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HYDROPHOBIC CHROMATOGRAPHY AND BIOANALYSIS OF SOME POLAR PYRIDINE DERIVATIVES USED AS ANTILIPOLYTIC AGENTS

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SUMMARY

The antilipolytic compound 5-fluoro-3-pyridinecarboxylic acid and 5-fluoro-3-hydroxymethylpyridine have been determined quantitatively in plasma (s_{rel} = 5–7% in the concentration range 1–20 μg/ml) by liquid chromatography on LiChrosorb RP-8 (5 μm) with phosphate buffer pH 3–4 as mobile phase, after precipitation of proteins and direct injection of the supernatant. Detection limits were 0.1–0.2 μg/ml. The chromatographic retention is explained by adsorption of the uncharged compounds on to the support complemented by ion-pair adsorption with buffer components at extreme pH values.

INTRODUCTION

It has been proposed that elevated levels of free fatty acids (FFA) in blood can be correlated with the occurrence of heart arrhythmias[1]. The administration of 5-fluoro-3-hydroxymethylpyridinium hydrochloride (FHP), which is metabolized in the body to 5-fluoro-3-pyridinecarboxylic acid (FPC), has been demonstrated to reduce elevated plasma FFA levels in healthy volunteers and in patients following acute myocardial infarction[2,3]. The FFA concentration is rapidly lowered after administration of FHP while the level of FPC, which is the pharmacologically active compound *in vitro*, remains low and no definite correlation between FFA and FPC levels has so far been established. Furthermore it is possible to recognize two sub-groups in treated patients. In the first group a constantly low level of FFA is observed during the whole of the treatment period, while the second group shows an adequate initial decrease in FFA-levels which, however, is gradually reversed during treatment until the initial values are almost reached within 4 h. These observations have initiated discussions on the possible existence of other active metabolites, on the possibility of *in vivo* activity of FHP although it is inactive *in vitro* and on the phenomenon of enzyme induction caused by FPC or some unknown metabolite.

The previous analytical method for FPC involves precipitation of proteins with

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acetone, extraction with chloroform in neutral solution and derivatization with BrCN followed by spectrophotometric determination. It is non-specific and probably gives the sum of all polar pyridines; consequently it may account for the bad correlation between the biological response and obtained concentrations of FPC.

The method presented in this paper is selective and sensitive and relies on high-performance hydrophobic liquid chromatography, which separates FPC and FHP from endogenous compounds and permits their individual determination by UV-photometric detection. The flexibility associated with the chromatographic system may also be useful in identifying unknown active metabolites. The chromatography of FPC, FHP and nicotinic acid on a reversed-phase ion-pair partition system has been described[4].

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of the following components: a Milton Roy Minipump with a pulse dampener (Laboratory Data Control Model 711-47), a UV detector with variable wavelength (Cecil model 212 equipped with a 10-μl flow-cell), a syringe loading sample injector (Rheodyne Model 7120 equipped with an 80-μl loop) and a recorder (Linear Model 252 or Tekman TE 200). The columns were of precision-bore 316 stainless-steel (Handy & Harman), 100 mm × 3.8 mm I.D. × $^1/_4$ in. O.D., and equipped with modified "low dead volume" Svagelok® connections as column end-fittings. A constant-pressure Haskel AO 15 gas amplifier pump was used for column packing.

Samples were mixed on a Fisons Whirlimixer and centrifuged in a Wifug-X-3. Photometric measurements were performed on a Zeiss DMR 21 spectrophotometer in 10-mm quartz cells, and pH measurements were made with a Radiometer PHM 62 digital standard pH meter. An ultrasonic bath (Bransonic 220) was used for homogenization and degassing of solvents.

Chemicals

LiChrosorb RP-8, 5 μm (Chrompack, Middelburg, The Netherlands), was used as the chromatographic support. 5-Fluoro-3-hydroxymethylpyridinium (FHP) hydrochloride, 5-fluoro-3-pyridinecarboxylic acid (FPC) and related compounds were used as obtained from the Department of Organic Chemistry, Astra Läkemedel AB. Ammonium sulphate, sulphuric acid, phosphoric acid, sodium dihydrogen phosphate and disodium hydrogen phosphate were all of pro analysi quality from E. Merck (Darmstadt, F.G.R.) and used as received. The water was deionized. The phosphate buffers (pH 2.35–7.03) had an ionic strength of 0.1 *M*.

Chromatographic technique

The columns were packed by the balanced-density slurry technique with 1,1,2,2-tetrabromoethane–tetrachloroethylene (3:7, w/w) as the dispersion medium and a pressure of 400 bar. After packing, the columns were washed with *n*-hexane, methylene chloride, methanol and water before applying the mobile phase. Mobile phases were aqueous phosphate buffer solutions with sodium as the cation. Equilibrium is obtained after the passage of 10–20 void volumes of mobile phase.

The mobile phases were degassed in an ultrasonic bath and allowed to stand overnight at ambient temperature (22°) before use. The void volume of mobile phase (V_m) was determined by the injection of potassium nitrate.

All reported chromatographic data are the means of duplicate or triplicate determinations.

Analytical method

Sample preparation. A 1-ml volume of blood plasma is acidified by the addition of 50 μl of 2.5 *M* sulphuric acid. In order to precipitate proteins, 2 ml of ammonium sulphate (45%) were added (or alternatively 0.25 g solid ammonium sulphate per ml plasma when it is important to avoid dilution), mixed on the Whirlimixer for 10 sec and the solution kept at room temperature for 10 min. After centrifugation at 4500 *g* for 10 min, 80 μl of the clear supernatant is injected on the chromatographic column.

Chromatographic system

Support: LiChrosorb RP-8, 5 μm. Mobile phase: phosphate buffer; ionic strength, 0.1 *M*; pH between 2.7 and 4.0, chosen according to the specific requirements of the analysis as discussed in Results and discussions. Flow-rate: 2.0 ml/min. The detector measures absorbance at 269 nm. For construction of standard curves, the peak heights obtained with 6–9 different concentrations of the samples in pooled plasma are plotted against the added concentrations.

RESULTS AND DISCUSSION

Chromatography

The principle of retention of ionogenic substances in liquid chromatography with nonpolar stationary phases and neat aqueous solutions as mobile phases has been treated exhaustively by Horváth *et al.*[5]. The chromatographic process is viewed as a reversible association of the solute with the hydrocarbonaceous ligand, and it is assumed that the process with both neutral and ionized solutes is solely determined by solvophobic interactions[6], which means that ionic or hydrogen bonding forces do not play any role in the association between the solute and stationary phase. The interactions are described by simple equilibrium constants. It has been shown that hydrophobic supports adsorb components of the mobile phase and the sample molecules may then be retained by interaction with such an adsorbed layer[7,8]as an alternative to the hydrocarbonaceous ligand. It is assumed in this paper that the retained species is the uncharged compound or the ion-pair of the charged species with buffer components, and furthermore that the number of available adsorption sites is unlimited.

Dependence of the capacity ratios on pH

The influence of pH on the capacity ratios for FHP, FPC and some related pyridine derivatives is illustrated in Fig. 1, and their chemical structures are given in Fig. 2. The appearances of the pH profiles are in good accord with the proposed retention mechanism. The capacity ratio of FHP attains a constant level at high pH and decreases at low pH. Since a constant level is not attained at low pH it is assumed that

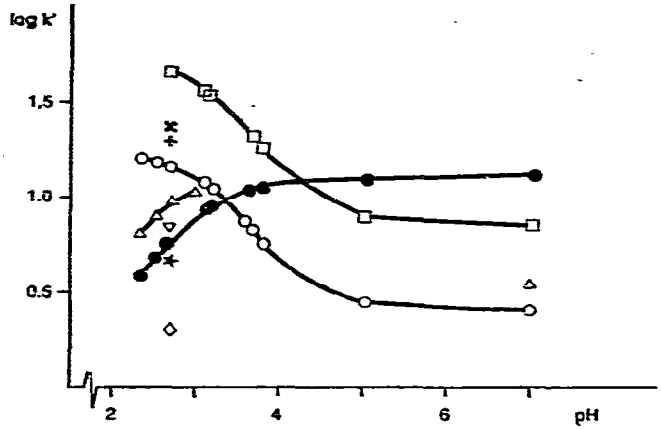
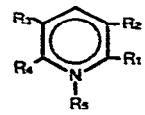


Fig. 1. Dependence of retention on pH for some pyridine derivatives: ●, FHP; ○, FPC; □, CPC; △, FPA; ×, BHP; +, CHP; ▽, 2-CPC; ★, 6-FPC; ◇, FPOC.



Compound	R_1	R_2	R_3	R_4	R_5
FHP	H	CH_2OH	F	H	–
FPC	H	COOH	F	H	–
CPC	H	COOH	Cl	H	–
FPA	H	CH_2COOH	F	H	–
BHP	H	CH_2OH	Br	H	–
CHP	H	CH_2OH	Cl	H	–
2-CPC	Cl	COOH	H	H	–
6-FPC	H	COOH	H	F	–
FPOC	H	COOH	F	H	O

Fig. 2. Chemical structures.

ion-pair adsorption is negligible in this case and it can be shown that the following equation is then valid

$$1/k' = \frac{1}{\varphi \cdot K_F} + \frac{a_{H+}}{\varphi \cdot K_F \cdot K_s} \qquad (1)$$

where φ is the phase volume ratio, K_F the equilibrium constant for the retention of the uncharged compound, K_s the acid dissociation constant, and k' the capacity factor.

A computation of $1/k'$ against a_{H+} gives a reasonably linear relationship (Fig. 3) except for the lowest pH which gives too high a k' value, possibly because adsorption of the ion-pair starts to have an influence in this region. From the intercept and slope, values of $\varphi \cdot K_F = 12.1$ and $K_s = 1.74 \cdot 10^{-3}$ ($pK_s = 2.76$) are obtained.

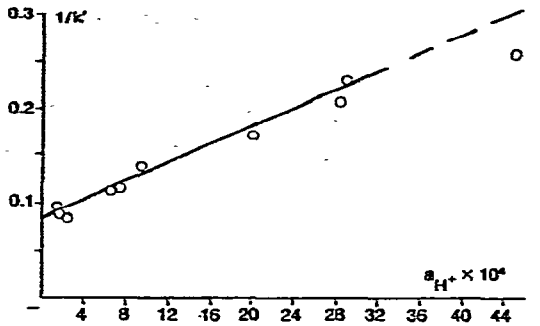


Fig. 3. Retention of FHP as a base. Computation of eqn. 1 ($r = 0.9915$).

The found pK_a value is in the expected range since pyridine has $pK_a = 5.1$ and the electronegative fluoro-substituent will decrease the electron density at the nitrogen; 3-chloropyridine, for example, has $pK_a = 2.84$ (ref. 9).

The other compounds in Fig. 1 are all amphoteric and their retention behaviour seems to follow the predictions for zwitterions. A closer look at the pH profile for FPC shows that the capacity ratio is high at low pH, decreases with increasing pH and finally seems to attain a new lower constant level at high pH. At intermediate pH the influence of possible ion-pair equilibria should be at a minimum, and in this region the equation below might be valid:

$$\frac{1}{k'} = \frac{1}{\varphi \cdot K_{HZ}} + \frac{K_2}{\varphi \cdot K_{HZ} \cdot a_{H+}} \quad (2)$$

K_{HZ} is the equilibrium constant for retention of the neutral form of the zwitterion and K_2 is the second acid dissociation constant.

The graphical computation of $1/k'$ obtained in the pH range 3–3.8 against $1/a_{H+}$ is shown in Fig. 4. The fit to the straight line is very good ($r = 0.9991$) and from the intercept and slope values of $\varphi \times K_{HZ} = 15.7$ and $K_2 = 2.64 \times 10^{-4}$ ($pK_2 = 3.58$) are obtained. The found pK_2 value is also in this case in the expected range since 4-pyridinecarboxylic acid has $pK_2 = 4.89$ (ref. 9) and the electron attracting properties of the fluorine atom will increase the acid strength. The $1/k'$ values at high pH fall far under the straight line, indicating the influence of ion-pair adsorp-

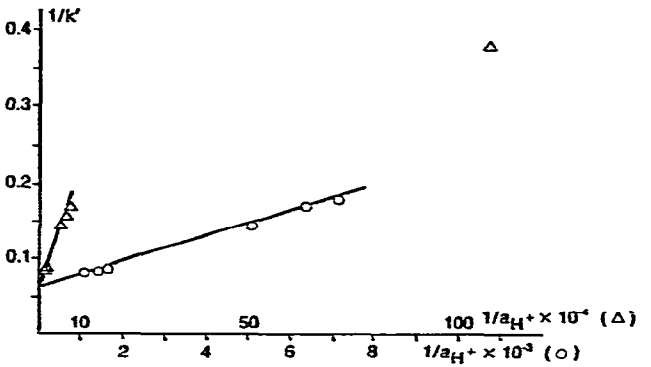


Fig. 4. Retention of FPC as a zwitterion. Computation of eqn. 2 ($r = 0.9991$, lower line).

tion which increases the retention in this pH range (≥ 5); insufficient data are, however, so far available to permit an evaluation of the constants.

5-Chloro-3-pyridinecarboxylic acid (CPC) is more hydrophobic than FPC and is consequently retained to a greater degree both as zwitterion and ion-pair, and the pH profile is rather similar to FPC. 5-Fluoro-3-pyridineacetic acid (FPA) is also more hydrophobic than FPC as reflected in the higher retention at high pH. However, the presence of the methylene group attached to the pyridine ring reduces the electron effects of both the fluorine atom and carboxylic group, which effects the acid strengths so that both pK_1 and pK_2 will increase. The chromatographic retention will then start to decrease at a higher pH than for FPC due to the formation of the protonated cation, as is observed.

A thorough discussion of the relation between chemical structure and chromatographic retention obviously necessitates a knowledge of the complete pH profiles of the compounds, and an accurate comparison is preferably based on equilibrium constants determined for each compound. This will, however, require much work and in this limited study capacity factors were determined for a few other related compounds only at one pH (= 2.7), see Fig. 1. 5-Chloro-3-hydroxymethylpyridine (CHP) and 5-bromo-3-hydroxymethylpyridine (BHP) probably have pK_s values that are very close to that of FGP, and their capacity ratios are in the expected order with the 5-bromo-compound as the most hydrophobic being retained to the largest extent. 2-Chloro-(2-CPC) and 6-fluoro-3-pyridinecarboxylic acid (6-FPC) have much lower capacity ratios than their 3-substituted analogues (CPC and FPC), probably as consequence of their lower pK_s values. The low retention of 5-fluoro-3-pyridinecarboxylic acid-N-oxide (FPOC) is probably mainly a consequence of its higher polarity compared to FPC. Nicotinic acid has a very low retention over the whole pH range tested (2–7), its capacity factor never exceeds 1.6 and in chromatograms obtained from the analysis of plasma (see below) it will be eluted with interfering components in the front and be indistinguishable.

Column performance

Some typical data on column characteristics with a mobile phase of pH 4 (used in the bioanalysis as shown below) are illustrated in Table I. The efficiencies are moderate, $h = 20$; this is, however, not a consequence of non-ideal thermodynamic behaviour of the compounds but of a non-optimal packing procedure of the column since the test compound, anisole, gives a similar efficiency.

TABLE I

COLUMN PERFORMANCE

Support: LiChrosorb RP-8, 5 μm in a 100 × 4.0 mm column. Mobile phase: phosphate buffer, pH 4.03 (ionic strength 0.1 *M*); flow-rate, 2 ml/min.

Compound	*k'*	*h**	$h_c/h_{anisole}$**	*Asymmetry factor*
FPC	4.46	20.4	1.19	1.4
FHP	9.89	19.8	1.15	1.5

* h = Reduced plate height = HETP/d_p, where HETP = height equivalent to a theoretical plate, and d_p = particle diameter.

** The efficiency with anisole was determined with a mobile phase of water–methanol (6:4, v/v).

Bioanalysis

FPC and FHP are small polar compounds that are difficult to extract quantitatively into an organic solvent. The strategy in achieving an analytical method for determinations of the two compounds in plasma was therefore the direct injection of the biological fluid after precipitation of the proteins. The two compounds have UV-absorbance maxima at *ca.* 269 nm with molar absorbances in the region of 6000. All chromatograms shown below were run at this wavelength.

Optimization of the pH of the mobile phase

As illustrated in Fig. 1, the selectivity between FPC and FHP is rather large both at high and low pH, but the choice of the best chromatographic conditions will to a large extent be governed by the appearance of the blank chromatograms. A series of blank chromatograms run at different pHs are demonstrated in Fig. 5. At low and high pH, endogenous compounds interfere and at pH $\geqslant 5$ the disturbances are especially serious, and both FPC and FHP more or less disappear among the multiplicity of peaks due to the endogenous compounds. At low pH only the peak of FHP is disturbed, and the possibility of analyzing FPC alone remains. The best conditions for analysis occur at intermediate pH (3–4). In this range there is one main endogenous peak with retention characteristics similar to FPC and FHP; its capacity factor decreases slightly with increasing pH. In this range a reversal of the order of retention of FPC and FHP takes place at pH *ca.* 3.4 (*cf.* Fig. 1), the capacity factor of FPC decreasing with increasing pH. Depending upon the aim of the actual study, it is thus possible to choose, by adjusting the pH of the mobile phase, whether FPC or FHP has a low capacity factor, an important parameter regarding the detection limit.

Quantitative determinations

In order to illustrate the performance of the system in quantitative determinations a mobile phase of pH 4 was chosen. A representative sample chromatogram is illustrated in Fig. 6. A part of the sample is lost by adsorption and/or occlusion to to protein precipitate. When quantitations were performed on eight samples the recoveries of FPC and FHP were 66.5 $\pm$ 4.5% and 73 $\pm$ 4.2% respectively relative to a standard curve obtained from pure buffer solutions. Some quantitative determinations (Table II) yielded precisions (s_{rel}) of 5–7% in the concentration range 1–20 μg/ml and an acceptable accuracy. Under these conditions, the limits of detection, defined as twice the baseline noise are, 0.1 and 0.2 μg/ml for FPC and FHP respectively.

Studies on the bioanalysis of other compounds[10,11] have shown an improved precision in quantitative determinations by use of internal standards that are chemically closely related to the drugs of interest. Some chromatograms (Fig. 7) of spiked plasma samples indicate the possibility of using either 5-fluoro-3-pyridineacetic acid (FPA) at a mobile phase pH of *ca.* 3, or 5-chloro-3-pyridinecarboxylic acid (CPC), at a pH of *ca.* 4, as internal standards for FPC and FHP.

In blank chromatograms obtained from citrated plasma, the fronts were broader and contained peaks that interfere with the compounds of interest at some mobile phase pH values. Therefore the use of heparinized plasma is recommended for quantitative work.

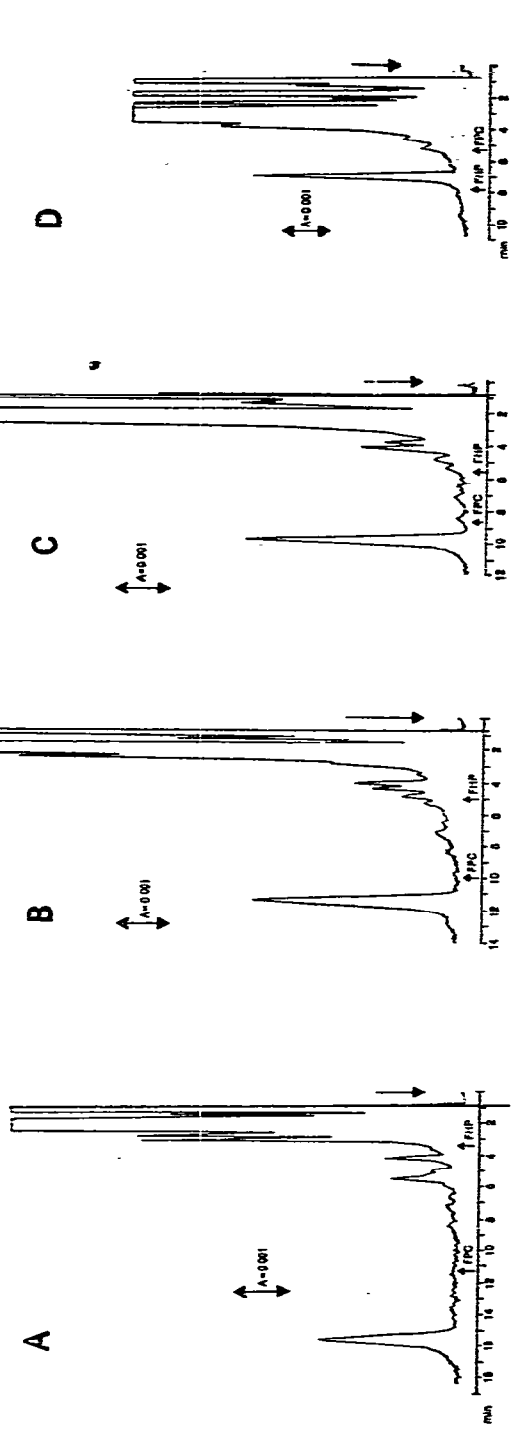


Fig. 5. Blank chromatograms with mobile phases of different pH. Analysis performed according to *Analytical method*. The arrows indicate the retention times for FPC and FHP respectively. pH = 2.35 (A), 2.82 (B), 3.05 (C), 3.66 (D), 4.03 (E), 5.03 (F) and 7.03 (G).

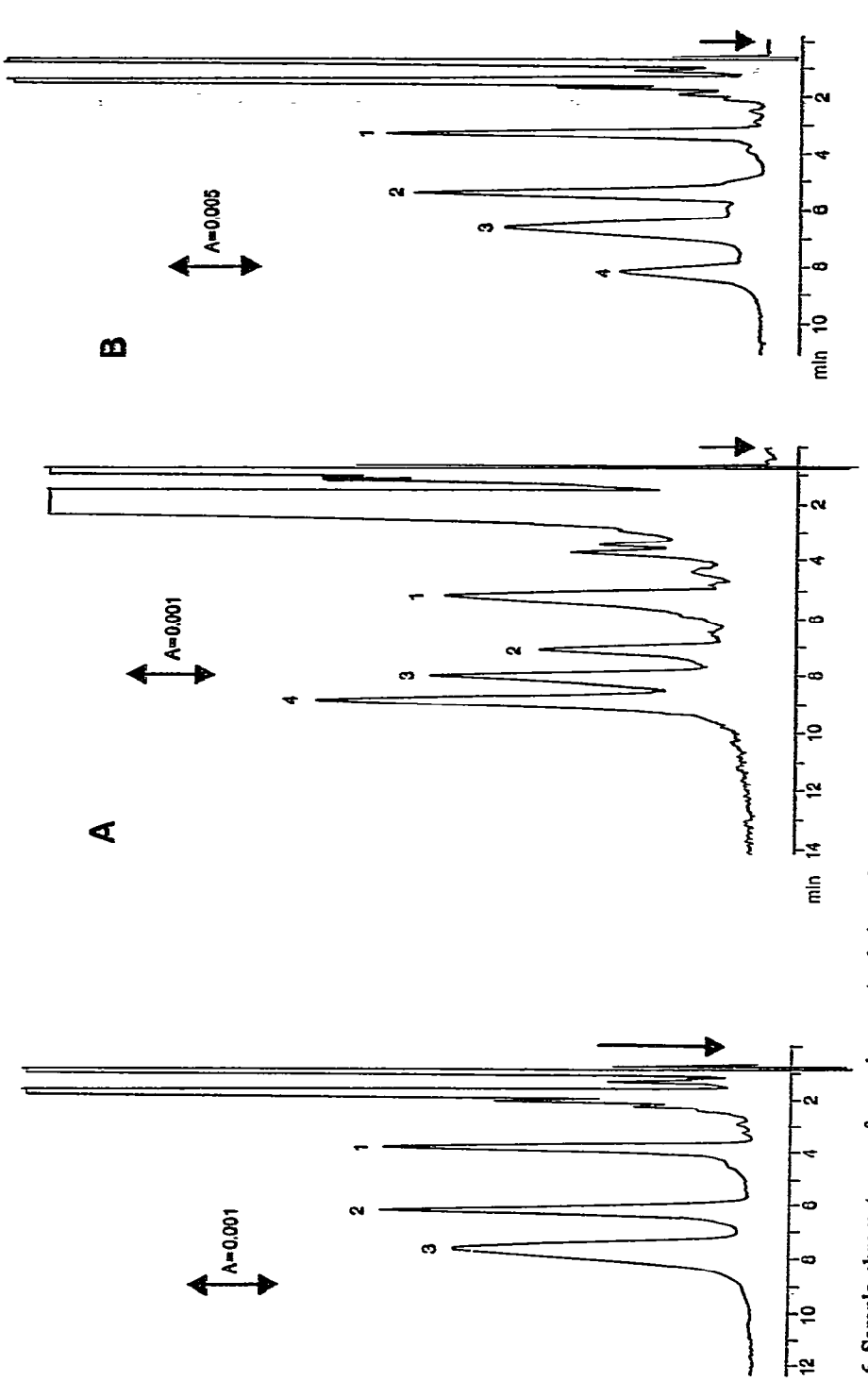


Fig. 6. Sample chromatogram from plasma. Analysis performed according to *Analytical method*. Mobile phase: phosphate buffer, pH 4.03. Peaks: 1 = FPC, 10.6 μg/ml; 2 = endogenous substance; 3 = FHP, 20.8 μg/ml.

Fig. 7. Sample chromatograms from plasma with potential internal standards. Analysis performed according to *Analytical method*. A, mobile phase, phosphate buffer, pH 3.05; concentrations in plasma, 5.1 μg/ml of each compound. Peaks: 1 = FHP; 2 = FPA, internal standard; 3 = FPC; 4 = endogenous substance. B, mobile phase, phosphate buffer, pH 4.01. Peaks: 1 = FPC, 8.5 μg/ ml; 2 = endogenous substance; 3 = FHP, 16.7 μg/ml; 4 = CPC, internal standard, 6 μg/ml.

TABLE II

QUANTITATIVE DETERMINATIONS IN PLASMA

Analysis performed according to *Analytical method*. Chromatographic conditions as in Fig. 6; $n = 9$.

Compound	*Amount (μg/ml)*		s_{rel} (%)
	Added	*Found*	
FPC	1.07	0.93	7.16
	10.7	10.4	4.73
FHP	2.08	2.05	5.72
	20.8	20.6	5.70

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