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Simultaneous determination of enalapril, felodipine and their degradation products in the dosage formulation by reversed-phase high-performance liquid chromatography using a Spherisorb C_8 column

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Abstract

A reversed-phase high-performance liquid chromatographic (RP-HPLC) method was developed and validated for the simultaneous determination of enalapril and its two degradation products, enalaprilat (diacid, hydrolytic degradation product of enalapril) and enalapril-DKP (cyclization of enalapril); and felodipine and its degradation product, H152/37 (oxidation of felodipine) in the combined enalapril/felodipine (5 mg/5 mg) formulation. Using a Spherisorb C_8 column with a CH_3CN–0.001 *M* KH_2PO_4 (pH 2) (35:65, v/v) mobile phase, these compounds were well separated from each other, and also from maleic acid and the excipients in the formulation. The method was demonstrated to be precise, accurate, specific and robust. Optimization of the separation in terms of mobile phase composition is crucial to the method development, which is discussed in detail. It is proposed that under the experimental conditions used, the retention of felodipine, DKP and H152/37 is governed by the reversed-phase partitioning process whereas that of enalapril and diacid is governed by both the partitioning and the cation-exchange process with residual silanols.

1. Introduction

Development of combination drugs has become a routine practice in pharmaceutical industry. An example is the development of the enalapril (1)/felodipine (4) (5 mg enalapril maleate/5 mg felodipine) combination formulation (see Fig. 1 for structures of the compounds). Enalapril is a pro-drug, which is hydrolyzed to enalaprilat (diacid) in vivo. The diacid acts as an angiotensin-converting enzyme inhibitor [1]. Felodipine is a calcium blocker [2]. Both are effective drugs for treating hypertension [1,2]. The antihypertensive effect and tolerance of the combined low doses of enalapril maleate and felodipine (5 mg/5 mg daily) have been evaluated and shown to be improved [3,4].

There are two general approaches in the methods development for combination drugs. One is to develop separate analytical methods for each of the active substances and their degradation products, and the other is to develop a single method for the simultaneous determination of all the active substances and their degradation products. Since the first approach is very time-consuming, the second ap-

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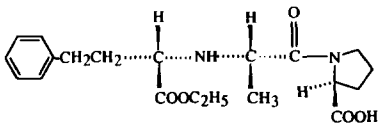

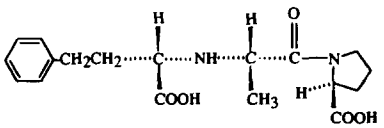

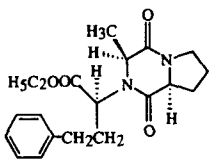

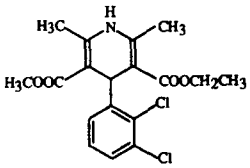

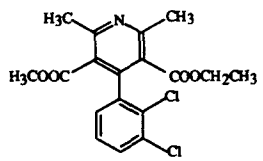

Fig. 1. Structures of the compounds of interest.

proach was attempted in the present study. This is challenging, since, during stability testing of tablets exposed to high temperature and/or high humidity, it was observed that enalapril formed two degradation products, diacid (enalaprilat, 2, hydrolysis degradation product) and enalapril-DKP (3, cyclization degradation product) [5]; and felodipine formed one degradation product, H152/37 (5, photo- or thermal oxidation degradation product) [6]. This approach has been accomplished by an isocratic, reversed-phase high-performance liquid chromatographic (RP-HPLC) method, which uses a Spherisorb C_8 column (5 μm particle size, 250 × 4.6 mm I.D.) with a CH_3CN–0.001 *M* KH_2PO_4 (pH 2) (35:65, v/v) mobile phase. The method can simultaneously determine both active substances and the three degradation products by separating them from each other and from maleic acid and the excipients of the formulation. The method was validated according to the USP validation guidelines, and was demonstrated to be precise, accurate, specific and robust.

The mechanistic aspects of the method have been investigated. The retention of enalapril-DKP, felodipine and H152/37 is governed by the reversed-phase partitioning process, while that of enalapril and diacid is governed by both the reversed-phase mechanism and the cation-exchange mechanism. The latter is associated with the type of the amino acids, the pH-dependent protolytic equilibria, and other secondary equilibria such as solvation. As demonstrated in detail below, the success of the method should be attributed to the selectivity due to the distinct separation mechanisms of these compounds.

2. Experimental

2.1. Chemicals and reagent

Enalapril/felodipine (5 mg/5 mg) combination tablets and placebo tablets were manufactured by Merck Research Laboratories (West Point, PA, USA). The standards of enalapril maleate (1, MK-0421), felodipine (4, MK-0218), and their degradation products, diacid (enalaprilat 2), enalapril-DKP (3) and H152/37 (5) of pharmaceutical grade were manufactured by Merck Research Laboratories (Rahway, NJ, USA). Potassium phosphate monobasic (99.8%, certified A.C.S.) and acetonitrile (Optima) were purchased from Fisher Scientific (Philadelphia, PA, USA). All solvents and reagents were used as received without further purification. Deionized water with at least 18 MOhm purified by a Milli-Q system was used for the mobile phase, and the sample and standard preparations.

2.2. Equipment

The development and validation work was performed on a Hewlett-Packard (HP) 1090 system equipped with a Spectra Physics (SP) 100 variable-wavelength UV detector. The column used is Spherisorb C_8 column (5 μm particle size, 250 × 4.6 mm I.D.) purchased from Phase

Separations. The column temperature is 40°C. The packing material has the following characteristics: pore size, 80 Å; pore volume, 0.5 ml/g; surface area, 220 m^2/g; carbon load, 6%, monomeric; and bonded phase coverage, 2.51 μmole/m^2.

2.3. Mobile phase, standard, and sample preparations

The mobile phase was prepared by first preparing a solution of 0.001 *M* KH_2PO_4, then adjusting its pH to 2 with phosphoric acid, and finally mixing the solution with acetonitrile in a volume ratio of 65:35 (v/v), respectively. Mobile phases of other concentrations were made by the same procedure. The diluent (50% CH_3CN and 50% 0.001 *M* KH_2PO_4 (pH 2)) was also prepared by this procedure. The standard solution was prepared by dissolving an appropriate amount of reference standard in the diluent to yield the desired assay concentration (0.1 mg/ml for enalapril maleate and felodipine (100% standard), and 0.001 mg/ml for the three degradation products diacid, DKP and H152/37 (1% standard). Tablet samples of enalapril/felodipine (5 mg/5 mg) formulation were prepared in an appropriate volume of the diluent by stirring until tablet(s) was completely dissolved. A portion of the resulting solution was centrifuged in a microcentrifuger for 3 min and the clear supernatant was transferred to an HPLC vial for analysis.

2.4. Assay conditions and procedure

The HPLC system (including column) was equilibrated with the mobile phase at a flow-rate of 2.5 ml/min (pressure ca. 3000 psi) by injecting the standard solution until reproducible injections (R.S.D. $<2\%$) were observed prior to the sample analysis. Standard and sample solutions (injection volume, 50 μl) were injected directly. The detection was by UV absorption at 215 nm. Chromatograms were recorded by the Multichrom 2.0 program (Fisons Instruments, Danvers, MA, USA) using a VG computer.

3. Results and discussion

3.1. Optimization of the method

The HPLC conditions were developed on the basis of the existing method for the determination of enalapril maleate drugs, in which a Lichrosorb RP-8 column (10 μm particle size, 300 × 4.6 mm I.D., E.S. Industries or Phenomenex using RP-8 packing material made by E. Merck) was used with a CH_3CN–0.02 *M* potassium phosphate (pH 2.25) (45:55, v/v) mobile phase [5,7]. Under these conditions, enalapril and its degradation products eluted in the first half of the chromatogram, while felodipine and its degradation product eluted in the second half of the chromatogram, which formed a good base for further optimization. However, recently, E. Merck has changed the manufacturing process of the packing material. The Lichrosorb columns with the new "RP-8" material showed chromatographic difficulties in the separation of enalapril and its two degradation products in the combination drug. Thus, several other C_8 columns were evaluated. Among them, Spherisorb C_8 columns were found to give similar and more rugged separation conditions with better column-to-column reproducibility.

As shown in Fig. 2, good resolutions between maleic acid, diacid, enalapril-DKP, enalapril, H152/37, felodipine, and an UV-absorbing excipient are achieved using the conditions described in the Experimental section.

The mobile phase CH_3CN–0.001 *M* KH_2PO_4 (pH 2) (35:65, v/v), characterized by the low concentration and the low pH of the phosphate solution, was obtained from an optimization process based on several considerations.

The low pH is critical to the determination of enalapril and diacid. Enalapril is a dipeptide with a proline peptide bond, which has *trans* and *cis* conformations due to its partial double bond character. The enalapril peak could be distorted or even splitted into two peaks at high pH. At low pH, it gives a sharper, single peak shape because the proline peptide bond is partially protonated, which decreases its partially double bond character, and increases the relaxation rate

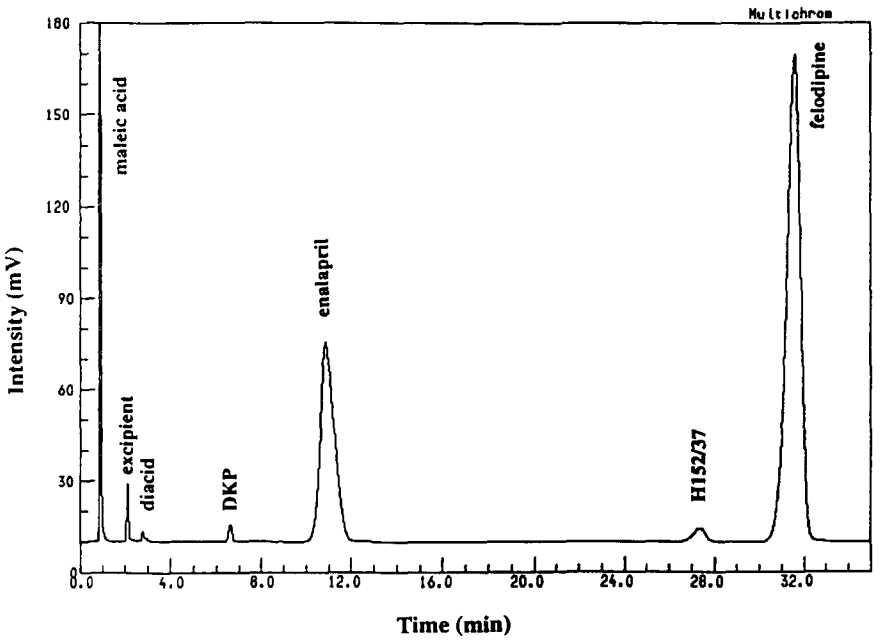

Fig. 2. Typical chromatogram showing the separation of enalapril, felodipine, their degradates and an UV-absorbing excipient by the use of the HPLC conditions listed in the text.

of the *cis* and *trans* isomerization [8]. Similarly, diacid has a much sharper peak shape at lower pH.

In addition to peak shape, the retention of enalapril and diacid is greatly influenced by pH. Since the pK_a values of enalapril are 2.97 (the carboxyl group) and 5.35 (the amine group) at 25°C [5], enalapril possesses a net positive charge at pH 2. Diacid also has a net positive charge at pH 2 because its two carboxyl groups have pK_a values between 2 and 3, and its amine group has a pK_a value between 5 and 6 [5]. As is well known, there are unreacted silanol groups in the bonded C_8 phase, which can have a density as high as 8 μmole/m^2, higher than that of the bond phase of the Spherisorb column (2.51 μmole/m^2). Most of these silanols are acidic with pK_a values between 5 and 7. However, some silanols can have lower pK_a values. At pH 2, although most of the silanol groups are protonated, some silanols could be deprotonated. These deprotonated silanols can interact with the positively charged enalapril and diacid through hydrogen-bonding and cation-exchange interactions [9–11]. When pH is increased from pH 2, the cation-exchange process will be influenced due to the change in the ionization status of the silanols, enalapril and diacid. In turn, the retention of enalapril and diacid will be influenced. Clearly, the pH is a good means to control the retention of enalapril and diacid.

The control of the retention of enalapril by adjusting the pH is important in achieving good resolution between enalapril and H152/37 and between diacid and the UV-absorbing excipient. As shown in Fig. 3a, when the pH increases from 1.8 to 3, the k' of enalapril increases much faster than that of H152/37. At about pH 3, it becomes overlapping with H152/37. Also, as shown in Fig. 3b, when the pH increases from 1.8 to 3, the k' of the excipient remains relatively unchanged; the k' of diacid first increases, which results in better separation between diacid and the excipient, and then decreases, which results in partial overlapping of the two species at pH 3.

Besides pH, the salt concentration is another parameter controlling the retention of enalapril and diacid. The salt concentration was varied with the pH of its solution always adjusted to 2. The low concentration (0.001 *M* KH_2PO_4) was

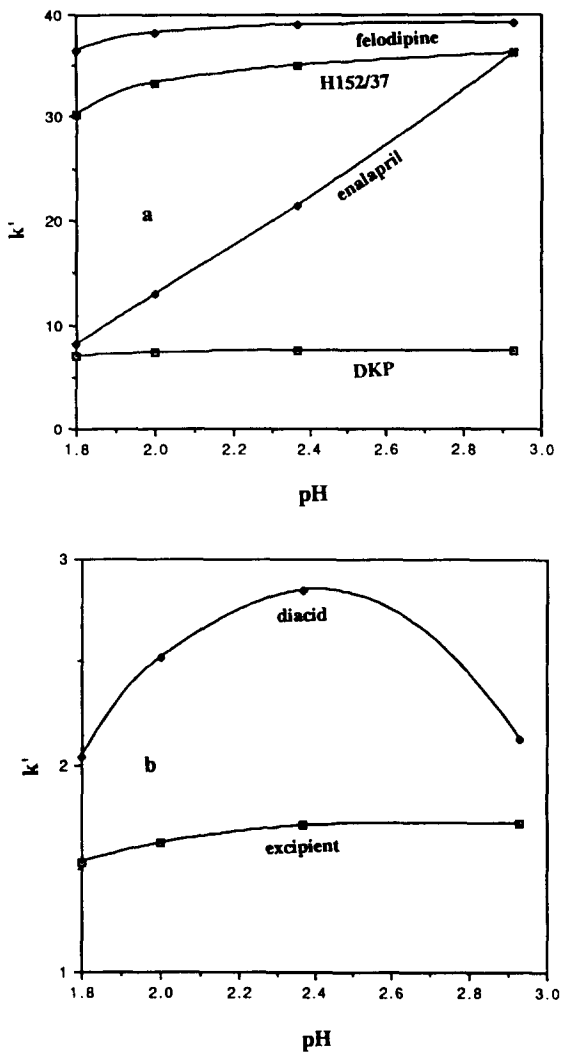

Fig. 3. Plots of k' as a function of pH of phosphate solution in the mobile phase.

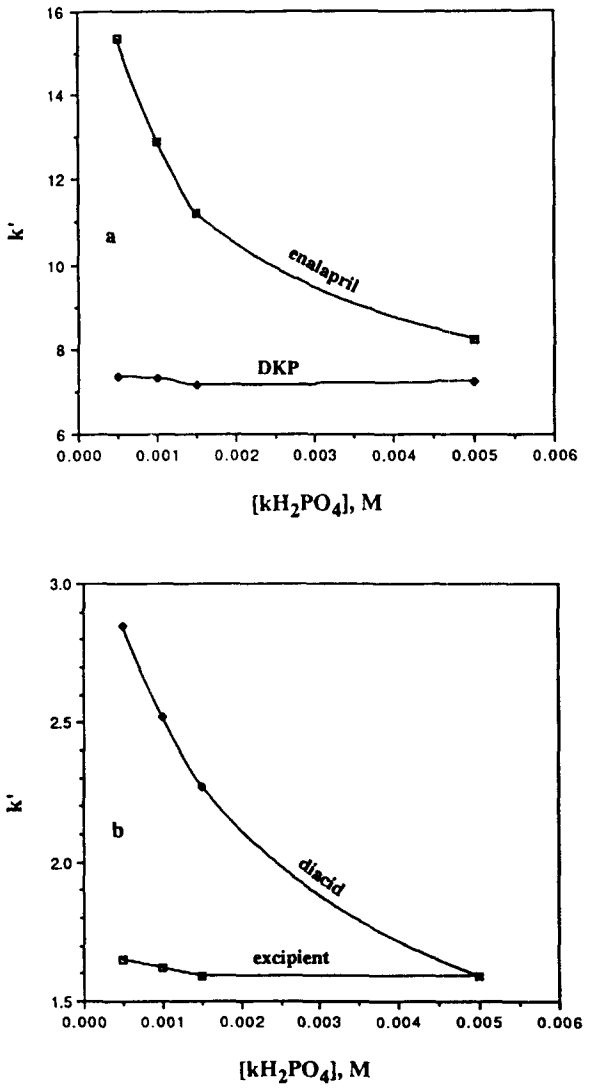

Fig. 4. Plots of k' as a function of $[KH_2PO_4]$ in the mobile phase.

found to be important for the rugged separation of enalapril and DKP. The k' of enalapril decreases while that of DKP remains relatively unchanged as the salt concentration increases (Fig. 4a). This can be attributed to the ionic strength effect of added potassium cations, which can shield the deprotonated silanols. Another explanation is the ion-pair formation between the enalapril cation and phosphate, which increases as the salt concentration increases. Since the ion-pair does not participate in the cation exchange with silanol groups, the k' of enalapril decreases. However, since phosphate is very hydrophilic, and its ion-pairing with an amine cation in aqueous solutions has never been reported, this is very unlikely. Since the k' of enalapril is affected, whereas the k' of DKP is not affected by the salt concentration, enalapril moves closer to DKP at higher salt concentrations, and starts overlapping with DKP at concentrations above 0.005 M KH_2PO_4. When the salt concentration further increases, the

elution order of enalapril and DKP will be reversed. Low salt concentration is also crucial for the separation of diacid from the excipient. It was observed that the k' of diacid decreases as the salt concentration increases (see Fig. 4b), probably due to a similar mechanism as for enalapril. At higher concentrations (0.005 M KH_2PO_4 and above), diacid co-elutes with the excipient.

DKP, felodipine and H152/37 are neutral in the pH range 1.8–3, and thus their retentions are not significantly affected by the salt concentration or pH. The controlling factor for these compounds is the percentage of acetonitrile, which has a significant effect on their k'. As demonstrated in Fig. 5a, the k' of these compounds decreases as the percentage of acetonitrile increases. It can be seen that felodipine and H152/37 have very similar k' values. They co-elute at higher percentages of organic modifier ($>40\%$ acetonitrile). A solvent strength of 35% acetonitrile is used in the mobile phase to separate them. Although their separation is better using a low percentage of organic modifier ($<35\%$ acetonitrile), the total run time will become unfavorably longer. The retention of enalapril and diacid is also affected by the percentage of acetonitrile, but to a much smaller extent (see Fig. 5b).

In summary, by variation of the salt concentration, pH and organic modifier in the mobile phase, the resolution of the compounds of interest is optimized on the Spherisorb column.

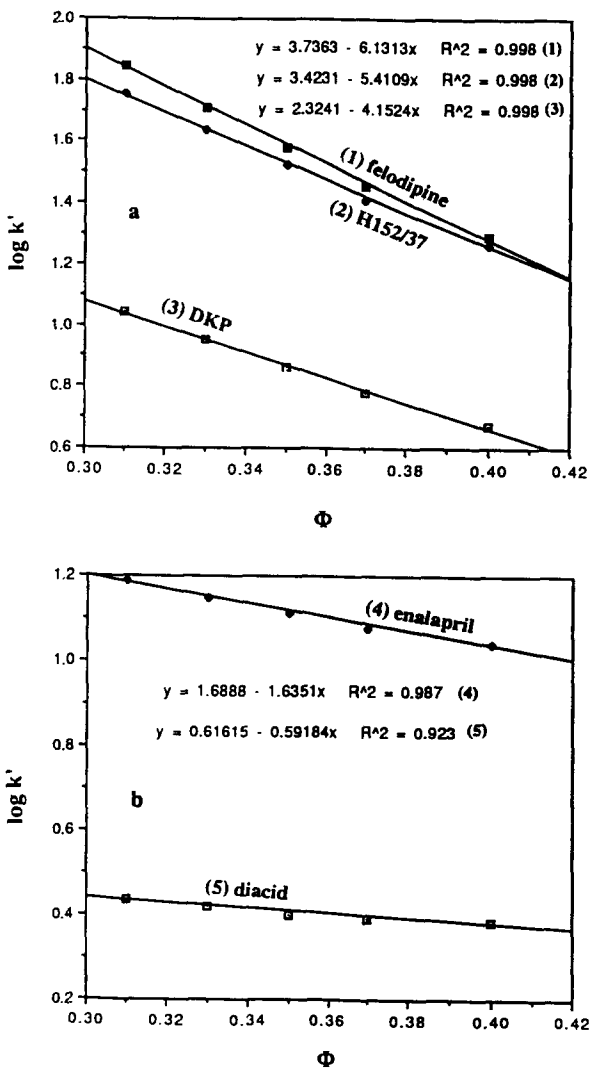

Fig. 5. Plots of $\log k'$ vs. percentage of acetonitrile (Φ) in the mobile phase.

3.2. Validation of the method

The method was validated according to the USP guidelines [12]. The results are all satisfactory.

Accuracy

The accuracy of the method was determined by investigating the recovery of enalapril maleate and felodipine in duplicate at 5 levels ranging from 50% to 150% of the method concentration (0.1 mg/ml) from solid spiked placebo tablets. The results indicate recoveries ranging from 98.6% to 102.1% with a mean of 100.7% (R.S.D. = 1.3%, $n = 10$) for enalapril maleate, and ranging from 99.7% to 102.0% with a mean of 100.2% (R.S.D. = 1.7%, $n = 10$) for felodipine. The accuracy was also measured for the degradation products by investigating the recovery of diacid, DKP and H152/37 in duplicate at 6 levels ranging from 0.1% to 3% from solution spiked placebo tablets. The results indicate acceptable recoveries ranging from 88.1% to 116.9% with a mean of 101.0% (R.S.D. = 8.1%, $n = 12$) for diacid, from 83.6 to 97.5%

with a mean of 93.0% (R.S.D. = 5.3%, $n = 12$) for DKP, and from 83.6 to 97.5% with a mean of 93.0% (R.S.D. = 5.3%, $n = 12$) for H152/37, respectively.

Precision

The measurement precision was determined by performing ten replicate injections of standard solution containing enalapril maleate, felodipine, diacid, DKP, and H152/37 [0.1 mg/ml for enalapril maleate and felodipine (the method concentration), and 1 μg/ml for diacid, DKP and H152/37 (1% of the method concentration)]. The measurement precision of all species was excellent with R.S.D. < 1% ($n = 10$) by either peak area or peak height measurement.

The method precision was determined by the analysis of 10 replicate samples. The R.S.D.s (4.2% for enalapril maleate and 1.3% for felodipine) are satisfactory.

Linearity

The detector responses were found to be linear for enalapril and felodipine both in the absence and in the presence of placebo over a concentration range of 50% to 150% of the method concentration (0.1 mg/ml) by peak area measurement. The correlation coefficients (r^2) were all 1.000. The bias contributed by placebo interference is negligible. The detector responses were also linear for diacid, DKP and H152/37 in the presence of placebo over a concentration range of 0.1% to 3%. The correlation coefficients (r^2) were all greater than 0.998.

Limit of detection and limit of quantitation

The limits of detection (LOD) were determined to be 0.02%, 0.01% and 0.02% for diacid, DKP and H152/37, respectively, based on a signal-to-noise ratio of 3. The limit of quantitation was determined to be 0.1% for the three degradation products. As shown above, recovery of these degradation products from 3.0% down to 0.1% was acceptable and the detector response was linear from 3.0% to the 0.1% level. In addition, measurement precision of the three degradation products at the 0.1% level was also acceptable (R.S.D. < 10%, $n = 10$).

Robustness

The method conditions are robust. Good resolutions (> 1.3) of the seven compounds of interest are obtained on small changes in the mobile phase (ranges: pH, from 1.8 to 2.4; KH_2PO_4 concentration, from 0.0005 to 0.0015 M; and percentage of acetonitrile, from 33% to 37%), and on small changes in temperature (range: 30°C to 50°C). The diacid, enalapril-DKP and H152/37 are the frequently observed degradation products during long term stability testing (25°C/60% rh or 30°C/60% rh for a long period of time), and thus they were used in the method validation. However, under certain accelerated stability and severe stress testing, some other degradation products, such as diastereoisomers of enalapril, diastereoisomers of diacid, and diacid-DKP could be formed. Since the method conditions are robust, they can be easily adjusted to separate these degradation products. Other possible impurities, such as the two symmetrical esters of felodipine formed in the synthesis process, c ı also be easily separated.

3.3. Further elucidation of separation mechanisms

Despite the many studies on the reversed-phase separation theory, there still seems to be considerable uncertainty as to the mechanism of the overall process. It appears that under the conditions used in the present method, the retention of enalapril-DKP, felodipine and H152/37 is governed by the reversed-phase partitioning process, while that of enalapril and diacid is governed by both the reversed-phase partitioning and the cation-exchange mechanism with residual silanols. The latter is associated with the type of the amino acids, the pH-dependent protolytic equilibria, and other secondary equilibria such as solvation, as briefly discussed in the optimization process.

It is observed that the elution of DKP, felodipine, and H152/37 follows quite well the regular reversed-phase behavior with increasing

content of the organic modifier, i.e. k' decreases inversely proportional with increasing volume fraction of acetonitrile (Φ) while the other conditions remained unchanged. A number of useful empirical relationships between k' and the solvent strength (percentage of organic modifier) can be found in the literature, the simplest equation being [13]:

$$\log k' = \log k'_w - S\Phi \quad (2)$$

where S is the slope of the curve, and k'_w is the value of k' in pure water. By plotting $\log k'$ vs. Φ, as shown in Fig. 5, the values of $\log k'_w$ and S are obtained (see Table 1). The fitting for DKP, felodipine and H152/37 in the plots is excellent ($r^2 = 0.998$), while some curvature was observed for the fitting of enalapril ($r^2 = 0.987$) and diacid ($r^2 = 0.923$). The deviation from linearity for these two compounds is attributed to the silanol interactions, i.e. the cations of these compounds interact with accessible silanols present in the packing via a cation-exchange mechanism in addition to the reversed-phase mechanism [14] (see below).

The slope S is an important parameter from both the practical and the theoretical point of view. Practically, S for two adjacent peaks (for example, the felodipine and H152/37 peaks) determines the selectivity as a function of a change in the percentage of organic modifier. Theoretically, S gives insight into the retention process. The parameter S is not merely a solvent–stationary phase parameter. It varies with solute structure. It increases with molecular size, and also with increasing hydrophobicity, which, in the present study, follows the order: felodipine > H152/37 > DKP&z.Gt;enalapril > diacid. The values of k'_w are estimated by the extrapolated intercept, which are also important. The value of k'_w is also dependent on the solute's structure and is suggested to be the best hydrophobic parameter [15]. As shown, the k'_w values follow the same order as the S values, confirming the hydrophobicity order of these compounds under the conditions employed.

Table 1
Log k'_w, S, and r^2 values

Species	log k'_w	S	r^2
Felodipine	3.74	6.13	0.998
H152/37	3.42	5.41	0.998
DKP	2.32	4.15	0.998
Enalapril	1.69	1.64	0.987
Diacid	0.62	0.59	0.923

To further explore the cation-exchange mechanism of the retention of enalapril and diacid, the relationship between the k' values of these compounds and the concentration of $K^+H_2PO_4^-$ is examined by the equation developed by Regnier et al., which is based on a non-mechanistic model to investigate charge–charge and other interactions between solutes and the surfaces of ion-exchange packing materials [16]:

$$\log k' = 2Z \log (1/([X^+Y^-])) + \log K_Z \quad (3)$$

where the parameter Z measures the number of charges interacting between the surface of the ion exchanger (e.g., residual silanol groups) and the solute ion (e.g., the enalapril and diacid cation), and $[X^+Y^-]$ is the concentration of the salt used as a displacing agent (e.g., $K^+H_2PO_4^-$) and $\log K_Z$ is a constant. The equation shows that if obeying the ion-exchange mechanism, the k' of enalapril (or diacid) should be a function of the concentration of $K^+H_2PO_4^-$, which decreases with the increasing of the concentration of $K^+H_2PO_4^-$. This is in good agreement with the experimental observations shown in Fig. 4, where the k' is plotted against $[K^+H_2PO_4^-]$. Using these experimental data and the data for felodipine and H152/37, the values of Z (Z = slope/2) (see Table 2) are derived by plotting $\log k'$ against log of $1/[K^+H_2PO_4^-]$ in Fig. 6. The fittings of the plots for DKP, felodipine and H152/37 are very poor, indicating that their retention does not follow the ion-exchange process. On the other hand, the fittings of the plots for enalapril ($r^2 = 0.998$) and diacid ($r^2 = 0.986$) are fairly good, in consistency with the suggestion that their retention follows cation-exchange mechanism with silanol groups. The

Table 2
Log k'_y, Z, and r^2 values

Species	log k'_y	Z	r^2
Felodipine	1.56	0.0031	0.256
H152/37	1.45	0.012	0.799
DKP	0.839	0.0039	0.364
Enalapril	0.289	0.14	0.998
Diacid	−0.385	0.13	0.986

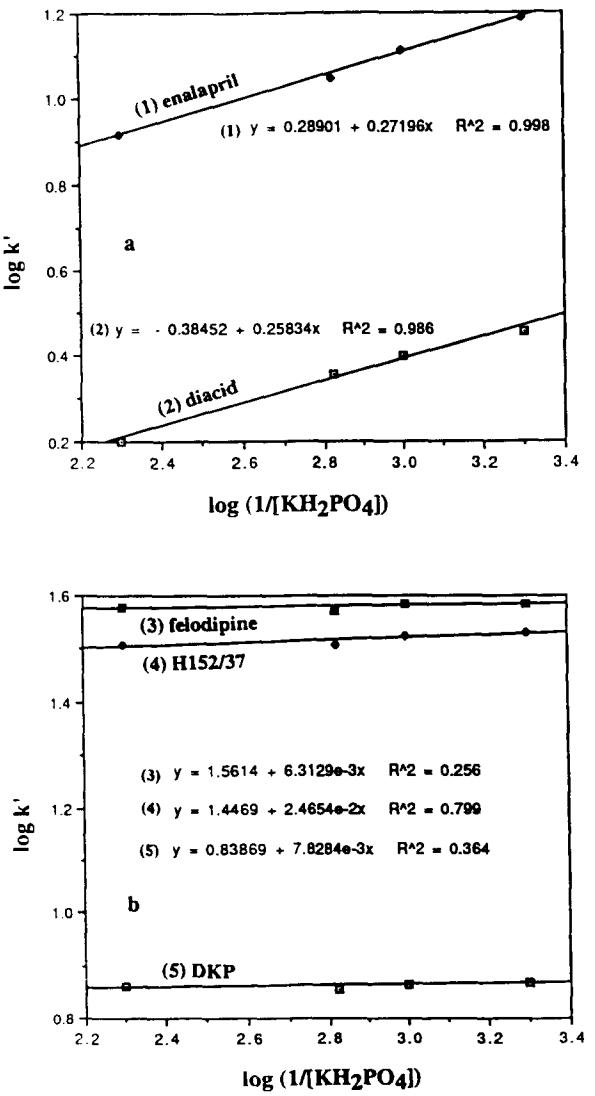

Fig. 6. Plots of log k' vs. log $(1/[KH_2PO_4])$.

exceedingly small Z values for DKP, felodipine and H152/37 also indicate no charge–charge interaction of these species with residual silanol groups. The Z values for enalapril and diacid are derived to be 0.14 and 0.13, respectively, which represents the charge of these species in the interaction with silanol groups according to the Regnier model. The charges deviated from the "net charge", which indicates that only a fraction of the enalapril or diacid surface interacts with the silanol groups like proteins [16], probably due to the charge asymmetry of these species, the counter-ion effect and solvation of ions. From the above discussion, it is clear that the separation mechanisms of enalapril and diacid and that of enalapril-DKP, felodipine and H152/37 are distinct, which induces selectivity, and facilitates the simultaneous determination of these compounds.

4. Conclusions

Simultaneous determination of enalapril, felodipine and their degradation products in the enalapril/felodipine (5 mg/5 mg) formulation has been accomplished by reversed-phase HPLC using a Spherisorb C_8 column. The retention of felodipine, DKP and H152/37 follows the reversed-phase partitioning process whereas that of enalapril and diacid follows both the partitioning and the cation-exchange process with residual silanols. The selectivity induced by the distinct separation mechanisms facilitates the simultaneous determination of these compounds. This study gives an example of a contribution of silanol interaction in the reversed-phase mode.

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