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ANALYSIS OF ACENOCOUMARIN AND ITS AMINO AND ACETAMIDO METABOLITES IN BODY FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Acenocoumarin and its acetamido metabolite, after extraction at pH 4.4, were analysed by isocratic reversed-phase high-performance liquid chromatography using aqueous acetonitrile (pH 4.90) as eluent. Warfarin was used as internal standard. The amino metabolite, after back-extraction into 0.5 *N* HCl, was derivatized by diazotization and heat treatment. The resulting product was analysed by the same reversed-phase system.

The sensitivity of the method for acenocoumarin and its amino metabolite was in the range of 20 ng/ml. To achieve this sensitivity for the analysis of the acetamido compound, the acetonitrile content of the eluent had to be lowered. The assay was applied to the analysis of plasma samples of patients under acenocoumarin therapy. The disposition of the amino compound in rats was investigated.

INTRODUCTION

Acenocoumarin (Sintrom^{R}) belongs to the 4-hydroxycoumarin congeners in use clinically as oral anticoagulants. Pharmacologically acenocoumarin is classified as a short-acting anticoagulant [1]. The compound differs chemically from warfarin by its 4′-nitro group (Fig. 1). Potentially, this nitro group is vulnerable to biotransformation (i.e. reduction) and, by using ^{14}C-labeled acenocoumarin Dieterle et al. [2] could show the formation of the amino and the corresponding acetamido metabolite in man. In mice these metabolites appeared to be more potent as anticoagulants than acenocoumarin [2]. In rats the amino metabolite was the most potent [3]. The significance of these metabolites for the pharmacodynamics of acenocoumarin in man, although stressed recently by Godbillon et al. [4], has not yet been established. Further investigation of the clinical relevance of the formation of these metabolites in

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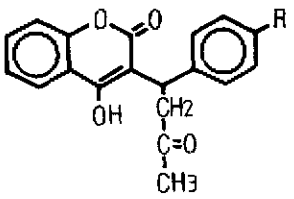

R = NO_2	acenocoumarin
R = NH_2	amino
R = $NHCOCH_3$	acetamido
R = H	warfarin

Fig. 1. Chemical structures of acenocoumarin, the amino and acetamido metabolites, and warfarin.

a broader group of subjects [5] requires an easy and reliable assay method for acenocoumarin and its amino and acetamido metabolites. This paper describes the application of high-performance liquid chromatography (HPLC) to analyse the compounds in body fluids.

EXPERIMENTAL

HPLC instrumentation and chromatographic conditions

The HPLC system consisted of a Waters M-6000A pump equipped with a U6K injector. Ultraviolet (UV) absorbance at 303 nm was monitored with a Pye-Unicam LC UV detector, and the signal was processed with a Waters computing integrator. A LiChrosorb RP-8 column (particle size 5 μm, 150 mm × 4.6 mm I.D.; Chrompack, Middelburg, The Netherlands) was used in combination with a guard column (ODS pellicular material; 50 mm × 2.1 mm I.D.). The mobile phase consisted of 0.1% acetic acid—acetonitrile–ethyl acetate (100:90:1, v/v) adjusted to pH 4.90 with 1 *N* ammonia. The flow-rate was 1.5 ml/min.

Materials

Acenocoumarin was a gift of Ciba-Geigy, The Netherlands. The amino derivative was prepared by catalytic (Pt) hydrogenation of acenocoumarin. The acetamido derivative was prepared by treating the amino derivative with acetic anhydride. The identity of the products was verified by comparison with authentic material obtained from Ciba-Geigy, Basel, Switzerland. Warfarin was obtained from Sigma (St. Louis, MO, U.S.A.).

Sample preparation

To 1 ml of plasma in a centrifuge tube, 500 ng of warfarin (10 μl of a 50 μg/ml solution in water), and 1 ml of concentrated citric acid—phosphate buffer, pH 4.40, were added. Extraction was made twice with 5 ml of dichloromethane—*n*-pentane (1:1, v/v). The combined organic phase was extracted with 0.2 ml of 0.5 *N* hydrochloric acid. Collection of the organic phase was performed by freezing the water phase.

The organic phase, containing acenocoumarin, acetamido derivative, and warfarin, was evaporated to dryness at 35°C under a stream of nitrogen. The residue was dissolved in 100 μl of eluent. The acid phase, containing the amino compound, was processed as follows: after removing remnants of organic phase by a stream of nitrogen at 35°C (takes about 5 min), 500 ng of warfarin (10 μl of a 50 μg/ml solution in water) and 200 μl of sodium nitrite (2% solution in

water) were added. After standing for 5 min at room temperature to complete the diazotization, the mixture was heated at 65°C for 30 min.

Together with the amino sample, calibration points were processed as follows: to 200 μl of 0.5 *N* hydrochloric acid containing 50, 100, 500 and 1000 ng of the amino compound in centrifuge tubes, warfarin (500 ng) and 200 μl of the nitrite solution were added. The mixtures were heated for 30 min at 65°C. Following the heat treatment, the reaction mixtures were extracted with 5 ml of the dichloromethane–*n*-pentane extraction solvent. After evaporation to dryness, the residue was dissolved in 100 μl of eluent. Of both residues a 20-μl volume was injected.

RESULTS AND DISCUSSION

Chromatography

Because of the acidic character of the 4-hydroxycoumarins, the reversed-phase system described in the literature for these drugs, i.e. warfarin [6], phenprocoumon [7], and acenocoumarin [8], basically consists of an acidic eluent. The amino metabolite, however, also posesses a basic function. This

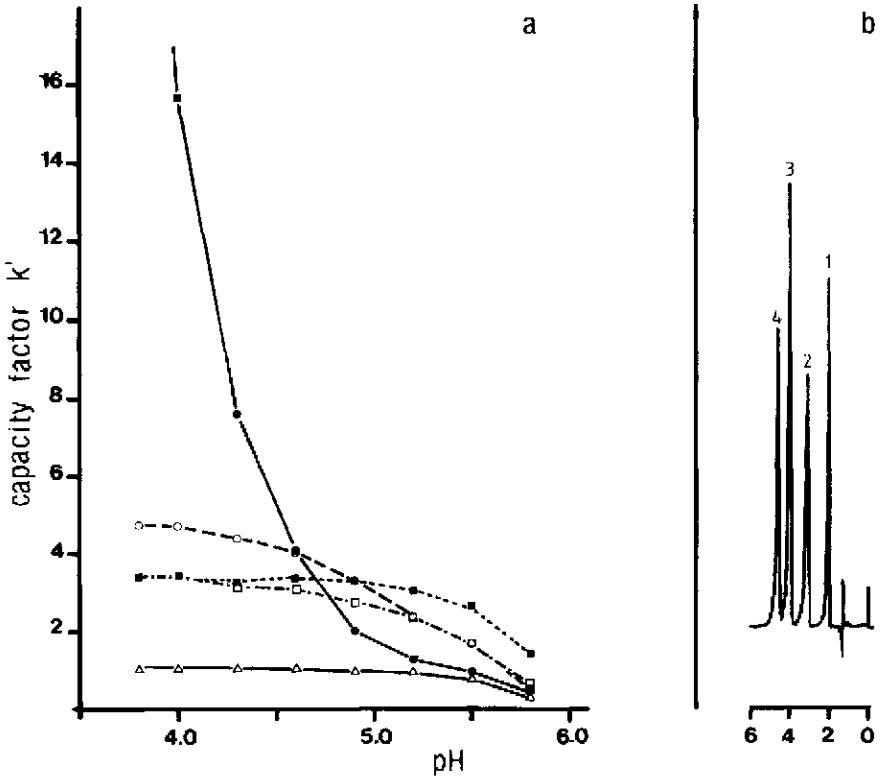

Fig. 2. (a) The dependency of the retention time (capacity factor *k'*) on the pH of the eluent. (□), acenocoumarin; (●), amino metabolite; (△), acetamido metabolite; (○), phenprocoumon; (■), warfarin. (b) Chromatogram of a mixture of acetamido metabolite (1), amino metabolite (2), acenocoumarin (3), and warfarin (4). The peaks represent 500 ng. Detector sensitivity, 0.08 a.u.f.s. Times scale in min.

makes the elution of the compound strongly pH-dependent. Fig. 2a shows that by raising the pH of the eluent the capacity factor of the amino metabolite decreased from "infinity" at pH 3.80, i.e. the pH of the eluent without addition of ammonia, to $k' = 0.4$ at pH 5.80. The same was observed when 0.01 *M* citric acid was used instead of 0.1% acetic acid. With sulfuric acid it was found that the retention time varied inversely with the concentration of the sulfuric acid in the eluent. These observations suggest ion-ion interactions between the stationary phase (LiChrosorb 5 RP-18) and the protonated amino metabolite. At pH 4.9 optimal separation was obtained for acenocoumarin its metabolites and warfarin, the latter being chosen as internal standard (Fig. 2b).

Sample preparation

Having once obtained optimal chromatographic conditions it was found that the recovery of the amino metabolite following straightforward sample preparation (i.e. extraction followed by evaporation of the organic phase) was erratic, apparently due to decomposition of the compound during evaporation of the organic phase. Several experimental variations were tried to solve the problem. Acid back-extraction of the amino metabolite from the organic phase followed by diazotization and heat treatment gave reliable results. Under the conditions used (see Methods) only one acid-extractable reaction product was formed (Fig. 3).

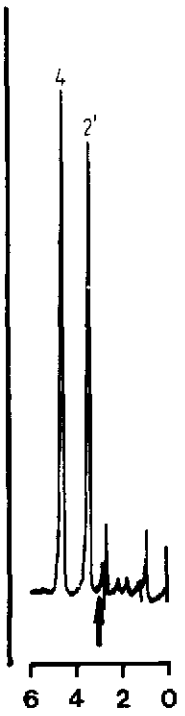

Fig. 3. Chromatogram of the extract of the diazotization reaction of the amino compound. Peak 2' represents the reaction product, peak 4 represents warfarin. The reaction was performed with 500 ng of the amino compound. (For reaction details, see Methods). The arrow indicates the position of the amino compound in the chromatogram. Detector sensitivity, 0.02 a.u.f.s. Time scale in min.

The precise identity of the product is not yet clear. In view of the way aromatic diazonium salts react, it is reasonable to think of either 4′-chloro- or 4′-hydroxywarfarin. The UV absorption spectrum of the compound showed a resemblance to that of warfarin. The reaction conditions, especially the reaction temperature, appeared to be critical. Temperatures above 75°C resulted in the formation of other products at the cost of the product just discussed. At temperatures lower than 60°C the reaction rate was slow. At a reaction temperature of 65°C about 60% of the amino derivative was converted to the product within 30 min; at 90 min this amounted to about 80%. Because of the specificity of the procedure, and because calibration points were always processed in parallel with the samples, a 30-min reaction time was found to be sufficient for the assay. The calibration curves (peak area ratio of product to warfarin vs. the amount of amino derivative) were found to be linear over the entire concentration range tested; up to 5 μg of amino metabolite. A typical regression is given by $y = (13.4 \cdot 10^{-4})x - (0.1 \cdot 10^{-4})$, $s = 0.01$, $r^2 = 0.999$, where y is the peak area ratio and x is the amount (ng) of the amino metabolite in the reaction mixture.

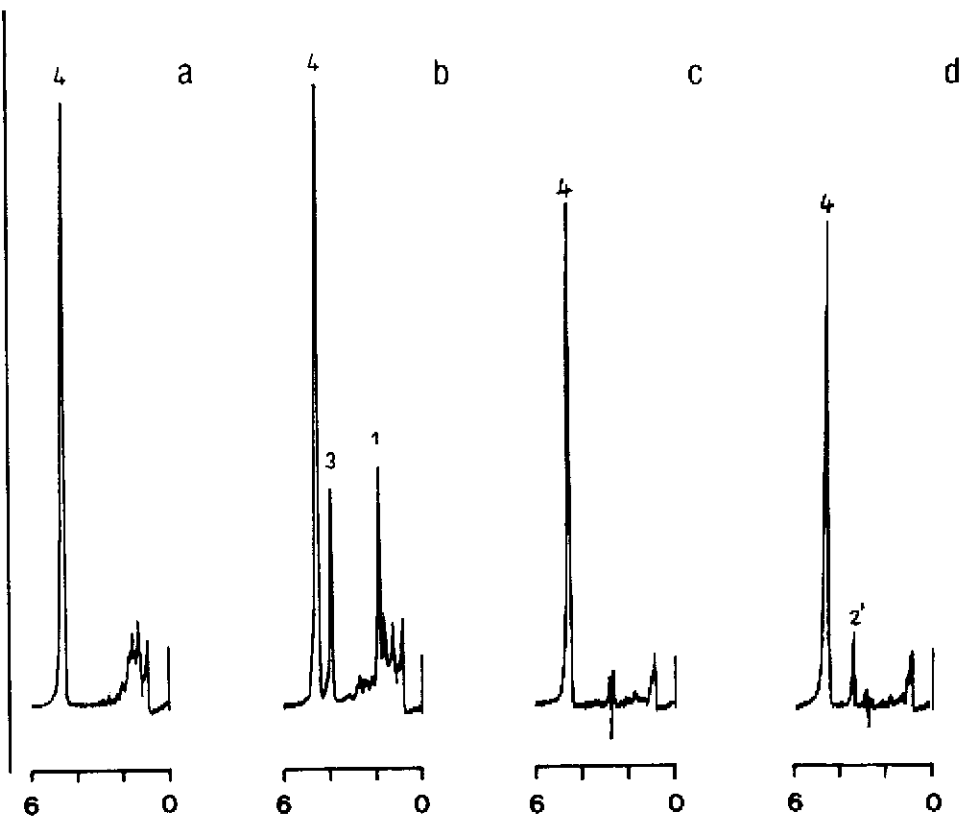

Fig. 4. Chromatograms of blank human plasma and of a plasma sample spiked with 100 ng of acenocoumarin and its amino and acetamido metabolites. (a) The organic phase of the blank. (b) The organic phase of the spiked plasma. (c) The "amino phase" of the blank. (d) The "amino phase" of the spiked plasma. The peaks represent: acetamido metabolite (1); the diazotization produce (2′); acenocoumarin (3); warfarin (4). Detector sensitivity, 0.02 a.u.f.s. Time scale in min.

Specificity and reproducibility

Fig. 4 shows examples of chromatograms of extracts obtained from pooled human plasma containing 100 ng/ml of acenocoumarin and its metabolites. For acenocoumarin and the amino derivative the procedure appeared to be highly specific, allowing the quantitation of concentrations of at least 25 ng/ml. For the acetamido metabolite, at low plasma concentrations (less than about 100 ng/ml) the system appeared to be less reliable because the compound eluted in the front where plasma constituents may also be present (Fig. 4b).

This problem can be surmounted by enhancing the resolution of the chromatographic system for the acetamido derivative, i.e. by decreasing the acetonitrile content of the eluent to 50 volume parts. Results of experiments testing the accuracy and precision of the assay are given in Table I. Using the internal standard procedure, the recoveries of acenocoumarin and the acetamido compound were about quantitative. Absolute recoveries of both the compounds and the internal standard warfarin, as measured by using ^{14}C-labeled compounds, were found to be about 90%. For the amino derivative, as calculated from the calibration points, about 80% recovery was obtained (Table I).

TABLE I

RECOVERY FOR ACENOCOUMARIN AND ITS METABOLITES

Results are expressed as mean ± S.D. (n = 4).

Plasma conc. (ng/ml)	Acenocoumarin	Amino metabolite*	Acetamido metabolite
50	96 ± 5	74 ± 3	92 ± 6**
100	98 ± 4	82 ± 10	95 ± 10**
200	103 ± 7	80 ± 2	92 ± 8
500	90 ± 3	76 ± 5	100 ± 8

*Calculated from calibration points processed along with the diazotization reaction (see methods).

**By analysis with the "slow" eluent (see text).

To test the applicability of the procedure for the amino and acetamido compounds, the former was given to three rats in a 2 mg/kg dose subcutaneously. Blood samples (0.2 ml) were obtained via an indwelling catheter in the femoral artery. Urine was collected for 48 h. The blood concentration—time profiles are shown in Fig. 5. Acetylation of the amino compound was rapid and extensive, the first blood sample (10 min after administration) already showed the presence of the acetamido metabolite. In urine about 10% of the dose was excreted unchanged and about 40% as the acetamido metabolite. The blood half-life was about 8 h for the amino compound.

Plasma samples of 27 patients anticoagulated with acenocoumarin were analysed (the patients were under control of the Thrombosis Department of the University Hospital). In all but two samples acenocoumarin was analysed, concentration range 14—154 ng/ml. In none of the samples could the

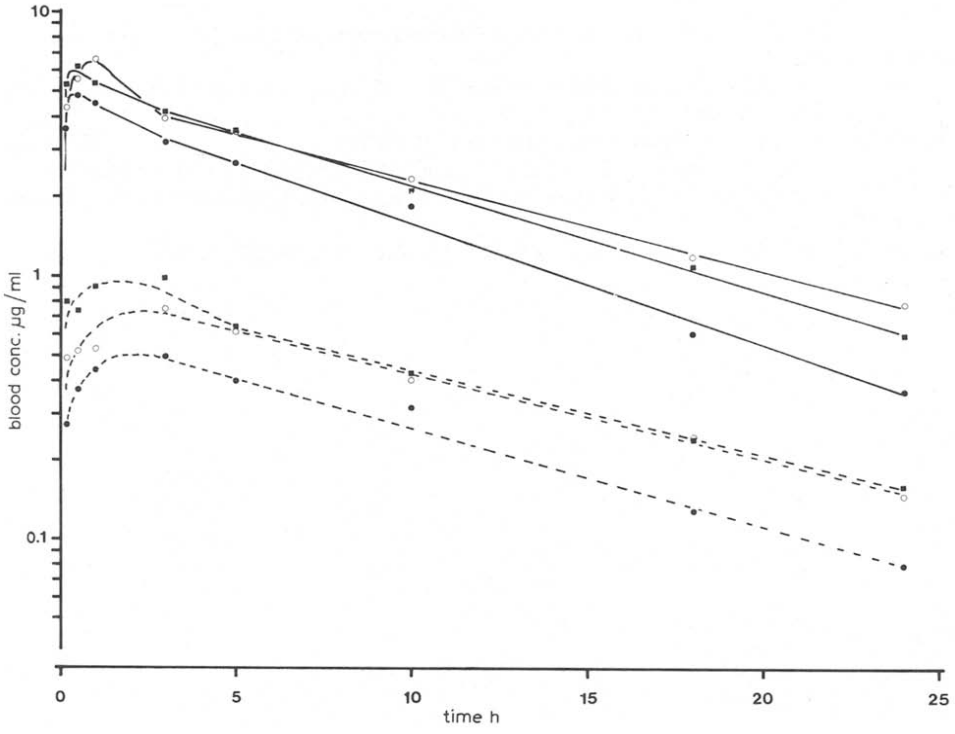

Fig. 5. Individual blood concentration — time profiles of the amino compound (——) and its acetylated derivative (- - - - -) in three rats. The amino compound (2 mg/kg) was administered subcutaneously.

acetamido metabolite be detected, and in three samples only was the amino metabolite analysed. These data do not fully agree with the results of Dieterle et al. [2], who for two volunteers showed plasma levels of the amino metabolite to exceed those of acenocoumarin. However, as optimal time schedules of dosing and sampling were not under control in our patients, the results do not give a correct insight into the clinical relevance of the formation of both the metabolites. Controlled clinical pharmacokinetic and pharmacodynamic studies are in progress at the moment.

CONCLUSIONS

The procedure presented permits the analysis of body fluids (plasma, urine) for acenocoumarin and its amino and acetamido metabolites with sufficient accuracy necessary for clinical pharmacological studies of the oral anticoagulant.

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