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Determination of pK_a values of some antihypertensive drugs by liquid chromatography and simultaneous assay of lercanidipine and enalapril in their binary mixtures

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ABSTRACT

In this study, pK_a values were determined using the dependence of the retention factor on the pH of the mobile phase for three ionizable substances, namely, enalapril, lercanidipine and ramipril (IS). The effect of the mobile phase composition on the ionization constant was studied by measuring the pK_a at different methanol–water mixtures, ranging between 50 and 65% (v/v), using LC–DAD method. Two simple, accurate, precise and fully validated analytical methods for the simultaneous determination of enalapril and lercanidipine in combined dosage forms have been developed. Separation was performed on an X-Terra RP-18 column (250 mm × 4.60 mm ID × 5 μm) at 40 °C with the mobile phase of methanol–water 55:45 (v/v) adjusted to pH 2.7 with 15 mM orthophosphoric acid. Isocratic elution was performed in less than 12 min with a flow rate of 1.2 mL min^{-1}. Good sensitivity for the analytes was observed with DAD detection. The LC method allowed quantitation over the 0.50–20.00 μg mL^{-1} range for enalapril and lercanidipine. The second method depends on first derivative of the ratio-spectra by measurements of the amplitudes at 219.7 nm for enalapril and 233.0 nm for lercanidipine. Calibration graphs were established for 1–20 μg mL^{-1} for enalapril and 1–16 μg mL^{-1} lercanidipine, using first derivative of the ratio spectrophotometric method. Both methods have been extensively validated. These methods allow a number of cost and time saving benefits. The described methods can be readily utilized for analysis of pharmaceutical formulations. The methods have been applied, without any interference from excipients, for the simultaneous determination of these compounds in tablets. There was no significant difference between the performance of the proposed methods regarding the mean values and standard deviations.

1. Introduction

The angiotensin-converting enzyme inhibitory drugs (ACE inhibitors) are widely used for the treatment of many cardiovascular conditions including mild to moderate hypertension and heart failure, either alone or in conjunction with other drugs [1]. Enalapril (ENA), [(2*S*)-1-[(2*S*)-2-[[(1*S*)-1-(ethoxycarbonyl)-3-phenylpropyl] amino] propanoyl]pyrrolidine-2-carboxylic acid (*Z*)-butenedioate] an ACE inhibitor, is a pro-drug. It is converted to its active metabolite, di-acid enalaprilat [2,3] and used as its maleate salt. In ENA one carboxylic group is esterified, while the second may be engaged in zwitterionic structure with the protonated basic nitrogen, depending on pH (Fig. 1). In this aspect, accurate knowledge of acidic and basic pK_a is required to assess the molecular species/pH profile.

Lercanidipine hydrochloride (LER) [2-[(3,3-diphenylpropyl) methylamine]-1,1-dimethylethyl methyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5 pyridine carboxylic ester hydrochloride] is a dihydropyridine calcium channel blocker (Ca antagonist) with actions similar to those of nifedipine. LER is member of new third generation Ca antagonist used in treatment of hypertension because of its selectivity and specificity on the smooth vascular cells [4,5]. It is administered orally as tablet dosage form. The chemical structure of LER is characterized by the presence of a side chain containing a 3,3-diphenylpropylmethylamine-2-methyl-2-propyl group that was introduced to improve the lipophilic properties and the activity duration of the drug. From a physico-chemical point of view, LER is slightly soluble in water, but it is more soluble in some widely used solvents as well as ethanol and methanol (MeOH), or mixture water–organic solvents.

Ramipril (RAM) (2-[*N*-[(*S*)-l-ethoxycarbonyl-3-phenylpropyl-lalanyl]-(1*S*,3*S*,5*S*)-2-azabicyclo [3-3-0]-octane-3-carboxylic acid, see Fig. 1) is also an orally active inhibitor of ACE, which is a pro-

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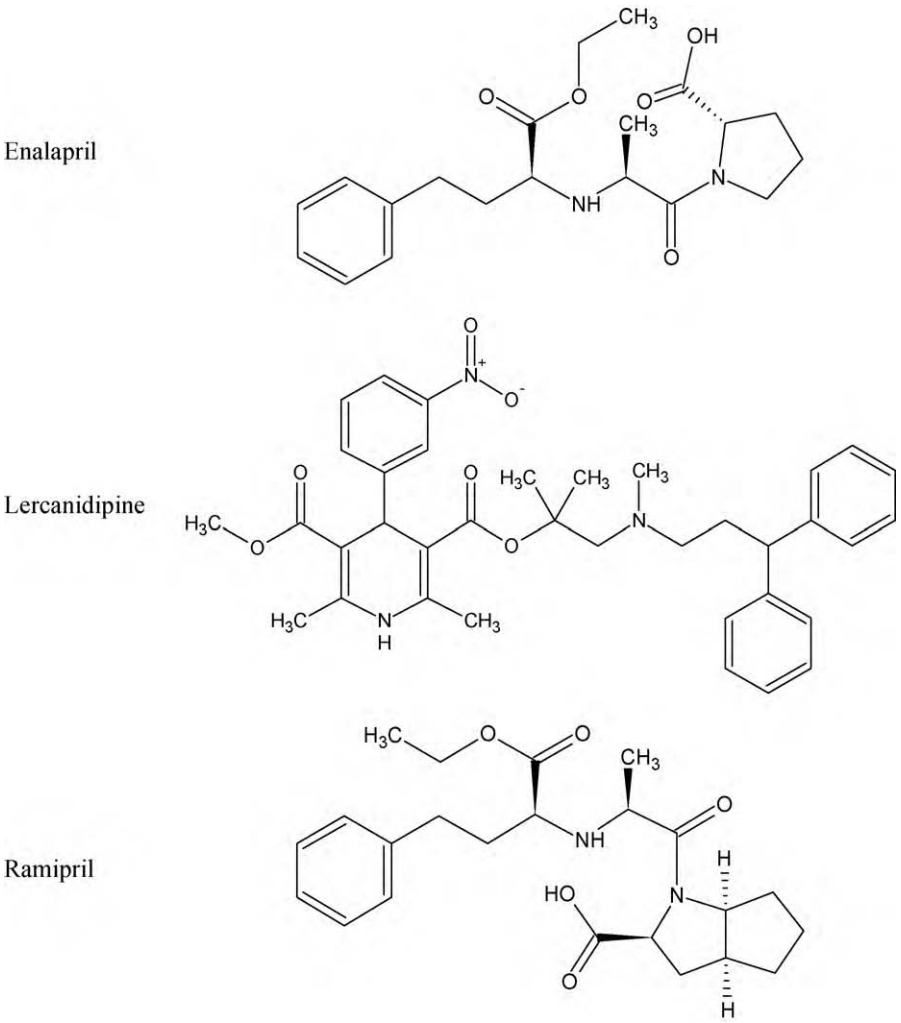

Fig. 1. Structures of compounds studied.

drug used in the treatment of all forms of hypertension, heart failure and following myocardial infarction to improve survival in patients with clinical evidence of heart failure [1–4]. In this study, RAM is used as an internal standard during separation studies because of its shorter elution time and similar structure.

The ionization constant is an important physico-chemical parameter of a drug and the knowledge of this parameter is of fundamental importance in a wide range of applications and research areas. The chromatographic retention and electrophoretic behavior of ionizable compounds strongly depend on the pK_a of the compound and the mobile phase pH. A satisfactory knowledge of the acid–base behavior of substances in hydro-organic media such as methanol–water is therefore essential to predict the influence of pH on selectivity and on retention in LC and also to optimize analytical procedures for the separation of ionizable compounds by different techniques [6,7]. Although methanol–water mobile phases have been used in RP-LC separation procedures, the pK_a values of ENA, LER and RAM have not yet been determined in methanol–water binary mixtures.

A methodological approach of choice is pK_a estimation by isocratic reversed-phase LC [8,9]. In fact, as most of the organic compounds tend to be poorly soluble in water, the classical potentiometric techniques for studying acid–base equilibrium are not practical. Another advantage of the LC method is that it only requires a small amount of sample. The determination of pK_a values by RP-LC is based on the relationship between the retention factors and the pH values of the mobile phase [10,11]. This procedure is limited by working pH range of the LC column, the optimum conditions being when the pK_a corresponds to the equilibrium between a neutral species and a charged species (this is, $H_2A^+ \leftrightarrow HA$, $HA \leftrightarrow A^-$, or $B \leftrightarrow HB^+$).

Several analytical methods have been reported in the literature for the analysis of ENA and LER, individually, in pharmaceutical dosage forms. The techniques include spectrophotometry [12], atomic absorption spectroscopy [13] and the number of high performance liquid chromatographic (LC) methods have also been reported for these drugs using ultraviolet (UV) as well as mass (MS) detectors [14–18]. Some of the reported methods require solid-phase extraction or expensive equipments, which are not economically feasible for routine use in pharmacokinetic and pharmaceutical studies, where numerous samples should be analyzed.

Owing to the widespread use of LC in routine analysis, it is important that specific LC methods are developed and that these are thoroughly validated [19–22]. LC–UV detection offers important advantages, such as rapid set-up of instrumentation, versatility and low cost, and has proved to be a valuable method in the quality control of drug compounds.

The ratio-spectra derivative spectrophotometric method permits the determination of a component in their binary mixtures at the wavelengths corresponding to a maximum or minimum and also the use of the peak-to-peak measurements between consecutive maximum and minimum. Moreover, the presence of a number

of maxima and minima is another advantage by the fact that these wavelengths give an opportunity for the determination of the active compound in the presence of other active compounds and excipients which possibly affect the analysis [22–24].

The pK_a values of studied compounds are either not known accurately or not available at all. Only a limited number of studies related to pK_a values of studied compounds are found in the literature [25–27]. This paper focuses on the determination of pK_a values of LER, ENA and RAM (IS) in several MeOH–water mixtures, 50, 55, 60 and 65% v/v, in order to overcome the lack of information related with the acid–base equilibria of this kind of compounds by means of chromatographic measurements. Although ENA and LER are commonly used in dual drug therapy as one potent antihypertensive preparation, no studies were found for the simultaneous determination of ENA and LER using neither LC nor spectrophotometric methods. Using the optimum separation conditions which were obtained from pK_a studies, the simultaneous determination of ENA and LER was also aimed to study. Thus, two simple, accurate, precise and fully validated analytical methods for the simultaneous determination of ENA and LER in combined dosage form have been developed.

2. Experimental

2.1. Chemicals and reagents

All chemicals and solvents were of analytical-reagent grade and harmaceutically active compounds used in this study were kindly supplied as follows: LER from (Fako Pharm. Ind.); ENA maleate and ramipril (IS) from (Nobel Pharm. Ind.). Methanol (MeOH) HPLC grade from Merck (Darmstadt, Germany) and orthophosphoric acid (min. 85%) were from Riedel (Riedel-de Haen, Germany). Sodium hydroxide was purchased from Merck. Hydrochloric acid (Titrisol) and potassium hydrogen phthalate (dried at 110 °C before use, Fluka), were used. All stock solutions of hydrochloric acid, potassium hydroxide and potassium hydrogen phthalate were prepared by water. Water, with conductivity lower than 0.05 μS cm^{-1} was obtained with a Zeneer Power I (Human Corp.)

Stock standard solutions of LER, ENA and RAM were prepared in MeOH at concentrations of approximately 200 mg L^{-1}. Working solutions were diluted with the corresponding mobile phase to 10 mg L^{-1}. All stock and working solutions were protected from light and stored in fridge at about 4 °C. The dead time (t_o) was measured by injecting uracil solution [0.01% (v/w), in water] which was established for each mobile phase composition and pH studied.

2.2. Apparatus

The LC analysis was carried out on a Shimadzu HPLC system with a pump (LC-20 AD), a DAD detector system (SPD-M 20A) and column oven (CTO 20 AC). This equipment has a degasser system (DGU 20 A). The system operates at 240 nm for LER, at 215 nm for ENA maleate and at 210 nm ramipril. An X-Terra RP-18 (250 mm × 4.60 mm ID × 5 μm) column was used as stationary phase at 40 °C. Mettler Toledo MA 235 pH/ion analyzer with Hanna HI 1332 Ag/AgCl combined glass electrode was used for pH measurements.

A Shimadzu 1601 PC double beam spectrophotometer equipped with 1.0 cm quartz cells with a fixed slit width (2 nm) was used for the spectrophotometric measurement, coupled an IBM-PC computer running spectrophotometric software Shimadzu UVPC software.

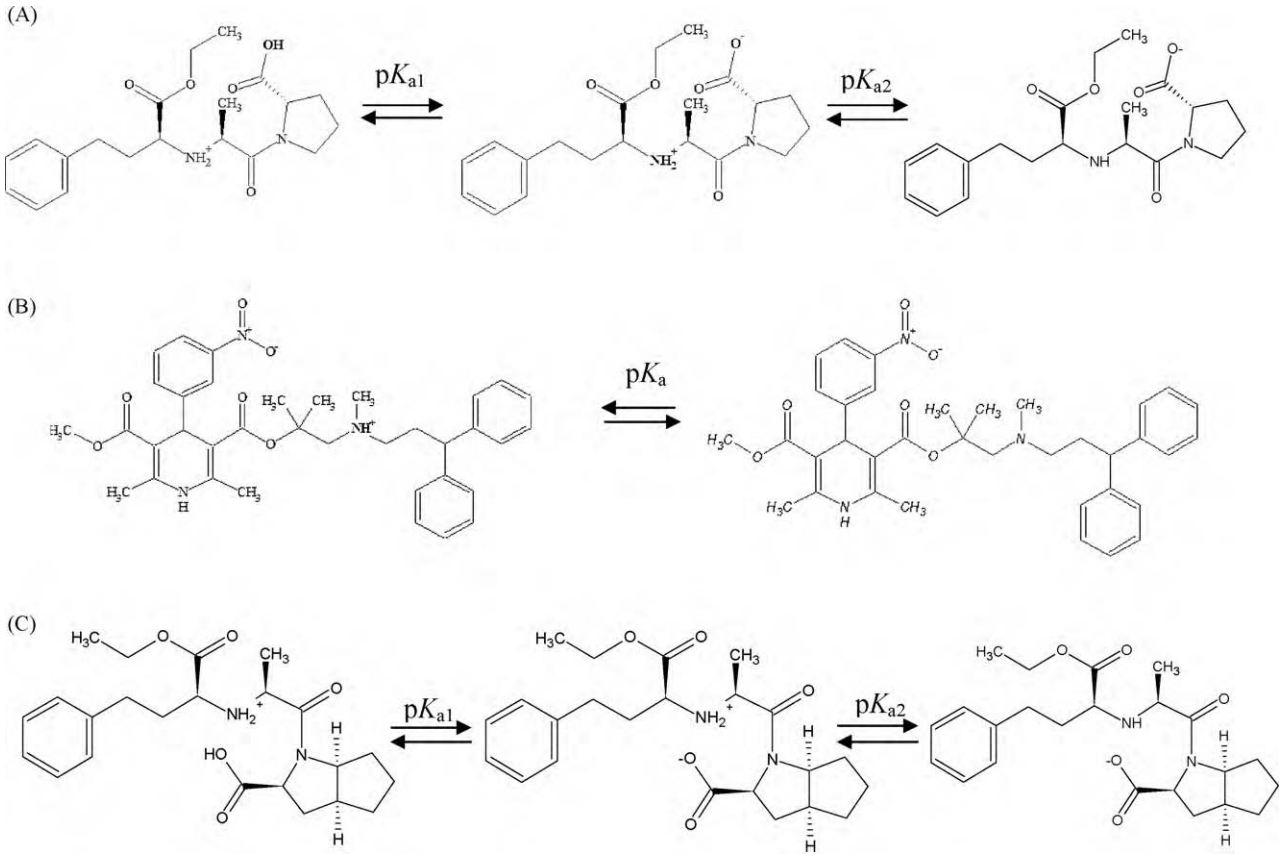

Fig. 2. Dissociation equilibria of studied compounds (A) ENA, (B) LER, and (C) RAM.

Table 1
The retention factors of studied compounds in different MeOH ratios calculated by NLREG program in MeOH–water media.

Compounds	50% (v/v) MeOH			55% (v/v) MeOH			60% (v/v) MeOH			65% (v/v) MeOH		
	k_{-1}	k_0	k_1	k_{-1}	k_0	k_1	k_{-1}	k_0	k_1	k_{-1}	k_0	k_1
ENA	0.041 (0.046)	0.832 (0.030)	1.352 (0.009)	0.215 (0.027)	0.551 (0.026)	0.820 (0.007)	0.119 (0.021)	0.394 (0.013)	0.520 (0.002)	0.038 (0.006)	0.208 (0.004)	0.367 (0.001)
LER	–	–	–	–	–	–	–	–	–	–	1.661 (0.121)	31.860 (0.307)
RAM	0.566 (0.075)	2.217 (0.081)	3.527 (0.022)	0.672 (0.027)	1.759 (0.023)	2.032 (0.009)	0.510 (0.026)	0.826 (0.021)	1.320 (0.016)	0.264 (0.007)	0.641 (0.014)	0.815 (0.034)

The values in parentheses are standard deviations.

2.3. Chromatographic procedure

Throughout this study, the mobile phases assayed were MeOH–water at 50, 55, 60 and 65% v/v, containing 15 mM phosphoric acid. The pH of the mobile phase was adjusted between 2.2 and 9.0 by the addition of sodium hydroxide. The flow rate was maintained at 1.2 mL/min and injected volume was 20 μL.

The column was pre-conditioned during at least 1 h at low flow rate (0.5 mL min^{-1}) with mobile phase at the corresponding pH before the first injection. For each compound, the retention time values, t_R, were determined from three separate injections for each mobile phase composition and pH considered.

The chromatographic retention of ionizable compounds is strongly dependent on the pH of the mobile phase. Thus an accurate measurement and control of mobile phase pH is required, in many instances, for efficient separations of ionizable compounds by LC [28].

Several procedures can be used to measure to pH of the mobile phase. The most common procedure is to measure the pH of the aqueous buffer before mixing it with the organic modifier, ${}^{w}_{w}$pH. A more rigorous procedure, recommended by the IUPAC, is to measure the pH of the mobile phase after mixing the aqueous buffer and the organic modifier. In this instance, the electrode system used to measure pH can be calibrated either with aqueous buffers, ${}^{s}_{w}$pH, or with buffers prepared in the same solvent composition used as mobile phase, ${}^{s}_{s}$pH. This requires knowledge of the pH value of reference buffers prepared in different aqueous–organic solvent mixtures [29].

As pH values have been previously determined in MeOH–water mixtures for the primary standard series of substances proposed by NIST, in accordance with the IUPAC rules, ${}^{s}_{s}$pH values in MeOH–water mixtures can be measured. In this study potassium hydrogen phthalate solutions (0.05 mol kg^{-1}) dissolved in the appropriate MeOH–water medium were used as primary standard buffer [30]. In general, each methanol contents for the chromatographic retention were studied from acid to basic pH. pH measurements were performed in triplicate to ensure stability and reproducibility of the mobile phase system.

In the study of dissociation equilibria by reversed-phase liquid chromatography, the compounds under study (ENA and RAM) can be considered to be amphoteric compounds throughout the working pH range. The overall dissociation process can be shown as:

$$H_2B^+ \overset{K_{a1}}{\rightleftarrows} HB + H^+ \overset{K_{a2}}{\rightleftarrows} B^- + 2H^+ \quad (1)$$

The molecular form HB ionizes in a cationic form, H_2B^+, as the pH decreases, and ionizes in an anionic form, B^-, as the pH increases. Fig. 2 shows the two-step dissociation pathway of compounds. K_{a1} and K_{a2} are the dissociation constants of the carboxylic acid and amino groups, respectively.

The expression of the observed retention factors ($k = t_R - t_o/t_o$, where t_R is the analyte retention time and t_o is the void volume) can be given by

$$k_{obs} = \frac{k_0 + k_{-1}(K_{a1}/[H^+]) + k_1([H^+]/K_{a2})}{1 + (K_{a1}/[H^+]) + ([H^+]/K_{a2})} \quad (2)$$

where k_0, k_{-1} and k_1 are the retention factors of the neutral, the anionic, and the cationic forms of the ampholyte and K_{a1} and K_{a2} are the corresponding acid dissociation constants, respectively.

As can be seen from Fig. 2, LER is a weak monoprotic base and the ionization process can be written as:

$$BH^+ \overset{K_a}{\rightleftarrows} B + H^+ \quad (3)$$

The observed retention factors can be described as a function of retention factors of the neural and ionized species.

Table 2
The pK_a values of studied compounds predicted by ACD Lab and obtained by chromatographic method in MeOH–water media at 40 °C.

Compounds	Literature values	ACD Lab	NLREG			
			50% (v/v) MeOH	55% (v/v) MeOH	60% (v/v) MeOH	65% (v/v) MeOH
ENA	2.85 ± 0.14[a]; 3.00[b]	3.17 ± 0.20	2.81 ± 0.06	2.99 ± 0.06	3.07 ± 0.09	3.15 ± 0.06
	5.37 ± 0.02[a]; 5.40[b]	5.42 ± 0.39	4.49 ± 0.09	4.34 ± 0.10	4.15 ± 0.09	4.01 ± 0.03
LER	–	8.41 ± 0.50	–	–	–	6.32 ± 0.02
RAM	3.31[c]	3.17 ± 0.20	2.93 ± 0.05	3.17 ± 0.07	3.28 ± 0.09	3.34 ± 0.04
	5.75[c]	5.43 ± 0.39	4.34 ± 0.08	4.31 ± 0.08	4.27 ± 0.08	4.25 ± 0.08

[a] Values are obtained from Ref. [25].
[b] Values are obtained from Ref. [26].
[c] Values are obtained from Ref. [27].

The molar activity coefficients, γ, were calculated using the classical Debye–Hückel expression, in which the A and a_o B values for each methanol contents were taken from Ref. [31]. The ionic strength, I, of the mobile phase used can be calculated from charge and mass balances at each mobile phase composition, the analytical concentration of the acid in the mobile phase and the pH values and activity coefficients, involving the use of an iterative calculation [32].

The pK_a values of studied compounds were determined from k/pH data pairs by means of the non-linear regression program NLREG [33]. This is a general non-linear least-squares regression program in which set of initial parameters are iteratively refined until a minimum of an objective function is attained. In our case, these parameters correspond to the thermodynamic pK_a values and the retention factors of the species; the objective function, U_m, is defined as the sum of the squared differences between the experimental and predicted values of the retention factors obtained in each mobile phase composition and pH.

$$U_m = \sum_{j=1}^{n_s} (k_{i,exp} - k_{i,calc})^2 \quad (4)$$

where n_s indicates the number of solutions, $k_{i,exp}$ the experimental value of the retention factor for solution, i, and $k_{i,calc}$ the calculated value. The predicted retention factors are calculated from the dissociation constants and individual retention factors, the measured pH of mobile phases, and calculated activity coefficients from the ionic strength. Initial estimates for pK_{a1} and pK_{a2} can be obtained from the literature values. The retention factors of the neutral, anionic, the cationic forms of studied compounds in different MeOH ratios calculated by NLREG program in MeOH–water media are given in Table 1. As can be deduced from Table 1, the experimental and obtained values by NLREG are in good agreement.

2.4. Preparation of standard solutions

Stock solutions (100 μg mL^{-1}) of LER, ENA and RAM (IS) were prepared in the methanol. All solutions were protected from light and were used within 24 h to avoid decomposition. The concentration of LER and ENA were varied in the range of 0.5–20 μg mL^{-1} and the concentration of IS was maintained at a constant level of 1.0 μg mL^{-1}. The calibration curves for LC analysis were constructed by plotting the ratio of the peak area of the drug to that of internal standard against the drug concentration.

The standard solutions were prepared individually by dilution of the stock solutions with methanol for the ratio derivative spectrophotometric (DD_1) experiments to reach concentration range of 1–20 μg mL^{-1} for ENA and 1–16 μg mL^{-1} for LER. According to the theory of the ratio-spectra derivative method [22–24], the stored spectra of the standard solutions of ENA were divided, wavelength-by-wavelength, by a standard spectrum of LER (10 μg mL^{-1} in methanol). Then, the 1st derivatives of the above ratio-spectra were recorded and the values of the derivatives were measured ($\Delta\lambda$ = 4 nm) at suitably selected wavelengths in the range of 200–410 nm and plotted against the corresponding concentration to obtain the calibration graph. The amplitudes at 219.7 nm were measured and found to be linear to the concentration of ENA.

For LER, the stored UV absorption spectra of standard solutions of LER were divided wavelength-by-wavelength, by a standard spectrum of ENA (10 μg mL^{-1} in methanol). The 1st derivative was calculated for the obtained spectra with $\Delta\lambda$ = 4 nm. The amplitudes at 233.0 nm were measured and found to be linear to the concentration of LER.

2.5. Analysis of tablets

For both methods, 10 tablets labeled to contain 10.0 mg of ENA and 10.0 mg of LER and excipients were weighed and finely powdered. An accurate weight of the powder equivalent to one tablet content was accurately weighed, transferred into a 100 mL calibrated flask, diluted with methanol, stirred for about 10 min and then completed to volume with the same solution. This solution was filtered and the filtrate was collected in a clean flask. After filtration, appropriate solutions were prepared by taking suitable aliquots of clear filtrate (for LC study, adding of the constant amount of IS) solution and diluting them with mobile phase and MeOH for LC and spectrophotometric methods, respectively, in order to obtain a final solution. The contents amount of ENA and LER were calculated from the corresponding regression equations.

2.6. Recovery studies from tablets and laboratory-made mixtures

In order to demonstrate the applicability of the method, the recovery tests were also carried out by analyzing synthetic mixtures of the LER and ENA. After five repeated experiments, the recoveries from these synthetic mixtures were calculated for each compound.

To verify the accuracy of the method, recovery experiments were performed by adding a known amount of pure drug to pre-analyzed tablets. Known amounts of the pure drug (and at a constant level of IS for LC) were added to LER and ENA tablet formulation and the mixtures were analyzed. The percent recovery was calculated by comparing the concentration obtained from spiked samples with the actual added concentration. Thus, the effect of common excipients in tablet formulation on chromatograms (e.g., tailing, broadening) and spectrums was investigated. Recovery experiments from tablets also showed the reliability and suitability of the method.

3. Results and discussion

3.1. Determination of dissociation constants (pK_a)

Before method development is taken up, it is generally important to know various physico-chemical parameters like pK_a,

solubility, wavelength maximum etc. of drug active compounds in question. The knowledge of pK_a is more important than other parameters because of the absorption, evaluation and bioavailability of drugs in the body. Also, it is important as the most of the pH-related changes in retention occur at pH values within p$K_a \pm 1.5$. The ionization value helps in selecting the pH of the buffer to be used in the mobile phase [15].

Most of the ACE inhibitors contain both proton acceptor and proton donor groups, which may be ionized and/or protonated. Dissociation equilibria of ENA, LER and RAM are shown in Fig. 2. ENA and RAM are characterized by the presence of an ionizable carboxylic group. pK_a values of this carboxylic acid (proline) moiety vary in the range of 3.0–4.5. ENA and RAM also contain a basic secondary amine functional group, which may be protonated at suitable pH.

In this study, the retention factors were determined for each mobile phase composition and pH studied. The examples of dependences of the retention factors on the pH value in the mobile phase (65% v/v) are given in Fig. 3.

Typical sigmoidal curve was obtained for LER in Fig. 3B, showing the dependence of the analyte retention factors upon the pH of the mobile phase. However, Fig. 3A and C correspond to ampholytic compounds. In general, the retention of the investigated analytes increased with increasing pH values.

The pK_a values obtained from chromatographic data in several MeOH–water mixtures are given in Table 2. The Table 2 gives the pK_a values reported in the literature, together with those predicted by the program ACD/pK_a DB [34]. ACD/pK_a DB is a software program that calculates accurate acid–base ionization constants under 25 °C and zero ionic strength in aqueous solutions for almost any organic structure. This program uses fragment methods to build a large number of equations with experimental or calculated electronic constants to predict aqueous pK_a values.

As can be seen in Table 2, the pK_a values for LER in 50, 55, and 60% (v/v) MeOH media could not be calculated from retention factors due to the insufficient retention times. It is known that one of the most important factors in determining pK_a is the reaction medium. The pK_{a1} values of ENA and RAM obtained in MeOH–water binary mixtures increase and pK_{a2} values decrease with percentage of MeOH. These variations could be explained by the fact that there is preferential solvation in these media that is related to the structural features of these binary mixtures. It has been found that especially pK_{a2} values of a given compounds show a linear relationship with the mole fraction of methanol. The intercept, slope and correlation coefficients for ENA 5.614, −3.722, 0.991 and RAM 4.554, −0.713, 0.992 are given, respectively. These results indicate that, in general, the assumption of a linear relationship between pK_{a2} values of studied compounds against the mole fraction of methanol is a good approximation in this interval.

3.2. Simultaneous assay of ENA and LER in their combined dosage forms

The LC method that we report provides a simple procedure to simultaneously determine the concentrations of ENA, LER and IS in drug formulations by DAD detection at 215, 240 and 210 nm, respectively. To develop an efficient and reproducible method, different mobile phase compositions and ratios (50, 53, 55, 60 and 65% v/v) were employed. Four different types of columns such as Synergy Max-RP (5 μm, 150 mm × 4.6 mm ID), Symmetry Shield C-8 (5 μm, 150 mm × 3.9 mm ID), YMC Pack ODS-AM (5 μm, 150 mm × 4.6 mm) and X Terra C-18 (5 μm, 250 mm × 4.6 mm ID) were tested in order to find the best resolution and the peak shape of the studied compounds. X Terra C-18 has been selected and used successfully as a stationary phase for the simultaneous determination of ENA, LER and RAM. This column has an extended pH stability,

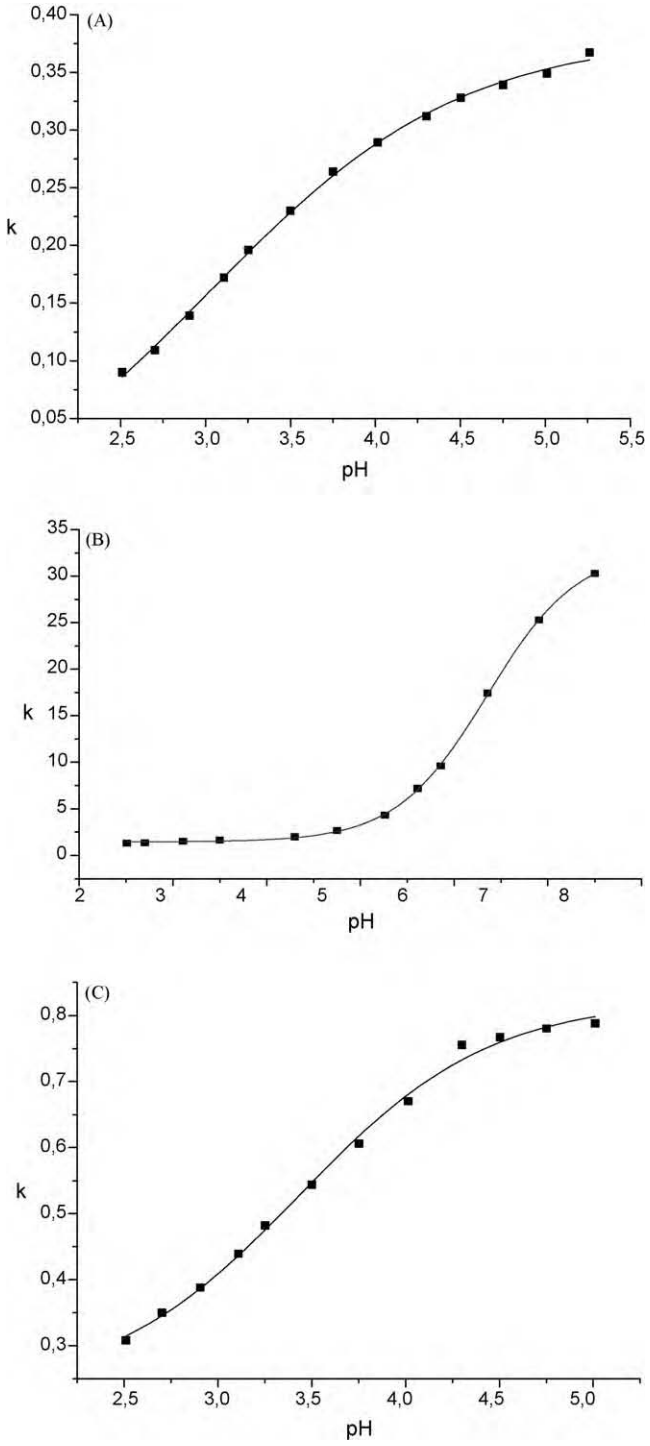

Fig. 3. Plot of chromatographic retention factor, *k*, vs. the pH of mobile phase with 65% (v/v) of acetonitrile. (A) ENA, (B) LER, and (C) RAM.

to be thermally more stable, and to be more efficient than classical silica-based packing. In contrast with classical silica-based packing, this stationary phase is based on silica gel containing methyl groups to reduce the number of silanol groups on the silica gel surface. The organo-silane substituent contains a polar carbamate group, and it is claimed that this stationary phase is more stable both chemically and thermally.

The influence of pH on the mobile phase and column temperature were examined in order to optimize the chromatographic conditions for RP-LC determination of ENA, LER and IS. Preliminary

Table 3
Statistical evaluation of the calibration data of ENA and LER by RP–LC and first derivative of the ratio spectrophotometric methods.

Compounds	LC–UV		First derivative of the ratio spectrophotometry	
	Enalapril	Lercanidipine	Enalapril	Lercanidipine
Retention time (min)/wavelength (nm)	2.76 min	11.15 min	219.7 nm	233 nm
Linearity range ($\mu g\,mL^{-1}$)	0.50–20.00	0.50–20.00	1.00–20.00	1.00–16.00
Slope	0.5310	0.9170	0.0061	0.0440
Intercept	−0.0475	−0.3064	0.0022	−0.0060
Correlation coefficient	0.999	0.999	0.999	0.999
SE of slope	6.00×10^{-3}	1.00×10^{-1}	6.21×10^{-5}	1.87×10^{-4}
SE of intercept	6.60×10^{-2}	1.00×10^{-3}	7.12×10^{-4}	1.71×10^{-3}
Limit of detection	0.124	0.058	0.085	0.062
Limit of quantification	0.372	0.175	0.259	0.190
Within-day precision (RSD%)	0.168	0.118	0.014	0.002
Between-day precision (RSD%)	0.196	0.311	0.019	0.003

Table 4
Determination of ENA and LER in laboratory-made mixtures.

LC–UV						First derivative of the ratio spectrophotometry					
Compounds ($\mu g\,mL^{-1}$)		Found		Recovery %		Compounds ($\mu g\,mL^{-1}$)		Found		Recovery %	
ENA	LER	ENA	LER	ENA	LER	ENA	LER	ENA	LER	ENA	LER
1	10	1.029	9.968	102.90	99.68	8	10	8.00	10.07	100.00	100.70
5	10	5.000	9.957	100.00	99.57	10	10	10.22	10.03	102.20	100.30
10	10	10.014	10.015	100.14	100.15	16	10	16.16	10.09	101.00	100.90
20	10	19.994	10.002	99.97	100.02	20	10	19.96	10.13	99.80	101.30
Mean recovery				100.75	99.855					100.75	100.80
RSD%				1.423	0.275					1.100	0.424
Bias%				−0.75	0.14					−0.75	−0.80
10	1	9.999	1.015	99.99	101.5	10	8	10.10	8.16	101.00	102.00
10	5	10.004	4.982	100.04	99.64	10	10	10.06	10.21	100.60	102.10
10	10	10.014	10.015	100.14	100.15	10	16	10.22	16.15	102.20	100.93
10	20	9.994	20.000	99.94	100.00	10	20	10.09	20.19	100.90	100.95
Mean recovery				100.03	100.32					101.23	101.49
RSD%				0.085	0.810					0.732	0.652
Bias%				−0.30	−0.32					−0.23	−1.49

pK_a studies show us the studied compounds are protonated form below pH 3. Four pH values (2.5, 2.7, 3.0 and 3.5) were tested and pH 2.7 was selected as optimum value with best peak asymmetry and retention values. pH of the mobile phase has always been adjusted with 15 mM orthophosphoric acid. The column temperature was set between 25 and 40 °C. 40 °C was selected because shorter analysis time and improved peak shapes. Finally, the mobile phase MeOH–water 55:45 (v/v) with 15 mM H_3PO_4 (at pH 2.7) at a flow rate of 1.2 mL min^{-1} was most suitable carrier for LC analysis.

In LC methods, precision and accuracy can often be enhanced by the use of an appropriate internal standard, which also serves to correct for fluctuations in the detector response. One of the main reasons for using an internal standard is for samples requiring significant pretreatment or preparation. Ideally, an internal standard should display similar physico-chemical properties to the analytes. Generally, sample preparation steps including reaction, filtration, precipitation, extraction may cause unexpected results because of the sample losses. When added prior to sample preparation, a properly chosen internal standard can be used to compensate for these sample losses. The IS is a different compound from the analytes, but one that is well resolved in the separation. Several compounds were tested as possible ISs: quinapril, lisinopril, bisoprolol, atenolol and ramipril. Ramipril (RAM) was chosen as the IS because it showed a shorter retention time with better peak shape and better resolution from the investigated compounds peaks, compared with other potential internal standards. Also, the chemical structure of RAM is similar to ENA (structures differ in only one ring). After

Table 5
Results of the assay and the recovery analysis of ENA and LER in pharmaceutical dosage forms.

Compounds	LC–UV		First derivative of the ratio spectrophotometry	
	Enalapril	Lercanidipine	Enalapril	Lercanidipine
Labeled claim (mg)	10	10	10	10
Amount found[a] (mg)	10.02	10.00	10.03	9.98
RSD (%)	0.201	0.203	1.471	0.513
Bias (%)	−0.24	−0.04	−0.32	0.22
t-Value	$t_{calculated}$: 0.89	$t_{calculated}$: 0.32	$t_{theoretical}$: 2.31	$t_{theoretical}$: 2.31
F-Value	$F_{calculated}$: 0.03	$F_{calculated}$: 0.10	$F_{theoretical}$: 2.60	$F_{theoretical}$: 2.60
Added (mg)	10	10	10	10
Found[a] (mg)	10.02	10.01	10.02	9.99
Recovery (%)	100.24	100.09	100.20	99.86
RSD% of recovery	0.203	0.202	0.724	0.075
Bias (%)	−0.24	−0.09	−0.20	0.14

[a] Obtained from five experiments.

determining the optimum conditions, a satisfactory resolution was obtained in a short analysis time (12 min). For all compounds, sharp and symmetrical well-resolved peaks were obtained.

The USP suggests that system suitability tests be performed prior to analysis [35,36]. The parameters include tailing factor, retention factor, theoretical plate number, retention time, asymmetry factor, selectivity and RSD% of peak height or area for repetitive injections. Typically, at least two of these criteria are required to demonstrate system suitability for the proposed method. Some of the tests were carried out on freshly prepared standard solutions including two compounds and the IS. Tailing factors of 1.08, 1.13, and 1.10 were obtained for ENA, RAM (IS) and LER, respectively. The theoretical plate numbers (N) were 2575 for ENA, 5778 for LER and 3457 for IS. The selectivity factors were 3.19 and 5.34 for ENA and LER, respectively. The chromatographic conditions described ensured adequate retention and resolution for all analytes. The retention times of ENA, IS and LER were 2.76, 3.91 and 11.15 min. The variation in retention time for five replicate injections of all compounds reference solutions gave RSDs of 0.903% for ENA, 0.899% for IS, and 0.172% for LER. The results obtained from the system suitability tests satisfy the USP requirements.

The calibration curves and equations for ENA and LER in the mobile phase and drug samples were calculated by plotting the peak area ratio of compound to IS vs. concentration of compound in the range of 0.50–20,000 $\mu g\,mL^{-1}$ for both compounds in the mobile phase (Table 3). These results showed highly reproducible calibration curves with correlation coefficients of >0.999. The low SE values of the slope and the intercept show the precision of the proposed method. The LOD and LOQ were calculated from the following equations and using the standard deviation (s) of response and the slope (m) of the corresponding calibration curve [37,38]

$$\mathrm{LOD} = 3.3s/m; \quad \mathrm{LOQ} = 10s/m \tag{5}$$

Precision and reproducibility of the method were assessed by performing replicate analysis of standard solutions in the mobile phase. Repeatability and reproducibility were characterized for different concentrations and given by mean recovery and RSD% (Table 4). Based on these results, there was no significant difference for the assay, as tested by within-day (repeatability) and between-day (reproducibility).

In order to demonstrate the accuracy, validity and applicability of the proposed LC method, recovery tests were carried out by analyzing in the synthetic mixtures of ENA and LER, which reproduced different composition ratios (Table 5).

The simultaneous determination of ENA and LER were also realized with the first derivative of the ratio spectrophotometric method (DD_1), which is proposed for the comparison of the proposed RP-LC method. To optimize the simultaneous determination of ENA and LER using DD_1 method, it is necessary to test the influence of the variables: divisor standard concentration, $\Delta\lambda$ and smoothing function. All these variables were studied in detail. The influence of $\Delta\lambda$ for obtaining the first derivative of the ratio-spectra was tested and $\Delta\lambda = 4$ nm was selected as optimum value. The calibration graphs of each drug at selected wavelengths were achieved by plotting the values of the first derivative of the ratio-spectra ENA/LER and LER/ENA, with variable concentrations of ENA and LER. The proposed method is applicable over the ranges 1–20 $\mu g\,mL^{-1}$ for ENA and 1–16 $\mu g\,mL^{-1}$ for LER.

As shown in Fig. 5A, the zero-order spectra of pure drugs were found to be overlapping, making simultaneous determination difficult. It can be seen that the absorption spectra of ENA and LER are overlapped and as a result, the determination of the two drugs was not possible for reliable direct absorbance measurements. In Fig. 5B, the series of the ratio-spectra of ENA/LER and LER/ENA was shown. In Fig. 5C, the corresponding first derivative of the ratio-spectra of Fig. 5B was shown. For calibration graph, the wavelengths were

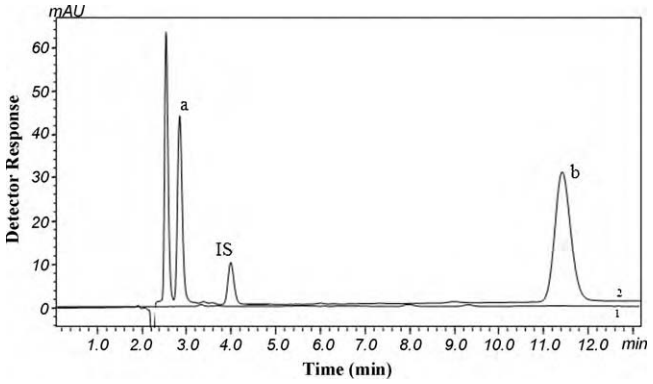

Fig. 4. Chromatogram obtained from tablet dosage forms (ZANIPRESS®). (1) Mobile phase and (2) mobile phase containing 10.0 $\mu g\,mL^{-1}$ ENA (a), 10.0 $\mu g\,mL^{-1}$ LER (b), and 2.0 $\mu g\,mL^{-1}$ RAM (IS). The monitoring wavelength was 210 nm.

selected which exhibited the best linear response to the analyte concentration, i.e. in the first derivative mode 219.7 nm for ENA and 233.0 nm for LER. The calibration graphs of each drug at both wavelengths were achieved by plotting the values of the first derivative of the ratio-spectra ENA/LER and LER/ENA, with variable concentrations of ENA and LER. The characteristic parameters and necessary statistical data of the regression equations, LOD and LOQ values, repeatability and reproducibility data are compiled in Table 3. The LOD and LOQ values were calculated as described in the previous section. Within-day and between-day variability were characterized by R.S.D. (%) and by the difference between theoretical and measured concentrations. There was no significant difference for the assay, which was tested within-day and between-day (Table 3). In order to demonstrate the validity and applicability of the proposed DD_1 method, recovery studies were performed by analyzing in synthetic mixtures of ENA and LER, which reproduced different composition ratios (Table 4).

When working on synthetic mixture, results encourage the use of the both proposed methods described for the simultaneous assay of ENA and LER in commercial tablet dosage forms. Both methods can be used for the simultaneous determination of ENA and LER in the presence of each other and without prior separation of the excipients. Each tablet contains the active ingredients 10 mg of ENA as enalapril maleate, 10 mg LER as lercanidipine hydrochloride and the inactive ingredients. Removal of the excipients before analysis was found to be unnecessary.

Fig. 4 shows a typical chromatogram obtained follow by analysis of ENA and LER in tablets ZANIPRESS®) with IS. As shown in Fig. 4, the substances were eluted, forming well shaped, symmetrical single peaks, well separated from the solvent front. No interfering peaks were obtained in the chromatogram due to tablet excipients. The utility of all of the proposed methods was verified by means of replicate estimations of pharmaceutical preparations and results obtained were evaluated statistically (Table 5). Results obtained from proposed methods of the analysis of both drugs in tablets indicate that the proposed techniques can be used for simultaneous quantitation and routine quality control analysis of this binary mixture in pharmaceuticals. A comparison with an official reference determination method has not yet been possible in any pharmacopoeia and literature, since no other procedure for the simultaneous quantitation of ENA and LER from pharmaceutical formulations has been reported so far.

Proposed LC results were compared with the ratio derivative spectrophotometric results. According to the Student's t-test and variance ratio F-test, the calculated t- and F-values were less than the theoretical values in either test at the 95% confidence level. This indicates that there is no significant difference between the

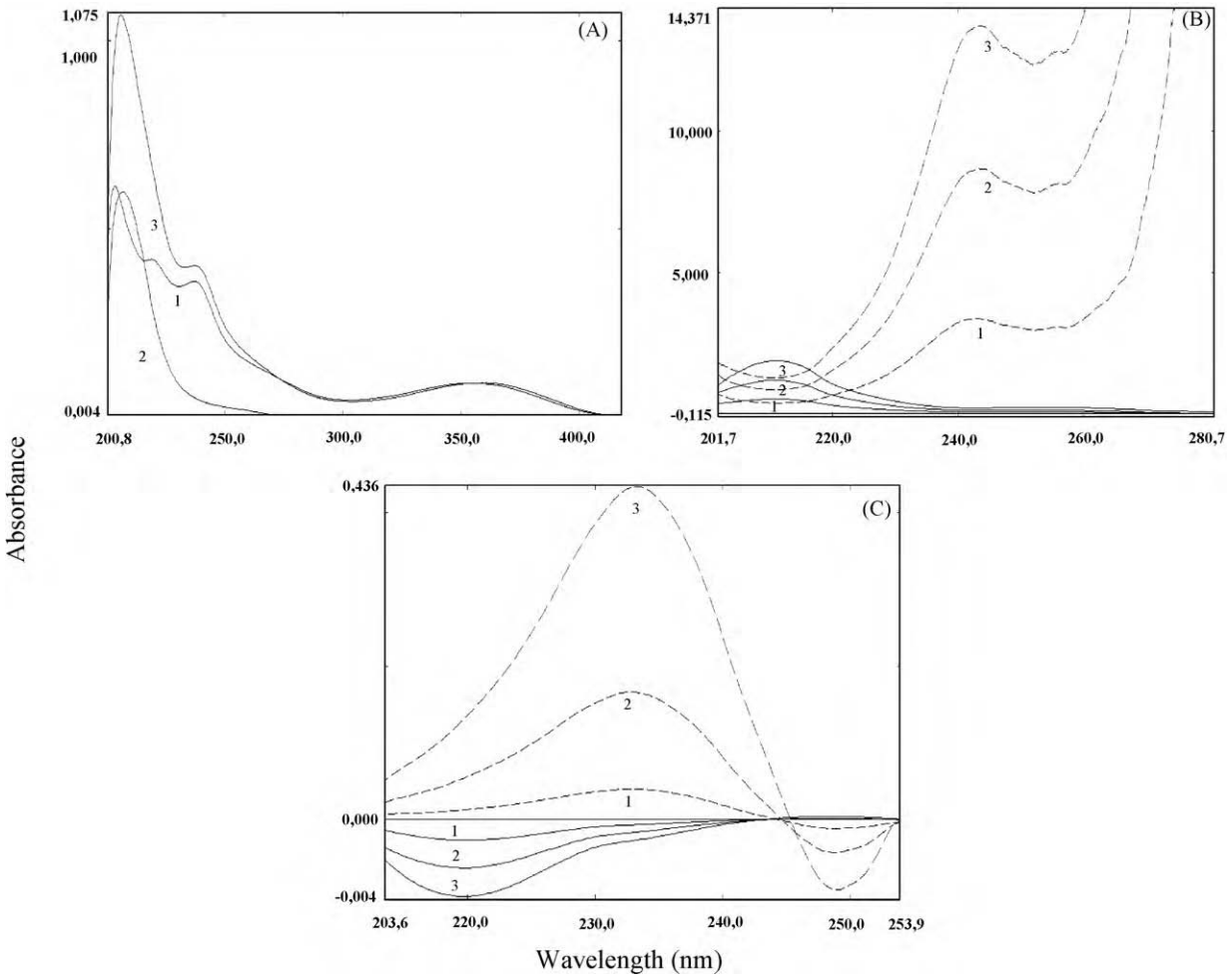

Fig. 5. (A) Zero order absorption spectra of (1) 10 μg mL^{-1} LER; (2) 10 μg mL^{-1} ENA; (3) a mixture of LER and ENA in methanol. (B) Ratio-spectra. (C) First derivative of the ratio-spectra of LER (1) 1 μg mL^{-1}; (2) 4 μg mL^{-1}; (3) 10 μg mL^{-1} (dashed lines) divisor ENA (10.0 μg mL^{-1}) and ENA (1) 4 μg mL^{-1}; (2) 10 μg mL^{-1}; (3) 16 μg mL^{-1} (continuous lines) divisor LER, (10.0 μg mL^{-1}) in methanol.

performance all of the proposed methods as regards to mean values and standard deviations (Table 5).

Recovery studies were realized from the tablets for accuracy and precision of the proposed techniques. The recovery of the procedure was carried out by spiking the already analyzed samples of tablets with the known concentrations of standard solutions of ENA and LER. The results of the recovery analysis for all techniques are shown in Table 5. It is concluded that the proposed methods are sufficiently accurate and precise in order to be applied to pharmaceutical dosage forms. High percentage recovery data shows that all of the proposed methods are free from the interferences of the excipients used in the formulations.

4. Conclusions

The pH of the mobile phase can be a useful tool when attempting to optimize selectivity during method development, especially for ionogenic compounds. Dependence of retention of drug active compounds on pH of the mobile phase was determined using LC methodology and the results were applied for the determination of pK_a values. This work presents the first study dealing with the determination of pK_a values of ENA, LER and RAM by chromatographic method in MeOH–water binary mixtures. The lipophilicity, solubility and permeability of drug compounds are pK_a dependent. The pK_a value can affect drug receptor binding. Therefore, the dissociation constants for studied compounds are useful for facilitating occurrence, effects, and control of ACE inhibitors in scientific studies for investigators.

Based on LC with DAD detection, the presented method for the simultaneous analysis of ENA and LER in combined dosage forms has given good results in terms of sensitivity and precision. Using described chromatographic conditions ENA, LER and RAM (IS) were well separated. The proposed method gives good resolution between selected compounds and IS within a short analysis time (<12 min). There was no significant difference for the assay tested within-day and between-day. The method developed would serve as a versatile analytical tool suitable for the simultaneous analysis of these drugs and would be of interest for quality control and clinical monitoring laboratories. High percentage recovery shows that the methods are free from the interferences of the endogenous substances in tablet forms.

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