proposed to all the experimental retention data avaliable for irbesartan, losartan, telmisartan and valsartan (coefficients describe chromatographic retention in terms of Eq. (10) for bases, and in terms of Eq. (7) for acidic compounds).

Substance	C_{H2A+}	e_{H2A+}	C_{HA}	e_{HA}	SSQ[a]	RRMSD[b] (%)	N
Irbesartan	−28.588	34.746	−14.124	18.110	0.091	6.685	18
Losartan	−30.587	37.525	−9.371	11.894	0.017	3.086	18
Telmisartan	−56.720	69.277	−15.619	20.228	0.171	5.232	18
Substance	C_{HA}	e_{HA}	C_{A-}	e_{A-}	SSQ[a]	RRMSD[b] (%)	N
Irbesartan	−10.556	13.461	−7.149	8.549	0.021	3.075	21
Losartan	−6.900	8.596	−5.928	6.715	0.004	2.450	21
Telmisartan	−9.008	11.821	−8.992	10.510	0.090	5.398	15
Substance	C_{H2A}	e_{H2A}	C_{HA-}	e_{HA-}	SSQ[a]	RRMSD[b] (%)	N
Valsartan	−10.877	14.014	−5.196	6.270	0.046	2.705	21
Substance	C_{HA-}	e_{HA-}	C_{A-}	e_{A-}	SSQ[a]	RRMSD[b] (%)	N
Valsartan	−5.020	6.440	−44.844	53.978	0.027	6.621	18

[a] Sums of squares of the residuals = $\Sigma(k_{obs} - k_{pred.})^2$.

[b] Relative root mean squared differences = $100\sqrt{\sum (k_{obs.} - k_{pred.})^2 / \sum (k_{obs.})^2}$.

Table 3
The retention, selectivity and separation factor values for ARA-IIs at 50% at pH 4.0.

Compounds	α	$\alpha - 1/\alpha$	k_2	k_2/k_2+1	N	$1/4\sqrt{N}$	R_s
Losartan/telmisartan	1.540	0.351	1.548	0.608	7919	22.247	4.739
Irbesartan/losartan	1.194	0.162	1.848	0.649	8154	22.575	2.380
Valsartan/irbesartan	1.473	0.321	2.723	0.731	9184	23.958	5.627

Table 4
System suitability parameters.

Parameters	Observed value					Recommended value
	Telmisartan	Losartan	Irbesartan	Valsartan	Naproxen (I.S)	
Retention factor (k)	1.12	1.43	1.67	2.72	2.35	>1
Asymmetry factor (A)	0.96	0.97	0.99	0.96	1.04	0.95–1.20
Selectivity factor (α)	2.11	1.65	1.41	1.16		>1
Theoretical plates (N)	7111	7919	8154	9184	7621	>2000
RSD% (for retention time)	0.55	0.48	0.73	0.82	0.30	≤1
RSD% (for peak area)	0.16	0.19	0.42	0.59	0.43	≤1

Table 5
Statistical evaluation of the calibration data of ARA-IIs by RP-LC.

Sample	Linearity range (μg/mL)	Slope	Intercept	S.E. of slope	S.E. of intercept	Correl. coeff.	Detection limit (μg/mL)	Quantitation limit (μg/mL)
Losartan	20–150	0.047	0.145	0.001	0.057	0.999	5.394	16.346
Valsartan	20–200	0.060	0.132	0.001	0.076	0.999	5.619	17.028
Irbesartan	50–400	0.026	0.122	0.001	0.090	0.999	15.100	45.758
Telmisartan	30–100	0.046	−0.077	0.001	0.060	0.999	4.151	12.580

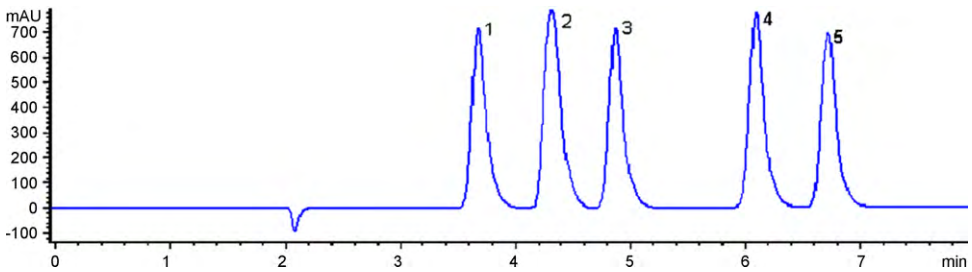


Fig. 3. Chromatogram of standard mixture. 1, Telmisartan (80 μg/mL); 2, losartan (50 μg/mL); 3, irbesartan (80 μg/mL); 4, naproxen (80 μg/mL); 5, valsartan (80 μg/mL). Experimental conditions as in chromatographic procedure.

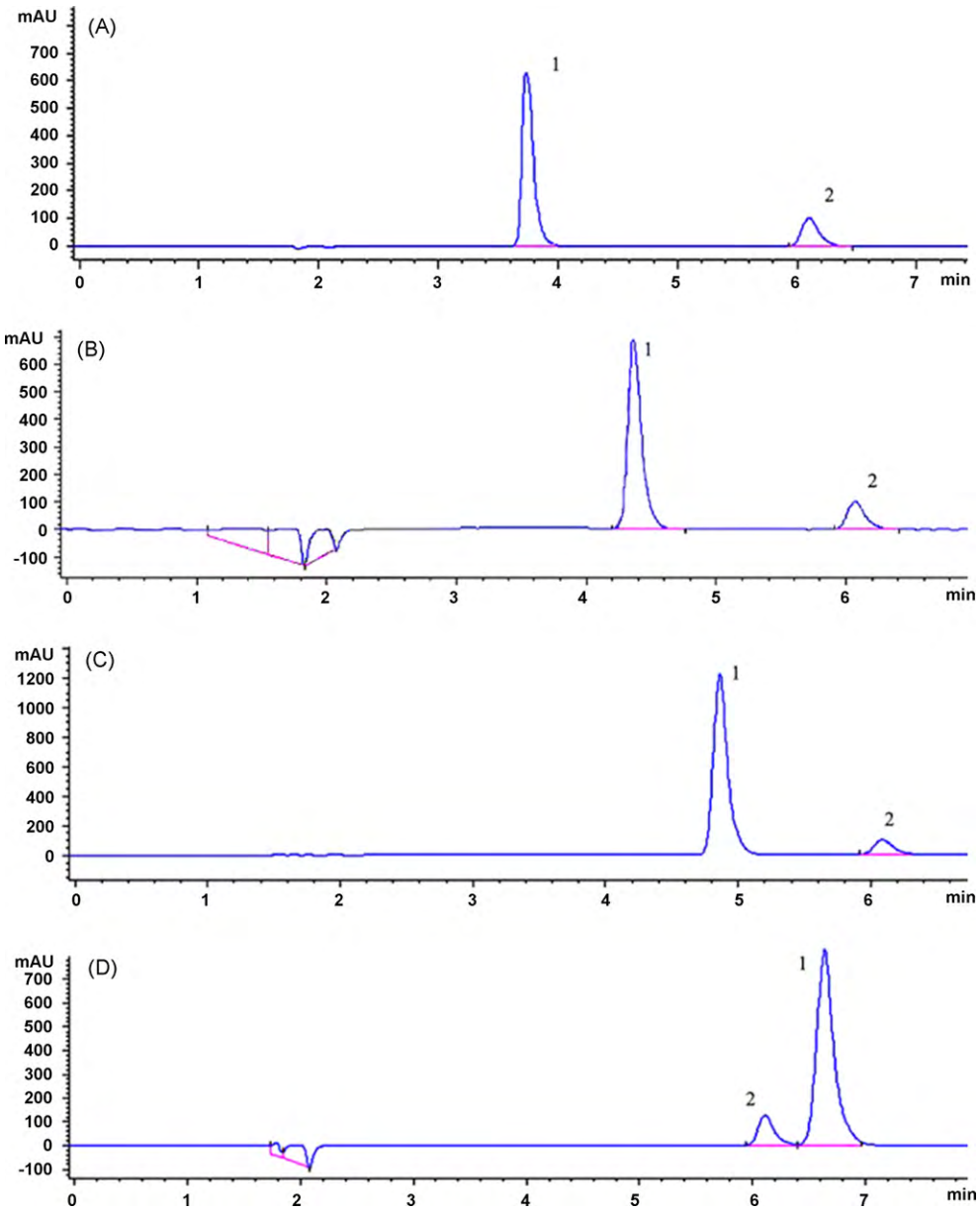


Fig. 4. (a) Chromatogram of angiotensin II receptor antagonist and naproxen (I.S.) in pharmaceutical dosage form. (A) 1, Telmisartan; 2, naproxen (I.S); (B) 1, Losartan; 2, naproxen (I.S.). (C) 1, Irbesartan; 2, naproxen (I.S.); (D) 1, Valsartan; 2, naproxen (I.S). Experimental conditions as in chromatographic procedure.

nal standard is critical for the assay method. The chemical and physical properties of internal standard should resemble those of the component to be investigated as far as possible. Several compounds were tested as possible I.S.: the most suitable was naproxen (Fig. 3). In fact, the chemical structure of naproxen is not similar to the investigated compounds. However, it was chosen as the I.S. because it showed a shorter retention time with a better peak shape and a better resolution from the investigated compounds peak, compared with other potential I.S.

According to US Pharmacopoeia 24th, method <621> [29], system suitability tests are an integral part of a liquid chromatographic method. System suitability tests are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. System suitability was checked by evaluating different parameters such as retention factor, theoretical plate number, retention time, asymmetry factor, selectivity and RSD% of peak height or area for repetitive injections. System suitability tests were carried out on freshly prepared standard stock solutions of ARA-IIs. The results from system suitability tests are presented in Table 4 for each drug. The results obtained from system suitability tests are in agreement with the USP requirements. Representative chromatograms of the compounds and naproxen (I.S.) in the optimized condition are presented in Fig. 3.

Finally, using the conditions above, a satisfactory chromatographic peak resolution was obtained in a short analysis time as can be seen in Fig. 3. For all compounds, sharp and symmetrical single peaks were obtained with good resolution.

The calibration curves and equations for telmisartan, losartan, irbesartan and valsartan were calculated by plotting the peak area ratios of ARA-IIs to I.S. *versus* concentration of the compounds in the range of 20–150 μg/mL for losartan; 20–200 μg/mL for valsartan; 50–400 μg/mL for irbesartan and 30–100 μg/mL for telmisartan (Table 5). These results showed highly reproducible calibration curves with correlation coefficients of >0.999. The low values of SE of slope, intercept and >0.999 correlation coefficient for all com-

Table 6
Results of the assay and the recovery analysis of ARA-IIs in pharmaceutical dosage forms.

	Losartan	Irbesartan	Valsartan	Telmisartan
Labeled claim (mg)	50	150	80	80
Amount found (mg)[a]	50.51	149.24	80.73	80.51
RSD%	1.24	1.22	1.63	0.77
Bias %	−1.03	0.51	−0.91	−0.64
Added (mg)	50	150	80	80
Found (mg)[a]	50.49	151.13	80.38	79.40
Recovery %	100.97	100.76	100.48	99.25
RSD% of recovery	0.86	0.67	1.76	0.76
Bias %	−0.97	−0.76	−0.48	0.75

[a] Each value is the mean of 5 experiments.

pounds established the precision of the proposed methods. The LOD and LOQ were calculated from the following equations by using the standard deviation (s) of response and the slope (m) of the corresponding calibration curve [30].

$$\mathrm{LOD} = 3.3\frac{s}{m} \quad (12)$$

$$\mathrm{LOQ} = 10\frac{s}{m} \quad (13)$$

Accuracy, precision and reproducibility of the proposed method were evaluated by performing replicate analysis (five replicates) of the standard solutions in mobile phase. Within-day and between-day variability were determined by using two different concentrations. The within-day and between-day precision, accuracy and reproducibility were calculated as RSD% and mean value. The intra-day variation was between 0.23 and 1.15, RSD% values. Intermediate precision was determined by replicate analysis over 3-day period and RSD% values were 0.77 and 1.56. Precision, accuracy and reproducibility results demonstrate good precision, accuracy and reproducibility of the method.

3.1. Analysis of commercial samples

When working on standard solutions and according to the validation parameters, results encourage the use of the proposed method described for the assay of telmisartan, losartan, irbesartan and valsartan in their pharmaceutical dosage forms. On the basis of above results, the proposed method was applied to the direct determination of ARA-IIs in their tablet dosage forms, using the related calibration straight lines without any sample extraction or evaporation other than filtration and adequate dilution steps. The results obtained from the analysis of tablet dosage forms are summarized in Table 6. The quantities found were in conformity with the values claimed by the manufacturers.

There is no official method present in any pharmacopoeias (e.g., USP, BP or EP) related to pharmaceutical dosage forms or bulk drugs of ARA-II compounds. For checking the accuracy, precision and selectivity of the proposed method and in order to know whether the excipients in pharmaceutical dosage forms show any interference with the analysis, the proposed method was evaluated by recovery tests after addition of known amounts of pure drug compound to various pre-analyzed formulations of related ARA-II compound. Recovery experiments were realized by using the standard addition method. These results showed that the proposed method had adequate precision and accuracy and consequently can be applied to the determination of ARA-II compounds without any interference from inactive ingredients used in the selected formulation (Table 6). Hence, the accuracy was determined by using the difference between nominal and measured concentration (bias %). It is concluded that the method is sufficiently accurate and precise in order to be applied to tablet dosage forms. Chromatograms obtained from pharmaceutical dosage form samples (with I.S.) are shown in Fig. 4.

4. Concluding remarks

Optimizing separation in order to find robust conditions is an important part of the method development for ionizable compounds. The combined effect of the two factors (solvent percentage and pH of the mobile phase) on the retention behavior of four ARA-IIs was investigated. The pH was selected to be in the range 2.75–8.0. Acetonitrile percentages of the mobile phases were selected in order to get convenient retention times. pK_a values and limiting retention factors of these compounds were calculated chromatographically using equations derived. The pH and acetonitrile percentage for optimized condition were predicted by using these equations. The method was applied to pharmaceutical formulations containing a single active ingredient. Method validation produced excellent results for linearity, precision, accuracy, limit of quantitation and limit of detection.

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