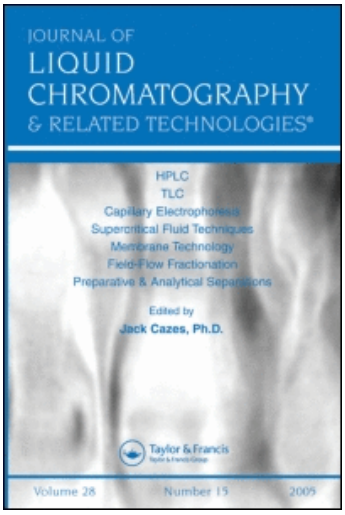

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QUANTITATIVE DETERMINATION OF ACENOCOUMARIN IN ANTICOAGULATED PATIENTS

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ABSTRACT

A new procedure of pre-concentration on Tenax GC followed by high performance liquid chromatography analysis has been developed for the quantitative determination of acenocoumarin in human plasma. The recovery of acenocoumarin was greater than 90% over a concentration range of 0.10 to 1.00 µg/ml and the limit of quantitation by the assay was 10 ng/ml of plasma. This method allows quantitative determinations in patients under acenocoumarin therapy and can be used as a routine clinical monitoring.

INTRODUCTION

Acenocoumarin (3-(α-acetonyl-p-nitrobenzyl)-4-hydroxycoumarin) (Sintrom®), derivative of the 4-hydroxycoumarin, is an orally administered anticoagulant.

It is used clinically as a vitamin K_1 antagonist for prophylaxis and treatment of tromboembolic disorders (1). Because of its clinical importance, analytical technics such as spectrophotometry (2), thin-layer chromatography (3), gas-liquid chromatography (4) and high-performance liquid chromatography (5,6) have been developed.

Some of these methods lacked specificity (7), sensitivity or implied a lengthy extraction procedure with derivatization of the samples (3).By now, HPLC is the best method for the separation and quantitation of coumarin anticoagulants because of its simplicity, specificity and sensitivity (8-10).

Previously, we have reported a simple HPLC procedure for the quantitation of warfarin and phenprocoumon in human urine and plasma (11). It is based on the hydrophobic property of a porous polymer, Tenax GC, allowing a pre-concentration of the anticoagulants prior to HPLC analysis.

We applied this original method, with a few modifications, to the quantitative determination of acenocoumarin in plasma. In this paper, we report preliminary clinical data obtained in anticoagulated patients.

MATERIALS AND METHODS

Apparatus

The liquid chromatograph was a Hewlett Packard (model 1084 B), equipped with an automatic sampling system and a LC terminal (model 79850 B).

The analytical column was a Waters Assoc. μ Bondapak C_{18} (10 μ particle size, stainless steel, 30 cm. x 1/4 inch.), equipped with a pre-column packed with Vydac-RP (30-44 μm).

The mobile phase was a mixture of ethanol-water (containing 0.1% of acetic acid 98%) (1:1 v/v). The system was operated at a flow rate of 1.4 ml/min. at ambiant temperature. All solvents were bidistillated. Acenocoumarin was detected at 313 nm.

Compounds

Tenax GC (60-80 mesh) was provided by Applied Sciences Labs (State College, Pa, USA). Acenocoumarin (Sintrom® Mitis) was provided by Geigy.

Procedure

Plasma (citrated 3.8%) were obtained from patients hospitalized in the Unité Coronaire (Dr. Bernard, Hôpital Universitaire St.Pierre, Bruxelles).

The samples were acidified at pH 4 (with acetic acid 98%) and injected through an injection valve (Pharmacia SRV-4) on a column (Pharmacia SR 10/50, 50 cm.x 10 mm I.D.), packed with Tenax GC (3g.), with a piston pump (FMI RS-SY) equipped with a micrometric flow adjustment. The flow was maintained constant at 2 ml/min. Water soluble compounds, non retained on Tenax GC, were eliminated by washing the column with 50 ml. of bidistillated water. Elution of the anti-coagulant adsorbed on Tenax GC was obtained with ethanol (50 ml).

The ethanol phase was evaporated in vacuo and the residue was dissolved in 500 µl of ethanol. The column, washed with 50 ml of water, was ready to use.

Coagulation test

The anticoagulant activity was evaluated in citrated plasma by the thrombotest of Owren and expressed as prothrombin complex activity (percent of control).

RESULTS AND DISCUSSION

Plasma (2 ml) was spiked with acenocoumarin to give concentrations of 0.10, 0.25, 0.50 and 1.00 µg/ml.

TABLE I

Recovery of Acenocoumarin added to Human Plasma

Concentration µg/ml plasma	Acenocoumarin recovered (µg/ml) Mean ± S.E.M.	n
1,00	0,97 ± 0,06	6
0,50	0,46 ± 0,04	6
0,25	0,24 ± 0,02	4
0,100	0,097 ± 0,008	4

Each determination was performed in duplicate.

The extraction efficiency was evaluated by comparing peak area obtained after extraction of spiked plasma to those obtained from corresponding concentrations of standard solutions. In the studied concentration range, recoveries are greater than 90% (Table I). A typical HPLC elution pattern of acenocoumarin is illustrated in Figure 1.

Figure 1 A shows a chromatogram obtained from blank plasma and Figure 1 B from plasma to which acenocoumarin has been added. At the retention time of acenocoumarin, 5,2 min., there is no interference plasma constituents. Figure 1 C shows the elution profile of plasma from an anticoagulated patient.

Application of the HPLC method for the estimation of acenocoumarin plasma levels in 8 patients, is given in Table 2.

These data show great individual variations in plasma levels corresponding to a similar anticoagulant

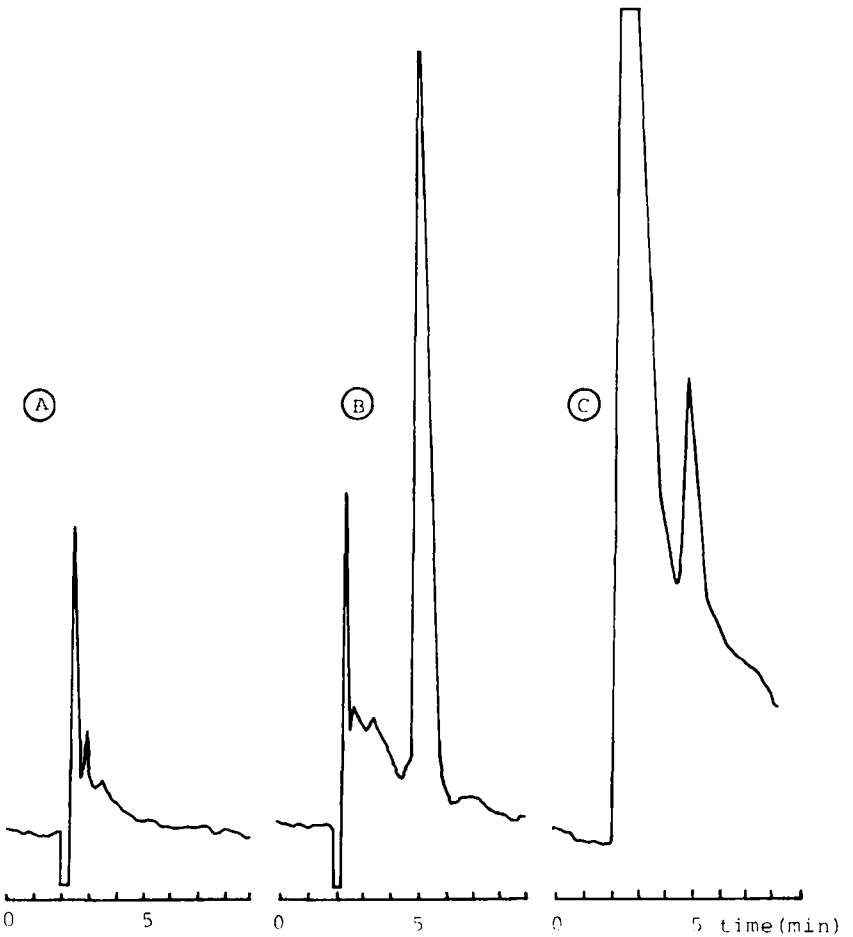

Figure 1 : Chromatographic separation from (A) control human plasma, (B) human plasma spiked with acenocoumarin, (C) , plasma from an anti-coagulated patient.

TABLE 2

Patient	Sex	Age	L.D.	P.C.A.%	AC ng/ml	M.D.	P.C.A.%	AC ng/ml
B.S.	M	35	0.185	32	69.8	0.026	13	31.0
C.	M	54	0.163	50	131.5	0.028	11	18.2
V.	M	60	0.175	65	-	0.019	11	22.6
G.S.	M	60	0.187	21	406.2	0.034	13	189.4
W.T.	M	50	0.219	24	62.3	0.032	8	91.2
W.R.	M	47	0.200	15	270.6	0.038	11	80.2
Ed.A.	M	52	0.193	33	344.6	0.053	12	212.4
Bd.	M	53	0.132	25	150.5	0.023	11	48.2

L.D. = loading dose = total mg/kg of acenocoumarin administered over the first 3 days of oral anticoagulation therapy.

M.D. = mean maintenance dose = mean mg/kg/day of acenocoumarin.

P.C.A. = prothrombin complex activity determined by Owren's thrombotest.

AC = acenocoumarin.

response. At this time, no correlation was found between the prothrombin time and acenocoumarin levels.

In conclusion, we describe here a simple and sensitive HPLC method, coupled with a pre-concentration and cleanup procedure, allowing the quantitative determination of acenocoumarin in plasma from anticoagulated patients. This procedure is rapid and can be used as a routine clinical monitoring with a sensitivity in the nanogram range.

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