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Elimination of peak splitting in the liquid chromatography of the proline-containing drug enalapril maleate

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ABSTRACT

Enalapril maleate contains alanylproline dipeptide and can be split into two or more peaks when separated by reversed-phase liquid chromatography. The dependence of the shape, retention or width of peaks of enalapril and its impurities on temperature, pH, ionic strength and presence of ion-pairing compounds was studied. The optimum separation conditions involve a state where the rotational rates are much higher than that of the separation process, *i.e.*, at a higher temperature and lower pH. Enalapril is eluted as a single peak even at room temperature at higher concentrations of both cetyltrimethylammonium bromide and sodium dodecyl sulphate in the mobile phase.

INTRODUCTION

Enalapril maleate ((*S*)-1-{N-[1-(ethoxycarbonyl)-3-phenylpropyl]-L-alanyl}-L-proline, (*Z*)-2-butenedioate (1:1) salt, ENM) [1] is a salt of enalapril (EN) and maleic acid (MA). EN is a pro-drug which is hydrolysed to enalaprilate (DIAC), acting as an inhibitor of enzyme angiotensin convertase. It is indicated for the treatment of renivascular hypertension. There are two major potential impurities in the substance besides the parent compound: DIAC, the free acid produced by hydrolysis of EN, and diketopiperazine (DKP), a cyclization product [1]. The structures of these compounds are shown in Fig. 1.

The configuration of a peptide bond can be either *trans* or *cis* [2] (Fig. 2). Nearly all amino acid residues in proteins are in the *trans* configuration, in which steric repulsion is minimized. With proline, the *cis* configuration is likely to occur as the *trans* configuration because the amide nitrogen is part of a ring.

Recently, reversed-phase liquid chromatography of some proline–containing dipeptides [3] and medium-sized peptides [4] has been studied. It was shown that the peak shape is dependent on temperature, flow-rate and pH. Some chromatographic properties of ENM were published by Ip and Brener [1] but more detailed studies are still lacking.

The presence of both a cationic surfactant and an alkane sulphonate in the mobile phase at concentrations close to saturation causes the formation of a liquid ionic multilayer. This was confirmed by the decrease in the void volume of the column [5–7].

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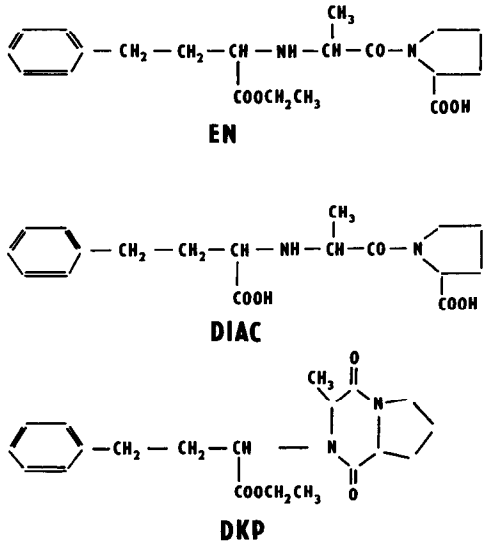

Fig. 1. Structural formulae of EN and its impurities DIAC and DKP.

In this work, the influence of temperature, pH, ionic strength and the presence of cetyltrimethylammonium bromide (CTAB) and sodium dodecyl sulfate (SDS) on the chromatographic behaviour and especially the peak shape and width of ENM and its impurities was studied in order to suppress the negative manifestation of conformational changes of EN and to elute it as a single peak.

EXPERIMENTAL

Enalapril maleate [99% by high-performance liquid chromatography (HPLC)], DIAC (98%) and DKP (95%) were prepared in Research Institute of Pure Chemicals (Lachema, Brno, Czechoslovakia). SDS was obtained from Lachema and acetonitrile (LiChrosolv) and CTAB from Merck (Darmstadt, F.R.G.). All other chemicals were of analytical-reagent grade.

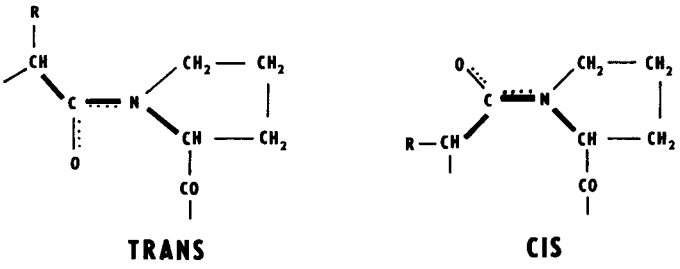

Fig. 2. Configurations around peptides bonds involving proline. The peptide bond interconverts between *trans* and *cis* configurations.

Chromatographic separations were performed on a Hewlett-Packard (Palo Alto, CA, U.S.A.) equipped with a Model 7125 10-μl six-port injection valve (Rheodyne, Cotati, CA, U.S.A.) and a Model 1040 diode-array detector (Hewlett-Packard). The analytical columns used were 250 × 4 mm I.D., packed with Silasorb

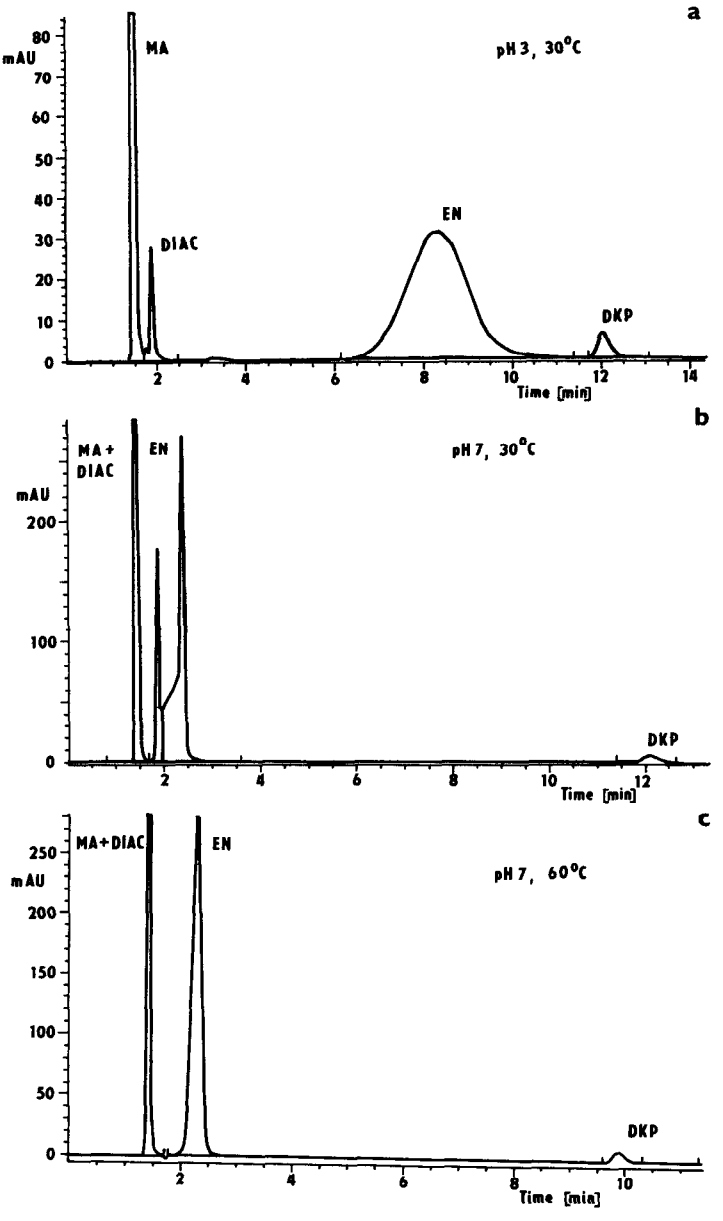

Fig. 3. Effects of pH and temperature on the elution of EN, DIAC and DKP. Mobile phase, 0.05 *M* phosphate buffer (pH 7.0 and 3.0)–acetonitrile (7:3, v/v); column temperature, 30 and 60°C; column packing, Silasorb C_{18}; flow-rate, 1.5 ml/min; volume injected, 10 μl; sample, ENM and DIAC (0.5 mg/ml) and DKP (0.05 mg/ml); detection, UV, 215 nm.

SPH C_{18}, 7.5 μm (Lachema), and 100 × 4.6 mm I.D., packed with Spherisorb ODS, 5 μm (Philips Analytical, Cambridge, U.K.).

RESULTS AND DISCUSSION

Influence of various parameters on chromatographic behaviour

Influence of pH. At lower pH of the mobile phase, the C–N bond loses its partially double-bond character, which restricts free rotation around the peptide bond owing to partial protonation of the imide group on proline [8]. The increase in the relaxation rate of isomerization of proline containing peptides results in a better peak shape in LC [3]. As far as DKP is concerned, its peak shape and retention are not seriously dependent on either pH or temperature because of the cyclic character of the molecule and because of the amino group being sterically blocked and shielded even from interactions with silanol groups. The influence of pH of the mobile phase on peak shape is illustrated in Fig. 3a and b, which show chromatograms obtained at pH 3 and 7. At higher pH, EN is cleaved into two peaks corresponding to the two conformational isomers. At pH 3 the rate of isomerization is higher and ultimately both isomers are eluted as a single broad peak. The dissociation of the EN carboxylic group is also lower and the hydrophobic interactions increase.

The most significant differences in the retention (Fig. 4) of all the compounds occur at lower pH values of the mobile phase. With decreasing pH, EN changes its net charge from negative to positive at pH 4.2 (its isoelectric point) [9]. Residual highly acidic sites of a silica-based reversed-phase stationary phase [10] interact with the positively charged molecule of EN and increase its retention. In such a way the ion-exchange mechanism significantly influences the retention of EN. With regard to the working pH range of the stationary phase and the resolution of MA and DIAC peaks, we chose pH 3 as the optimum.

Influence of temperature. The rate of isomerization rises with increasing temperature as the activation energy of the peptide bond in the molecule containing

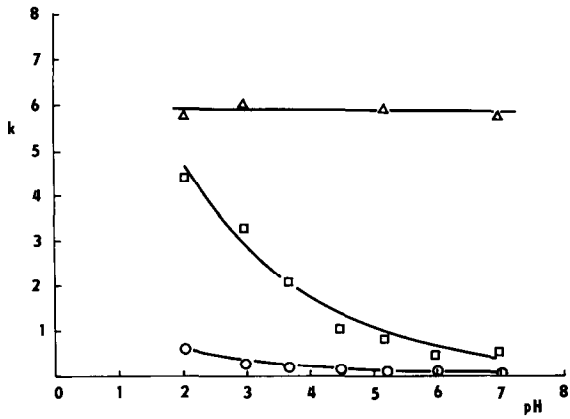

Fig. 4. Dependence of capacity factor (k) on pH for (□) EN, (○) DIAC and (△) DKP. The pH of the phosphonate buffer varied from 2 to 7. The retention time of the first-eluted peak (MA) was used for the calculation of k. Column temperature, 60°C; other conditions as in Fig. 3.

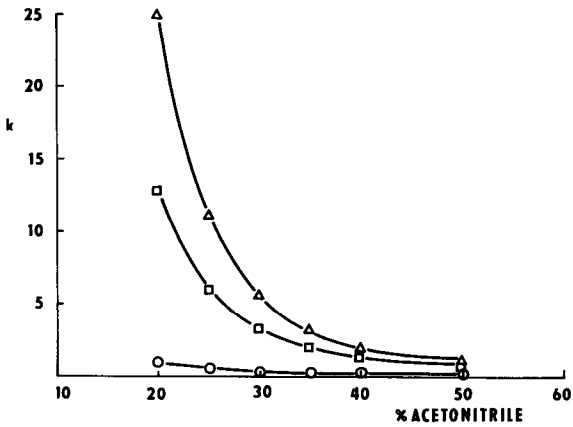

Fig. 5. Dependence of capacity factor (k) of (□) EN, (○) DIAC and (△) DKP on acetonitrile concentration in the mobile phase. 0.05 M phosphate buffer (pH 3) was used for mobile phase preparation. Column temperature, 60°C; other conditions as in Fig. 3.

alanylproline is relatively high, *ca.* 20 kcal/mol [3]. The influence of temperature on the retention of chromatographed compounds is not significant, but the peak shape can be improved (Fig. 3).

Influence of organic solvent concentration. The retentions of solutes increase with decreasing organic solvent concentration; see Fig. 5, where the dependence of capacity factor (k) on acetonitrile concentration in the mobile phase is shown.

Influence of phosphate buffer concentration. The concentration of buffer in the mobile phase plays a more significant and a different role in the separation of all the solutes studied (Fig. 6) in comparison with the above-described influence of the concentration of the organic modifier. A low buffer ionic strength allows an increase in interactions of the positively charged molecule of EN (at pH 3) with the negatively

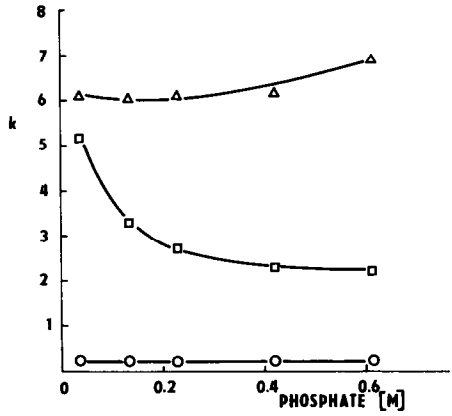

Fig. 6. Dependence of capacity factor (k) of (□) EN, (○) DIAC and (△) DKP on phosphate buffer (pH 3) concentration. Column temperature, 60°C; other conditions as in Fig. 3.

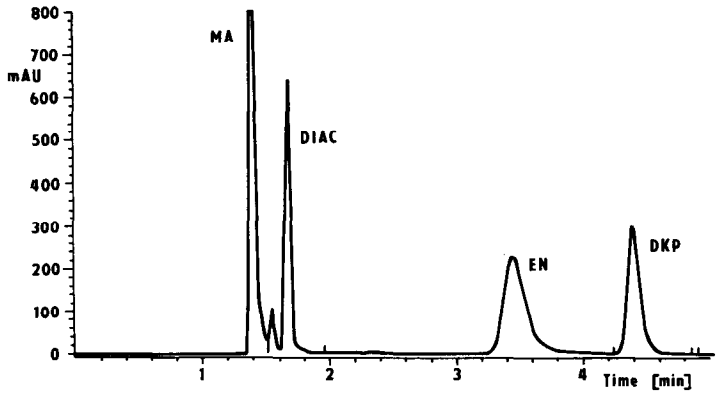

Fig. 7. Chromatogram of ENM, DIAC and DKP under the optimum conditions. Mobile phase, 0.05 *M* phosphate buffer (pH 3)–acetonitrile (6:4, v/v); column temperature, 60°C; concentration of compounds, 0.5 ml/min; other conditions as in Fig. 3.

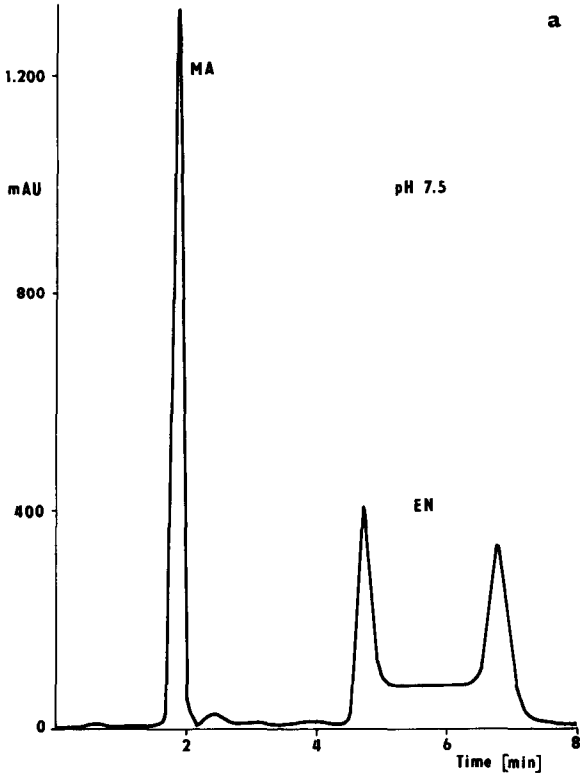

Fig. 8.

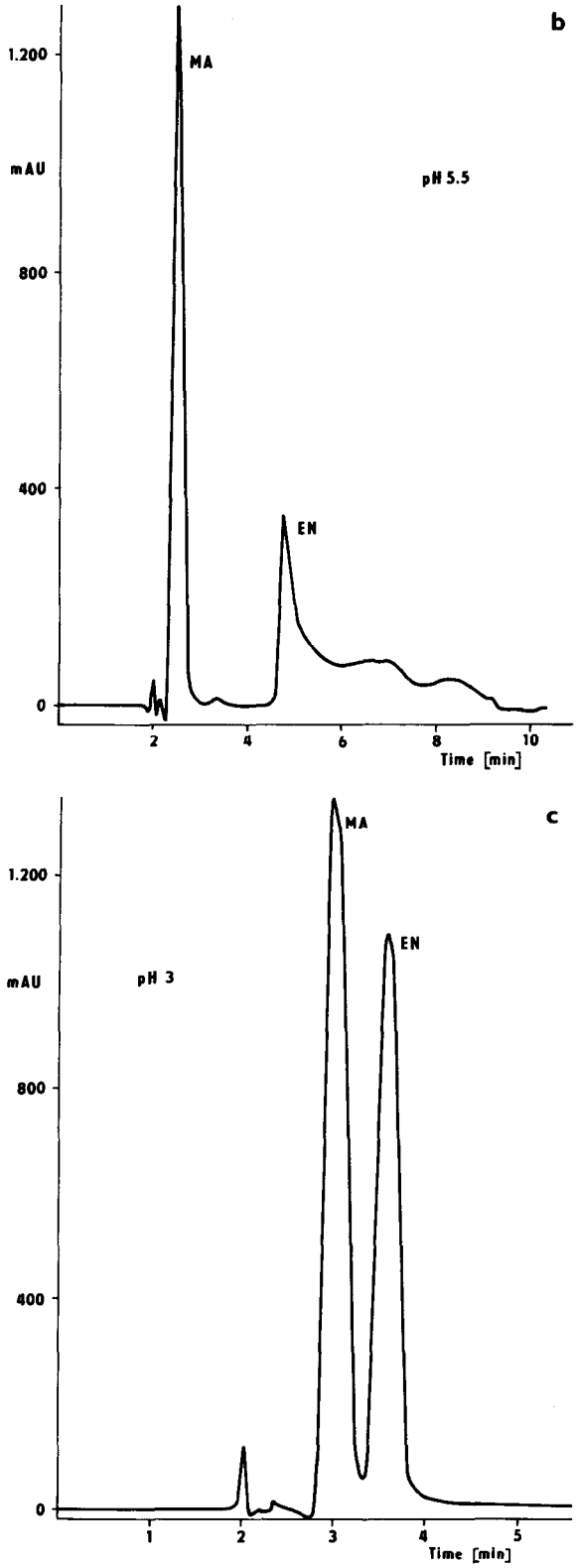

Fig. 8. Effect of pH in the presence of SDS and CTAB on the elution of MA and EN. Mobile phase, 0.05 *M* phosphate buffer (pH 7, 5.5 and 3)–methanol (4:6 at pH 7, otherwise 3:7, v/v) with 0.1 m*M* SDS and 0.1 m*M* CTAB. Column packing, Spherisorb ODS; temperature ambient; flow-rate, 0.5 ml/min; injection volume, 10 μl; other conditions as in Fig. 3.

charged sites of the silica matrix of the reversed-phase sorbent and the retention of EN also increases. The retention of EN may increase to such an extent that the order of elution of EN and DKP may change at a concentration of phosphate buffer below 0.02 *M*. With the increase in ionic strength of the mobile phase, the ion-exchange interaction decreases. On the other hand, the retention of negatively charged molecules of DIAC and DKP is only slightly influenced by interactions with anionic adsorption sites in the chromatographic system used.

In this way, both pH and ionic strength are significant parameters for control of the retention and selectivity of the separation of the examined compounds. A chromatogram of a mixture of all the compounds studied under the optimum conditions, *i.e.*, 60°C, pH 3 and a 40% content of acetonitrile and 0.05 *M* phosphate buffer, is shown in Fig. 7. All the compounds are well separated in 5 min.

Influence of SDS and CTAB. The rotational changes in the molecule of EN in comparison with non-rotating DKP result in a great difference in their peak shapes and widths at lower temperature (Fig. 3). This difference can be decreased significantly by the addition of SDS and CTAB to the mobile phase (Fig. 8). The reduction of the EN peak splitting in the presence of both CTAB and SDS in the mobile phase can be explained by the existence of ionic multilayers of CTAB and SDS in the stationary phase. The high concentration of ionic groups in the stationary phase decreases the electrostatic interactions within the EN molecule in the adsorbed state, which can speed up the transition between the two EN isomers. In this way the use of both CTAB and SDS in the mobile phase at concentrations close to saturation acts in the same direction as the increase in the separation temperature.

The pH of the mobile phase containing CTAB and SDS had a dramatic influence on the elution profile, as shown in Fig. 8. A single, symmetrical peak of EN can be obtained at low pH even at the laboratory temperature (25°C).

CONCLUSIONS

A decrease in the pH of the mobile phase, an increase in the column temperature and/or the addition of ion-pairing agents can eliminate peak splitting and significantly improve peak shape. The optimum peak shape is achieved at a lower pH (3) of the mobile phase and either at a higher temperature (60°C) or with the addition of both CTAB and SDS to the mobile phase. The resolution and retention of all the compounds studied can be influenced by, in addition to the organic modifier, the pH and ionic strength as the additional ion-exchange mechanism plays a significant role in the chromatographic system used. All these observations can help in the further development of chromatographic separations of proline-containing peptides.

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