Journal of Chromatography, 145 (1978) 332—335
Biomedical Applications

CHROMBIO. 106

Note

Quantitative and qualitative analysis of the anticoagulant acenocoumarol in human plasma

GODFRIED M.J. VAN KEMPEN
Biochemical Laboratory, Psychiatric Institute "Endegeest", Oegstgeest (The Netherlands)

and

ELLY A.M. KOOT-GRONSVELD and FREDERIK A. DE WOLFF*
Laboratory of Toxicology, University Hospital, Leiden (The Netherlands)

(Received August 2nd, 1977)

Recently we described a method for the determination of the coumarin anticoagulant phenprocoumon (Liquamar, Marcoumar). The reasons presented by us for that investigation are also valid for another coumarin anticoagulant, acenocoumarol, namely to obtain information on the presence of intoxication, malabsorption, non-compliance or hereditary resistance, and on the pharmacokinetics of the drug.

As, in contrast to phenprocoumon, acenocoumarol does not display natural fluorescence because of its nitro group, a reduction step and a labelling step were introduced which resulted in the formation of a fluorophore. This enables us to use the plasma as its own blank by omission of the reduction step. Only other compounds containing nitro groups might interfere with the assay.

MATERIALS AND METHODS

Acenocoumarol (Sintrom) and its reduced derivative "aminosintrom" [(CGP 8 435, 3-(*a*-acetonyl-4-aminobenzyl)-4-hydroxycoumarin)] were gifts from Ciba-Geigy (Arnhem, The Netherlands). Fluorescamine (Fluram) was purchased from Hoffmann-La Roche (Mijdrecht, The Netherlands). All other chemicals were of analytical grade. Thin-layer chromatography, fluorescence densitometry, preparation of blood plasma, and thrombotest determinations were performed as described previously [1], with the exception of the development of the thin-layer plates which will be described in the final procedure.

*To whom reprint requests and correspondence should be addressed.

RESULTS

Extraction

Of a number of organic solvents examined, the best results were obtained with a mixture of light petroleum (b.p. 40—60%) and dichloromethane. Recovery for the extraction of 50 ng/ml acenocoumarol, added to blank plasma, was 68 ± 1.2% (mean ± S.E.M., n=7).

Reduction

After extraction and evaporation the nitro group was reduced to an amino group by tin (II) chloride. The efficiency of the reduction was 77 ± 1% (mean ± S.E.M., n=7), as compared to known amounts of aminosintrom. No destruction of aminosintrom was observed under the experimental conditions.

Formation of the fluorophore and thin-layer chromatography

After evaporation of the reaction mixture the residue was dissolved in toluene which is used instead of the commonly employed ethanol since tin(II) chloride, which interferes with the chromatography, is insoluble in toluene. Formation of the fluorophore with fluorescamine was performed by derivatization at the origin of the thin-layer plate by developing with a fluorescamine solution [2].

Procedure

To 1 ml of plasma in a Sovirel culture tube (110 × 16 mm) with a PTFE-lined screw cap, add 1 ml of 0.1 mole/l sodium acetate buffer (pH 4.0) and 1 g NaCl. After mixing, add 7 ml of light petroleum (b.p. 40—60°)—dichloromethane (1:1, v/v). Treat in the same manner a calibration series containing 0, 0.1, 0.5 and 0.9 mg acenocoumarol per litre added to blank plasma. Shake the tubes mechanically for 15 min and centrifuge at maximum speed in a clinical centrifuge for 10 min. Filter the upper layer in conical tubes through Whatman No. 1 PS phase-separating filter paper that has been washed thoroughly with chloroform to prevent the impurities present in the paper [3] interfering with the subsequent chromatographic procedure. Wash the filters with 1—2 ml of the solvent mixture. Evaporate the filtrate in a hot water bath under a stream of nitrogen. Dissolve the residue in 70 µl of ethanol and add 20 µl of 30 m-moles/l $SnCl_2$ in 0.4 mole/l acetate buffer (pH 4.0). Mix thoroughly and incubate for 30 min at 60°. Evaporate again under nitrogen and add 90 µl of toluene. Centrifuge and spot 30 µl of the supernatant solution on a thin-layer plate (maximally 13 spots per 20 cm × 20 cm plate). Place the plate in a tank containing a 1-cm depth of derivatizing reagent, prepared by dissolving 10 mg of fluorescamine in 20 ml acetone and then adding hexane to a final volume of 100 ml. After the solvent front has moved at least 10 cm, remove the plate and evaporate the solvent. Then develop the plate in ethyl acetate—methanol—triethanolamine (70:30:3, v/v/v), until the solvent has ascended 10 cm from the starting point. After drying at room temperature, inspect the plate under ultraviolet light at 366 nm. Mark the position and diameter of the spots with a pencil at both vertical rims of the plate (R_f 0.35). Scan the plate with a Vitatron TLD 100 Flying Spot densitometer. The instrument settings are: mode,

lin II+; level, e 7; zero, C 7; damping, 2; span, 7—10; lamp, Hg; diaphragm, 0.50 mm; primary filter, 366 nm; secondary filter, U12, cut off below 540 nm; scanning speed, 1 cm/min; integrator sensivity, position 7.

Draw a calibration curve by plotting the number of integrator units vs. concentration; calculate from this straight line unknown concentrations by interpolation. Use as a blank the unknown serum to be investigated submitted to the same procedure but without addition of the reducing reagent.

The lower limit of detection under the conditions presented above is about 0.010 mg/l. The determination is reproducible to about 3%. Nine samples can be analyzed within 5 h.

When acenocoumarol was given to patients in the evening and the blood was drawn the following morning for a thrombotest and acenocoumarol assay, plasma concentrations between 0.015 and 0.080 mg/l were observed.

Qualitative demonstration of acenocoumarol in plasma

The formation of a fluorescent product from acenocoumarol can be used in the screening of a suspected case of intoxication with anticoagulant coumarin derivatives. Plasma extracts can be chromatographed on thin-layer plates with chloroform—methanol (97:3, v/v) as described by Daenens and Van Boven [4]. Dicoumarol (R_f 0.29), warfarin (R_f 0.45) and phenprocoumon (R_f 0.55) are visible under ultraviolet light at 254 nm. It is possible to visualize also acenocoumarol (R_f 0.40) on this plate by spraying with the buffered tin(II) chloride solution, followed, after incubation of the plate for 30 min at 60° in a closed tank saturated with water vapour, by spraying with a fluorescamine solution in acetone. The fluorescent product is visible under light of 366 nm.

Interference by other compounds

For those compounds that contain no nitro group, a correction is made by using as the blank a non-reduced extract from the same plasma. Only the few nitro compounds that are in use as therapeutic agents may cause serious interference. We submitted all the drugs containing nitro groups known to us to the same procedure as acenocoumarol and observed the following R_f values: acenocoumarol, 0.35; chloramphenicol, 0.34; nitrazepam, 0.28; hydroxymethylnitrofurantoin, 0.28; clonazepam, 0.23; nitrofurantoin, invisible.

Hydroxymethylnitrofurantoin is not extracted from plasma under the circumstances used here. The only serious interference may be caused by chloramphenicol, the presence of which is known in most cases.

Stability of acenocoumarol in serum

When a serum spiked with 100 ng/ml acenocoumarol was kept at −20° or +4° for one week, no decrease in concentration was observed. Kept on the laboratory bench for one week the concentration of acenocoumarol was diminished by about 30%.

DISCUSSION

We have developed a highly specific and sensitive method for the determination of acenocoumarol in human plasma. The specificity is a result of the use

of a reduction step, necessary for the development of a fluorophore, which eliminates interference from all compounds not containing nitro groups, if the proper blank is used. The fluorogenic labelling enhances the sensitivity of the method, which is necessary in view of the very low blood levels of acenocoumarol under therapeutic circumstances. A major advantage is the use of derivatization at the origin of the thin-layer plates, as derivatizing by spraying lowers both sensitivity and reproducibility. However, the procedure of reduction on the plate, followed by spraying with fluorescamine can be useful to visualize acenocoumarol next to other coumarin derivatives which display natural fluorescence. So far, no correlation can be demonstrated between thrombotest values and acenocoumarol concentration, unlike our recent findings with phenprocoumon [5].

In summary, this method is useful for the simultaneous qualitative and quantitative analysis of acenocoumarol in human plasma in the therapeutic as well as in the toxic range.

ACKNOWLEDGEMENTS

This study was initiated by Professor Dr. E.A. Loeliger. The investigations were financially supported by Ciba-Geigy B.V., Arnhem, The Netherlands.

REFERENCES

1 F.A. de Wolff and G.M.J. van Kempen, Clin. Chem., 22 (1976) 1575.
2 H. Nakamura and J.J. Pisano, J. Chromatogr., 121 (1976) 33.
3 A.F. Rosenthal, M.G. Vargas and C.S. Klass, Clin. Chem., 22 (1976) 1756.
4 P. Daenens and M. van Boven, J. Chromatogr., 57 (1971) 319.
5 F.A. de Wolff and G.M.J. van Kempen, in W.A.M. Duncan and B.J. Leonard (Editors), Clinical Toxicology, Excerpta Medica, Amsterdam, Oxford, 1977, pp. 189–191.