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Optimization and validation of a capillary zone electrophoretic method for the analysis of several angiotensin-II-receptor antagonists

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Abstract

We optimized a capillary zone electrophoretic method for separation of six angiotensin-II-receptor antagonists (ARA-IIs): candesartan, eprosartan, irbesartan, losartan potassium, telmisartan, and valsartan. A three-level, full-factorial design was applied to study the effect of the pH and molarity of the running buffer on separation. Combination of the studied parameters permitted the separation of the six ARA-IIs, which was best carried out using 60 m*M* sodium phosphate buffer (pH 2.5). The same system can also be applied for the quantitative determination of these compounds, but only for the more soluble ones. Some parameters (linearity, precision and accuracy) were validated.

Keywords: Validation; Pharmaceutical analysis; Experimental design; Angiotensin II receptor antagonists

1. Introduction

The angiotensin-II-receptor antagonists (ARA-II) are safe and effective agents in the treatment of hypertension and heart failure, either alone or in conjunction with diuretics. They have been proposed as alternatives to the more traditional angiotensin-converting enzyme (ACE) inhibitors because they selectively block the angiotensin type 1 (AT_1) receptor, which is responsible for vasoconstriction, and salt and water retention. The angiotensin type 2 (AT_2) receptor, which is thought to have cardio-protective effects and inhibitory effects on growth, is left unaffected [1–4]. Until now, there have been six ARA-IIs available on the market: candesartan, eprosartan, irbesartan, losartan potassium, telmisartan, and valsartan. Candesartan, irbesartan, losartan potassium, and valsartan contain a biphenyltetrazole moiety, whereas telmisartan contains a structurally related biphenylcarboxylic acid moiety. The structure of eprosartan differs from the others. Candesartan is orally administered as the pro-drug candesartan cilexetil, which is completely converted to the active compound candesartan during absorption from the gastrointestinal tract. Losartan potassium is also converted into a more active drug during metabolism in the liver. However, losartan potassium is not a classic pro-drug because it possesses significant ARA activity of its own. All the other ARA-IIs are

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active on their own and do not require metabolism into active molecules [4–6].

Until now, high-performance liquid chromatography (HPLC) has been the major technique used for the determination of the different ARA-IIs, but these studies have usually been limited to the determination of a single component [7–21]. One study has reported the determination of five ARA-IIs by HPLC [22]. Capillary electrophoresis (CE) offers an alternative technique. Although analysis by means of CE has been achieved for losartan potassium [23], the literature shows no selective single method able to separate and quantify the ARA-IIs.

The aim of the present study was therefore to develop a selective capillary zone electrophoretic method capable of separating the six ARA-IIs: candesartan, eprosartan, irbesartan, losartan potassium, telmisartan, and valsartan. A statistical experimental design was used for the optimization of the method [24,25]. After preliminary investigations to adjust the experimental domain under study, a three-level full-factorial design was applied to study the impact of two parameters on the retention of these compounds [26]. The parameters studied were the pH and the molarity of the running buffer. Afterwards, the usefulness of the system for the quantitative determination of these compounds in their pharmaceutical formulation was investigated, and the most important parameters for quantitative analysis were validated.

2. Experimental

2.1. Instrumentation and electrophoretic procedure

Experiments were performed on a Crystal CE (Thermo Capillary Electrophoresis, Franklin, USA), equipped with PC 1000 software installed on a Dell computer with an OS/2 operating system. A fused-silica capillary was used, 85 cm (33 cm to the detector) $\times$50 μm I.D. The Crystal CE was temperature controlled at 25 °C for the tray, and at 30 °C for the capillary. The sample solutions were injected by pressure (50 mbar) for 5 s. Each solution was at least three times injected. A constant voltage of 25 kV was applied, and UV absorbance at 214 nm was employed for detection by means of a variable-wavelength UV detector (Spectra FOCUS detector, Spectra-Physics, San Jose, CA, USA). The pH measurements were performed on a calibrated Metrohm 744 pH Meter (Herisau, Switzerland).

2.2. Reagents

Sodium dihydrogenphosphate monohydrate (analytical-reagent grade) was obtained from Merck (Darmstadt, Germany). Phosphoric acid (85%, w/w) was obtained from UCB (Leuven, Belgium), hydrochloric acid (37%, w/w) from Panreac (Barcelona, Spain), and meglumin (analytical-reagent grade) from Fluka (Buchs, Switzerland). The excipients (microcrystalline cellulose, lactose, pregelatinized starch, pregelatinized maize starch, magnesium stearate, hydroxypropylcellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, sorbitol, polyvidone, silicon dioxide, titanium dioxide, and poloxamer 188) are commercially available products that meet the requirements of the European Pharmacopoeia.

Candesartan was obtained from AstraZeneca (Mölndal, Sweden), eprosartan from Solvay (Weesp, Netherlands), irbesartan from Sanofi-Synthelabo (Gentilly Cedex, France), losartan potassium from Merck Sharp & Dohme (MSD, Rahway, NJ, USA), telmisartan from Boehringer Ingelheim (Ingelheim, Germany) and valsartan from Novartis (Basel, Switzerland). The chemical structures of the ARA-IIs are represented in Fig. 1.

The commercially available drugs Teveten (Solvay), Aprovel (Sanofi-Synthelabo), Cozaar (MSD), and Micardis (Boehringer Ingelheim) were used for quantitative determinations.

All solutions were prepared with distilled water obtained from deionized water.

2.3. Running buffers

During the development of the method, sodium phosphate buffers of different pH were used. In the pH range 2.0–3.0, a mixture of a phosphoric acid solution and a sodium dihydrogenphosphate solution was used. A 60-m*M* sodium phosphate buffer (pH 2.5) was finally chosen as the running buffer. It was prepared by adjusting the pH of a 60-m*M* sodium

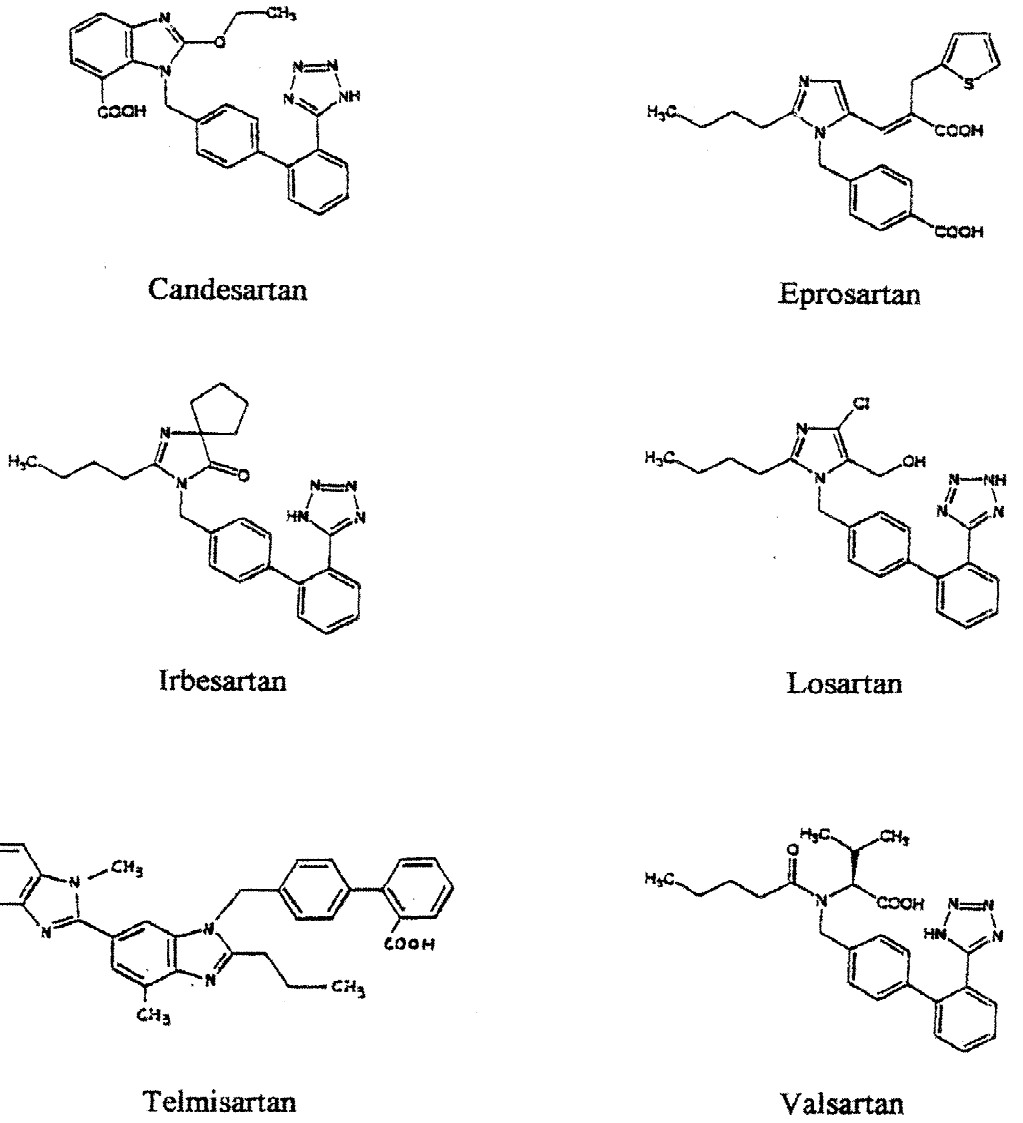

Fig. 1. Chemical structures of the angiotensin-II-receptor antagonists.

dihydrogenphosphate solution to pH 2.5 by the addition of 60 m*M* phosphoric acid solution.

2.4. Internal standard solutions

For quantitative determination of the ARA-IIs, another ARA-II was always used as an internal standard. Selection had to be made based on the substance to be examined. Although each ARA-II can be combined, losartan potassium was mostly chosen as the internal standard because of its good solubility. For the determination of losartan potassium, another ARA-II must be used: in this investigation, irbesartan. An appropriate amount of the compound (Table 2) was dissolved in 10 ml of 1 *M* HCl and diluted to 100.0 ml with water.

2.5. Choice of solvent

The running buffer cannot be used as a solvent for

Table 1
Reference solutions for the quantitative determination

Reference substance	Reference solution (mg/50 ml)	Diluted reference solution (mg/ml)
Eprosartan mesylate	±35	±0.20
Irbesartan	±35	±0.20
Losartan potassium	±35	±0.20
Telmisartan	±30	±0.17

the preparation of reference and sample solutions because of the poor solubility of the ARA-IIs. Taking the acidic medium in which the experiments are performed into account, 1 *M* HCl was added to dissolve the active substances, and the solutions were then diluted with water. However, candesartan and valsartan remained poorly soluble and were not amenable to quantitative determination.

2.6. Reference solutions for the experimental design

Reference solutions of the six compounds were prepared at 300 μg ml^{-1} in a 1 *M* HCl:water (1:9) mixture.

2.7. Reference solutions for the quantitative determination

Reference solutions were prepared by weighing accurately an appropriate amount of the corresponding reference substance, dissolving it in 10 ml 1 *M* HCl and diluting to 50.0 ml with water. An appropriate volume of each solution was mixed with 10.0 ml of the internal standard solution and diluted to an appropriate concentration with 0.1 *M* HCl (Table 1).

2.8. Sample preparations for the quantitative determination

A minimum of 20 tablets of each compound were weighed, ground, and mixed. The requisite amount of the powder was mixed with 10 ml 1 *M* HCl and diluted to 100.0 ml with water. A suitable volume of the filtrate was mixed with 10.0 ml of the appropriate internal standard solution and diluted to the required concentration with 0.1 *M* HCl (Table 2).

All samples and buffers were filtered by passing them through 0.45-μm membrane filters (Millipore, Bedford, MA, USA).

Table 2
Sample preparation for the quantitative determination

	Average mass (mg)	Sample solution (mg powder /100 ml)	Internal standard solution (mg/ml)	Diluted sample solution (mg active substance/ml)
Eprosartan mesylate (Teveten) 735.82 mg tablets	987.0	ca. 66	Losartan: 0.25	±0.20
irbesartan (Aprovel) 300 mg tablets	598.1	ca. 105	Losartan: 0.25	±0.20
losartan potassium (Cozaar) 50 mg tablets	152.9	ca. 160	Irbesartan: 0.55	±0.20
telmisartan (Micardis) 80 mg tablets	479.2	ca. 170	Losartan: 0.40	±0.17

2.9. Experimental set-up and analysis of results

The set-up of the design and the statistical analysis of the response variables were supported by the statistical graphics software system ''STATGRAPHICS Plus'' version 4.1 (STSC, Rockville, MD, USA).

3. Results and discussion

Until now, the literature has shown no selective capillary electrophoretic method able to separate and quantify the ARA-IIs. Capillary zone electrophoresis (CZE) is the simplest mode of CE, and the most widely used. Therefore, CZE was investigated as a separation method while the experimental design was applied to optimize the separation conditions.

3.1. Screening phase

Several parameters were considered. From preliminary results, it was found that the factors most affecting the response migration time were the pH and the molarity of the running buffer. The pH of the running buffer plays an important role because it influences the separation by affecting the charge of the compounds as well as the electroosmotic flow. Different concentrations of the running buffer were tested to optimize the separation. Selection of the experimental domain was made from prior experience and knowledge of the separation system. The voltage initially was also considered, but it was found to have less influence on the selectivity of the separation and was kept constant at 25 kV.

3.1.1. Selection of the pH

Because of the amphoteric character of the ARA-IIs, their retention is greatly influenced by pH, which determines whether these compounds are negatively or positively charged. This offers the possibility of using either an acidic or an alkaline running buffer. From pH 7.5 and up, the six ARA-IIs can be divided into two groups that can be separated from each other: telmisartan, irbesartan, and losartan potassium on one hand, and eprosartan, valsartan and candesartan on the other. In the first group, no baseline separation between the three ARA-IIs could be achieved, while in the second group, eprosartan and valsartan co-eluted. Telmisartan gave bad peak symmetry.

Considering that the aim of this study was to develop a CZE method to separate these compounds, the alkaline medium was abandoned and an acidic medium was investigated. The best peak shapes and shortest analysis times were obtained in the pH range of 2.0–3.0. Therefore, the measurements were performed at three pH levels (2.0, 2.5, and 3.0). Because of the low solubility of candesartan and valsartan in this medium, these two compounds were not included in the experimental design. Moreover, for all possible experimentally different conditions in the studied domain, candesartan and valsartan do not interfere with the other four ARA-IIs. They migrate last and are well separated from eprosartan, irbesartan, losartan potassium, and telmisartan (Figs. 2 and 3). The determination and quantification of candesartan and valsartan must be carried out using a buffer with a higher pH and with the addition of sodium dodecyl sulfate (paper in preparation).

3.1.2. Concentration of the running buffer

In earlier investigations, the molarity of the sodium phosphate buffers varied from 20 to 80 m*M*. When the concentration of the electrolyte increased, the selectivity of the separation improved and the migration times increased. If concentrations above 80 m*M* were used, a high current was generated. Because of the optimum balance in ionic strength, the concentration of the running buffer was tested at three levels (40, 60, and 80 m*M*) for optimization purposes.

3.2. Response surface design

To establish the influence of the two parameters and their interaction on the separation, a three-level full-factorial design was applied. This design requires nine runs. The experimental matrix included two extra experiments at the central level of the design to obtain an estimate of experimental variance. Thus, the entire design required 11 runs. The individual runs of the design were carried out in a randomized sequence. Randomization offers some

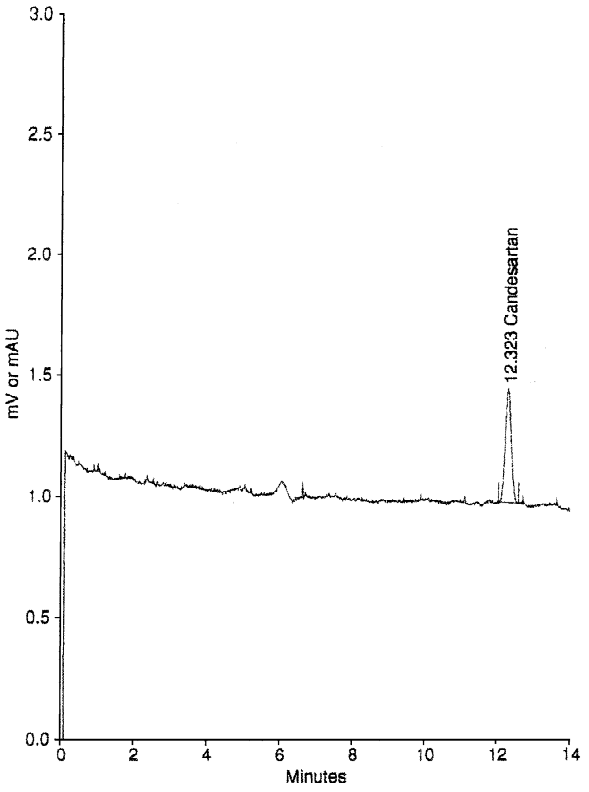

Fig. 2. Electropherogram of the determination of candarsartan using a fused-silica capillary 85 cm (33 cm to the detector) ×50 μm I.D., and 60 m*M* sodium phosphate buffer (pH 2.5) as the running buffer. The applied voltage is 25 kV and detection is at 214 nm.

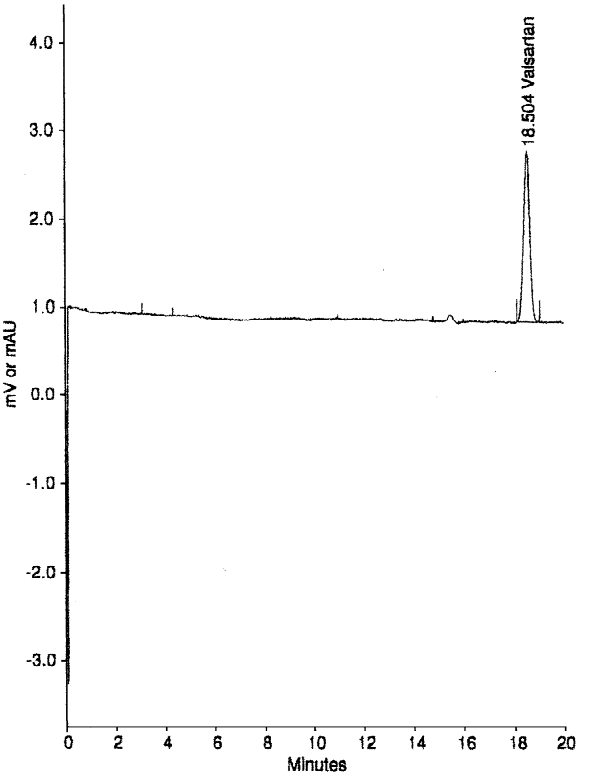

Fig. 3. Electropherogram of the determination of valsartan using a fused-silica capillary 85 cm (33 cm to the detector) ×50 μm I.D., and 60 m*M* sodium phosphate buffer (pH 2.5) as the running buffer. The applied voltage is 25 kV and detection is at 214 nm.

assurance that uncontrolled variation of factors, other than those studied, will not influence the estimations. Replicate measurements ($n=3$) were performed to verify if retention times were stable and the capillary was well equilibrated after tuning to new electrophoretic conditions.

The measured responses were the migration times of eprosartan (t_E), irbesartan (t_I), losartan potassium (t_L), and telmisartan (t_T). In Table 3, the measured migration times (t) for each run of the design are compiled.

3.2.1. Regression modelling

From the 3^2 design for each response, the following model was determined:

$$y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2$$

where y is the measured response (migration time) for each compound; b_0 is the intercept; b_i are the regression coefficients; X_i are the values of the independent electrophoretic variables (X_1, pH; and X_2, molarity of the running buffer).

To obtain a good separation of compounds, an adequate difference in migration time is needed. The minimal time difference or the time difference of the two worst separated peaks (Δt_{min}) is especially important. Therefore, we were interested in the domain(s) where Δt_{min} was maximal.

First, the measured migration times for each ARA-II were modelled. Then the responses were predicted for all possible, experimentally different conditions in the studied domain. Subsequently, for each situation, the migration times of the compounds were sorted, the difference in migration time of the successive pairs of peaks (t_i) was calculated, and

Table 3
Measured response variables

Run	pH	Molarity of the running buffer (m*M*)	t_E	t_I	t_L	t_T
1	2	40	5.86	5.78	5.93	4.32
2	2	60	5.97	5.87	6.05	4.35
3	2	80	6.53	6.39	6.64	4.66
4	2.5	40	7.34	6.92	7.82	5.25
5	2.5	60	7.75	7.26	8.31	5.44
6	2.5	60	7.66	7.20	8.19	5.39
7	2.5	60	7.61	7.16	8.15	5.35
8	2.5	80	7.89	7.39	8.48	5.47
9	3	40	9.21	7.89	10.30	6.29
10	3	60	9.47	8.09	10.93	6.27
11	3	80	9.64	8.20	11.29	6.26

Migration times (t) are exprerssed in minutes.
E, I, L and T in the subscript refer to the first letter of the corresponding ARA-II.

Δt_{min} was selected. Finally, all Δt_{min} were plotted, and the region(s) where Δt_{min} was maximal were investigated.

The contour plot of Δt_{min} as a function of the pH and molarity of the running buffer, is shown in Fig. 4, while the contour plot of the longest migration time (t_{max}) is given in Fig. 5. As the pH and the molarity of the running buffer increased, Δt_{min} also increased. The progressive general increase of the minimal difference in migration time paralleled an increase of the analysis time. In this case, the region with an optimum balance between Δt_{min} and the analysis time must be sought to obtain baseline separation of the four ARA-IIs within an acceptable analysis time. Because of the good peak shape of these compounds, a baseline separation can be expected with a predicted value of Δt_{min}: 0.5. Therefore, the region with this value was selected (Fig. 4). In this area, the longest migration time is situated between 8 and 9 min, and is thus also

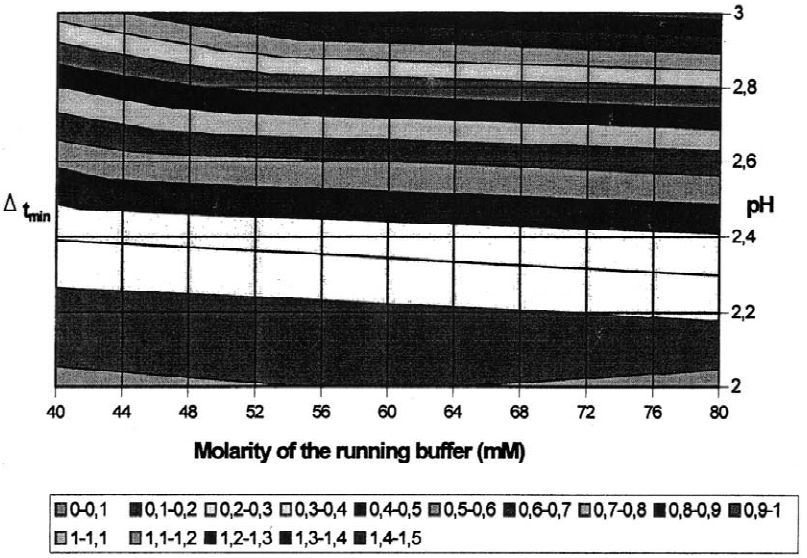

Fig. 4. Contour plot of Δt_{min} as a function of the pH and molarity of the running buffer.

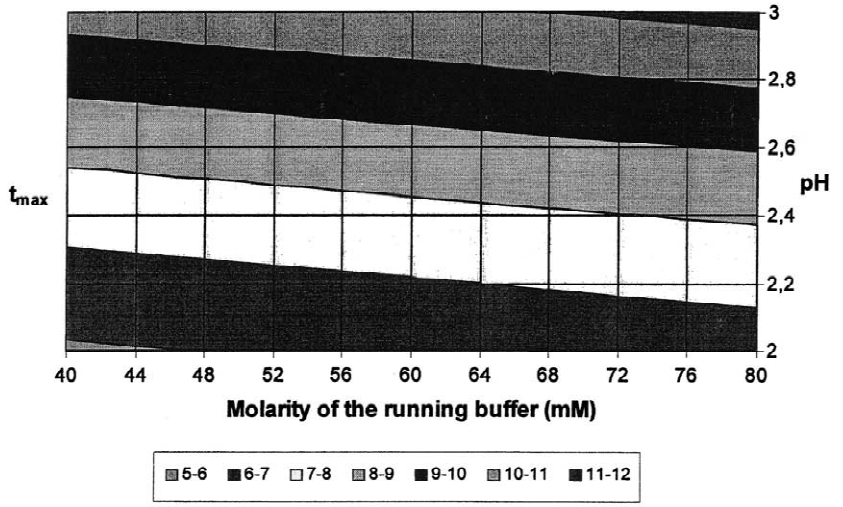

Fig. 5. Contour plot of the longest migration time (t_{max}) as a function of the pH and molarity of the running buffer.

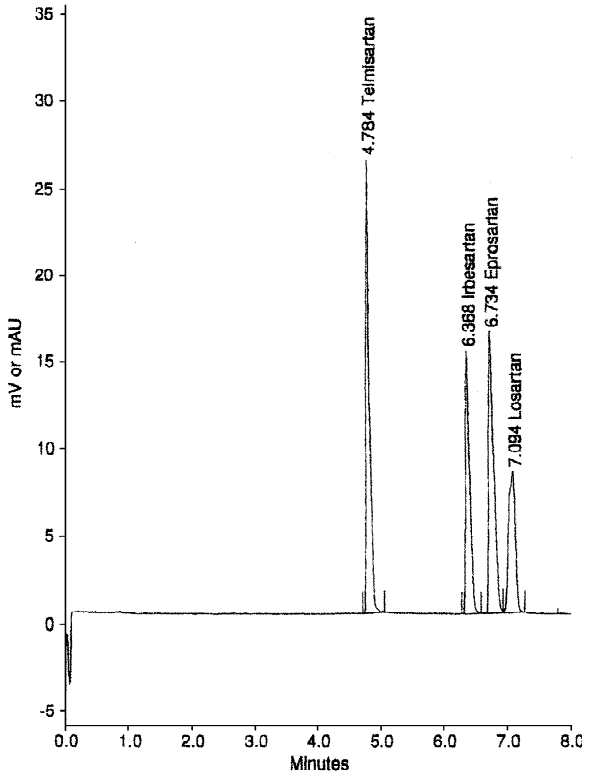

Fig. 6. Electropherogram of a mixture of several ARA-IIs using a fused-silica capillary 85 cm (33 cm to the detector) ×50 μm I.D., and 60 m*M* sodium phosphate buffer (pH 2.5) as the running buffer. The applied voltage is 25 kV and detection is at 214 nm.

acceptable (Fig. 5). Therefore, the best combination seems to be pH 2.5 and 60 m*M*. Applying these conditions leads to an adequate separation because the peak symmetry for all ARA-IIs is acceptable. A typical electropherogram obtained applying these optimized conditions (60 m*M* sodium phosphate buffer, pH 2.5) is presented in Fig. 6.

3.2.2. Quantitative determination in pharmaceutical formulations

The same system (60 m*M* sodium phosphate buffer, pH 2.5) may be applied for the quantitative determination of eprosartan, irbesartan, losartan potassium, and telmisartan in tablets and capsules (Figs. 7–10). Using different placebo mixtures it was demonstrated that the following excipients do not adversely affect the results: microcrystalline cellulose, lactose, pregelatinized starch, pregelatinized

Table 4
Linearity

	Concentration range (mg/ml)	Correlation coefficient (r^2)
Eprosartan mesylate	0.07–0.35	0.9997
Irbesartan	0.06–0.30	1
Losartan potassium	0.06–0.30	0.9999
Telmisartan	0.05–0.24	0.9998

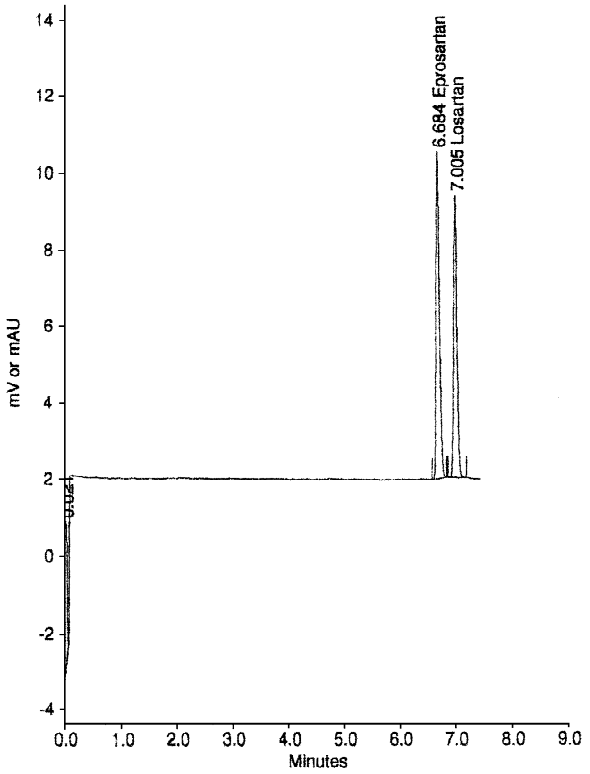

Fig. 7. Electropherogram of the quantitative determination of eprosartan mesylate (Teveten) on a fused-silica capillary 85 cm (33 cm to the detector) ×50 μm I.D. Conditions: 60 m*M* sodium phosphate buffer (pH 2.5) as running buffer; applied voltage, 25 kV; detection at 214 nm.

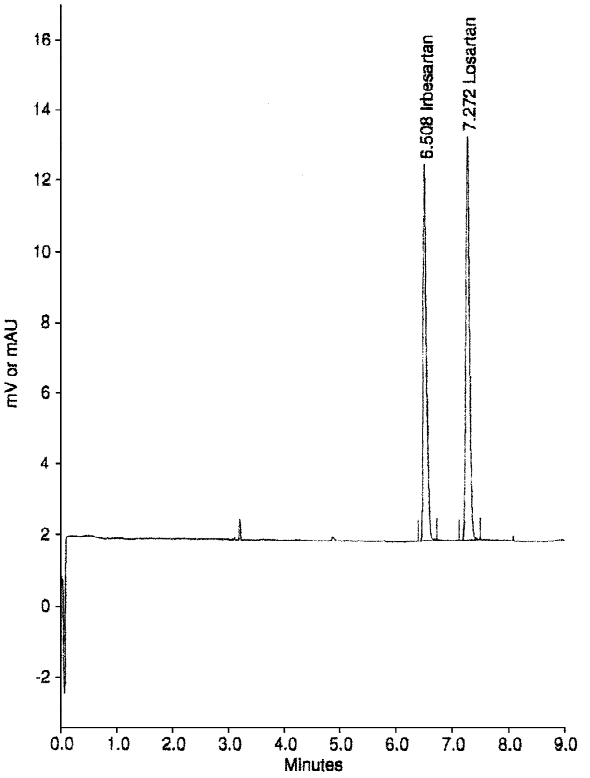

Fig. 8. Electropherogram of the quantitative determination of irbesartan (Aprovel) on a fused-silica capillary 85 cm (33 cm to the detector) ×50 μm I.D. Conditions: 60 m*M* sodium phosphate buffer (pH 2.5) as running buffer; applied voltage, 25 kV; detection at 214 nm.

maize starch, magnesium stearate, hydroxypropylcellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, sorbitol, meglumin, polyvidone, silicon dioxide, titanium dioxide, or poloxamer 188.

3.2.3. Validation of the method

3.2.3.1. Linearity

The detector responses were found to be linear for the different components in the concentration range,

Table 5
Precision (repetability) of the total analysis of 10 replicate samples

Substance to be examined	Theoretical amount (mg/tablet)	Amount found	Relative standard deviation (%, $n=10$)
Eprosartan mesylate (Teveten)	735.82	732.16±9.32 mg or 99.5%	1.27%
Irbesartan (Aprovel)	300	300.37±2.78 mg or 100.1%	0.93%
Losartan potassium (Cozaar)	50	50.43±0.39 mg or 100.9%	0.77%
Telmisartan (Micardis)	80	80.90±0.71 mg or 101.1%	0.88%

Table 6
Repetability of 10 consecutive injections of the same sample

Sample solution	Relative standard deviation (%, $n=10$)
Eprosartan mesylate	0.67
Irbesartan	0.39
Losartan potassium	0.26
Telmisartan	0.47

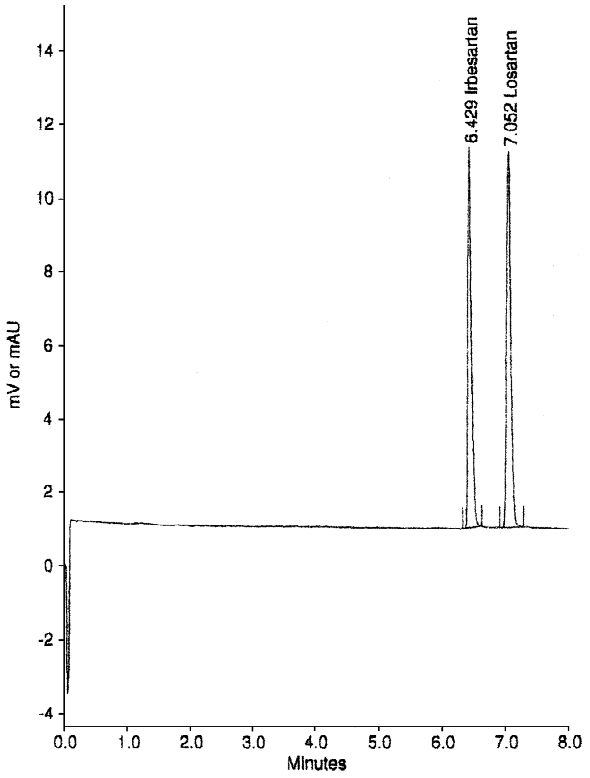

Fig. 9. Electropherogram of the quantitative determination of Losartan potassium (Cozaar) on a fused-silica capillary 85 cm (33 cm to the detector) ×50 μm I.D. Conditions: 60 m*M* sodium phosphate buffer (pH 2.5) as running buffer; applied voltage, 25 kV; detection at 214 nm.

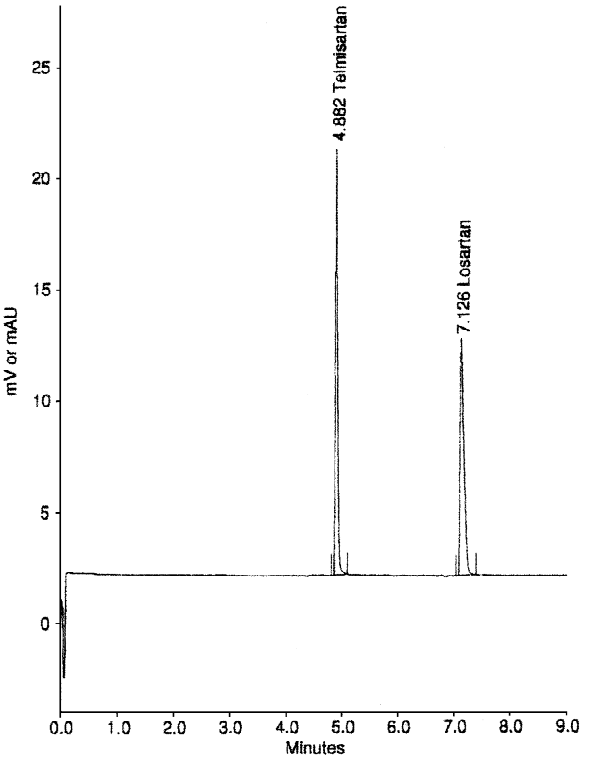

Fig. 10. Electropherogram of the quantitative determination of telmisartan [Micardis] on a fused-silica capillary 85 cm (33 cm to the detector) ×50 μm I.D. Conditions: 60 m*M* sodium phosphate buffer (pH 2.5) as running buffer; applied voltage, 25 kV; detection at 214 nm.

as described in Table 4. The amount of the internal standard was adjusted according to the concentration range used. Regression analysis data for the calibration curves were calculated using the peak areas.

3.2.3.2. Precision

The precision (repeatability) was determined by the total analysis of 10 replicate samples under the

Table 7
Accuracy

	Recovery placebo +80% ($n=3$)	Recovery placebo +100% ($n=3$)	Recovery placebo +120% ($n=3$)
Eprosartan mesylate	100.9±0.4%	97.5±0.1%	98.1±0.6%
Irbesartan	101.7±0.4%	101.7±0.9%	99.7±0.7%
Losartan potassium	100.7±1.1%	99.1±0.9%	99.5±0.9%
Telmisartan	101.4±0.4%	99.8±0.5%	99.6±0.6%

same operating conditions, by the same analyst, and on the same day. The mean value of the concentration and the relative standard deviation are summarized in Table 5.

The error of the equipment, the accuracy of electrophoretic separation, and the relative standard deviations of the peak area ratios were determined by performing 10 consecutive injections of the same sample (Table 6).

3.2.3.3. Accuracy

The accuracy of the method was determined by investigating the recovery of each component at three levels, ranging from 80 to 120% of the theoretical concentration, from placebo mixtures spiked with the active substance (Table 7).

4. Conclusions

The above results demonstrate that capillary zone electrophoretic separation of four angiotensin-II-receptor antagonists (ARA-IIs) can be achieved using a 60-m*M* sodium phosphate buffer at pH 2.5.

Applying the optimized conditions, the two other ARA-IIs, candesartan and valsartan, can also be identified, so this system can be applied successfully to the identification of these six compounds. Because of the poor solubility of candesartan and valsartan, the same system can only be applied for the quantitative determination of eprosartan, irbesartan, losartan potassium, and telmisartan in pharmaceutical formulations. The possibility of simultaneous quantification and identification of the active ingredient in the finished product is therefore very attractive.

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