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Note

Determination of anticoagulants in serum by column liquid chromatography

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A rapid and reliable determination of anticoagulants in blood serum is urgently needed for the control of the concentration of these drugs during the treatment of heart and arterial diseases. In the past mainly fluorimetric procedures have been used, which failed to be specific unless combined with thin-layer chromatography[1]; more recently, gas chromatographic methods have been described. Mundy *et al.*[2] pointed out that these gas chromatographic determinations were not suitable and this has been confirmed by experiments in our laboratory.

The first liquid chromatographic investigations were performed by Vesell and Shively[3] using a pellicular packing material, and similar work was carried out later by Mundy *et al.*[2]. Fasco *et al.*[4] and Vanhaelen-Fastré and Vanhaelen[5] presented a method based on reversed-phase liquid chromatography.

The aim of our study was to achieve an efficient separation and a rapid quantification of some commonly used anticoagulants using a versatile selective mobile phase system in liquid–solid chromatography on silica. The procedure presented here may serve as a basis for the development of a routine analytical method.

EXPERIMENTAL

The anticoagulants used in this study are listed in Table I.

All solvents were of pro analysi grade and obtained from Merck (Darmstadt, G.F.R.). A Kipp 771 liquid chromatograph (Kipp & Sons, Delft, The Netherlands) equipped with a Zeiss PM2A variable-wavelength UV detector (Carl Zeiss, Oberkochen, G.F.R.) operated at 281 nm has been used throughout this study. The packing material was LiChrosorb SI 60 (Merck) with a mean particle diameter of 5 μm.

The extraction procedure was similar to the procedure described by De Wolff and Van Kempen[1] and is outlined in Table II.

The columns (300 mm $\times$ 4.6 mm I.D.) were packed by means of a balanced-density slurry technique. Plate numbers achieved with these columns ranged from 10,000 to 15,000.

The composition of the mobile phase, which consisted of dichloromethane, ethanol and water, was varied in order to find optimal separation conditions, as has been described elsewhere[6]. Capacity ratios (k) were calculated from the retention

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TABLE I

ANTICOAGULANTS USED

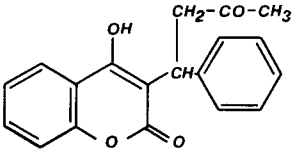
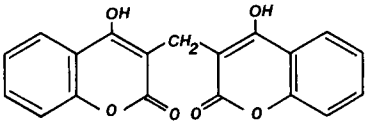
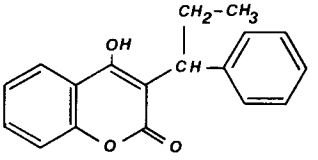
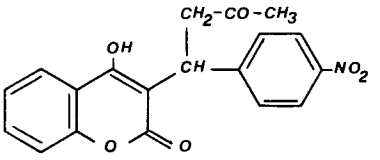
Common name	*Manufacturer*	*Systematic name and structure*
Warfarin	Sigma (St. Louis, Mo., U.S.A.)	4-Hydroxy-3-(3-oxo-1-phenylbutyl)-2*H*-chromen-2-one 
Dicoumarol	Sigma	4,4′-Dihydroxy-3,3′-methylenedi-(2*H*-chromen-2-one) 
Marcoumar	Hoffmann-La Roche (Nutley, N.J., U.S.A.)	4-Hydroxy-3-(1-phenylpropyl)-2*H*-chromen-2-one 
Sintrom	Ciba-Geigy (Summit, N.J., U.S.A.)	4-Hydroxy-3-[1-(4-nitrophenyl)-3-oxobutyl]-2*H*-chromen-2-one 

TABLE II

SCHEME FOR EXTRACTION OF ANTICOAGULANTS FROM SERUM

Stage	*Operations*
Extraction	(1) Mix 2 ml of serum with 0.2 ml of 5 *N* hydrochloric acid and homogenize. (2) Add 10 ml of trichloromethane and homogenize for 3 min. (3) Centrifuge for 10 min at 2000 rpm.
Concentration	(4) Collect 8 ml of the organic phase (lower layer). (5) Evaporate the solvent to dryness in a stream of nitrogen at 60°.
Sample preparation	(6) Dissolve the residue in 0.5 ml of eluent and ultrasonicate for 1 min.

times of the components and an unretarded compound (benzene). Samples were injected by means of an injection valve with a 50-μl loop.

RESULTS AND DISCUSSION

The optimal eluent composition for the separation of a test mixture containing all four anticoagulants in Table I was found to be dichloromethane–ethanol–water (98.8:1.0:0.2, v/v/v).

A chromatogram of the test mixture is shown in Fig. 1. As far as the eluent composition is concerned, it is necessary to work outside the miscibility gap of the dichloromethane–ethanol–water phase diagram[6]. In order to ensure stable separation conditions, it is also necessary to control carefully the water and ethanol contents.

The overall extraction efficiency is determined by, among other factors, the pH and the protein content of the aqueous phase, the type of organic phase and the mixing conditions. The composition of the aqueous phase also determines whether only the free drugs or also the originally protein-bound drugs will show up in the

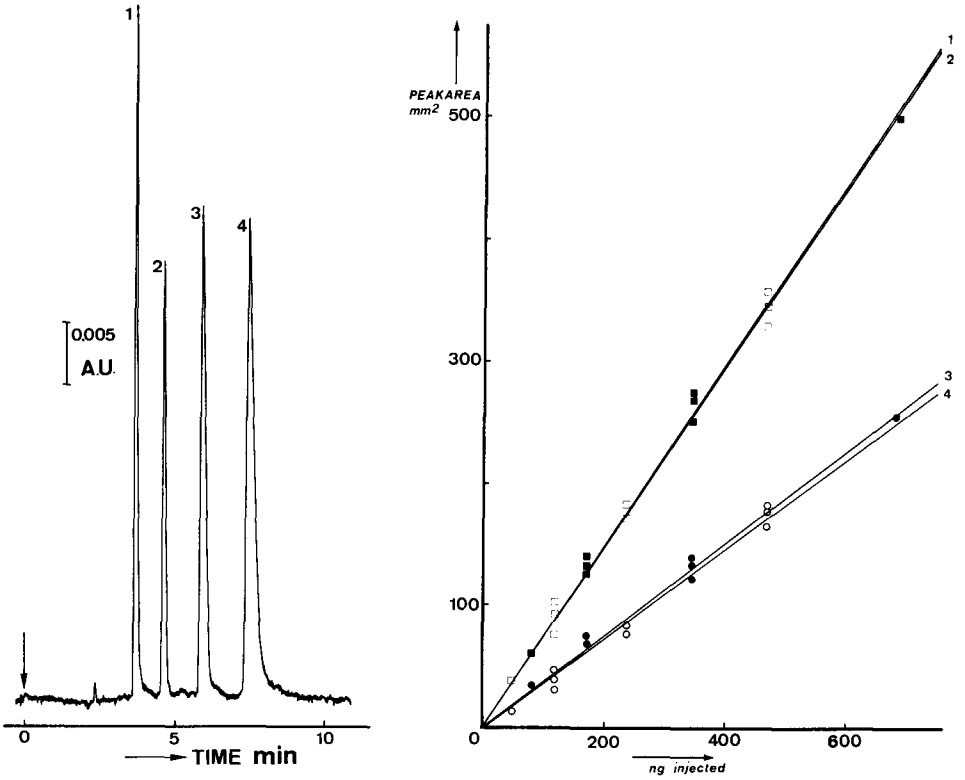


Fig. 1. Chromatogram of a test mixture of anticoagulants. Column: 300 × 4.6 mm I.D., LiChrosorb SI 60, $\bar{d}_p = 5\,\mu$m. Eluent: dichloromethane–ethanol–water (98.8:1.0:0.2, v/v/v). Flow-rate: 1.5 $cm^3 \cdot min^{-1}$. Detection: UV, 281 nm. Components: 1, Marcoumar ($k = 0.5$); 2, warfarin ($k = 0.9$); 3, sintrom ($k = 1.5$); 4, dicoumarol ($k = 2.1$). Volume injected: 50 μl.

Fig. 2. Calibration graphs. 1, Sintrom serum extract (■), correlation coefficient 99.6%; 2, sintrom test mixture (□), correlation coefficient 99.7%; 3, marcoumar serum extract (●), correlation coefficient 99.5%; 4, marcoumar test mixture (○), correlation coefficient 99.5%.

Fig. 3. Chromatogram of bovine serum extract. Conditions as in Fig. 1. Components: 1, marcoumar, 185 ng injected, 1.54 μg/ml in serum; 2, sintrom, 267 ng injected, 2.03 μg/ml in serum.

organic phase. The calibration graphs in Fig. 2 show that the overall recoveries of marcoumar and sintrom, dissolved in human serum and extracted as described in Table II, were 100 ± 3% and 102 ± 3%, respectively. The correlation coefficients for the calibration graphs were better than 99.5%.

We used the serum calibration graphs in Fig. 2 to determine the concentrations of sintrom and marcoumar in bovine serum samples. A typical chromatogram is shown in Fig. 3.

Anticoagulant concentrations ranged from 0.5 to 2.5 μg/ml in serum. The reproducibility of our analysis was within 3%, derived from the standard deviation of the recovery experiments. This method can be used for concentrations down to 0.1 μg/ml in serum. The minimal detectable amount for each of the four drugs studied was 2 ng. The presence of acetylsalicylic acid did not interfere with our anticoagulant determination[1].

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