Journal of Chromatography, 124 (1976) 331–335

CHROM. 9188

GAS CHROMATOGRAPHIC DETERMINATION OF ACENOCOUMARIN IN HUMAN PLASMA

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(Received February 17th, 1976)

SUMMARY

A method based on solvent extraction, formation of a fluorinated derivative and quantitation by gas–liquid chromatography with electron-capture detection has been developed for the determination of acenocoumarin in plasma.

The specificity and sensitivity of the procedure appear to be satisfactory for drug level measurements in human plasma. Its relative simplicity permits its use in routine analysis.

INTRODUCTION

A knowledge of the pharmacokinetic profile is useful for a more rational therapeutic approach with several classes of compounds. For anticoagulants, complete characterization of the pharmacokinetic behaviour has been achieved only for warfarin[1,2], and this information has improved the understanding of its interactions with other drugs[3,4].

Acenocoumarin [3-(*α*-acetonyl-*p*-nitrobenzyl)-4-hydroxycoumarin] (Sintrom) is an anticoagulant drug widely used in Europe, and its pharmacokinetic profile has not yet been defined. Our interest in the possible interactions between acenocoumarin and antipyretic and antirheumatic drugs prompted us to study plasma levels of acenocoumarin in course of chronic treatment.

As all of the methods available in the literature[5–7] were lacking either in sensitivity or in specificity, we felt the need to develop a sensitive and specific gas–liquid chromatographic (GLC) method with electron-capture detection (ECD) which would give the sensitivity and specificity required for the accurate evaluation of the kinetic behaviour of this drug. The technique reported here appears to meet these requirements.

EXPERIMENTAL

Standards and reagents

Acenocoumarin (A) was kindly supplied by Ciba-Geigy (Milan, Italy).

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Clonazepam (CLZ) (Rivotril), kindly supplied by Roche (Milan, Italy), was used as the internal standard. Other reagents were acetone RP, ethylene chloride RP, anhydrous sodium carbonate RP, all supplied by Carlo Erba (Milan, Italy), and pentafluorobenzyl bromide (PFBB), supplied by I.C.N. Pharmaceuticals, Plainview, N.Y., U.S.A. Thin-layer chromatography (TLC) was conducted on silica gel 60-F254 plates (0.25 mm layer thickness) (Merck, Darmstadt, G.F.R.).

Apparatus

A Carlo Erba Fractovap G-1 gas chromatograph equipped with a ^{63}Ni ECD was used.

The chromatographic column was a glass tube (60 cm × 4 mm I.D.) packed with 100–120-mesh Chromosorb Q coated with 3% OV-17 (Applied Science Labs., State College, Pa., U.S.A.), conditioned for 1 h at 250° (at a flow-rate of nitrogen of 40 ml/min), 4 h at 320° (no nitrogen) and 24 h at 285° (at a flow-rate of nitrogen of 40 ml/min). The operating conditions were: carrier gas (nitrogen) flow-rate, 20 ml/min; scavenger gas (nitrogen) flow-rate, 60 ml/min; column temperature, 285°; injection port temperature, 290°; and detector temperature, 290°. The ECD was used with a pulse current at an excitation voltage of 50 V and a pulse interval of 30 msec.

Determination of standard external calibration graph

Acenocoumarin was dissolved in acetone (5 μg/ml) and 40 μl of an acetone solution of CLZ as internal standard were added to individual aliquots covering the range from 100 to 400 ng (in triplicate). The samples were dried under a stream of nitrogen. The reaction was performed as described by Kaiser and Martin[8] for warfarin with some modifications. A 0.5-ml volume of PFBB (1% solution in acetone) and 20 mg of anhydrous sodium carbonate were added to the dry residue. The tubes were capped, shaken on a mixer for about 10 sec and incubated at 70° for 60 min. The solution was then evaporated to dryness under a stream of nitrogen. To the dry residue, 0.5 ml of acetone was added and the tubes were shaken for 10 sec on a mixer; the acetone layer was then transferred into another series of test-tubes and concentrated under nitrogen to 100–200 μl. A 1-μl volume of this solution was injected into the gas chromatograph.

Extraction procedure

To increasing amounts of acenocoumarin, from 100 to 400 ng, plus 200 ng of CLZ (dry residue) in 20-ml glass test-tubes, 1 ml of Tris buffer (0.2 *M*, pH 8.4) was added. The tubes were shaken for about 10 sec on a mixer, then 2 ml of human plasma and 0.2 ml of 0.5 *M* zinc sulphate solution were added. The samples were then extracted with 10 ml of ethylene chloride, shaking them horizontally at room temperature for 20 min. After centrifugation at 800 *g* for 5 min, the aqueous phase was removed. A 8.5-ml volume of the organic phase was transferred into another test-tube and dried in a thermostatic bath at 50° under a gentle stream of nitrogen. The dry residue was then processed as described above.

Thin-layer chromatography

Standard solutions of acenocoumarin, its pentafluorobenzyl (PFB) derivative and PFBB were spotted on silica gel plates. The plates were developed by the ascending

technique for a distance of 17 cm in the solvent system acetone–ethylene chloride (1:10).

The developed materials were rendered visible with a short-wavelength (252 nm) UV lamp. Under these conditions, for acenocoumarin $R_F = 0.12$ and for its PFB derivative $R_F = 0.53$; no spot was seen for PFBB alone. On the plate where the PFB derivative was run, no other spots were detectable, indicating a high derivatization yield. The zones corresponding to acenocoumarin and its PFB derivative were scraped into test-tubes containing 2 ml of methanol. After shaking for 10 min in a horizontal position and centrifuging for 5 min at 800 *g*, 1.5 ml of the methanolic solution were transferred to another test-tube and dried under a stream of nitrogen. A 1-μl volume of an acetone solution of the dry residue was injected into the gas chromatograph.

The acenocoumarin sample did not give any detectable peak, while the PFB derivative gave a single and well defined peak with the same retention time as that given by the external and internal standards.

RESULTS AND DISCUSSION

The reaction of acenocoumarin with PFBB, under the conditions used, was practically complete, as demonstrated by the TLC assay, and the PFB derivative is stable at least for 72 h at room temperature. The internal standard, CLZ, on the contrary, does not react with PFBB under the conditions used.

In order to determine the optimal reaction time, we prepared a series of samples, containing a known amount of acenocoumarin, that were incubated with PFBB for different periods of time. CLZ was added as internal standard. As shown in Fig. 1, the formation of the PFB derivative does not increase after 45 min.

Fig. 2 shows a typical gas chromatogram of the PFB derivative and of CLZ obtained from plasma extract; the retention times were 1.5 and 4.5 min, respectively. The calibration graph, obtained by plotting the ratios of the peak area of the PFB derivative to that of CLZ against known amounts of acenocoumarin added to the plasma, is shown in Fig. 3. Good linearity is obtained for concentrations from 100 to 400 ng/ml in plasma. The minimal detectable amount was 500 pg. The recovery of the

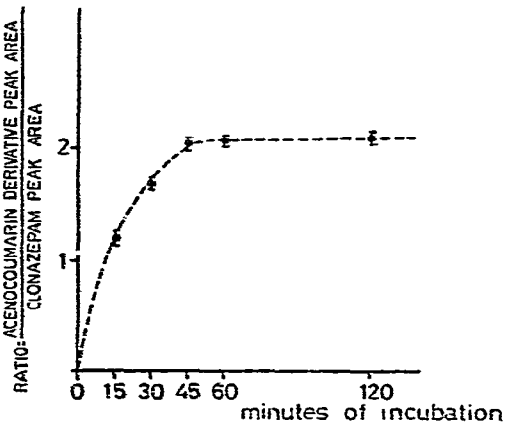

Fig. 1. Effect of reaction time on the formation of the PFB derivative of acenocoumarin.

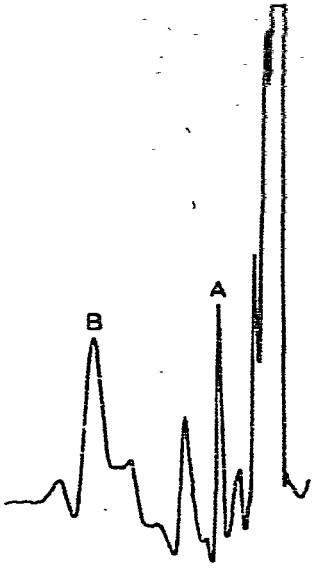

Fig. 2. Gas chromatogram of 300 ng of acenocoumarin (PFB derivative) (B) and of 100 ng of clonazepam (A) obtained from a plasma extract and dissolved in 100 µl of acetone.

extraction procedure was checked by comparing a standard external with an internal calibration graph, using CLZ as an external standard; the value is about 75%.

Administration of acenocoumarin (5 mg orally in two doses with a 12-h interval) to two patients for 2–3 weeks gave steady-state levels varying between 20 and 70 ng/ml in plasma, as shown in Fig. 4.

It should be stressed that the levels found are much lower than those found by Blatrix *et al.*[9] using a spectrophotometric method; this difference may be due to the fact that, with our method, we measure only intact acenocoumarin, without metabolites, which probably are measured in the spectrophotometric method.

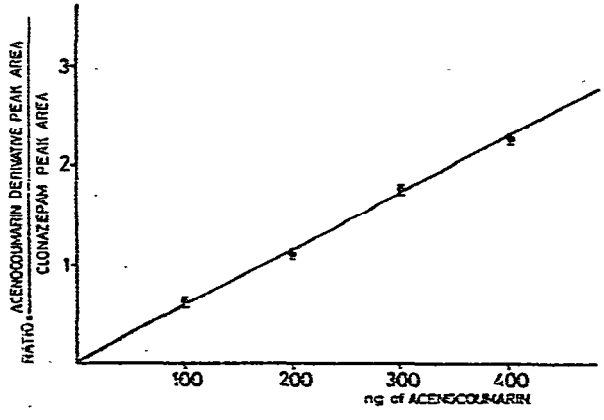

Fig. 3. Standard calibration graph for acenocoumarin added to the plasma and carried through the analytical procedure.

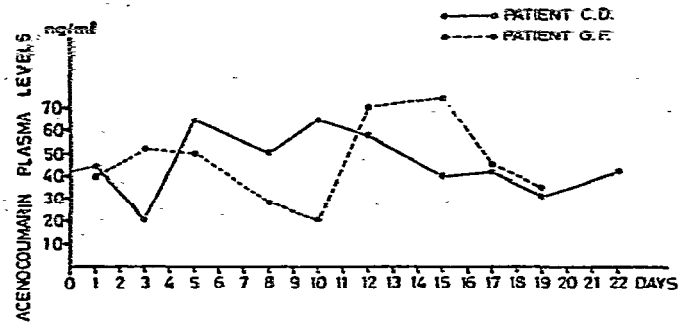

Fig. 4. Plasma levels of acenocoumarin in two patients during chronic therapy. The daily dose was 5 mg of acenocoumarin orally (2.5 mg twice a day).

The wide fluctuation of the plasma levels during the "steady state" might be explained both by a low compliance and an erratic absorption owing to the presence of food in the gastrointestinal tract, or by an inappropriate dosage interval that is inadequate compared with the apparent plasma half-life of acenocoumarin. From preliminary results, acenocoumarin levels appear to be strictly correlated with prothrombin time and plasma levels of 40–60 ng/ml seem to be sufficient to maintain a prothrombin activity of about 20%.

CONCLUSIONS

The method described, with its simplicity and specificity, could be used for the routine adjustment of dosage in patients undergoing chronic treatment in order to avoid wide fluctuations of plasma levels and consequently of anticoagulant activity. Because of its high sensitivity, it can also be used for single and multiple dose pharmacokinetic studies. Further observations along these lines are now in progress.

ACKNOWLEDGEMENTS

We thank to Dr. Caruso for kindly supplying plasma samples and Dr. de Gaetano for performing prothrombin time determinations.

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