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Note

Quantitation of acenocoumarol in plasma by reversed-phase high-performance liquid chromatography

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Acenocoumarol (Sintrom) is a short-acting synthetic oral anticoagulant belonging to the mono-coumarin class. It is structurally related to warfarin and phenprocoumon which are relatively longer-acting oral anticoagulants [1]. Acenocoumarol is used clinically in the prophylaxis and treatment of venous thrombosis and pulmonary embolism and as an adjunct in the treatment of coronary occlusion and transient cerebral ischemic attacks. Recently, there has been considerable interest in the anticoagulant activity [2] and metabolism [3] studies of acenocoumarol. For the investigation of acenocoumarol pharmacokinetics, it is desirable to have a simple, sensitive and specific quantitation procedure. In 1968, a pharmacokinetic study in man was performed using a photometric assay [4] which apparently lacked specificity [3]. In 1977, a gas—liquid chromatographic procedure [5] for quantitating acenocoumarol was reported, but the technique contained a lengthy extraction procedure and diazomethane derivatization of the sample prior to chromatographic analysis. More recently, a sensitive and specific thin-layer chromatographic method for the quantitative and qualitative analysis of acenocoumarol was reported [6]. The technique required the reduction of the nitro group and derivatization in the formation of a fluorophore.

High-performance liquid chromatography (HPLC) has been shown to be a useful tool for the separation and quantitation of coumarin anticoagulants because of its simplicity, specificity and sensitivity [7—10]. Previously, we have reported a simple and specific HPLC procedure for the quantitation of warfarin in human plasma [11]. The technique was found to be generally applicable to the mono-coumarins (warfarin, acenocoumarol, phenprocoumon) and in this paper, we describe an adaptation for the analysis of acenocoumarol. With the incorporation of a highly sensivity UV detector (Vari-

Chrom; Varian, Palo Alto, Calif., U.S.A.), as low as 125 ng/ml of the drug in plasma can be quantitated with a coefficient of variation (C.V.) of 4.7%. Methylated warfarin [(3-α-acetonylbenzyl)-4-methoxycoumarin] is used as an internal standard.

EXPERIMENTAL

Apparatus

The liquid chromatograph was a Varian-Aerograph Model 4100, equipped with a positive displacement pump capable of developing a pressure up to 5000 p.s.i., a stop-flow injection port and a variable-wavelength UV absorbance detector (Vari-Chrom, Varian) operated at 305 nm and attenuated to 0.05 a.u.f.s. The column (25 cm × 2.2 mm I.D., stainless steel) was packed with 10 μm LiChrosorb RP-2 using a high-pressure (5000 p.s.i.) balanced-density slurry technique [12]. A pre-column (5 cm × 2.2 mm I.D.) packed with Vydac-RP (30—44 μm) was used to trap eluent insoluble materials. The mobile phase contained 0.75 g ammonium acetate per 100 ml of a mixture of acetonitrile—water—acetic acid (37:62:1, v/v). The system was operated at a flow-rate of 40 ml/h at ambient temperature.

Materials

Methylated warfarin was synthesized by reacting warfarin (Aldrich, Milwaukee, Wisc., U.S.A.) with an ethereal alcoholic solution of diazomethane (Diazald; Aldrich) giving quantitative yield of the product [13]. A solution of methylated warfarin (240 ng/ml) was prepared in *n*-butyl chloride. Acenocoumarol (Ciba-Geigy, Basle, Switzerland) was neutralized with 0.1 *N* NaOH and a stock solution (40 μg/ml) in distilled water was prepared and appropriate dilutions were made for the construction of standard curves. Ammonium acetate was obtained from BDH (Toronto, Canada). Glass distilled acetonitrile, *n*-butyl chloride and acetone were supplied by Caledon Labs. (Georgetown, Canada). LiChrosorb RP-2 was purchased from Aviation Electric (Montreal, Canada).

Plasma level study

Acenocoumarol (neutralized with 0.1 *N* NaOH) dissolved in water was administered orally to two rabbits at a dose of 1 mg/kg. Blood samples (3—4 ml) were drawn from the ear vein, using heparinized syringes, at 0, 1, 3, 5, 7, 10 and 24 h after drug administration. The blood samples were centrifuged and the plasma collected and stored at −20° and analyzed within three weeks.

Extraction and analysis procedure

To 0.5—1.0 ml of plasma (spiked or from dosed animals) in a conical glass-stoppered centrifuge tube (15 ml) were added 1 ml of 3 *N* HCl and 5 ml of *n*-butyl chloride solution containing methylated warfarin as internal standard. The sample was agitated at slow speed in a horizontal shaker for 10 min. After centrifugation at 2000 *g* for 10 min, about 4 ml of the organic layer was transferred into another conical glass-stoppered centrifuge tube (5 ml) and the solution evaporated to dryness under a stream of dry nitrogen at room

temperature. The side of the tube was washed with 1 ml of acetone and evaporated to dryness. The residue was dissolved in 30 μl of acetonitrile or dioxane, mixed in a Vortex mixer for 15 sec, and aliquots (5—10 μl) were chromatographed. Drug concentration was estimated by comparing the drug:internal standard peak height ratio to that of a standard constructed from pooled human plasma spiked with 0.125, 0.25, 0.5, 1.0 and 2.0 μg/ml of acenocoumarol.

Evaluation of extraction efficiency

Plasma was spiked with acenocoumarol to give concentrations of 0.25, 0.5, 1.0 and 2.0 μg/ml, extracted with *n*-butyl chloride containing methylated warfarin (1.2 μg) and chromatographed as described above. Care was taken to recover all of the organic extract. The extraction efficiency was evaluated by comparing peak height obtained after extraction of spiked plasma to those obtained from corresponding concentrations of standard solutions.

RESULTS AND DISCUSSION

With a few modifications, the HPLC technique reported earlier for the quantitation of warfarin [11] was adapted to the analysis of acenocoumarol in plasma. A mobile phase of ammonium acetate in acetonitrile—water—acetic acid was used in place of a dioxane—water mixture because: (a) it gave a more stable column peak retention over a long period of use, and (b) it eliminated the use of dioxane which had to be redistilled prior to use. *n*-Butyl chloride (5 ml) was used for extraction instead of ethylene chloride (25 ml) as it gave a cleaner extract and, because of its greater volatility, was easily evaporated at room temperature. The sensitivity of the method was greatly improved by the incorporation of a highly sensitive variable wavelength UV detector.

Fig. 1A shows a chromatogram obtained from blank plasma and Fig. 1B from plasma to which acenocoumarol and methylated warfarin had been added. The retention times for acenocoumarol and methylated warfarin were 5.5 and 9 min, respectively. The extraneous peaks from the plasma had retention times which were <4.5 min and therefore did not interfere with the assay. Fig. 1C shows a chromatogram of a plasma extract containing 170 ng/ml of acenocoumarol from a 10-h sample (1 ml) of a rabbit after receiving a single oral dose (1 mg/kg) of the drug.

The efficiency of the extraction procedure is depicted in Table I. The average mean recoveries of acenocoumarol and methylated warfarin from plasma were 79.74 ± 9.6 and 81.31 ± 5.7%, respectively (0.25 to 2 μg/ml range). The accuracy of the analysis is shown in Table II. Results are based on at least 4 determinations of each concentration (0.125 to 2 μg/ml). The peak height ratio of acenocoumarol and methylated warfarin was used as an index for quantitation. A linear response was obtained with a mean slope of 1.68 ± 0.05. The overall C.V. over the range was 4%. A larger C.V. was obtained when estimating below 0.125 μg/ml.

Application of the HPLC method for the estimation of acenocoumarol plasma levels in the rabbit is described in Fig. 2. The figure shows the plasma

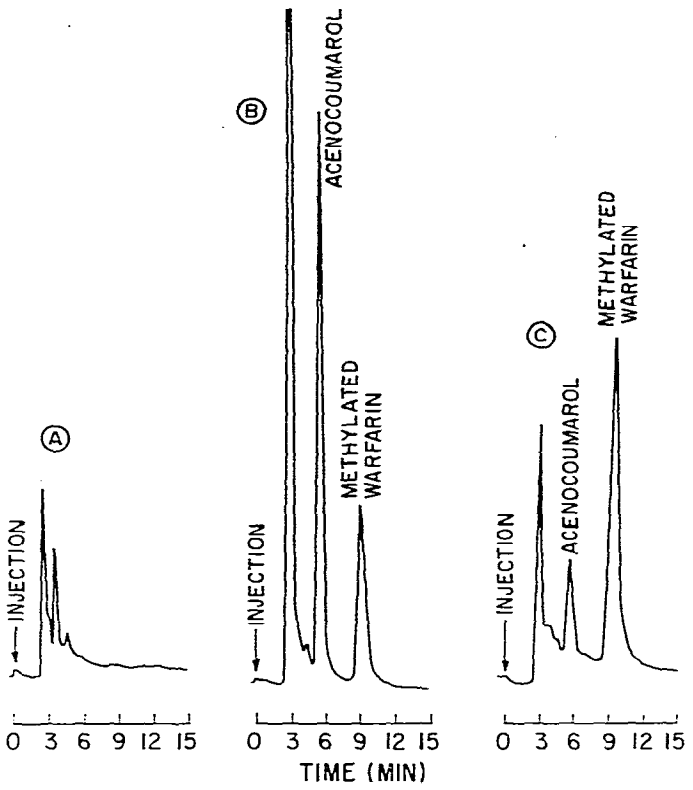

Fig. 1. Chromatographic separation from (A) control human plasma, (B) pooled human plasma spiked with acenocoumarol, and (C) 10 h plasma sample from a rabbit given 1 mg/kg of per oral acenocoumarol. HPLC system: column, 25 cm × 2.2 mm I.D., packed with LiChrosorb RP-2; mobile phase, 0.75 g ammonium acetate per 100 ml acetonitrile—water—acetic acid (37:62:1); flow-rate, 40 ml/h.

TABLE I

RECOVERY OF ACENOCOUMAROL AND METHYLATED WARFARIN FROM PLASMA

Values in brackets are percentage recoveries; $n = 3$ for each estimation.

Acenocoumarol added to 1 ml plasma (μg)	Acenocoumarol recovered Mean ± S.D. (μg)	Methylated warfarin added to 1 ml plasma (μg)	Methylated warfarin recovered Mean ± S.D. (μg)
0.25	0.17 ± 0.03 (68.8)	1.2	0.92 ± 0.18 (76.7)
0.5	0.38 ± 0.03 (75.1)	1.2	0.93 ± 0.08 (77.2)
1.00	0.90 ± 0.03 (90.4)	1.2	1.07 ± 0.06 (88.9)
2.00	1.69 ± 0.16 (84.7)	1.2	0.99 ± 0.18 (82.5)

TABLE II

HPLC ESTIMATION OF ACENOCOUMAROL ADDED TO PLASMA

Acenocoumarol added to 1 ml plasma (μg)	*n*	Mean peak height ratio of acenocoumarol:methylated warfarin	S.D.	C.V.	Slope*
0.125	4	0.21	0.01	4.7	1.68
0.250	5	0.44	0.01	2.3	1.76
0.500	5	0.84	0.03	3.5	1.68
1.000	5	1.61	0.07	4.3	1.61
2.000	5	3.33	0.17	5.1	1.67

*Peak height/concentration.

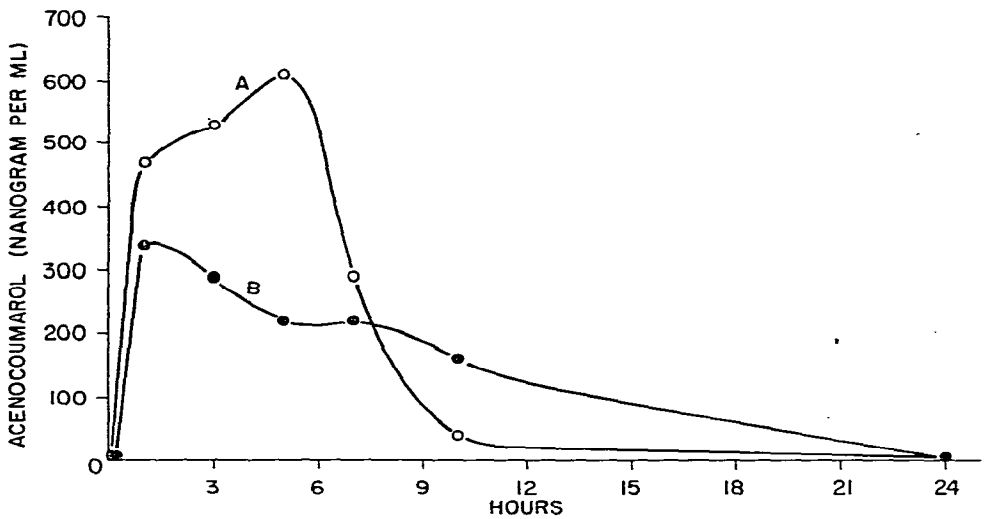

Fig. 2. Plasma acenocoumarol levels in two rabbits during 24 h following a single oral dose of 1 mg/kg acenocoumarol.

profile over 24 h of two rabbits after each received a single oral dose (1 mg/kg) of acenocoumarol. The peak acenocoumarol plasma level in rabbit B (340 ng/ml) was attained within 1 h, but that of rabbit A (610 ng/ml) was reached only after about 5 h. The technique is of sufficient sensitivity for the estimation of acenocoumarol plasma levels in humans after therapeutic doses since Dieterle et al. [3], using ^{14}C-acenocoumarol, reported plasma concentrations in the mid-nanogram range in human subjects who had received a low therapeutic dose of the drug.

In summary, a simple and sensitive HPLC procedure for the estimation of acenocoumarol in plasma has been developed. Previous quantitation of the coumarin in biological samples relied mainly on the use of gas—liquid chromatography [5] which required an elaborative purification step and a derivatiza-

tion procedure. The use of reversed-phase HPLC provides a simpler means of analysis for the anticoagulant, with a simple and direct cleanup procedure, no derivatization of sample prior to chromatographic analysis, and a sensitivity limit in the nanogram range.

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