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Purity Evaluation of Aprotinin by High Performance Liquid Chromatography

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A separation to the baseline isocratic technique has been developed for the evaluation of the purity of aprotinin by internal normalization. The column used was a 30 nm pore diameter butyl-bonded silica stationary phase. An exact relationship was found to give an adequate description of the retention of aprotinin using acetonitrile concentrations of 17–22% and $NaClO_4$ concentrations of 5–50 mmol. It can be used to optimize the mobile phase content for the particular user column and to compensate for possible variations in the retention time of aprotinin, analysed on different batches of butyl-bonded stationary phases.

INTRODUCTION

Aprotinin is a protease inhibitor with several trade names [e.g. Trasilol (Bayer, Leverkusen, Germany), Gordox (Gedeon Richter, Budapest, Hungary), Aprotil (DF "Sofarma" Sofia, Bulgaria)]. It is a peptide with molecular mass of about 6500. It consists of 58 amino acids. The pI value is above 9, due to the presence of a terminal arginine amino acid residue (Reinbolt, 1983).

Although all three modes, i.e. reversed phase (e.g. Mabichi and Nakahashi, 1981; Gazdag and Szepesi, 1981; Raspi *et al.*, 1990), ion exchange (Mabichi and Nakahashi, 1981; Hefti, 1982) and gel (e.g. Mabichi and Nakahashi, 1981), of high performance liquid chromatography (HPLC) have been applied to aprotinin, there is very little information on aprotinin purity evaluation by HPLC. There are also no systematic studies on the influence of the different chromatographically significant factors on the aprotinin retention.

According to Gazdag and Szepesi (1981), separation of aprotinin from its impurities can be achieved under conditions which differ from those for the other studied peptides; the pH of the mobile phase is neutral, while for the others its value is below 3. Even at the optimum conditions the separation is not baseline. The latest results (Raspi *et al.*, 1990) concerning the determination of aprotinin in plasma again showed that the valleys between the peaks do not reach the baseline.

The aim of this study was to develop a better HPLC separation of aprotinin from its impurities. The method should also serve for routine analysis of the active ingredient in pharmaceutical formulations and for stability analysis.

EXPERIMENTAL

Apparatus. The analyses were performed on a Perkin-Elmer (Norwalk, USA) Model LC-4 equipped with a Rheodyne syringe-loaded sample injector Model 7125 with a 20 μL loop, a Perkin-Elmer LC-75 UV absorption variable wavelength detector and a Perkin-Elmer Sigma-10 integrator. The column used was an LC-304 (Supelco, Bellefonte, PA) (250 mm × 4.6 mm, particle size 5 μm and pore size 30 nm).

Standards and reagents. Acetonitrile (AcCN), methanol (MeOH), trifluoroacetic acid (TFA), orthophosphoric acid, triethylamine (TEA) and $NaClO_4$ were purchased from Merck (Darmstadt, Germany). AcCN was gradient grade, MeOH was HPLC grade and all other chemicals were analytical grade. Water was glass-distilled in-house.

Formulations (Gordox and Trazilol injections) were purchased on the open market. Aprotil was kindly supplied from DF "Sofarma", Sofia, Bulgaria (Kamburov *et al.*, 1990).

Aprotinin reference substance. A 1.5 mg/cm^3 stock solution of Aprotinin (NOVO) was prepared. All the solutions, stored at −20 °C, are stable for several weeks.

Sample preparation. Formulations were diluted (if necessary) to a concentration of 0.5 mg/cm^3 and 20 μL of the solution was injected directly in duplicate onto the column. Working standard solutions containing 0.05–1.5 mg/cm^3 aprotinin were prepared by dilution of the stock solution.

Chromatographic conditions. The mobile phases were composed of 0.1% TFA in water or in $NaClO_4$ solutions with different molarity (1–50 mmol) and acetonitrile mixed at

Table 1. Experimental and predicted retention times, t_R(min), of aprotinin peaks at different % AcCN, $NaClO_4$ molarities and mobile phase flow-rates

% AcCN	$NaClO_4$ (mol/L × 1000)	Retention time (min) Experimental	Predicted
Flow-rate 1.0 mL/min			
17	5	17.1	16.3
19	10	14.4	14.7
20	18	17.5	18.0
21	20	13.6	14.4
22	31	17.0	16.2
23	40	13.0	13.3
23	50	17.4	17.4
Flow-rate 0.7 mL/min			
22	31	22.9	24.1
Flow-rate 0.5 mL/min			
22	31	32.1	34.5

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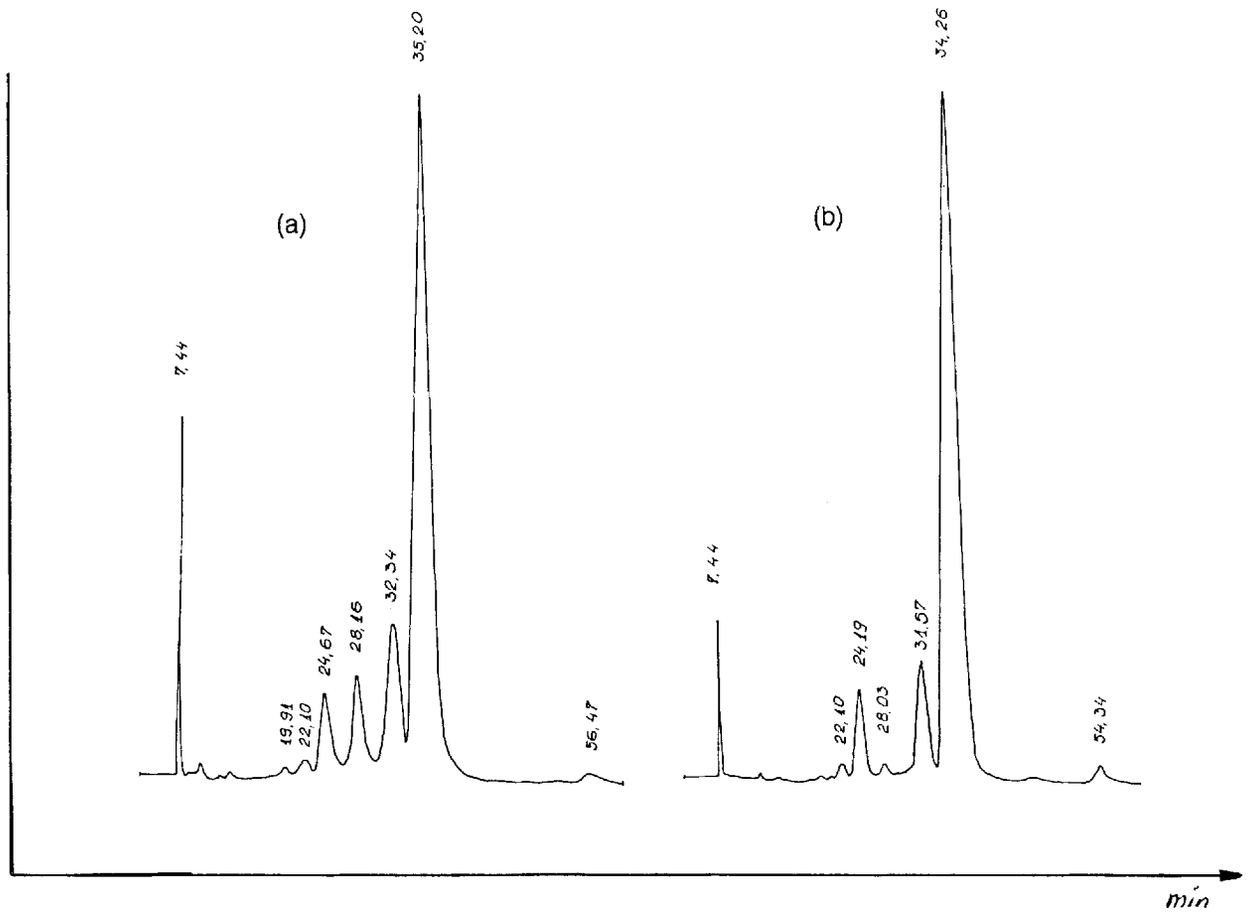

Figure 1. Chromatogram of aprotil NOVO (a), and NIHFI substance (b). Peaks with $t_R = 34$–35 min are aprotinin. Detector at 214 nm, flow-rate 0.5 mL/min, AcCN 22% in $NaClO_4$ solution 0.031 mol/L.

various ratios (v/v) to obtain optimum chromatographic separation.

The mobile phase was filtered through a Whatman 0.5 μm PTFE filter (Maidstone, UK) and degassed by an ultrasonic bath. The flow-rate varied from 1.5 to 0.5 mL/min and the detector wavelength was set at 214 nm. The injection volume was 20 μL. All samples were eluted isocratically. Quantitation of aprotinin in formulations was performed by an external standard technique.

RESULTS AND DISCUSSION

Influence of organic modifier

Acetonitrile (AcCN), and methanol (MeOH) were checked as organic modifiers of 0.1% CF_3COOH. Binary mixtures have been also used. In all cases the combinations of two organic modifiers improved the separation of the peaks eluted before the main peak (aprotinin); however, the tailing remained unacceptably high. The asymmetry factor was above 5.

Influence of aqueous pH and ionic strength

Two regions of pH—below 3.0 and about 7.0±0.5—have been studied using TFA and TEA/H_3PO_4 or potassium phosphate buffers for pH corrections. In all cases the peak shape at acidic pH was best.

To monitor the ionic strength of the mobile phase 0–50 mmol/L KH_2SO_4 and *in situ* obtained TEA phosphate as well as $NaClO_4$ were used. The best asymmetry factor was obtained with $NaClO_4$. All further experiments were carried out with AcCN and $NaClO_4$.

Both AcCN and perchlorate concentrations in the mobile phase were found to be critical for the retention (t_R) of aprotinin, for its peak symmetry and for its separation from the nearest peak of impurity. Their influence was therefore studied carefully and an equation was created from 24 experiments with 17–23% AcCN and 5–50 mmol/L $NaClO_4$ solution:

$$t_R = 300.85 - 28.73(\%) + 6.1(\text{mol}) + 0.6861(\%)^2 - 0.259(\%).(\text{mol}) + 0.003(\text{mol})^2 \quad (1)$$

This equation has a regression coefficient of 0.999 and the standard deviation (SD) = ±50 s (Table 1). Here (mol) is the molarity of the $NaClO_4$ solution multiplied by 1000. The equation permits the calculation of the necessary concentration of $NaClO_4$ solution for the percentage of AcCN suitable for the particular column and task.

Influence of the mobile phase velocity

The influence of the mobile phase velocity in the region 0.5–1.5 mL/min was checked. A velocity of 0.5 mL/min allowed a baseline separation at concentrations of aprotinin in sample solutions below 1.0 mg/cm^3. The peak-area did not change more than 2.0% in the same concentration region and at flow-rates of 0.5–1.0 mL/min. The calculated number of theoretical plates for the peak of aprotinin is about 6000.

Figure 1 shows two chromatograms obtained on the LC-304 column under the following conditions: mobile phase, acetonitrile: 0.031 mol/L $NaClO_4$ in water (22:78); flow-rate, 0.5 mL/min; 20 μL of 1.0 mg/cm^3 aprotinin solution. The retention time of aprotinin varies from 34 to 36 min. The analysis of a sample was completed in 60 min. The linearity was verified at 0.025–1.0 mg/cm^3. The limit of detection at a signal-to-noise ratio of 2 was 5 μg/mL. The asymmetry factor was about 2.5.

The proposed method is flexible; it is well governed by both acetonitrile and $NaClO_4$ content and by using equation (1) isoelutropic mobile phases could be prepared to compensate for the variations between the different butyl 30 nm stationary phases.

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